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The unfolded protein response and HLA-B27 misfolding: implications for ankylosing spondylitis

Andrew James Lemin

A thesis submitted for the degree of Doctor of Philosophy

School of Biological and Biomedical Sciences

University of Durham

August 2010

Material Abstract

The unfolded protein response (UPR) detects the presence of misfolded proteins in the endoplasmic reticulum (ER) and subsequently relieves ER stress by increasing the folding capacity of the ER. The secretory pathway substrate HLA-B27 is highly associated with the chronic inflammatory disease ankylosing spondylitis (AS) and has a tendency to misfold in the ER. Here, we show that overexpression of HLA-B27 and non-disease associated HLA-B7 in immortalised cell lines leads to heavy chain misoxidation, which is accompanied by upregulation of *BiP* and splicing of *XBP1*, a key step in the IRE1 pathway of the UPR which is increasingly being linked with intestinal inflammation.

We also demonstrate that different cell lines respond to different ER stress stimuli in distinct ways. We establish that HT1080 cells inefficiently induce a UPR in response to tunicamycin and that this has consequences for cell survival. However, inefficient activation of the UPR in HT1080 cells can be overcome by secondary signals, since co-administration of the tyrosine kinase inhibitor genistein leads to activation of *XBP1*. Furthermore, we show that genistein can inhibit UPR induction of *BiP* in response to a range of ER stresses indicating that the cancer drug genistein can inhibit or activate the UPR depending on the environment and cell type. This has implications for inflammatory disease since regulation of the UPR is important in determining a cell's tendency towards apoptosis.

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Abbreviations

AD	Activation domain
AS	Ankylosing spondylitis
ASK1	Apoptosis signal-regulating kinase 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
β_2m	β_2 microglobulin
B27-Tg	HLA-B27/β_2m transgenic
BIM	BCL-2-interacting mediator of cell death
BiP	Immunoglobulin binding protein
BMDM	Bone marrow derived macrophage
BSA	Bovine serum albumin
CHOP	CCAAT/Enhancer-binding protein homologous protein
CP4H	Collagen prolyl-4 hydroxylase
CREB	cAMP response element-binding protein
DBD	DNA binding domain
DMSO	Dimethyl sulfoxide
DSS	Dextran sodium sulphate
DTT	Dithiolthreitol
EDEM	ER degradation enhancing α-mannosidase-like protein
eIF2α	Eukaryotic initiation factor 2 alpha

ERAAP	ER amino peptidase associated with antigen presentation
ERAD	Endoplasmic reticulum-associated degradation
ERSE	ER stress response element
Ero	Endoplasmic reticulum oxidoreductin
FAD	Flavin adenine dinucelotide
FCS	Foetal calf serum
GCN2	General control non-derepressible-2
GRP	Glucose regulated protein
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GT	UDP-Glc:glycoprotein
glucosyltransferase	
HC	Heavy chain
HLA	Human leukocyte antigen
Hsp	Heat shock protein
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRE1	Inositol-requiring kinase 1
JNK	c-Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
KIR	Killer immunoglobulin receptor

LIR	Leukocyte immunoglobulin receptor
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mRNA	Messenger ribose nucleic acid
NEM	N-Ethylmaleimide
NF-κB	Nuclear factor kappa B
NF-Y	Nuclear factor Y
NK	Natural killer
NMR	Nuclear magnetic resonance
NR	Non-reducing
NRF2	Nuclear factor erythroid 2-related factor 2
ORF	Open reading frame
pAb	Polyclonal antibody
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
PDILT	Protein disulfide isomerase-like protein of the testis
PDIp	Pancreas-specific protein disulfide isomerase
PERK	RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase

pK_a	The negative logarithm of the acid dissociation constant
PPIase	*cis-trans* peptidyl prolyl isomerase
R	Reducing
RAC	Ribosome associating complex
ReA	Reactive arthritis
RIPA	Radio immunoprecipitation assay
RNase A	Bovine pancreatic ribonuclease A
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
SpA	Spondyloarthropathy
SREBP	Sterol response element binding protein
SRP	Signal recognition particle
TAP	Transporter associated with antigen processing
Tm	Tunicamycin
TNF	Tumour necrosis factor
TRAF2	TNF receptor associated factor 2
UPR	Unfolded protein response
UPRE	Unfolded protein response element
WB	Western blot

WT	Wild-type
XBP1	X-box binding protein 1

Declaration

I declare that the composition of this thesis, and all the data presented herein, is the result of my own work. No part of the materials offered has previously been submitted for a higher degree. This body of work has been achieved under the supervision of Dr. Adam. M. Benham.

The copyright of this thesis rests with the author. No quotation from it should be published without the prior written consent and information derived from it should be acknowledged.

Andrew J Lemin

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THESIS

1. SUMMARY

1. Summary

The major histocompatibility complex (MHC) class I heavy chain human leukocyte antigen (HLA)-B27 is highly associated with the development of the chronic inflammatory group of diseases known as the spondyloarthritides (SpA). HLA-B27 is unusual since it can form homodimers both at the cell surface and in the endoplasmic reticulum (ER). Homodimer formation of HLA-B27 in the ER is believed to be a consequence of the propensity of HLA-B27 to misfold. Recently, induction of the unfolded protein response (UPR) has been associated with HLA-B27 misfolding in the B27/human β_2-microglobulin (hβ_2m) transgenic rat model. UPR induction has been linked to the pathogenesis of inflammatory diseases since UPR activation can lead to activation of nuclear factor (NF)-κB, a master regulator of inflammation, immune responses and apoptosis and c-Jun N-terminal kinase (JNK).

Our results demonstrate that overexpression of the MHC class I heavy chains HLA-B27 and HLA-B7 lead to misoxidation and the formation of high molecular weight, disulfide-linked complexes. Formation of misoxidised class I heavy chains (cI HC) is accompanied by *XBP1* splicing, a key step in the activation of the IRE1 branch of the UPR. This is important in the context of SpA since *XBP1* activation is increasingly being linked with gut inflammation, a component of SpA. Furthermore, I demonstrate misoxidation-induced upregulation of *BiP*, a major target of the UPR indicating that misoxidation of HLA-B heavy chains can trigger downstream effects. Stable transfection of HLA-B27 in C58 cells however does not lead to *XBP1* splicing

but may decrease the sensitivity of these cells to secondary stress implying that chronic misfolding may pre-sensitise cells to further bouts of ER stress.

I also describe cell specific differences in the UPR induction in response to the pharmacological agent tunicamycin, which triggers ER stress by inhibiting glycosylation. HT1080 cells do not initiate *XBP1* splicing or *BiP* induction in the face of tunicamycin. Interestingly, HT1080 cells also do not initiate expression of CCAAT/Enhancer-binding protein (C/EBP) homologous protein (*CHOP*). Since *CHOP* is a proapoptotic transcription factor, I studied the effects of tunicamycin on cell survival of HT1080 and found that these cells exhibit increased cell survival over HeLa cells, which promote *CHOP* expression in the face of tunicamycin. However, the weak response of HT1080 to tunicamycin can be overcome by serum deprivation, indicating that HT1080 cells may have mechanisms of UPR regulation which are not as highly expressed in HeLa cells. Taken together, these results demonstrate that there are differences in how certain cells respond to different stimuli and that these responses can have an impact on cell survival.

Furthermore, I also demonstrate that UPR-driven transcriptional activation of *BiP* – a major downstream target of the UPR – by DTT, tunicamycin and thapsigargin can be inhibited with the use of the isoflavone genistein. Genistein has previously been demonstrated to inhibit *BiP* upregulation in response to thapsigargin but I demonstrate that genistein can inhibit transcriptional activation of *BiP* by a range of ER stresses in HeLa cells. Furthermore, I propose that this action is due to the tyrosine kinase inhibitory activity of genistein since the isoflavone diadzein, which lacks the tyrosine kinase inhibitory activity, does not regulate *BiP* expression in the

face of ER stress. Furthermore, I show that HT1080 cells can initiate tunicamycin-driven, but not thapsigargin- or DTT-driven *XBP1* splicing in the presence of genistein further indicating that there are cell specific differences in the regulation of the UPR. This has implications for SpA and inflammatory disease since regulation of the UPR is involved in controlling the cell's tendency towards survival or death.

2. GENERAL INTRODUCTION

2. General Introduction

2.1 *The molecular fate of polypeptides*

In order for proteins to carry out their intended task, they must acquire the correct three-dimensional structure necessary for their function. Evolutionary pressure, along with the vast number of possible unique protein structures, is responsible for generating the level of structural and functional diversity seen in the human proteome. The structure of a protein is determined primarily by its amino acid sequence; however, the majority of proteins require further processing to achieve functionality. A polypeptide chain of amino acids needs to be converted into a tightly-folded, compact, energetically-favourable structure where hydrophobic amino acids are buried within the soluble protein and hydrophilic residues are found in the solvent-accessible positions. This stable, 'correctly' folded, functional conformation is known as the native structure and will comprise secondary structural elements – α-helixes and β-sheets – as well as stabilising hydrogen bonds and electrostatic van der Waal's interactions. Oxidative folding and the addition of disulfide bonds contribute to the tertiary structure of secretory proteins (Sevier & Kaiser, 2002).

Achieving the native structure requires finding the state at which the protein is most stable under physiological conditions. According to Levinthal's paradox, it is impossible for a protein to systematically sample all of the possible confirmations before settling on the most stable (Dill & Chen, 1997). This is especially unlikely when you consider that protein folding events need to occur within a biologically practical timescale. As a result, the concept of energy landscapes, which describes

the free energy of a polypeptide chain as a function of its conformational state, was born. This theory describes the maturity of an unfolded polypeptide chain along an energy landscape to the final compact, native structure (Jahn & Radford, 2008).

Just as each protein will exhibit its own unique three-dimensional structure when at its native conformation, so too does each protein exhibit a unique energy landscape determined by the multitude of possible conformational states. Energy landscapes for small, relatively simple proteins appear funnel-shaped since many of the conformational options available lead reliably towards the native state. However, more complex proteins exhibit elaborate, highly dynamic energy landscapes where many intermediate conformations – or partially folded species – may journey towards or indeed away from the final native structure. These intermediates are sometimes referred to as on- or off-pathway species (Brockwell & Radford, 2007). An example of an energy landscape of a complex folding protein can be seen in Figure 1.

The energy landscape of a folding protein illustrates the wide variety of conformational states and folding pathways available to the polypeptide. However, the relative depth of each energy well is not simply dependent on the polypeptide sequence alone, but takes into consideration temperature, solution conditions and protein concentration (Jahn & Radford, 2008).

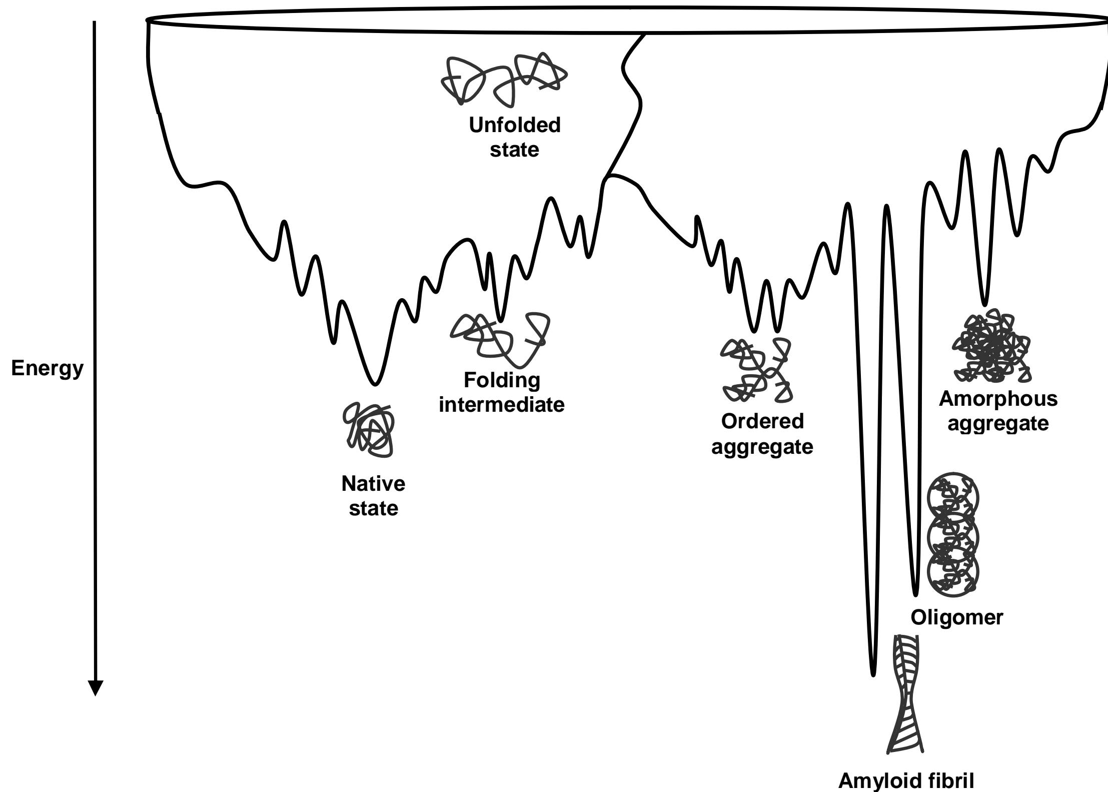


Figure 1. **A combined energy landscape diagram for both protein folding and protein aggregation.** Unfolded polypeptide chains progress along the energy landscape towards the compact native structure (Left hand side). Alternatively, polypeptide chains can form intermolecular protein associations, increasing the ruggedness of the energy landscape (Right hand side) and potentially resulting in the formation of low energy oligomeric and amyloid fibril states. Based upon a figure published by Jahn & Radford, 2008.

As polypeptide chains get larger, an assortment of factors, including an increased number of hydrophobic domains, may lead polypeptides to form large regions of non-native like structures, even when highly native-like segments have been formed (Dobson, 2004). This situation may trap the protein, albeit transiently, in a misfolded state. The appearance of species with a significant number of persistent non-native interactions is not considered part of the normal protein folding pathway since the architecture of these species is distinct from that of the native protein. However, a folding intermediate participating in the normal folding process of a protein is rather termed ‘unfolded’, not ‘misfolded’, even though they may contain non-native regions and similarly have higher free-energy than the native confirmation (Dobson, 2004).

An increase in population of a species with persistent non-native interactions, or indeed partially folded proteins, enhances the probability of species-specific intermolecular interactions between aggregation-prone regions (Ferguson *et al.*, 2006). The key residues for aggregate formation are thought to be different from those guiding correct folding of the polypeptide chain (Chiti *et al.*, 2002). However, certain key amino acids have been indentified which influence the on- or off-pathway fate of specific proteins (Wigley *et al.*, 2002) indicating the importance of the polypeptide sequence in defining its folding fate (DuBay *et al.,* 2004). Aggregate formation is nucleation-dependent and requires a transiently populated aggregation nucleus. The lag phase of protein aggregation – determined by the rate of nucleation – can be eliminated by the addition of pre-formed fibrils in a process known as ‘seeding’ (Harper & Landsbury, 1997). Once nucleus formation arises, aggregate growth occurs rapidly by the addition of further ‘monomers’. Although the formation of the nucleus is unfavourable, once underway, elongation of the aggregate is highly

favourable and rapidly leads to the formation of fibril structures with characteristic cross-β structures. However, the progression of amyloid fibril formation will depend on the specific energy landscape of that protein.

The formation of so-called amyloid fibrils represents an alternative, stable folding destination for polypeptides. The energy landscapes discussed earlier, therefore, are only part of the protein folding narrative, since the formation of a non-native, energetically-stable fibril, which is distinct from the native protein, can be achieved. Thus, a double-funnel diagram with two energetically favourable destinations seems more appropriate, since this view recognises the effects of aggregation on productive folding processes and the potential destinations of unfolded and misfolded intermediates (Fig. 1).

An increasing number of human diseases have been linked with protein misfolding, protein aggregation and amyloid fibril formation in different tissues and organs (Dobson, 2001). Termed 'protein misfolding' or 'protein conformational' diseases; these pathological conditions include, but are not limited to, neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Tan *et al.*, 2009; Meraz-Rios *et al.*, 2010), type II diabetes (Gotz *et al.*, 2009), cystic fibrosis (Chaudhuri & Paul, 2006) and α1-antitrypsin deficiency (Perlmutter, 2006). Some of these diseases, such as cystic fibrosis, are the consequence of specific misfolded proteins not being able to carry out their intended function (Thomas *et al.*, 1995) whereas the formation and build up of protein aggregates within cells or in the extracellular space characterises Alzheimer's and Parkinson's disease. Aggregate formation, in the case of the neurodegenerative disorders, confers cytotoxicity on

neuronal cells due to a gain-of-function (Caughey *et al.*, 2003). Inappropriate interactions of fibrillar aggregates may lead to sequestration of cellular proteins into disease-specific deposits such as inclusion bodies or plaques leading to cell death. However, in the case of Alzheimer's disease, smaller, multimeric aggregates have been described, which correlate with the early stages of the disease in a mouse model suggesting that early-stage aggregation of specific protein species can have pathological consequences (Berger *et al.*, 2007).

2.2 *Assisted protein folding*

Although it is possible, under optimised conditions, to reconstitute simple, monomeric proteins to their native conformation *in vitro*, this process is still too slow to occur in the cell. Renaturation of bovine pancreatic ribonuclease (RNase) – containing 124 amino acids and 8 sulfhydryl groups – under favourable pH, temperature and protein concentration still takes around 20 minutes after reduction of disulfide bridges and disruption of the tertiary structure (Goldberger *et al.*, 1963). It had been established that the synthesis of native RNase in a higher organism should take around 2 minutes (Dintzis, 1961; Cranfield & Anfinsen, 1963). Thus, it was realised very early on that cells must possess special mechanisms to increase the efficiency of protein folding. This realisation was further reinforced by observations that if RNase concentrations were increased to mimic physiological concentration, the yield of native protein was markedly low and that some chains become trapped with incorrect disulfide pairings (Anfinsen *et al.*, 1961; Goldberger *et al.*, 1963). The resulting non-native proteins were likely to be the result of incorrect formation of intrachain disulfides as well as interchain disulfides.

Since the intrinsic features of the polypeptide chain – despite being critical – do not seem to guarantee the reliable manufacture of functional proteins or preclude the threat of protein aggregation, the cell must possess a system that addresses these issues in the crowded cellular environment. The cell's quality control machinery ensures the correct folding of nascent polypeptides and the rapid removal and degradation of inappropriately folded, or misfolded, proteins. This multitude of folding enzymes and molecular chaperones prevent proteins getting trapped in off-pathway intermediates and inhibit unproductive interactions with other proteins, increasing the likelihood of reaching the native conformation.

2.2.1 *Cytosolic chaperone system*

Nascent polypeptides may interact with a number of different molecular chaperones. A chaperone is defined as a protein which interacts, stabilises or helps a non-native protein acquire their native conformation but is not incorporated into the functional structure. A protein destined for the cytoplasm will encounter two systems of chaperone action (Albanèse *et al.*, 2006) which act to a) stabilise nascent proteins on the ribosome and initiate folding (Kramer *et al.*, 2009) and b) aid downstream folding events to the native conformation (Bukau *et al.*, 2000; Hartl & Hayer-Hartl, 2002). Upon emergence from the ribosome, nascent polypeptides – in mammals – initially interact with the specialised ribosome-associated complex (RAC) comprising the heat shock protein 70 (Hsp70) homolog Hsp70L1 and the Hsp40 homolog Mpp11 (Otto *et al.*, 2005). The RAC provides a role similar to that which is provided by bacterial trigger factor (TF) in the early stages of prokaryotic protein folding (Chang *et al.*, 2007; Tang *et al.*, 2007). Together, these early-stage chaperones bind to hydrophobic regions, preventing compaction of the polypeptide

chain. This keeps the chain in an elongated state, delaying constructive folding events until sufficient structural information – inherent within the emerging polypeptide chain – is available.

2.2.1.1 *The Hsp70 system*

In higher eukaryotes, the constitutively expressed Hsp70 homolog, heat shock cognate 70 (Hsc70) mediates co- or post-translational folding of polypeptides through ATP-regulated binding cycles (Hartl & Hayer-Hartl, 2009). Members of the Hsp70 family are able to couple ATP hydrolysis to the binding and release of substrate molecules in a co-factor dependent manner. Binding of ATP to the N-terminal ATPase domain of Hsp70 family members causes a conformational change in the C-terminal domain, opening the peptide binding cleft, allowing the rapid binding and release of substrates. Hydrolysis of ATP to ADP however, closes the substrate binding cleft, resulting in an ADP-bound state that binds tightly to peptide substrates (McCarty *et al.*, 1995; Szabo *et al.*, 1994). In eukaryotes, the chaperone co-factor Hsp40 activates the ATPase activity of Hsc70, promoting the retention of substrates as well as targeting those substrates to Hsc70 (Tsai & Douglas, 1996). This system is analogous to the well-studied bacterial DnaK/DnaJ system (Hartl, 1996; McCarty *et al.*, 1995; Szabo *et al.*, 1994). The bacterial system also includes a nucleotide-exchange factor (GrpE in *E. coli*) co-factor which promotes the release of ADP from DnaK (Liberek *et al.*, 1991). Recent studies however, have highlighted a number of other regulatory co-chaperone components of the Hsc70/Hsp40 system including BAG-1 which stimulates Hsc70's ATP hydrolysis activity (Höhfeld & Jentsch, 1997) and Hip, an oligomeric protein which both stabilises the ADP-bound state of Hsc70 by interacting with its ATPase domain and binds directly to peptide

substrates, acting itself as a molecular chaperone (Höhfeld *et al.*, 1995). The regulated stabilisation and release of unfolded substrates within the Hsp70 system both prevents aggregation and aids in the formation of the native state.

2.2.1.2 *Chaperonins*

Downstream from the Hsp70 folding system are the chaperonins, a distinct group of sequence-related proteins characterised by their large, oligomeric, double-ring complexes of around 800 kD. Chaperonins promote substrate folding by global protein encapsulation and, like Hsp70, are ATP-regulated. The chaperonins can be split into two separate groups (Horwich *et al.*, 2007). Group I chaperonins, also known as Hsp60s, are found in bacteria, mitochondria and chloroplasts. They comprise seven rings and cooperate with members of the Hsp10 proteins to form the lid of a folding cage, also known as an Anfinsen folding cage (Saibil *et al.*, 1993). Group II chaperonins are cytosolic proteins with eight- or nine-membered rings. This group of chaperonins is Hsp10-independent and encapsulates its substrate using specialised α-helical extensions which are part of the ring structure (Hartl & Hayer-Hartl, 2009).

The best characterised group I chaperonin system is bacterial GroEL and its cofactor GroES (Hartl, 1996; Horwich *et al.*, 2007; Horwich *et al.*, 2009). GroEL is a tetradecamer assembled as two back-to-back rings, each with a central hydrophobic cavity which can bind non-native polypeptide substrates (Braig *et al.*, 1994). The co-chaperonin GroES associates with either end of the GroEL in an ATP-dependent manner, forming an enclosed cavity (Weissman *et al.*, 1996; Hunt *et al.*, 1996). Isolation of unfolded monomers, in this system, serves to protect them from

unproductive aggregation events, allowing folding to occur unimpaired by the crowded, outside environment. Accordingly, the main purpose of the chaperonin cavity is to prevent aggregation (Horwich *et al.*, 2009). Furthermore, the environment within the Anfinsen folding cage is critical in accelerating folding and rearrangement steps of non-native polypeptides (Tang *et al.*, 2006). This is believed to be down to the cavity lining which provides a non-stick surface which compels the non-native chain to "follow its intrinsic folding pathway without providing any general or substrate-specific direction to the process" (Horwich *et al.*, 2009). Nevertheless, not all proteins require entry into the GroEL/GroES system in addition to the DnaK/DnaJ-mediated folding. Ellis and Hartl used temperature-sensitive mutants to knock-out GroEL in *E. coli*; an experiment which indicated that a maximum of 30% of bacterial proteins require GroEL to reach their mature structure (Ellis & Hartl, 1996).

The group II chaperonins, found in eurkarya and archaea is represented by TCP-1 Ring Complex (TRiC) or chaperonin-containing TCP-1 (CCT). TRiC/CCT is arranged in a double-ring structure but, unlike group I chaperonins which are homo-oligomeric, each TRiC/CCT ring comprises eight different subunits. Each polypeptide-binding domain has evolved to bind a diverse set of polypeptide substrates (Kim *et al.*, 1994). Generally, TRiC/CCT substrates tend to be large hydrophobic proteins with the most abundant substrates being identified as the cytoskeletal proteins actin and tubulin (Lewis *et al.*, 1996; Yam *et al.*, 2008). In a key paper by Spiess and colleagues, the TRiC substrate VHL tumour suppressor was used to identify the binding sites in TRiC in order to further understand how the chaperonin engages its substrates (Spiess *et al.*, 2006). It was established that

different subunits in the complex are specialised to recognise distinct but overlapping substrates resulting in the ability to facilitate the folding of a range of eukaryotic proteins. However, despite recent advances in our understanding of the structural architecture of the group II chaperonins, the functional mechanisms underlying their chaperone activity are still not fully understood (Bosch *et al.*, 2000; Shomura *et al.*, 2004; Pappenberger *et al.*, 2006).

2.2.1.3 *Hsp90*

The cytosolic chaperone Hsp90 differs from other eukaryotic chaperones in its substrate specificity. Hsp90 is known to preferentially interact with signal transduction proteins, typified by the steroid hormone receptors and signalling kinases (Picard, 2002; Xu & Lindquist, 1993). Therefore, Hsp90 is essential for the maintenance of signal transduction networks. Unlike the Hsc70/Hsp40 system, Hsp90 family members do not interact with nascent polypeptide chains but rather bind downstream of the Hsc70/Hsp40 system, recognising a subset of client proteins that have neared their native conformation (Jakob *et al.*, 1995). However, much the same as Hsp70 family members, Hsp90 is ATP-dependent, relying on cycles of ATP hydrolysis to regulate the binding and release of substrates (Obermann *et al.*, 1998; Prodromou *et al.*, 2000). Loading of the substrate onto Hsp90 requires Hsp70 (Hutchinson *et al.*, 1994) and is aided by the co-chaperone Hsp-organising protein (Hop/p60) (Scheufler *et al.*, 2000).

Together, the Hsp70/Hsp40 system, chaperonins and Hsp90 provide an essential cytosolic system for proteins to reliably and efficiently traverse the energy landscape, preventing aggregation and guiding them towards the native conformation.

Mutational studies of key chaperones and co-factors reveal that many of them are lethal or associated with protein-conformational diseases (Senderk *et al.*, 2005; Chapman *et al.*, 2006; Polier *et al.*, 2008; Hainzl *et al.*, 2009), highlighting the importance of the cytosolic chaperone system.

2.2.1.4 *The bacterial periplasm*

Gram-negative bacteria also possess a vast cell membrane-bound compartment – the periplasm – which is specialised for protein biosynthesis of extracytoplasmic proteins (Pugsley, 1993). The periplasm, or periplasmic space, is bounded by the inner cytoplasmic membrane and the porous outermembrane of gram-negative bacteria. Gram-positive bacteria also possess a periplasm but this is located between the outer plasma membrane and an outer peptidoglycan layer. The periplasm in gram-positive bacteria also tends to be less pronounced. The two membranes which bound the periplasm in gram-negative bacteria are distinct in both composition and structure. The inner membrane is a phospholipid bilayer which contains integral membrane proteins which typically span the membrane as hydrophobic α-helices whereas the outer membrane is an asymmetric bilayer consisting of phospholipids and lipopolysaccharides (LPS) and contains integral membrane proteins which exist as cylindrical β-strand-containing β-barrels with a hydrophobic interior (Bos *et al.*, 2007).

The membranes which form the boundary of the periplasmic compartment contain many proteins which are essential for several cell functions including the sensing and signal transduction of environmental stimuli and the uptake and secretion of biological substrates (Facey & Kuhn, 2010). Since these proteins are translated in the

cytosol, they require coordinated targeting to the periplasm and subsequent translocation across the membrane – in the case of outer membrane proteins – or insertion into the membrane – in the case of inner membrane proteins. Furthermore, within the periplasm exists molecular chaperones which facilitate the folding and assembly of these proteins (Miot & Betton, 2004).

The synthesis of bacterial integral membrane proteins takes place on ribosomes in the cytosol. Proteins destined for outer- or inner-membrane insertion are recognised by the highly conserved signal recognition particle (SRP) shortly after synthesis. SRP delivers the polypeptide to the Sec translocase for transport across the inner membrane into the periplasm or for insertion into the inner membrane (Keenan *et al.*, 2001; Ullers *et al.*, 2003). Both these tasks are carried out by the SecYEG complex which has to switch between a transversal opening – for allowing secretory proteins in to the periplasm – and a lateral opening – to allow for the insertion of transmembrane proteins. SecA, the motor subunit of the SecYEG complex, is required for both the translocation of secretory proteins and the insertion of inner-membrane proteins (Facey & Kuhn, 2010). However, there is evidence of the insertion of small inner-membrane proteins occurring in a SecYEG-independent manner via YidC (Samuelson *et al.*, 2000; Chen *et al.*, 2002).

Secretory proteins, on the other hand, are targeted to the inner membrane translocation machinery by the cytoplasmic chaperone SecB. Much like SRP-mediated membrane targeting, SecB also scans the nascent polypeptide for a signal sequence which targets the polypeptide to the inner membrane. SecB, binds to the unfolded polypeptide after dissociation from TF, shortly after synthesis (Muller,

1996). Secretory proteins are then delivered to the SecYEG complex in a translocation competent conformation.

Chaperones and protein folding catalysts exist within the periplasm, which serve to promote the formation of native structures and prevent aggregation (Surrey & Jahnig, 1995). Most cytoplasmic chaperones utilise ATP to drive cycles of protein substrate binding and release, however, the periplasm lacks ATP. Few periplasmic chaperones have been identified thus far, but the general chaperone Skp is one which is well studied. The trimeric Skp chaperone can keep periplasmic substrates in a soluble and unstructured conformation by forming stable complexes with substrates, thus preventing aggregation (Bulieris *et al.*, 2003). Furthermore, the folding rate of the periplasmic substrate OmpA is increased in the presence of Skp. However, Skp null mutants only cause a moderate decrease in the levels of properly folded outer membrane proteins, indicating that other folding factors may be involved in outer membrane protein maturation (Chen & Henning, 1996). SurA is also a periplasmic chaperone, but exhibits *cis-trans* peptidyl prolyl isomerase (PPIase) activity - which catalyses the *cis-trans* isomerisation of peptide bonds. The null mutant of SurA too exhibits diminished levels of folded outer membrane proteins indicating a role in the folding and assembly of outer membrane proteins for SurA (Lazar & Kolter, 1996). The chaperone activity of SurA was inferred by the deletion of the PPIase I and PPIase II domains, which are responsible for the isomerase activity of the enzyme. This double mutant almost complements wild type function *in vivo*, indicating that the N- and C-terminal domains exhibit chaperone activity (Behrens *et al.*, 2001). Furthermore, SurA selectively binds outer membrane proteins since it preferentially recognises peptide sequences containing aromatic amino acids in the sequecne Ar-*X*-

Ar, which occur more frequently in membrane proteins than cytoplasmic and other soluble proteins (Bitto & McKay, 2003).

The periplasmic serine protease DegP (also called high temperature requirement A (HtrA) or protease Do) unusually also functions as a chaperone (Spiess *et al.*, 1999; Ortega *et al.*, 2009). DegP is an ATP-independent heat shock protein that can degrade and refold misfolded proteins in the periplasm. DegP can discriminate between misfolded and folded substrates due to the dimensions of its inner cavity which excludes folded substrates (Clausen *et al.*, 2002). DegP is required for bacterial survival at high temperatures and for the degradation of aberrantly folded periplasmic proteins (Lipinska *et al.*, 1988; Strauch & Beckwith, 1988). Interestingly, DegP can switch between its chaperone activity, at low temperatures, and protease activity, at high temperatures perhaps allowing it to respond to environmental changes (Skorko-Glonek *et al.*, 1995; Spiess *et al.*, 1999). The chaperone activity of DegP is physiologically relevant since the proteolytically inactive *degPS210A* mutant can complement the lethality of the *surA degP* double mutant (Rizzitello *et al.*, 2001). Taken together, these results suggest that a functional chaperone network exists in the periplasm that is essential for protein folding and degradation. Further work is required to identify additional members of this chaperone network.

In addition, protein disulfide isomerases exist within the periplasm to pass on disulfide bonds to substrate proteins, which provides them with structural stability. Protein disulfide isomerases and the formation of disulfide bonds will be examined in more detail when discussing protein folding in the endoplasmic reticulum. The

periplasmic disulfide isomerases DsbA and DsbC function together with the inner membrane proteins DsbB and DsbD to allow the oxidation, reduction and isomerisation of disulfide bonds in the oxidising environment of the bacterial periplasm. DsbA has two cysteine residues and is a potent catalyst of disulfide bond formation (Wunderlich *et al.*, 1993; Zapun and Creighton, 1994). When in an oxidised form (i.e. when there is an intramolecular disulfide bond between the two cysteines), DsbA is able to transfer its intramolecular disulfide bond to substrate proteins in the periplasm leading to reduction of DsbAs disulfide to two thiol groups (-SH). However, DsbA may catalyse the formation of non-native disulfides in substrates. If this occurs, DsbC-mediated isomerisation of these bonds allows for the rearrangement of disulfides and the formation of native structures (Zapun *et al.*, 1993; Rybin *et al.*, 1996; Sone *et al.*, 1997). Evidence of this comes from studying proteins targeted to the periplasm. Urokinase, a protein that contains 12 disulfide bonds is undetectable in the DsbC null mutant. However, the yield of alkaline phosphatase, which only contains two disulfide bonds, is only lowered by 15% (Rietsch *et al.*, 1996). After passing on the disulfide bond from DsbA to the substrate, the disulfide bond on DsbA is ‘recharged’ by DsbB which delivers electrons to the electron transport chain (Kobayashi *et al.*, 1997). DsbD, on the other hand, maintains DsbC and DsbG in their reduced forms – thus allowing them to carry out reduction and isomerisation of non-native disulfides – by receiving electrons from cytoplasmic thioredoxin (Chung *et al.*, 2000; Joly and Swartz, 1997). Together, periplasmic chaperones and protein disulfide isomerases function to form native disulfide bonds and assist in the folding and insertion of substrate proteins in the periplasm.

2.2.2 *Protein folding in the ER*

Protein folding in the endoplasmic reticulum (ER) has been studied extensively in the last two decades. The ER is similar to that of the bacterial periplasm since substrate proteins can undergo the addition of covalent disulfide bonds between two cysteine residues. However, the eukaryotic ER boasts a further feature which is not present in cytosolic and bacterial protein folding: the introduction of N-linked oligosaccharide chains to the folding protein. As a consequence, the ER requires its own specialised set of components to assist in the correct folding of substrate proteins in order to reach the native conformation (Jonikas *et al.*, 2009). However, since many of the principles of protein folding in the cytosol apply to protein folding in the ER, there also exists a set of ER-resident chaperones and folding enzymes with homology to cytosolic, mitochondrial and periplasmic folding factors (Christis *et al.*, 2008; Hartl & Hayer-Hartl, 2002).

A considerable proportion of the proteome is destined for the extracellular space (Clark *et al.*, 2003; Chen *et al.*, 2005). These proteins are intended for secretion by the cell into the extracellular environment or to be incorporated into membranes to act as cell surface receptors, for example. Similar to the periplasm, the signal peptide, located at the N-terminus of nascent polypeptides is recognised by the signal recognition particle (SRP) and directs the mRNA/ribosome/nascent polypeptide complex to the ER membrane. This enables cotranslational translocation into the ER via the Sec61 translocon, where newly synthesised secretory/membrane-bound proteins can acquire their native conformation (Sakaguchi, 1997; Walter & Johnson, 1994). However, recent evidence has highlighted the ability of mRNAs to localise to the ER membrane in the absence of SRP indicating intrinsic localisation

determinants within the mRNA sequence (Pyhtila *et al.*, 2008). Once all folding events are completed, native proteins are then trafficked to the Golgi where additional modifications may take place before being further compartmentalised or secreted.

2.2.2.1 *The endoplasmic reticulum*

The ER is a convoluted bilayer membrane enclosing a continuous lumenal space which provides an optimal environment for protein folding, disulfide bond formation, the removal of signal sequences, the acquisition of N-linked glycans and oligomeric assembly of proteins destined for the extracellular environment. Furthermore, the ER is responsible for the synthesis of glycosylphosphatidylinositol (GPI)-anchored proteins and fatty acylation of proteins. The ER is loaded with numerous chaperones and folding enzymes which outnumber the client proteins that require folding, reaching millimolar concentrations (Lyles & Gilbert, 1992). Activity of ER-resident chaperones and folding enzymes is highly dependent on the localised environment of the organelle.

The ER is the principal Ca^{2+} store of the cell and Ca^{2+} levels are regulated in an ATP-dependent manner by inositol 1,4,5-triphosphate receptor type 1, 2 and 3 and the ryanodine receptor Ca^{2+} release channels which allows the release of large quantities of the cation in response to extracellular stimuli (Meldolesi *et al.*, 1998; Berridge *et al.*, 2000). However, the Ca^{2+} channel inositol 1,4,5-triphosphate receptor type 1 (IP_3R1), which mediates Ca^{2+} release from the ER, is regulated by the ER protein disulfide isomerase-like protein ERp44 through direct binding to its third lumenal loop (Higo *et al.*, 2005). Regulating Ca^{2+} ion concentration in the ER is

crucial for productive protein folding since many chaperones, such as calreticulin, rely upon Ca^{2+} binding to regulate protein-protein interactions (Corbett *et al.*, 1999; Michalak *et al.*, 2009). Thus, perturbations in Ca^{2+} concentration crucially affect protein folding and maturation (Berridge *et al.*, 2003).

2.2.2.2 *Oxidative protein folding*

Anfinsen and colleagues in the 1960s showed that disulfide bond formation can occur spontaneously *in vitro* in the presence of O_2 (Anfinsen *et al.*, 1961). However, shortly after, it was observed that folding of secretory proteins was facilitated in the presence of microsomal extracts (Goldberger *et al.*, 1963). These vesicles are now known to contain machinery involved in protein folding as well as having the ability to generate a redox potential conducive to disulfide bond formation (Gething & Sambrook, 1992; Hwang *et al.*, 1992; Ruddon & Bedows, 1997). Thus, *in vivo*, the formation of disulfide bonds is an enzyme-catalysed event which takes place in the specialised environment of the ER.

Key to the conferment of disulfide bonds is the reduction/oxidation (redox) environment within the endoplasmic reticulum. In terms of redox potential, the ER is similar to the extracellular environment providing an appropriate folding location for proteins destined for the extracellular space since if a protein is unable to reach a stable formation in the ER, it is unlikely to be stable in the extracellular environment. Comparatively, the bacterial and eukaryotic cytosol are poor environments for the production of multiple disulfide bonds since these locations are highly reducing. The redox environment of the ER, however, is highly oxidising and is illustrated by the

ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG), which is ~3:1 in the ER compared with ~100:1 in the cytosol (Hwang *et al.*, 1992; Bass *et al.*, 2004).

The role of glutathione in the ER is still not fully understood. Oxidised glutathione was initially believed to provide oxidising equivalents for the formation of disulfide bonds via the oxidation of the oxidoreductase protein disulfide isomerase (PDI). However, it is now largely believed that the ER flavoprotein Ero1 is responsible for the oxidation of reduced PDI (to be discussed in more detail later). However, there is evidence of glutathione having a role in disulfide bond formation, since lowering the level of glutathione in the cell results in an increase in disulfide bond formation. This is accompanied by an increase in non-native disulfide bond formation and an increase in the time required for disulfide bond isomerisation (Chakravarthi & Bullied, 2004; Molteni *et al.*, 2004). These results may imply a role for reduced glutathione in disulfide bond isomerisation.

Furthermore, reduced glutathione can reduce the PDI family member ERp57 indicating that GSH may be involved in maintaining oxidoreductases in their reduced form allowing them to carry out the reduction and isomerisation of non-native disulfides in ER substrates (Fratelli *et al.*, 2002). In addition, glutathione can be found in mixed disulfides with proteins (Bass *et al.*, 2004). These are either formed during the reduction of substrate disulfides by GSH or the oxidation of substrate disulfides by GSSG (Cuozzo & Kaiser, 1999). Together, these results show that glutathione may have a direct or non-direct role in native disulfide bond formation and that this may influence the redox buffering system in the ER.In the highly oxidising environment of the ER, disulfide bond reduction and isomerisation is

crucial to avoid getting trapped in off-pathway intermediates on the way to the native conformation (Jansens *et al.*, 2002). It was therefore evident that in eukaryotes, the correct formation and arrangement of disulfide bonds was catalysed by an ER-resident enzyme (Givol *et al.*, 1964).

2.2.2.3 *Protein disulfide isomerase*

Protein disulfide isomerase, or PDI, mentioned earlier, catalyses the formation and isomerisation of disulfide bonds in the eukaryotic ER. PDI is a highly abundant ER-resident protein, making up approximately 0.8% of total cellular protein (Freedman *et al.*, 1994). PDI is highly conserved between species and is essential for eukaryotic oxidative protein folding as illustrated by its requirement for *S. cerevisiae* viability (Farquhar *et al.*, 1991).

Due to recent extensive research into PDI and its homologs, we now have a reasonably detailed understanding of the structure and enzymatic properties of this protein (Edman *et al.*, 1985; Xiao *et al.*, 2004; Gruber *et al.*, 2006; Tian *et al.*, 2006). PDI comprises four thioredoxin-like domains (**a**, **b**, **b'**, **a'**, an anionic tail domain called **c** and a linker region between **b'** and **a'** called **x**) (Fig. 2). The **a** and **a'** domains show both sequence- and structural-homology to thioredoxin – an αβ-fold with a mixed β-sheet core – (Ferrari *et al.*, 1998), each containing an independent active site. These active sites consist of two cysteine residues – in the sequence WCGHCK – which are responsible for PDIs enzymatic activity (Edman *et al.*, 1985). This short, cysteine-containing sequence is sometimes called the active-site motif. The **b** and **b'** domains do not have active sites. However, the **b** and **b'** domains may

be important for substrate recognition and peptide binding activities of PDI (Kemmink *et al.*, 1997; Klappa *et al.*, 1998a).

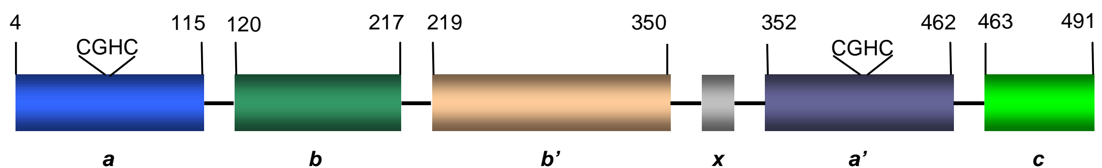


Figure 2. **PDI domain structure**. PDI consists of two active site thioredoxin-like domains ***a*** and ***a'*** and two non-reactive thioredoxin-like domains ***b*** and ***b'***. The boundaries for the domains ***a*** and ***b*** are those defined by NMR (Kemmink *et al.*, 1999).

The CXXC active-site motif is key to PDIs oxidase and isomerase activity. When the cysteines are oxidised, the disulfide can be transferred to a reduced protein substrate, reducing the disulfides of the active site. When the active site is in the reduced state however, substrate disulfides can be transferred to PDI and the active site becomes oxidised. The function of PDI-family members is defined by the biochemistry of the sequence both immediately surrounding and within the active site, as well as the residues and features of the three-dimensional space directly surrounding the active site. The pK_a value of the reactive N-terminal cysteine in the active site is 4.5. This value has been shown to be influenced by both the histidine residue in the thioredoxin fold and by partial positive charges from the N-terminus of an α-helix

close to the active site (Kortemme and Creighton, 1995). This generates a PDI disulfide which can promote the oxidation of substrate proteins.

The body of evidence indicating isomerases activity of PDIs is significant (Walker and Gilbert, 1997; Gilbert, 1998; Schwaller *et al.*, 2003). The process of isomerisation requires the breaking of a substrate disulfide for rearrangement. Thus, there is no net change in substrate redox state but rather the protein moves closer towards its native state. Therefore, isomerisation requires PDI to be in the reduced state so it may attack substrate disulfides, catalysing their rearrangement. Recent work from Kulp and colleagues highlighted an asymmetry in the functionality of PDIs active sites which contribute to its isomerase activity (Kulp *et al.*, 2006). Their studies showed that oxidation of the N-terminal active site, in the first catalytic domain (**a**), is subject to substrate-mediated inhibition whereas the C-terminal active site – in the **a’** domain – is readily oxidised. This asymmetry is not intrinsic to the individual active sites but rather occurs in the context of the full-length protein. The asymmetry of active site motifs permits the two thioredoxin-like domains to exhibit two distinct functional roles. The disparity in rates of oxidation ensures the **a’** domain is in the oxidised state, ready to promote substrate oxidation while the **a** domain is in the reduced state and can promote disulfide isomerisation. How PDI recognises non-native disulfides is still not fully understood but the requirement of the non-redox active domains (**b** and **b’**) for oxidase and isomerase activity on bovine pancreatic trypsin inhibitor (BPTI) (Darby *et al.*, 1998) may suggest that one or both of these domains is important in recognising non-native proteins requiring isomerase activity.

The crystal structure studies of PDI reveal that the two active sites are located on two flexible arms which are connected to a rigid base formed by the **b** and **b'** domains (Tian *et al.*, 2006; Tian *et al.*, 2008). This molecular flexibility can allow for different conformations, allowing the protein to accommodate an array of structurally diverse substrates. Furthermore, the flexibility of the two arms may be required for the isomerisation of non-native disulfides since it might ensure that the active sites can locate incorrect disulfide bonds in the substrate (Tian *et al.*, 2008).

2.2.2.3.1 *PDI homologs*

The protein disulfide isomerase family now extends to around 19 proteins (Appenzeller-Herzog & Ellgaard, 2008). The most well studied PDI family members are: ERp29 (Demmer *et al.*, 1997; Mkrtchian *et al.*, 1998; Hermann *et al.*, 2004; Rainey-Barger *et al.*, 2007; Barak *et al.*, 2009), ERp44 (Anelli *et al.*, 2002; Anelli *et al.*, 2003; Wang *et al.*, 2008), ERp57 (Oliver *et al.*, 1997; Oliver *et al.*, 1999; Antoniou *et al.*, 2002; Peaper *et al.*, 2005; Jessop *et al.*, 2006; Santos *et al.*, 2007; Zhang *et al.*, 2009), ERp72 (Mazzarella *et al.*, 1990; Shaiff *et al.*, 1992; Miyaishi *et al.*, 1998; Forster *et al.*, 2006; Menon *et al.*, 2007; Kozlov *et al.*, 2009), ERdj5 (Cunnea *et al.*, 2003; Dong *et al.*, 2008; Ushioda *et al.*, 2008; Thomas & Spyrou, 2009), PDIp (Desilva *et al.*, 1997; Volkmer *et al.*, 1997; Klappa *et al.*, 1998b; Ruddock *et al.*, 2000; Klappa *et al.*, 2001) and PDILT (van Lith *et al.*, 2005; van Lith *et al.*, 2007). Members of the PDI family are defined by the presence of the thioredoxin-like domain but vary greatly in the arrangement and number of these domains. The variation of domain arrangement is thought to modulate the function of PDI family members. The diversity of human PDI family member domain structure can be seen in table 1.

Table 1 – The structural diversity of some of the key members of the human PDI family

Member name	Accession Number	Length	ER localisation motif	Number of a-type domains	Active site sequence	Domain composition
PDI	P07237	508	KDEL	2	2x CGHC	**a-b-b'-a'**
ERp29	P30040	261	KEEL	0	-	**b-D**
ERp44	Q9BS26	406	RDEL	1	CRFS	**a-b-b'**
ERp57	P30101	505	QEDL	2	2x CGHC	**a-b-b'-a'**
ERp72	P13677	645	KEEL	3	3x CGHC	**a^{o}-a-b-b'-a'**
ERdj5	Q8IXB1	793	KDEL	4	CSHC, CPPC, CHPC, CGPC	**J-a''-b-a^{o}-a-a'**
PDIp	Q13087	525	KEEL	2	CGHC, CTHC	**a-b-b'-a'**
PDILT	Q8N807	584	KEEL	2	SKQS, SKKC	**a-b-b'-a'**
ERp18	O95881	172	EDEL	1	CGHC	**a**
ERp27	Q96DN0	273	KVEL	0	-	**b-b'**
EndoPDI/ ERp46	Q8NBS9	432	KDEL	3	3x CGHC	**a^{o}-a-a'**
P5	Q15084	440	KDEL	2	2x CGHC	**a^{o}-a-b**
PDIr	Q14554	519	KEEL	3	CSMC, CGHC, CPHC	**b-a^{o}-a-a'**
TMX	Q9H3N1	280	Unknown	1	CPAC	**a**
TMX2	Q9Y320	296	KKDK	1	SNDC	**a**
TMX3	FLJ20793	454	KKKD	1	CGHC	**a-b-b'**
TMX4	Q9H1E5	349	RQR	1	CPSC	**a**

The D-domain in ERp29 is a C-terminal α-helical domain. The J-domain in ERdj5 is a BiP-binding domain. The a^{o} and a'' domains are variants of the active site domains. Taken from data in Appenzeller-Herzog & Ellgaard, 2008 and Ellgaard & Ruddock, 2005.

Although the archetypal protein disulfide isomerase, PDI, is responsible for directly catalysing disulfide bond formation, it is not necessary for PDI family members to exhibit oxidase or isomerase function. Rather, membership into this group is dependent – amongst other things – on structural features as opposed to functional ones. Typical examples of this are ERp27 and ERp29 which are non-catalytic since neither contains catalytic **a**-type domains (Ferrari *et al.*, 1998; Alanen *et al.*, 2006). Most PDI family members however, do contain at least one **a**-type domain (Ellgaard & Ruddock, 2005) (Table 1).

PDILT, a testis-specific PDI homolog has the same domain organisation as PDI – **a-b-b'-a'** – but lacks the canonical CXXC active site motif – instead possessing an SXXC motif. Accordingly, PDILT has no oxidoreductase activity *in vitro* (van Lith *et al.*, 2005). Recently, PDILT has been shown to be involved in a specialised, spermatogenesis-specific, chaperone system with the testis-specific calnexin homolog, calmegin (van Lith *et al.*, 2007). Thus, although not redox active, PDILT still has a role in protein maturation. Similarly, ERp44 boasts a non-canonical active site motif – CRFS – and, despite covalently interacting with the ER oxidoreductase Ero1-Lα - which will be discussed in depth later – and substrate proteins, it is not fully understood whether it exhibits oxidoreductase activity (Anelli *et al.*, 2002; Anelli *et al.*, 2003). However, ERp44 has a role in PDI oxidation since it helps retain Ero1-Lα and Ero1-Lβ in the ER (Otsu *et al.*, 2006). Furthermore, as mentioned earlier, ERp44 can inhibit the Ca^{2+} channel IP_3R1, regulating ER Ca^{2+} levels (Higo *et al.*, 2005). Thus, in line with the divergent oxidoreductase capacity within this group of proteins, PDI family members show differences in their ability to complement a PDI-deficient yeast strain (Gunther *et al.*, 1993).

The majority of PDI family members are constitutively expressed, however, in addition to PDILT, a few other family members exhibit tissue-specific distributions. EndoPDI is a PDI-like protein containing three CXXC active site motifs and is highly expressed in endothelial cells (Sullivan *et al.*, 2003). EndoPDI expression is induced upon hypoxia and is thought to protect endothelial cells from hypoxia-induced apoptosis.

The pancreatic acinar cell-specific PDI, PDIp shares the same domain organisation as PDI but lacks the acidic C-terminal region (Desilva *et al.*, 1997; Volkmer *et al.*, 1997). PDIp has two canonical CXXC active site motifs but it is the **b**-like, non-redox active domains which have been the subject of extensive research. Soon after identification, PDIp was reported to bind both peptides and misfolded proteins – including those not containing any cysteine residues (Klappa *et al.*, 1998b). Furthermore, studies with peptide substrates revealed that tyrosine and tryptophan residues are the recognition motifs for binding to PDIp (Ruddock *et al.*, 2000). Studies using cross-linkers to trap non-peptide ligands showed that hydroxyl groups are the structural motifs for the binding of PDIp (Klappa *et al.*, 2001). However, studies comparing the **b'** domains – thought to be important for peptide binding – of PDI and PDIp reveal that they can interact with many of the same substrates indicating that there is an overlap in substrate specificity (Klappa *et al.*, 1995; Klappa *et al.*, 2001). This finding is strange since PDI has been shown to colocalise with PDIp in acinar cells (Dias-Gunasekara *et al.*, 2005). The existence of tissue-specific PDI homologs may be due to the requirement for the folding of tissue-specific proteins, however, it seems some functional redundancy does exist at least in the case of PDIp. Ruddock and colleagues postulate that co-expressed members of the PDI

family do not act on distinct proteins but rather cooperate by interacting with different parts of the polypeptide (Ruddock *et al.*, 2000).

ERp57, together with PDILT and PDIp, shares a common domain architecture with PDI. ERp57 is considered the closest known homolog of PDI, containing two canonical active site motifs (Freeman *et al.*, 1994; Koivunen *et al.*, 1996). Unlike PDI, however, ERp57 almost exclusively catalyses the disulfide bond formation of glycoproteins (Elliott *et al.*, 1997; Oliver *et al.*, 1997; Ellgaard & Frickel, 2003). The redox activity of ERp57 was investigated soon after its discovery and ERp57 was shown to exhibit thiol-dependent reductase activity and form mixed disulfides with glycoprotein substrates (Bourdi *et al.*, 1995; Hirano et al., 1995; Molinari & Helenius, 1999). However, the mechanism for the recognition of these specialist substrates is more complex. In 1999, Oliver and colleagues demonstrated the non-covalent binding of ERp57 to the ER-resident lectins calnexin and calreticulin (Oliver *et al.*, 1999), whilst further investigation by Russell and colleagues showed that the **b'** domain of ERp57 – thought to be involved in substrate binding – has been adapted to specifically interact with ER lectins (Russell *et al.*, 2004). Recently evidence suggests that the specificity of ERp57 for client proteins is determined by its interaction with the calnexin cycle and that these client proteins tend to share similar structural motifs (Jessop *et al.*, 2007; Jessop *et al.*, 2008). I shall talk about these two lectins in more detail later but in brief, calnexin and calreticulin are chaperones capable of recognising one or more monoglucosylated oligosaccharide side chains on unfolded proteins (Rodan *et al.*, 1996). Thus, ERp57 recognises glycoproteins in concert with the ER lectins calnexin and calreticulin to catalyse disulfide bond formation in monoglucosylated glycoproteins (Zapun *et al.*, 1998).

Also of note is the fact that ERp57 binds glycoproteins lacking any cysteine residues, indicating that the oxidoreductase may function as a more general chaperone within the ER lumen (Elliot *et al.*, 1997; Oliver *et al.*, 1997).

The most well-studied example of ERp57-mediated protein folding is in the maturation of the major histocompatibility complex (MHC) class I molecule. Here, ERp57, calnexin and calreticulin form part of a series of complexes that are responsible for the correct folding, oxidation and maturation of the immune molecule (Dick, 2004; Zhang & Williams, 2006). The folding and maturation of the MHC class I molecule as well as its role in the immune system will be discussed in greater detail later.

2.2.2.4 *ER oxidoreductin (Ero) protein*

The existence of a protein disulfide isomerase does not provide a complete model for the formation of correct disulfide bonds since disulfides requiring rearrangement may have been oxidised by another oxidation mechanism and, more importantly, an additional oxidant is required for the reoxidation, or recharging, of PDI itself.

An abundance of recent work provides evidence suggesting that glutathione is not essential for oxidative protein folding in the ER and that disulfide bond formation relies upon a different electron acceptor (Frand and Kaiser, 1998; Pollard *et al.*, 1998; Cuozzo and Kaiser, 1999). Using *S. cerevisiae* to genetically dissect oxidative protein folding, Frand and Kaiser and the Weissman lab isolated endoplasmic reticulum oxidoreductin 1 (*ERO1*) which encodes a novel but conserved ER membrane protein essential for the net formation of protein disulfide bonds (Pollard

et al., 1998; Frand & Kaiser, 1998). It was suggested that *ERO1* could introduce oxidising equivalents – utilised during disulfide bond formation – via its two pairs of conserved cysteines (CXXCXXC), since introduction of the thiol oxidant diamide restored viability in *ero1-1* mutants – defective in disulfide bond formation – and overexpression of *ERO1* confers resistance to otherwise toxic levels of the reductant dithiothreitol (DTT) (Frand and Kaiser, 2000).

Further investigation from this group led to the isolation of mixed disulfide intermediates between yeast Ero1p and Pdi1p which was presented in a key paper in 1999 (Frand and Kaiser, 1999). Direct, covalent interaction between the two ER-resident proteins was representative of the transfer of disulfide bonds from Ero1p to Pdi1p and the passing of oxidising equivalents to Pdi1p to sustain it in its disulfide form. Thus, the recharging of Pdi1p – that is, the direct oxidation of the reduced CXXC motif – is carried out by Ero1p.

As with PDI, the two active sites of Ero1p exhibit discrete properties which are crucial for functional activity. The N-terminal CXXCXXC active site – containing the so-called shuttle cysteines at Cys^{100} and Cys^{105} – is responsible for transferring oxidising equivalents to PDI (Frand & Kaiser 2000). Further mutational studies and the elucidation of the crystal structure of yeast Ero1p by Gross and colleagues revealed that the N-terminal active site motif is then likely to transfer electrons to the latter two residues – Cys^{352} and Cys^{355} – of the C-terminal CXXCXXC motif (Gross *et al.*, 2004; Sevier & Kaiser 2006). This is possible because of the presence of a disulfide relay where Cys^{100} and Cys^{105} are located on a flexible loop. This loop is

capable of changing conformation by 17Å, bringing Cys^{105} within disulfide-bonding distance of Cys^{352} (Gross *et al.*, 2004).

This paper, however, still left the question unanswered of how the C-terminal active site of Ero1p becomes reoxidised. In the bacterial periplasm, reoxidation of DsbB – the functional equivalent of Ero1p – occurs primarily via the transfer of electrons to molecular oxygen at the later stages of the respiratory electron transport chain via cytochrome *bd* or *bo* oxidase (Kobayashi and Ito, 1999; Kobayashi *et al.*, 1997). Ero1p however, uses a flavin-dependent reaction to pass electrons directly to molecular oxygen (Tu and Weissman, 2002). Thus, oxidative folding in yeast is dependent on the levels of cellular FAD (Tu *et al.*, 2000). Ero1p is a novel FAD-binding protein confirmed by the presence of an FAD-binding domain in close proximity to the C-terminal active site (Gross *et al.*, 2004). Under anaerobic conditions, excess free FAD cannot drive Ero1p-catalysed disulfide bond formation (Tu and Weissman, 2002). Thus, FAD is not thought to be the terminal electron acceptor. Rather, the bound FAD-cofactor can shuttle the electrons to molecular oxygen in a reaction that produces hydrogen peroxide in stoichiometric amounts to the disulfides formed (Tu & Weissman, 2002; Gross *et al.*, 2006). Interestingly, a few groups have shown that disulfide bond formation can proceed via FAD in anaerobic conditions, suggesting the existence of alternative electron acceptors (Gross *et al.*, 2006; Farrell & Thorpe, 2005).

2.2.2.4.1 *Human Ero protein*

Human homologs of the yeast Ero1p have recently been described and have been termed Ero1-Lα and Ero1-Lβ (Cabibbo *et al.*, 2000; Pagani *et al.*, 2000). Both

homologs are capable of rescuing the *S. cerevisiae ero1-1* thermosensitive mutant and catalysing the oxidation of substrate proteins (Cabibbo *et al.*, 2000; Pagani *et al.*, 2000; Mezghrani *et al.*, 2001). Both proteins contain the conserved CXXCXXC motif and are involved in the transfer of oxidising equivalents to PDI (Benham *et al.*, 2000; Cabibbo *et al.*, 2000; Dias-Gunasekara *et al.*, 2005; Mezghrani *et al.*, 2001; Pagani *et al.*, 2000). The CXXCXXC motif of Ero1-Lα is also important for the folding and structural integrity of Ero1-Lα as well as the stability of the Ero1-Lα-PDI complex (Benham *et al.*, 2000). Recent complementary studies by Ellgaard and colleagues and Bullied and colleagues highlighted the role of a non-catalytic disulfide bond between the redox active Cys^{94} residue and Cys^{131} in Ero1-Lα (Appenzeller-Herzog *et al.*, 2008; Baker *et al.*, 2008); the formation of which is responsible for regulating the redox activity of Ero1-Lα in an oxidising environment. This strong disulfide bond inactivates Ero1-Lαs redox activity until there is an increased demand for oxidative activity, as determined by the presence of reduced PDI (Sevier *et al.*, 2007). Hence, PDI is acting as a key regulator of ER redox homeostasis.

The reasons why higher eukaryotes contain two different Ero proteins, whilst *S. cerevisiae* only requires one, is not yet fully understood. Induction of the expression of Ero1-Lβ is induced by ER stress, possibly suggesting separate regulation pathways (Pagani *et al.*, 2000). However, Ero1-Lα and Ero1-Lβ do differ in their tissue distribution, with Ero1-Lα highly expressed in the oesophagus, whilst Ero1-Lβ is more abundant in the pancreas, stomach, testis and pituitary gland (Dias-

Gunasekara *et al.*, 2005; Pagani *et al.*, 2000). Thus, different human Ero proteins might participate in tissue-specific oxidation pathways.

2.2.2.5 *Glycosylation*

The second modification to polypeptides afforded by the endoplasmic reticulum, in addition to disulfide bond formation, is the addition of N-linked oligosaccharides to form so-called glycoproteins. Upon translocation, the oligosaccharyltransferase complex (OST) scans the polypeptide chain for asparagine residues in the sequence Asn-x-Ser/Thr. The 8 subunit OST enzyme covalently transfers a preformed triglucosylated oligosaccharide ($GlcNAc_2Man_9Glc_3$), known as the core oligosaccharide, from dolicholpyrophosphate to the Asn residue of the polypeptide (Abeijon and Hirschberg, 1992) (Fig. 3). Soon after, the terminal glucose is trimmed off by the membrane-bound α-glucosidase I (GI) enzyme (Kornfeld & Kornfeld, 1985). The remaining two glucose molecules are removed by the ER soluble glucosidase II (GII) enzyme leaving a $GlcNAc_2Man_9$ stalk. This, however, is not an irreversible step, since the ER enzyme UDP-Glc:glycoprotein glucosyltransferase (GT) is able to add a glucose unit onto the terminal mannose residue. Thus, GII not only trims glucose residues after GI trimming but removes single glucose units added by UDP-Glc.

The explanation for this deglucosylation, reglucosylation cycle was revealed in the mid 90s (Hebert *et al.*, 1995). Binding of the ER lectins calnexin and calreticulin to oligosaccharide side chains requires the trimming of the first two glucose units while the release of substrates from these lectins is determined by the trimming of the third glucose unit. Thus, glucose trimming by the α-glucosidases GI and GII and

reglucosylation by UDP-Glc serves to regulate the association of folding polypeptides with the ER lectins calnexin and calreticulin. Calnexin is a Ca^{2+} binding, type-I membrane protein of the ER. Calreticulin, on the other hand, is a soluble ER protein with both high and low affinity Ca^{2+} binding sites and a C-terminal KDEL signal (Michalak *et al.*, 1999; High *et al.*, 2000). A homolog of calnexin, calmegin is expressed in the testis and can interact with the non-redox active PDI homolog, PDILT (van Lith *et al.*, 2007).

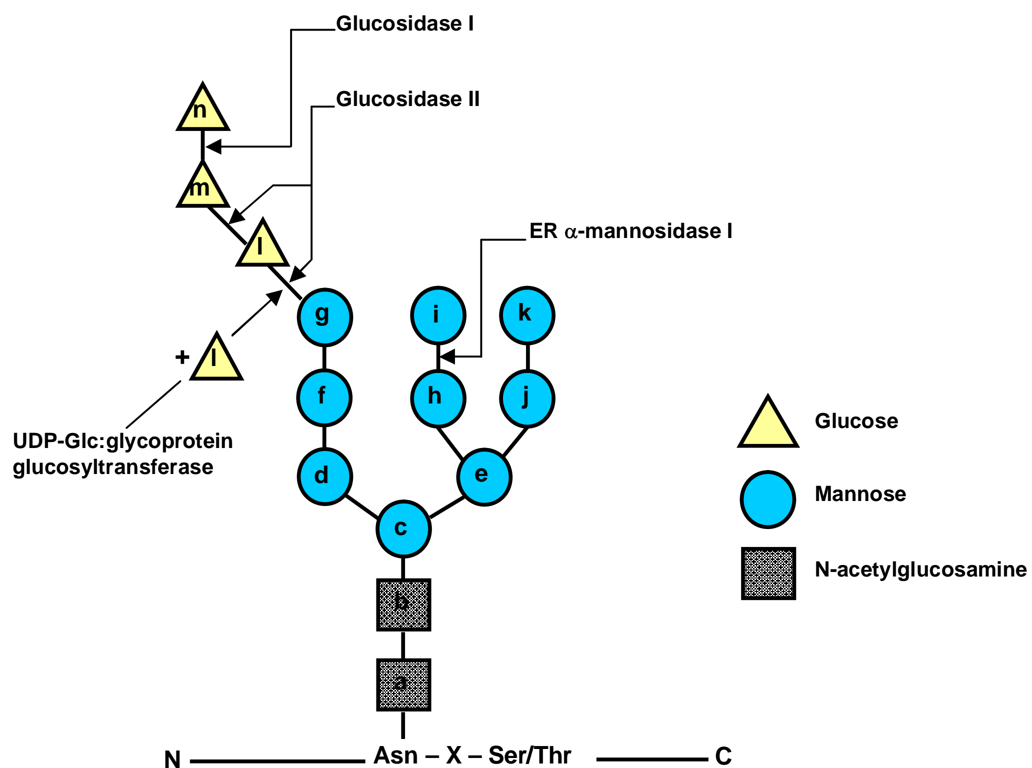


Figure 3. **The N-linked core oligosaccharide**. The core glycan has 14 saccharides: 3 glucose, 9 mannose and 2 N-acetylglucosamine. The terminal glucose (**n**) is removed by glucosidase I. The **m** and **l** glucose residues are then removed by glucosidase II. However, a glucose residue can be added to the terminal mannose residue, at position **l**, by UDP-Glc:glycoprotein glucosyltransferase (GT). The ER α-mannosidase I enzyme removes the terminal **i** mannose as a countdown timer for glycoprotein folding.

Regulation of lectin binding is important for the maturation of protein substrates since encouraging lectin binding allows the retention of the substrate protein in the ER lumen, preventing partially folded or misfolded proteins from traversing the secretory pathway. As such, the monoglucosylation/deglucosylation cycle has implications for protein quality control. Local structure around the glycan is responsible for determining GT association although it may recognise unstructured regions not in the vicinity of the N-glycan (Taylor *et al.*, 2004). If areas surrounding the glycan exhibit unstructured, non-native features they are targeted by GT (Trombetta & Helenius, 2000), allowing another round of lectin association which may promote constructive folding events. This cycle continues – if native portions of the unfolded protein exist – until the substrate has reached its conformational maturation. Thus, in proteins which normally undergo multiple lectin binding events, deletion of GT results in the premature release of substrates from calnexin and may contribute to lower folding efficiency. However, calnexin-substrates only requiring one binding events are not affected by GT knock-out (Solda *et al.*, 2007).

The addition of an oligosaccharide side chain to a newly synthesised polypeptide initially serves to increase the hydrophiliy of the unfolded protein. The consequential binding of calnexin/calreticulin prevents aggregation of the unstructured polypeptide chain and promotes ER retention. As previously mentioned, the ER oxidoreductase ERp57 is able to interact directly with glycoproteins as well as the ER lectins calnexin and calreticulin (Elliott *et al.*, 1997; Oliver *et al.*, 1997). The importance of these ER components is demonstrated by the fact that mouse knock-outs of calreticulin and ERp57 are both embryonic lethal (Mesaeli *et al.*, 1999; Garbi *et al.*,

2006; Solda *et al.*, 2006). Knock-out of calreticulin alone leads to disruption of heart development despite allowing normal development of the other embryonic organs. The calnexin knock-out leads to premature death due to impaired growth and motor disorders (Denzel *et al.*, 2002). However, knock-out studies with calnexin and calreticulin have demonstrated that they are unable to compensate for the loss of each other indicating that they have unique functions (Masaeli *et al.*, 1999; Nakamura *et al.*, 2001; Denzel *et al.*, 2002). Calreticulin has an important role to play in the modulation of Ca^{2+} homeostasis. Calreticulin-deficient cells have impaired Ca^{2+} homeostasis leading to a reduction in the free Ca^{2+} concentration in the ER whereas overexpression of calreticulin results in an increase in the ER concentration of free Ca^{2+} and an increase in the Ca^{2+} capacity of the ER (Bastianutto *et al.*, 1995; Mery *et al.*, 1996; Arnaudeau *et al.*, 2002; Molinari *et al.*, 2004).

2.2.2.6 *Chaperones of the ER*

In addition to calnexin and calreticulin, many other chaperones exist in the ER lumen to monitor and facilitate the folding of substrate proteins. Many members of the PDI family are postulated to have chaperone activity in addition to, or in lieu of oxidoreductase activity (Ferarri *et al.*, 1999). There are many chaperones which aid the folding of specific client substrates such as tapasin – which will be discussed later. Here I discuss the chaperone activities of PDI, BiP and Grp94.

2.2.2.6.1 *Chaperone activity of PDI*

Disulfide oxidoreductases should only require catalytic concentrations in order to facilitate disulfide bond formation or rearrangement. However, PDI exists at millimolar concentrations in the ER. Since studies show that PDI is essential for cell

viability and Δ*pdi1* null mutations of yeast are rescued by mammalian PDI with unconventional domain structure, it is possible that another function of PDI is essential for yeast viability (Farquhar *et al.*, 1991; Xiao *et al.*, 2001). The isomerase activity of PDI somehow requires the preferential recognition of unfolded proteins. Direct binding of PDI to unfolded substrates would then facilitate disulfide bond rearrangement as well as prevent unfolded proteins from participating in unconstructive intermolecular interactions.

LaMantia & Lennarz demonstrated the binding of unfolded proteins by PDI, suggesting that the oxidoreductase may demonstrate chaperone activity (LaMantia and Lennarz, 1993). Studies with the enzyme lysozyme – which is prone to aggregation – demonstrated that PDI did indeed exhibit chaperone activity since the recovery of soluble lysozyme – in optimised conditions – was increased in the presence of PDI – which prevented lysozyme aggregation (Puig and Gilbert, 1994). In addition to this, Puig and Gilbert discovered a novel anti-chaperone activity of PDI, where, at higher lysozyme concentrations and substoichiometric PDI concentrations, PDI facilitates the formation of large disulfide cross-linked lysozyme aggregates. It has been suggested that anti-chaperone activity is a consequence of multivalent binding of partially aggregated substrates to PDI (Primm *et al.*, 1996). This is perhaps not significant *in vivo* but it does indicate that the concentration of PDI in the ER is key to determining the fate of proteins between an off- and on-pathway destination. Thus, the unexpectedly high concentration of PDI is perhaps necessary to prevent potential anti-chaperone activity and help promote constructive oxidative events.

2.2.2.6.2 *BiP*

Immunoglobulin-binding protein (BiP), also known as glucose-regulated protein 78 (Grp78) is an abundant ER homolog of Hsp70 (Lee, 2001; Kleizen & Braakman, 2004; Hendershot, 2004). During translocation of nascent polypeptides into the ER, BiP is responsible for gating the translocon on the ER lumenal side, maintaining the permeability barrier of the translocon during the initial stages of translocation (Hamman *et al.*, 1998). BiP also seals translocons that are assembled even if they are not participating in translocation. This serves to preserve the environment in the ER. Furthermore, BiP may provide the driving force for post-translational translocation by acting as a molecular ratchet since multiple BiP molecules bind substrates at the translocon and prevent passive backwards movement through the translocation channel (Matlack *et al.*, 1999; Rapoport *et al.*, 1999).

BiP was initially discovered as a binder of immature immunoglobulin precursors (Haas & Wabl, 1983; Bole *et al.*, 1986) and later found to be essential for the assembly, transport and ultimate secretion of complete immunoglobulin molecules Hendershot *et al.*, 1987). Since BiP is a member of the Hsp70 family, it too possesses a conserved ATPase domain and hydrolyses ATP to regulate its function (Bukau & Horwich, 1998). The ATP-bound BiP molecule is open to protein binding, and hydrolysis of ATP to ADP closes the binding site onto the substrate. The ER-resident DnaJ ortholog ERdj3 has been detected in a BiP-Ig heavy chain complex, implicating it as a candidate BiP co-chaperone. There are several ER localized DnaJ proteins including ERdj1, ERdj3, ERdj4 and ERdj5. The nucleotide-exchange factor BiP-associated protein (BAP) induces ADP release so that ATP can bind, reopening the binding cleft (Chung *et al.*, 2002).

A key paper by Flynn and colleagues demonstrated the ability of BiP to distinguish unfolded and folded proteins (Flynn *et al.*, 1991). They showed that BiP binds to linear segments of polypeptides which contain non-native features. They suggested that the seven amino acid residues which make up the peptide binding site of BiP substitutes for the hydrophobic interior of a native globular protein, allowing the recognition of unfolded structures and the protection of exposed sections of newly translated polypeptides.

More recently, BiP has been demonstrated to have a key role in the response to the accumulation of unfolded proteins in the ER, allowing the detection and transduction of ER stress signals across the ER membrane (Malhotra & Kaufman, 2007). This will be discussed in further detail later.

2.2.2.6.3 *Grp94*

Glucose-regulated protein 94 (Grp94) – also known as Gp96, adenotin and endoplasmin – is an ER-resident member of the Hsp90 family. Grp94 has an essential role in the folding of a specific subset of client proteins such as Toll-like receptors, immunoglobulins and integrins (Melnick *et al.*, 1994; Argon *et al.*, 1999; Nigam *et al.*, 1994). Grp94 interacts with unfolded immunoglobulins after interaction with BiP to ensure correct folding of the substrate (Melnick *et al.*, 1992; Melnick *et al.*, 1994).

Cytosolic Hsp90 activity is aided by the co-chaperone Hsp-organising protein (Hop/p60) which mediates the association of Hsp90 with Hsp70 (Scheufler *et al.*,

2000). Currently, no Grp94 co-chaperones have been identified although many other ER chaperones have been detected in complexes with Grp94. This is not surprising since Grp94 lacks the C-terminal KEEVD domain necessary for recognition by TPR motif-bearing accessory proteins such as the co-chaperone Hop.

Grp94 does interact with adenosine nucleotides, but this association is very weak. It was, therefore, believed that Grp94 does not function as an ATPase (Wearsch & Nicchitta, 1997; Rosser & Nicchitta, 2000). The role of adenosine nucleotides in substrate binding was recently studied by Rosser and colleagues who demonstrated that in the presence of ATP, ADP or geldanamycin, Grp94-Ig heavy chain complexes remained stable indicating that the hydrolysis of ATP is not responsible for the release of client proteins from Grp94 (Rosser et al., 2004). However, recent evidence now suggests that Grp94 does have ATPase activity (Dollins *et al.*, 2007; Frey *et al.*, 2007).

The crystal structure of the Grp94 N-terminal regulatory domain bound to the adenosine derivative NECA shows structural divergence from other Hsp90 family members. A 5 amino acid insertion in a subdomain could potentially act as a ligand responsive conformational switch, altering the oligomerisation behaviour of Grp94 (Soldano *et al.*, 2003). Further crystallographic work from nearly full-length Grp94 has supported this finding (Dollins et al., 2007). Cytosolic Hsp90 complex recruitment is regulated by ligand binding, however, indicating potential functional similarities between Grp94 and Hsp90 (Whitesell *et al.*, 1994).

2.2.2.7 *ER-associated Degradation (ERAD)*

Those proteins stuck in off-pathway conformations pose a danger to the cell due to the risk of accumulation and aggregation or the binding of wild-type proteins as well as causing membrane damage, leakiness of organelles and loss of ion/small molecule gradients over these membranes. Although unfolded or misfolded proteins interact with ER chaperones to promote constructive folding events, eventually, if they do not reach their native conformation they are removed from the ER, possibly by retrotranslocation, and degraded in the cytoplasm in a process known as ER-associated degradation (ERAD) (Kostova & Wolf, 2003; Nakatsukasa & Brodsky, 2008). These 'terminally misfolded' proteins may have arisen as a consequence of mutations at translation, lack of co-factors or prosthetic groups or failure to oligomerise. In short, the aim of ERAD is to remove terminally unfolded/misfolded proteins from the ER to maintain ER homeostasis.

2.2.2.7.1 *ERAD substrate recognition*

The first step in the removal of terminally misfolded proteins is their detection. One of the key modes of highlighting proteins destined for retrotranslocation – at least for glycoproteins – is the trimming of N-glycans by ER-mannosidase I, since inhibition or removal of this enzyme halts glycoprotein degradation (Su *et al.*, 1993; Knop *et al.*, 1996; Vallee *et al.*, 2000). The Ca^{2+}-requiring enzyme is responsible for removing a single mannose residue – or more (Helenius, 1994; Hosokawa *et al.*, 2003) – from the core oligosaccharide resulting in a $GlcNAcMan_8Glc_{0\text{-}3}$ structure (Fig. 3). Since mannose removal is a slower process than glucose removal – mediated by GI and GII – mannose trimming by ER-mannosidase I acts as a countdown timer for glycoprotein folding which, if surpassed, results in degradation.

Although essential, the removal of this mannose residue is not sufficient for ERAD since correctly folded proteins also require mannose trimming before exiting the ER (Byrd *et al.*, 1982). Thus, there must exist other mechanisms – most likely involving an interplay between ERAD components, ER folding assistants and the biophysical properties of the substrate – to determine if proteins are to be retrotranslocated; especially since non-glycosylated proteins are not subjected to mannose trimming.

Once ER-mannosidase 1 has removed a single mannose residue, the efficiency of re-entry into the calnexin cycle is reduced and the binding of the putative lectin ER degradation enhancing α-mannosidase-like protein (EDEM) (Htm1/Mnl1 in yeast) is favoured. Through interaction with calnexin, terminally misfolded proteins are directly handed over to EDEM, diverting these substrates towards ERAD (Molinari *et al.*, 2003; Oda *et al.*, 2003; Cormier *et al.*, 2009). Furthermore, overexpression of EDEM1 reduces intervals of calnexin binding, accelerates the degradation of substrates and inhibits the formation of disulfide-linked dimers and complexes for clients of the calnexin cycle, thus maintaining the retrotranslocation competence of terminally misfolded proteins and preventing the formation of misfolded aggregates (Molinari & Helenius, 2000; Oda *et al.*, 2003; Hosokawa *et al.*, 2006). In a screen for EDEM-binding proteins, Ushioda and colleagues illustrated that the disulfide isomerase, ERdj5, binds to EDEM and accelerates the ERAD of substrates by facilitating the reduction of substrate disulfide bonds (Ushioda *et al.*, 2008). As such, EDEM seems to possess a new type of chaperone-like activity, preventing unconstructive covalent interactions and aggregation – essential since the misfolded protein needs to be retrotranslocated out of the ER – but in order to promote degradation rather than constructive folding events. It is unclear however, whether

EDEM facilitates degradation through the recognition of misfolded conformations/hydrophobic regions in addition to recognising specific oligosaccharide side chains. The three members of the EDEM family – EDEM1, EDEM2 and EDEM3 – share considerable homology to ER-mannosidase 1 in their lumenal domain but seem to lack the enzymatic activity to process α-1,2-mannose. However, overexpression of EDEM1 was found to result in mannose trimming and enhancement of ERAD (Hosokawa *et al.*, 2010). Furthermore, Htm1, the yeast orthalog of EDEM, exhibits α1,2-specific exomannosidase activity firstly requiring processing of the glycan by GI, GII and mannosidase I (Clerc *et al.*, 2009) and also possibly acts as a marker for Yosp9 activity by potentially recognising the presence of misfolded structures (Quan *et al.*, 2008). The homology between EDEM and ER-mannosidase 1 may be responsible for EDEMs ability to preferentially recognise mannose-trimmed substrates. EDEM1 is a membrane protein whereas EDEM2 and EDEM3 are located in the lumen of the ER (Olivari *et al.*, 2005; Mast *et al.*, 2005; Hirao *et al.*, 2006). However, only EDEM3 contains a KDEL ER retention signal; EDEMs 1 and 2 lack any discernable retention signal (Hirao *et al.*, 2006). Since EDEM does not interact with calreticulin, it is possible that further lectins will be discovered that link ERAD to calreticulin-mediated chaperone activity.

In addition to EDEM, a second class of lectin-like ERAD components exist. Yeast osteosarcoma 9 protein (Yos9p) – the yeast OS-9 ortholog – possesses a lectin-like domain but with similarities to the mannose 6-phosphate receptor (MPR) family – indicative of its function in mannose recognition. The protein contains a C-terminal HDEL ER retention signal and associates with the ER membrane (Friedmann *et al.*, 2002). More recent work has identified Yos9p as being able to bind misfolded

glycoproteins in an N-glycan-specific manner (Szathmary *et al.*, 2005). A genome-wide screen initially identified Yos9p as being essential for glycoprotein ER-associated degradation. This was further supported by the observation that Yos9p co-immunoprecipitated with the classic ERAD substrate misfolded carboxypeptidase Y (CPY) when exhibiting specific oligosaccharide architecture (Buschhorn *et al.*, 2004; Bhamidipati *et al.*, 2005; Szathmary *et al.*, 2005). Moreover, Bhamidipati and colleagues found that Yos9p could bind misfolded CPY *lacking* N-glycans. Although this seems contradictory to Szathmary and colleagues' work, Bhamidipati and colleagues used different methods. Furthermore, the concentrations of Yos9p used in the experiments of the latter reportedly exceed that of physiological Yos9p concentration which may have an influence of substrate binding.

The role of Yos9p in ERAD is still not fully understood but the observation that the binding of misfolded glycoproteins with Yos9p is reduced in the absence of EDEM (Htm1p) in yeast might suggest that Yos9p is part of the same degradation pathway as EDEM and perhaps exerts its function downstream of EDEM or forms a complex together with Yos9p which is able to stabilise the misfolded protein-Yos9p interaction. Due to the observation that altering conserved residues in the lectin-like mannose 6-phosphate homology (MRH) domain of Yos9p abolishes its ERAD function and that degradation of mutant glycoproteins is dependent on Yos9p, is was proposed by the Wolf laboratory that Yos9p-misfolded glycoprotein binding is mediated by an MRH-domain-N-glycan interaction (Szathmary *et al.*, 2005). However, this theory does not exclude the binding of misfolded glycoproteins to Yos9 in an N-glycan-independent manner. Yos9 has two mammalian homologs, OS-9 and XTP3-B, both of which contain MRH domains (Christianson *et al.*, 2008). OS-

9 and XTP3-B have been found in a complex with the ubiquitin ligase Hrd1 via the SEL1L adaptor protein as well as with ERAD substrates indicating that both homologs are involved in the ERAD of proteins in the mammalian system.

2.2.2.7.2 *Retrotranslocation and degradation*

Once the recognition of proteins destined for degradation has taken place, these ERAD candidates are then targeted to the putative dislocation and ubiquitination complex. The pore through which ERAD substrates are retrotranslocated is still being debated (Herbert *et al.*, 2010). Sec61, the main component of the protein translocation channel responsible for allowing the translocation of newly synthesised proteins into the ER, was shown to participate in the retrotranslocation of ER proteins. In mammalian cells, Sec61α has been coimmunoprecipitated with MHC class I heavy chains directed to ERAD by the cytomegalovirus (CMV) gene products US2/US11 (Wiertz *et al.*, 1996). Many further studies support a role for Sec61 in the retrotranslocation of ERAD substrates (Pilon *et al.*, 1997; Schmitz *et al.*, 2000; Oyadomari *et al.*, 2006; Scott & Schekman, 2008). However, the crystallographic structure of SecY/E, an archaeal ortholog of Sec61, revealed that the size limit for the pore diameter is small and seems unlikely to accommodate larger retrotranslocation substrates (Tirosch *et al.*, 2003; van den Berg *et al.*, 2004). Another candidate, Derlin1 (Der1p in yeast), which has four membrane spanning domains, has too been shown to be necessary for CMV-induced dislocation of MHC class I heavy chains from the ER (Hitt & Wolf, 2004; Lilley & Ploegh, 2004; Ye *et al.*, 2004). Overexpression of members of the Derlin protein family accelerated the degradation of ERAD substrates further supporting their putative role in retrotranslocation (Oda *et al.*, 2006). Derlin1 forms a complex with both ER lumenal

components and cytosolic proteins required for efficient ERAD including the homohexameric ATPase p97/VCP (cell division cycle 48 (Cdc48) in yeast) and VIMP (p97-interacting protein). The yeast system is better characterised and, in addition to Cdc48 and Der1p, other components of the retrotranslocation machinery have been identified and include: the dimeric cofactor Ufd1/Npl4, the membrane adapter protein Ubx2p, which contains a ubiquitin associating (UBA) domain, the E3 ubiquitin-protein ligase Hrd1p and lastly Usa1p, which stabilises the interaction between Hrd1p and Der1p (Lilley & Ploegh, 2004; Ye *et al.*, 2004; Carvalho *et al.*, 2006; Oda *et al.*, 2006; Bernardi *et al.*, 2008; Christianson *et al.*, 2008; Goder *et al.*, 2008). Thus, Derlin interacts with components of the ubiquitination and degradation-targeting machinery. Furthermore, in yeast, Yos9p has been shown to form a complex with Kar2 (the yeast homolog of BiP) – which has also been implicated in ERAD – and Hrd3p which stabilises Hrd1p in the multimeric ubiquitination complex ((Plemper & Wolf, 1999; Brodsky *et al.*, 1999; Kabani *et al.*, 2003; Denic *et al.*, 2006; Hedge *et al.*, 2006). Thus, it has been proposed that Yos9p recruits proteins, genuinely designated for ERAD, to the putative dislocation/ubiquitination complex providing a link between the recognition of aberrantly folded proteins and their retrotranslocation and subsequent ubiquitination as well as helping to eliminate indiscriminate, basal levels of degradation. In higher eukaryotes, many other factors have been reported to be important in assisting in the retrotranslocation of ER components including PDI and BiP (Gillece *et al.*, 1999; Molinari *et al.*, 2002; Forster *et al.*, 2006; Heiligenstein *et al.*, 2006; Hosokawa *et al.*, 2008)

Dislocation of ERAD substrates is aided by the Cdc48-Ufd1-Npl4 complex and precedes the addition of polyubiquitin to these proteins targeting them towards

proteasomal degradation (Ye *et al.*, 2001; Jarosch *et al.*, 2002). Ubiquitination requires a sequence of reactions starting with the activation of ubiquitin by the ubiquitin-activating enzyme (E1) which then transfers the activated ubiquitin to the ubiquitin-conjugating enzyme (Ubc or E2). The E3 ubiquitin ligase is responsible for conjugating the ubiquitin – via an isopeptide bond – to a target lysine residue. A number of E3 ligases involved in the ubiquitination of ERAD substrates have been identified (Kostova *et al.*, 2007). Hrd1p, a putative retrotranslocon, contains a cytosolic RING-H2 face that is stabilised by an interaction with the transmembrane Hrd3p (Bordallo *et al.*, 1998; Bays *et al.*, 2001). Hrd1p can interact with the E2 enzymes Ubc1p, Ubc6p or Ubc7p and is responsible for the selective ubiquitination of ER lumenal and membrane proteins (Friedlander *et al.*, 2000; Bays *et al.*, 2001; Sato *et al.*, 2009). Doa10 – also proposed as an alternative dislocation channel – is a further E3 ligase with a much broader a range of substrates than Hrd1p (Kostova *et al.*, 2007).

The fact that these E3 ligases are proposed to be putative retrotranslocons seems sensible, since housing the retrotranslocation and ubiquitination activities in the same enzyme may be the most efficient way of targeting ERAD substrates for proteasomal degradation (Vembar & Brodsky, 2008).

Once targeted for degradation, substrates are delivered to the proteasome possibly by Cdc48 and several proteasome-interacting factors including UBA domain- and UBL domain-containing proteins such as Rad23 and Dsk2, which have been shown to increase ERAD efficiency (Verma *et al.*, 2000; Medicherla *et al.*, 2004; Raasi & Wolf, 2007). Substrates are then deubiquitinated and degraded by the 26S

proteasome which may even be localised to the surface of the ER membrane (Rivett, 1993; Amerik & Hochstrasser, 2004).

ER-associated degradation of integral membrane proteins is more poorly understood than that of ER lumenal proteins (Nakatsukasa & Brodsky, 2008). The ER-associated, cytoplasmic Hsp40-Hsp70 chaperones have been shown to assist in the interaction of integral membrane proteins with E3 ligases, possibly indicating a role in their degradation (Nakatsukasa *et al.*, 2008). This is consistent with the several other studies which suggest that the cytoplasmic chaperone machinery plays an important role in the ERAD of membrane proteins (McClellan *et al.*, 2005; Carvalho *et al.*, 2006). However, further research is needed on the degradation of membrane proteins with few or no soluble domains.

2.3 *Unfolded Protein Response*

In the endoplasmic reticulum, much as in any organelle, an increase in population of a folding polypeptide species with persistent non-native interactions can have dire consequences for the cell. The accumulation of off-pathway intermediates can lead to the formation of intermolecular interactions and the development of protein aggregates. I have already discussed the intrinsic properties that chaperones exert on constructive protein folding and the prevention of aggregate formation. However, due to potential changes in the protein folding demand of the cell as well as the ER's susceptibility to perturbations caused by environmental factors, it is essential that the ER possesses a mechanism for detecting the folding competency and status of the organelle. Accordingly, the ER contains sensors that detect the folding capacity of the organelle and transduce that signal across the ER membrane and, where

appropriate, mount a suitable response to maintain ER homeostasis. This signalling network is known as the unfolded protein response (UPR).

The capacity of the ER protein folding machinery to meet the folding demand of ER proteins is regulated by coordinating the expression of many genes. The UPR responds to perturbations in ER homeostasis by coordinating a transcriptional – and additionally in higher eukaryotes, a translational – programme to restore protein folding efficacy. These perturbations can be the synthesis and accumulation of terminally misfolded proteins, environmental stresses such as heat shock or hypoxia or a sudden increase in protein synthesis. All of these perturbations result in an imbalance between the demand of folding proteins and the folding capacity of the ER and can collectively be referred to as 'ER stress', which is typically defined as 'a situation where the folding capacity of the ER is exceeded'. At this point, the cell must make a 'decision' on whether it is beneficial or detrimental to promote survival under these conditions. The mechanisms by which the cell switches between prosurvival a proapoptotic pathways are not fully understood but the role of the UPR in apoptosis and the respective merits of survival and death on the organism will be discussed further on in this chapter.

2.3.1 *The yeast UPR*

The story of the yeast UPR started with genetic screens of mutants defective in UPR carried out in the budding yeast *Saccharomyces cerevisiae.* As a result, in 1993, two separate groups independently isolated *IRE1* as a putative ER stress transducer (Cox *et al.*, 1993; Mori *et al.*, 1993). *Ire1Δ* mutants are unable to activate transcription of *KAR2* and *PDI1* which encode the yeast ER resident proteins BiP and PDI

respectively. Furthermore, *IRE1* was found to be essential for cell viability under stress conditions where unfolded proteins were allowed to accumulate in the ER; indicating a direct role for *IRE1* in the protection of cells from perturbation in ER homeostasis. Sequence data suggested that Ire1p was a single-spanning ER transmembrane protein kinase with an N-terminal region in the ER lumen, whilst further work illustrated that its cytosolic domain is composed of two functional domains, a serine/threonine protein kinase and an RNase L-like endoribonuclease domain (Bork & Sander, 1993; Mori *et al.*, 1993 Shamu *et al.*, 1994; Sidrauski & Walter, 1997).

Subsequent biochemical analysis of Ire1p revealed that after detecting the accumulation of unfolded proteins in the ER, Ire1p forms dimers or oligomers in the plane of the membrane inducing its kinase activity and causing *trans*-autophosphorylation of Ire1p (Shamu & Walter, 1996; Welihinda & Kaufman, 1996). This, in turn, induces the endoribonuclease activity of Ire1p (Sidrauski & Walter 1997). The sole target of Ire1p RNase activity is the constitutively-expressed *HAC1* mRNA (Cox & Walter, 1996; Sidrauski *et al.*, 1996; Kawahara *et al.*, 1997). Recently, crystal structure evidence for the early-stage formation of Ire1 oligomers was published (Lee *et al.*, 2008a; Aragòn *et al.*, 2009; Korennykh *et al.*, 2009; Wiseman *et al.*, 2010). These high-order, ‘supramolecular structures’ give rise to Ire1 foci to which *HAC1* mRNA is recruited via a conserved 3’ untranslated region (UTR) targeting element which promotes Ire1p activation and Ire1p-mediated UPR signalling (Aragòn *et al.*, 2009). Indeed, both *HAC1* and the metazoan equivalent, *XBP1*, have a tendency to be found associated with ER membranes (Diehn *et al.*, 2000; Stephens *et al.*, 2005; Yanagitani *et al.*, 2009).

In yeast, Hac1p (Homology to ATF and CREB 1 protein) – identified by three independent groups – is responsible for mediating transcription from unfolded protein response element (UPRE)-containing promoters (Mori *et al.*, 1996; Cox & Walter, 1996; Nikawa *et al.*, 1996). The UPRE is a single 22-bp element which is conserved among targets of the UPR and required for transactivation of target genes such as *KAR2*, *PDI1*, *EUG1*, *LHS1* and *ERO1* (Normington *et al.*, 1989; LaMantia *et al.*, 1991; Mori *et al.*, 1992; Tachibana & Stevens, 1992; Kohno *et al.*, 1993; Craven *et al.*, 1996; Pollard *et al.*, 1998; Frand & Kaiser 1998). The *HAC1* gene was identified as a homolog of the ATF/CREB family of basic leucine zipper (bZIP) transcription factors but unusually, transcripts of the gene vary depending on the presence or absence of ER stress (Bork & Sander, 1993; Nojima *et al.*, 1994; Sidrauski & Walter, 1997). Under stressed conditions, *HAC1* mRNA lacks a 252-bp region present in non-stressed cells. The endoribonuclease activity of Ire1p – together with an RNA ligase – is responsible for this non-canonical splicing event, rather than the conventional splicosome machinery. Removal of this intron takes place by Ire1p-mediated cleavage at two specific sites near the 3' end of the mRNA sequence on conserved, predicted stem-loop structures either side of the 252-bp fragment – which are then joined by tRNA ligase (Sidrauski *et al.*, 1996; Sidrauski & Walter, 1997; Kawahara *et al.*, 1998; Gonzalez *et al.*, 1999). The result of Ire1p-mediated splicing is the replacement of the 10-amino acid C-terminal tail encoded in the intron of unspliced *HAC1* mRNA (*HAC1*^{u}) with an 18-amino acid tail encoded by the second exon of spliced *HAC1* (*HAC1*^{i}). The splicing event does not affect the 220-amino acid N-terminal region which contains the DNA-binding domain.

Under non-stressed conditions, constitutively-expressed *HAC1^{u}* associates with polyribosomes to initiate translation but translation stalls on ribosomes possibly due to long-range base pairing between the 5' untranslated region (UTR) and sequences within the intron (Chapman & Walter, 1997; Mori *et al.*, 2000; Rüegsegger *et al.*, 2001). Splicing out of this 252-bp intron prevents this long-range base pairing and relieves *HAC1* from translational attenuation (Rüegsegger *et al.*, 2001). Moreover, Mori and colleagues showed that increased transcriptional activator activity was not solely due to an increase in translation efficacy (Mori *et al.*, 2000). They proposed that an observed 10-fold increase in the transcription activator activity of Hac1p^{i} over Hac1p^{u} was due to the proximity of a C-terminal activation domain (AD) to the DNA-binding domain (DBD). However, the C-terminal tail becomes more acidic in spliced Hac1p^{i}, leading to transcriptional activity.

Yeast cells that lack *HAC1* are unable to mount an unfolded protein response and, although viable, are sensitive to pharmacological agents which can induce ER stress and tend to die at ordinarily sublethal doses (Mori *et al.*, 1996). Thus, the transcriptional regulation of components involved in the promotion of protein folding and the restoration of ER homeostasis – and the regulation of Rpd3 histone deacetylase activity – is performed by Hac1p^{i}, which, through Ire1p-mediated regulation, and in conjunction with Gcn4p, responds to the protein folding capacity of the yeast endoplasmic reticulum (Travers *et al.*, 2000; Patil *et al.*, 2004; Schröder *et al.*, 2004).

2.3.2 *The mammalian UPR*

Whereas yeast cells have a single proximal stress sensor, the UPR in higher eukaryotes consists of three distinct pathways modulated through three transmembrane stress sensors, IRE1, PERK and ATF6. Similar to the yeast UPR, the mammalian UPR responds to perturbations in ER homeostasis and initiates a transcriptional programme to meet the requirements of the ER for protein folding. Transcriptional activation of specific genes involved in protein folding – such as chaperones and folding enzymes – and components of ER-associated degradation alleviates ER stress and restore ER homeostasis. Moreover, in addition to yeast, the mammalian UPR exerts attenuation of global protein translation to combat ER stress. This is achieved by inhibiting translation initiation and serves to decrease the load of newly synthesised proteins on the ER.

2.3.2.1 *IRE1*

IRE1 is highly conserved in evolution. In mammals, two homologs of yeast *IRE1*, which are encoded by different genes, have been identified (Tirasophon *et al.*, 1998; Wang *et al.*, 1998). IRE1α is expressed broadly in a range of tissues whereas IRE1β is expressed only in the epithelial cells of the gastrointestinal tract, particularly in colon and stomach. Deletion of the mouse homolog of IRE1α is embryonic lethal whereas IRE1β-deficient mice are viable but susceptible to environmental agents that promote colitis (Bertolotti *et al.*, 2001; Lee *et al.*, 2002). Similarly to Ire1p, both mammalian IRE1 proteins respond to ER stress by *trans*-autophosphorylation and dimerisation – which has recently been confirmed by solving the yeast cytosolic domain crystal structure – and can activate downstream target genes (Lee *et al.*, 2008a). Overexpression of either IRE1α or IRE1β is able to activate the BiP

promoter indicating a functional conservation between yeast and mammals – although BiP activation might be the result of IRE1α/β acting as an unfolded protein. Deletion of mouse IRE1α and/or IRE1β does not *prevent* transcriptional activation of BiP or the Hsp90 family-member Grp94, indicating that there are IRE1-independent pathways that can complement IRE1 in the mammalian UPR target gene regulation (Tirasophon *et al.*, 1998; Wang *et al.*, 1998; Urano *et al.*, 2000; Lee *et al.*, 2002).

The main substrate for the endoribonuclease activity of mammalian IRE1 is the bZIP transcription factor X-box binding protein 1 (*XBP1*) mRNA (Shen *et al.*, 2001; Yoshida *et al.*, 2001a; Calfon *et al.*, 2002; Lee *et al.*, 2002) (Fig. 4). Unlike *HAC1*, in which the DNA-binding domain (DBD) and transcriptional activation domain (AD) are in the same open reading frame (ORF), the pre-mRNA version of *XBP1* (*XBP1*^{u}) only contains the DBD (Yoshida *et al.*, 2001a). Splicing of *XBP1*^{u} joins two open reading frames allowing the translation of both the DBD and AD. The splice-site motif of *XBP1*^{u} is conserved from yeast further indicating that *XBP1*^{u} undergoes a non-conventional splicing event. Unlike *HAC1*, the intron removed from *XBP1*^{u} is small at only 26-bp compared with 252-bp. This is in line with the fact that apart from the DBD, *XBP1*^{s} and *HAC1*^{i} share little sequence homology.

Furthermore, in contrast to *HAC1*^{u} expression, which is subject to translational stalling, *XBP1*^{u} is constitutively translated as a non-functional protein, but is quickly degraded by the proteasome (Lee *et al.*, 2003). The purpose of this non-functional version of *XBP1* – besides providing a cytoplasmic source of a 'ready' downstream target for IRE1 without *de novo* transcription – has been investigated recently

(Tirosh *et al.*, 2006; Yoshida *et al.*, 2006b; Uemura *et al.*, 2009; Yoshida *et al.*, 2009). Tirosh and colleagues showed that increasing the stability of $XBP1^u$ protein augments the transcription of *XBP1*-specific and -non-specific target genes in response to ER stress, indicating that rapid degradation of $XBP1^u$ is required to prevent uncontrolled activation of the UPR (Tirosh *et al.*, 2006). Yoshida and colleagues, on the other hand, demonstrated that $XBP1^u$ protein acts as a negative regulator of the UPR by promoting the degradation of the transcriptionally active $XBP1^s$ and ATF6α proteins, preventing residual transcription of ER chaperone and ERAD components (such as EDEM) during the recovery phase. Thus, *XBP1* mRNA seems to encode a positive- and negative-regulator of mammalian UPR in $XBP1^s$ and $XBP1^u$.

Spliced XBP1 binds to a *cis*-acting ER stress-response element (ERSE), ERSE II and mammalian unfolded protein response element (UPRE) (Yamamoto *et al.*, 2004). XBP1 acts alone, as a homodimer, to bind promoter elements – as in the case of UPRE – or in heterodimers with other transcriptional activators such as AFT6α or NF-Y, which will be discussed in more detail later (Yoshida *et al.*, 1998; Yoshida *et al.*, 2001b, Yamamoto *et al.*, 2004; Yamamoto *et al.*, 2007). As a consequence, XBP1 transactivates the expression of components of the ER-associated degradation system including EDEM, Derlin1 and Derlin2, OS9, HERP and the DnaJ co-chaperones $p58^{IPK}$, HEDJ and MDG1, secretory pathway components, SEC23B, SEC24C, SEC61A, SEC61G, SRP54 and TRAM1, and condition- and tissue-specific targets such as the transcription factor Mist1 (Lee *et al.*, 2003; Yoshida *et al.*, 2003; Shaffer *et al.*, 2004; Oda *et al.*, 2006; Yoshida *et al.*, 2006a; Acosta-Alvear *et al.*,

2007). Thus, IRE1-mediated splicing of *XBP1* regulates a great number of downstream targets able to influence the protein folding capacity of the ER.

IRE1 exhibits further features, outside of mediating *XBP1* cleavage, which may have an influence on protein expression and cell fate. Overexpression of IRE1 can promote the cleavage of 28S ribosomal rRNA – although this may only be very minor – as well as mRNA encoding IRE1 itself, indicating that, under certain conditions, the endonuclease activity of IRE1 might have a wider range of substrates (Iwawaki *et al.*, 2001; Tirasophon *et al.*, 2000). This hypothesis was further supported by a recent systematic study which concluded that, under stressed conditions, IRE1 mediates the rapid degradation of a specific subset of mRNAs which encode proteins in the plasma membrane and other secreted proteins (Hollien & Weissman, 2006; Iqbal *et al.*, 2008; Han *et al.*, 2009; Hollien *et al.*, 2009). This was shown to be a rapid response to ER stress, which takes place much faster than expression changes mediated by XBP1. This makes sense since quickly attenuating the translation of proteins before they enter the secretory pathway may relieve acute ER stress while the XBP1-dependent pathway takes effect. It is not clear however, if oligomerisation and formation of IRE foci is necessary for ER-localised mRNA decay.

Additionally, IRE1 possesses features which may promote cell death in response to ER stress. When cells become stressed, IRE1 binds to the E3 ligase TRAF2 via the kinase domain of IRE1 (Fig. 4). This causes the activation of apoptosis signal-regulating kinase 1 (ASK1/MAP3K5) through the formation of a IRE1-TRAF2-ASK1 heterotrimer. As a result, the phosphorylation and consequential activation of

c-Jun N-terminal kinase (JNK) occurs (Urano *et al.*, 2000; Nishitoh *et al.*, 2002). The IRE1-TRAF2 interaction has, however, been demonstrated in the absence of IRE1 kinase activity indicating that that kinase activity is not essential for IRE1-TRAF2 heterodimer formation (Kaneko *et al.*, 2003). ASK1-deficient cells are resistant to ER stress-induced apoptosis and JNK activation, providing evidence that JNK is downstream of ASK1 activation (Nishitoh et al., 2002). ASK1 activation is also associated with amyloid beta-induced neuronal cell death further demonstrating how important the IRE1-TRAF2-ASK1 signalling pathway is in apoptosis (Kadowaki *et al.*, 2005). JNK activation promotes cell death under physiological stresses such as UV irradiation, thus JNK activation might too contribute to cell death in response to ER stress (Barr & Bogoyevitch, 2001) (Fig. 4). Bcl-2 is phosphorylated by ASK1 and JNK leading to Bcl-2 inactivation, and Bim, a Bcl-2 family member, is induced and phosphylated by JNK leading to the induction of apoptosis (Molton *et al.*, 2003). Furthermore, JNK is responsible for the induction of apoptosis by the proteasome inhibitor PS-341 through activation of the JNK/AP-1/CHOP signalling by inhibiting NF-κB and Bcl-2 (Nozaki *et al.*, 2001). The consequences of ASK1 and JNK activation, however, are most likely widespread and these factors may also be involved in prosurvival as well as proapoptotic pathways (Lin *et al.*, 2008; Kim *et al.*, 2008; Choi *et al.*, 2009). These are discussed in more detail in section 2.3.2.5 Apoptosis vs. Survival.

Integration of IRE1 signalling and activation of the caspase pathway may also promote cell death signalling. The caspases are a family of cysteine proteases which can act as effectors of apoptosis, mediating apoptotic signalling and inflammatory cytokine processing. Upon ER stress and the binding of TRAF2 to IRE1, the

TRAF2/procaspase-12 interaction is disrupted, causing the conversion of procaspase-12 to the mature form (Yoneda *et al.*, 2001). The effect of caspase-12 maturation in humans is unclear; however other caspases have been implicated in the ER stress response in humans (Hitomi *et al.*, 2004; Xue *et al.*, 2006). Lastly, *Bak*$^{-/-}$ *Bax*$^{-/-}$ cells are highly resistant to cell death including that induced by ER stress (Wei *et al.*, 2001; Hetz *et al.*, 2006). BCL-2 homologous antagonist/killer (BAK) and BCL-2-associated X protein (BAX) are members of the pro-apoptotic BCL-2 family which, possibly due to the release of ER lumenal Ca^{2+}, facilitate the exit of cytochrome *c* from the mitochondria into the cytosol, initiating apoptosis (Nutt *et al.*, 2002; Oakes *et al.*, 2005; Oakes *et al.*, 2006). Alternatively, the BCL-2 family member BIM may contribute to apoptosis as a result of its activation by phosphorylated JNK (Lei & Davis, 2003; Putcha *et al.*, 2003). Thus, IRE1 signalling has widespread effects that include the downstream regulation of genes involved in protein folding and ERAD, the selective degradation of secretory- and membrane-targeted mRNAs and the regulation of pro-apoptotic pathways.

A question which has thus far not been addressed in this thesis is how does the N-terminal region of IRE1 ‘sense’ ER stress? Concurrent with UPR studies on yeast was an investigation into the ability of several mutant versions of simian virus 5 haemagglutinin-neuraminidase glycoprotein to induce the UPR in mammalian fibroblast CV-1 cells (Ng *et al.*, 1992). When a mutant version of the glycoprotein – which does not bind BiP – accumulated in the ER, no induction of the UPR was detected. This is in contrast to other misfolded mutants of the glycoprotein, capable of binding BiP, which initiated a UPR. This lead to the hypothesis that UPR transducers detect changes in the concentration of free BiP in the ER or in the

concentration of BiP bound to unfolded proteins. Furthermore, the overexpression of BiP attenuates the induction of the UPR (Dorner *et al.*, 1992; Kohno *et al.*, 1993). Thus, the amount of free BiP in the ER negatively regulates the activation of the UPR (Shamu *et al.*, 1994; Liu *et al.*, 2003). This model states that, under non-stressed conditions, BiP is bound to the lumenal domain of IRE1, inhibiting dimer and oligomer formation and consequently downstream signalling. Under ER stress conditions however, the increased concentration of unfolded/misfolded proteins results in these proteins competing with the IRE1 lumenal domain for free BiP which will then bind to these off-pathway intermediates to prevent aggregation. This then promotes dimer/oligomer formation and IRE1-mediated signalling (Bertolotti *et al.*, 2000; Okamura *et al.*, 2000; Kimata *et al.*, 2003; Todd-Corlett *et al.*, 2007; Oikawa *et al.*, 2009). The relationship between the structural features of the lumenal domain of IRE1 and IRE1-mediated signalling is complex and not fully understood. Deletion of the subregion of the IRE1 lumenal domain responsible for BiP binding (sub region V) is dispensable for activation in the face of ER stress (Kimata *et al.*, 2004; Kimata *et al.*, 2007; Oikawa *et al.*, 2007). These results suggest that BiP binding and dissociation are not sufficient for IRE1 activation and that BiP may not be the primary determinant for activation of the UPR .

The crystal structure of lumenal regions of yeast Ire1 and human IRE1 have recently been determined by two separate groups (Credle *et al.*, 2005; Zhou *et al.*, 2006). Credle and colleagues determined the presence of a shared central groove in yeast Ire1 reminiscent of the peptide binding groove of major histocompatability (MHC) molecules. This grove is formed by two α-helices positioned on the β-sheet platforms of each monomer to form the walls of a deep groove that is connected to

the groove of the neighbouring monomer. Disruption of groove architecture by removing hydrophobic regions buried in the interface (F247A) and introducing bulk into the interface (T226W) for example, resulted in reduced *in vivo* Ire1 activity. This led the group to speculate that this groove – created by dimerisation of Ire1 monomers – might recognise flexible regions of unfolded proteins during Ire1 activation and that binding of unfolded proteins might contribute to the formation of higher-order structures of Ire1 in yeast (Credle *et al.*, 2005). On the other hand, Zhou and colleagues, who also detected an MHC-like groove upon IRE1 dimerisation, suggested that this groove was too narrow to accommodate peptides. Furthermore, the residues that were proposed to be involved in peptide binding are buried in the human IRE1 crystal indicating that these residues would not be involved in prptide binding. Additionally, the groove was oriented towards the membrane rather than the lumen and acess to the groove was blocked by an α-helix providing further evidence that direct binding of peptides is unlikely. Despite this, they demonstrated that an intact groove was necessary for both dimerisation and ER stress-induced UPR signalling. In accordance with this finding, other groups have provided evidence that IRE1 activation does not depend on the binding of a ligand (Liu *et al.*, 2002; Liu *et al.*, 2003; Oikawa *et al.*, 2009). Thus, Zhou and colleagues propose a ligand-independent model of IRE1 activation where BiP release is sufficient for dimerisation via an MHC-like groove while Credle and colleagues' data support a model where, in addition to BiP release, the binding of unfolded proteins to the groove of the Ire1 dimer may be required for oligomer formation; a ligand-dependent model.

Recently a two-step model for IRE1 regulation was proposed which suggested that both BiP dissociation and IRE1 binding of unfolded proteins are responsible for IRE1 activation (Kimata *et al.*, 2007). The first step in this model is the dissociation of BiP from IRE1 which causes oligomerisation of IRE1 while subsequent binding of unfolded proteins causes activation. However there is evidence that the enzymatic activity of IRE1 is determined by the oligomerisation state of IRE1 which would suggest that once oligomerisation has taken place, IRE1 becomes activated. A recent paper by Peter Walter's group, however, suggested that BiP binding to Ire1 modulates efferent signalling by sequestering inactive Ire1 molecules, which prevents oligomerisation and facilitates deactivation if the folding conditions within the ER permit (Pincus *et al.*, 2010). Furthermore, they propose that direct sensing of unfolded proteins is the primary trigger for Ire1 activation. In low level stress situations, BiP binding reduces the system's sensitivity, ensuring that Ire1 is only activated when the stress is sufficient to warrant a response. Thus, BiP-mediated modulation of Ire1 activity may allow Ire1 to efficiently counteract a large spectrum of stress magnitudes. This work was undertaken in yeast Ire1. However, the structure and function of Ire1 is conserved between yeast and mammals and when human IRE1 lumenal domain replaces the corresponding domain in yeast Ire1, yeast UPR function is retained (Liu *et al.*, 2000). Further work will be required to determine if BiP modulates the activation of IRE1 in higher eukaryotes.

Recently, the crystal structure of the cytoplasmic region of IRE1 was resolved, which provided an insight into the activation of the endoribonuclease activity of IRE1 (Lee *et al.*, 2008a; Korennykh *et al.*, 2009; Yanagitani *et al.*, 2009). The protein that Lee and colleagues crystallised had both kinase and endoribonuclease activity, was

phosphorylated on the activation loop – which usually occurs by *trans*-autophosphorylation – and was loaded with ADP. Thus, the crystallised protein is likely to represent activated IRE1. The structure reveals that IRE1 forms a symmetric dimer with both kinase and both nuclease domains facing back to back. Additionally, nucleotide binding is required for dimer formation Therefore, it is likely that ER stress induced dimer formation positions the cytosolic domains in proximity for *trans*-autophosphorylation of the kinase activation loop which then, upon nucleotide binding, is reconfigured, leading to the formation of the dimer seen in the crystal structure, which can now accommodate substrate mRNA for cleavage in the kinase extension nuclease (KEN) domain. Further work will be required to understand the relevance of dimer formation in mRNA cleavage. A summary of the IRE1 branch of the mammalian UPR is shown in Figure 4.

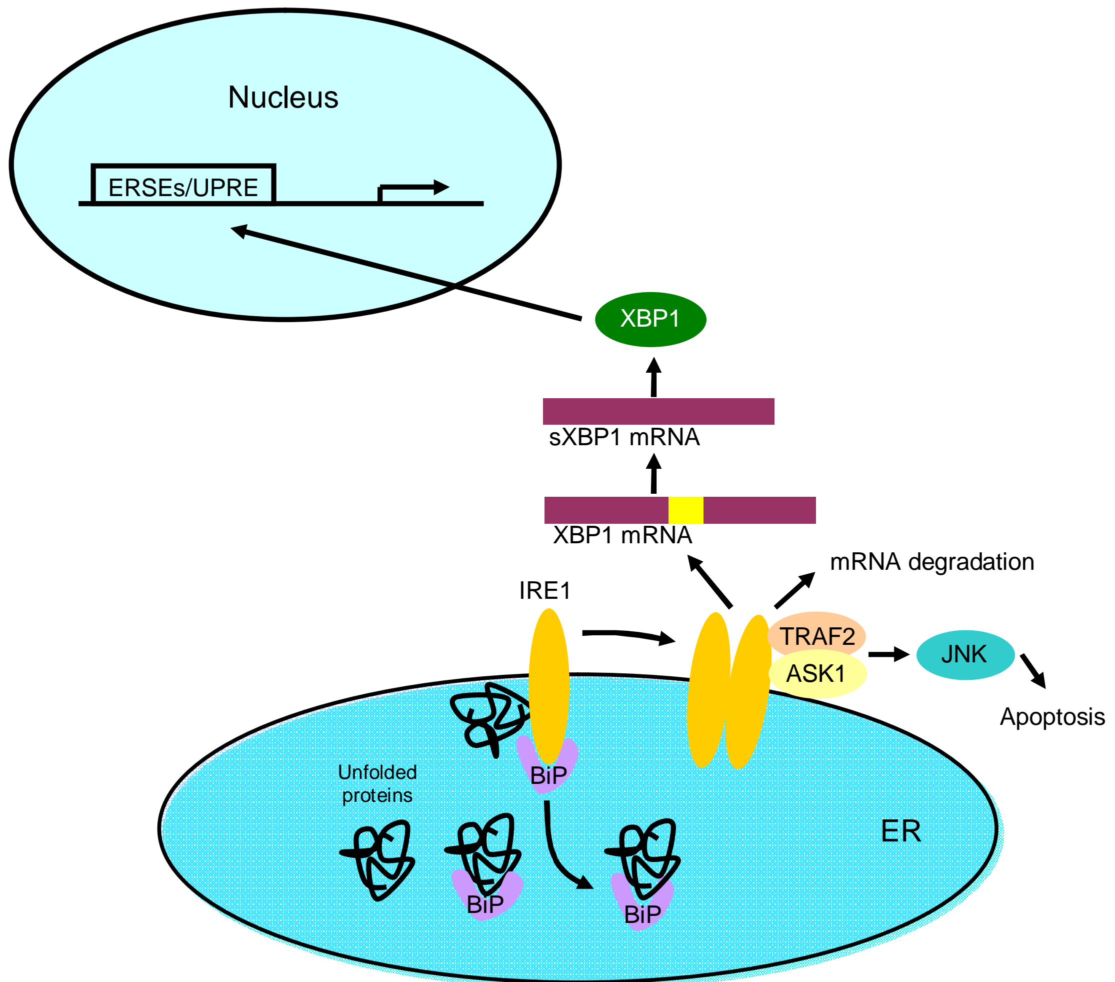


Figure 4. **The IRE1 branch of the UPR**. An increase in concentration of unfolded proteins in the ER causes the titration of BiP away from the lumenal portion of IRE1. Direct binding of IRE1 to unfolded proteins may also influence IRE1 activation. Once activated, IRE1 forms homodimers leading to activation of its endoribonuclease activity. Activation also allows the binding of TRAF2 and ASK to IRE1 which leads to JNK phosphorylation and downstream events which may lead to apoptosis. IRE1 is also involved in the rapid degradation of mRNA coding for membrane and secretory proteins. IRE1 RNase activity targets *XBP1* and removes a 26-bp intron which results in the translation of an active transcription factor (XBP1). This then travels to the nucleus to recognise ERSE I, ERSE II and UPRE promoter elements and upregulate target genes.

2.3.2.2 *PERK*

The second pathway in the metazoan UPR is the PKR-like endoplasmic reticulum kinase (PERK) pathway. PERK, like IRE1, is a type I transmembrane protein located in the ER membrane which too senses the accumulation of unfolded proteins in the ER lumen (Shi *et al.*, 1998; Shi *et al.*, 1999; Harding *et al.*, 1999; Harding *et al.*, 2000b; Harding *et al.*, 2001; Liu *et al.*, 2003). The lumenal domain of PERK, despite low sequence homology, is supposedly similar to that of IRE1 but the C-terminal cytosolic domain contains a eukaryotic translation initiation factor 2 alpha (eIF2α) kinase domain (Harding *et al.*, 1999; Ma *et al.*, 2002). Regulation of PERK activity is similar to that of IRE1 since binding of BiP to the PERK lumenal domain seems to keep the kinase in an inactive, monomeric state.

Dissociation of BiP from PERK triggers oligomerisation, then *trans*-autophosphorylation of a large number of residues and activation of the cytosolic domain (Bertolotti *et al.*, 2000; Ma *et al.*, 2002; Marciniak *et al.*, 2006). Phosphorylation of residues that lie in the kinase insert loop of PERK are thought to be responsible for selectively recruiting its substrate, eIF2α (Marciniak *et al.*, 2006). Further evidence for shared functionality of the IRE1 and PERK lumenal domain comes from domain swapping experiments where the PERK lumenal domain can replace the yeast Ire1 lumenal region and the lumenal domain of human IRE1β can replace the corresponding domain in mammalian PERK (Bertolotti *et al.*, 2000; Liu *et al.*, 2000). Ma and colleagues, however, did note an inconsistency with IRE1 function; their mutant, which lacks the BiP-binding domain (sub-region V) in the PERK lumenal region, was constitutively active, suggesting that BiP is essential for the negative regulation of PERK activity, contrary to that demonstrated by Kimata

and colleagues for IRE1, which may mean that the PERK lumenal domain is unable to directly bind unfolded proteins (Ma *et al.*, 2002).

Under ER stress conditions, *Perk*$^{-/-}$ cells are defective in eIF2α phosphorylation, polyribosome deaggregation and inhibition of amino acid incorporation into proteins (Harding *et al.*, 2000b). Accordingly, activated PERK phosphorylates eIF2α on a serine at position 51, mutations of which ($eIF2\alpha^{S51A}$) leave cells extremely sensitive to the lethal effects of ER stress (Scheuner *et al.*, 2001). Phosphorylation of eIF2α makes it an inhibitor of the GTP exchange factor eIF2B, attenuating the recycling of eIF2. The eIF2 complex is essential for the synthesis of new proteins since it recruits the initiator methionyl tRNA to ribosomes at the commencement of protein translation (Anderson & Kederscha, 2002; Kolitz & Lorsch, 2010). Therefore, Ser51 phosphorylation of eIF2α inhibits this activity and thus reduces global protein translation. As a result, cells are able to confer immediate protection on the ER by adjusting the level of new protein synthesis in response to a decrease in folding capacity. If eIF2α phosphorylation is not tightly regulated however, protein levels could fall below that required for efficient cellular function. Accordingly, growth arrest and DNA damage-inducible gene 34 (GADD34), a protein phosphatase regulatory subunit, is also induced by activated PERK and its downstream targets (Novoa *et al.*, 2001; Jiang *et al.*, 2004; Marciniak *et al.*, 2004). GADD34 dephosphorylates eIF2α to oppose PERK activity and prevent over attenuation of translation or to limit the time window of translational arrest.

Whereas during ER stress and consequential eIF2α phosphorylation the majority of transcripts undergo translational inhibition, a small subset of transcripts are preferentially translated (Harding *et al.*, 2000a; Mikulits *et al.*, 2000; Lu *et al.*, 2004; Vattem & Wek, 2004; Yoshimura *et al.*, 2008; Zhou *et al.*, 2008). The stress-inducible transcription factor *ATF4* is the best studied of these preferentially translated transcripts. *ATF4* contains multiple upstream ORFs that precede the *ATF4* ORF (Harding *et al.*, 2000a). Under non-stressed conditions, when ribosomal assembly is efficient, this feature of *ATF4* mRNA inhibits its translation since ribosomes bind and initiate translation at the decoy ORFs. However, when ribosomal assembly is impaired – as is the case in PERK-mediated eIF2α phosphorylation – ATF4 translation is enhanced because the ATF4 start codon is favoured over the decoy ORFs.

ATF4, like XBP1, is a bZIP transcription factor that recognises specific promoter elements and upregulates genes involved in protein folding, ERAD and indeed upregulates other bZIP transcription factors (Harding *et al.*, 2003; Oyadomari & Mori, 2004). Additionally, ATF4 induces the antioxidant response, protecting cells against oxidative stress as well as upregulating genes involved in amino acid metabolism (Okada *et al.*, 2002; Harding *et al.*, 2003). Furthermore, ATF4 induces the induction of 4E-binding protein 1 (4E-BP1), inhibiting translation through the inhibition of the 5'-cap binding protein eukaryotic translation initiation factor 4E (eIF-4E) which may contribute to cell cycle arrest during ER stress (Yamaguchi *et al.*, 2008). ATF4 can homodimerise or form heterodimers with other bZIP transcription factors such as ATF6, XBP1 and other members of the C/EBP family, and binds to the nutrient-sensing response element (NRSE)-1 and -2 during both ER

stress and nutrient deficiency (Barbosa-Tessmann *et al.*, 2000; Bruhat *et al.*, 2002; Chen *et al.*, 2004).

One of the best studied targets of ATF4 transcription activator activity is CHOP/GADD153 promoter (Fawcett *et al.*, 1999; Harding *et al.*, 2000a; Scheuner *et al.*, 2001; Ma *et al.*, 2002). ATF4, however, is not the only inducer of CHOP expression since other components of the UPR as well as other pathways have been shown to activate CHOP (Wang & Ron, 1996; Wang *et al.*, 1998; Yoshida *et al.*, 2000). CHOP is considered a proapoptotic component of the UPR since *chop*$^{-/-}$ cells are protected from the lethal consequences of ER stress (Zinszner *et al.*, 1998; Oyadomari *et al.*, 2002b). Moreover, CHOP inhibits the protective, anti-apoptotic factor BCL-2, promoting, among other things, formation of the apoptosome (McCullough *et al.*, 2001; Ma *et al.*, 2002). In addition, CHOP is responsible for the translocation of BAX from the cytosol to the mitochondria as well as the promotion of apoptotic caspase activity and the induction of proapoptotic Tribbles related protein 3 (TRB3) (McCullough *et al.*, 2001; Gotoh & Mori, 2004; Ohoka *et al.*, 2005). CHOP also upregulates ERO1α which leads to the promotion of oxidising conditions in the ER and a potential increase in the levels of reactive oxygen species (ROS) (McCullough *et al.*, 2001; Marciniak *et al.*, 2004).

Interestingly, whereas the induction of CHOP and GADD34 expression by ER stress requires PERK, induction by amino acid starvation is PERK-independent. This is because amino acid-induced eIF2α phosphorylation requires the eIF2 kinase GCN2 rather than PERK (Harding *et al.*, 2000b; Novoa *et al.*, 2001). GCN2 is induced by a mechanism whereby uncharged tRNA binds to regulatory regions homologous to

histidyl-tRNA synthestase (HisRS) enzymes (Wek *et al.*, 2006). Indeed, GCN2 responds to other forms of stress such as UV irradiation and proteasomal inhibition, suggesting that ER stress-transducer-independent pathways of protein translational attenuation exist, and that inhibition of protein synthesis is a common response to diverse forms of stress (Jiang & Wek, 2005a; Jiang & Wek, 2005b; Wek *et al.*, 2006).

Recently, a new target of PERK phosphorylation activity was identified. NRF2, under non-stressed conditions, is held in an inactive complex with the cytosolic cytoskeletal anchor protein KEAP1. Phosphorylation of NRF2 by PERK results in disruption of this complex and NRF2 moves to the nucleus where, in a heterodimer with ATF4, activates, among other things, the transcription of antioxidant genes and genes involved in protein folding, ERAD and immune responses (Cullien & Diehl, 2006).

A further mechanism by which PERK-mediated- and GCN2-mediated-eIF2α phosphorylation can have an effect on transcriptional activity is related to its general attenuation of protein synthesis. Besides the many effects that have been discussed, translational attenuation can preferentially reduce the expression of regulatory components that are rapidly turned over. An example of this is the regulation of the transcription factor, and master regulator of inflammation, the immune response, cell proliferation and apoptosis, NF-κB. Under non-stressed or non-inflammatory conditions, NF-κB is bound in the cytosol to inhibitors of NF-κB (IκB) such as IκBα. Triggering of the inflammatory response – due to the detection of cytokines such as tumour necrosis factor alpha (TNFα) – causes rapid phosphorylation of IκBα

by IκB kinase (IKK). The effect of serine 32 phosphorylation is to target the inhibitor for ubiquitination and subsequent proteasomal degradation, releasing NF-κB and facilitating its translocation to the nucleus to regulate target genes. Reduction in global protein synthesis is accompanied by a significant reduction in the translation of IκBα (Deng *et al.*, 2002; Jiang & Wek, 2005a). Therefore, eIF2α phosphorylation may promote the NF-κB-mediated transcriptional activator activity. Furthermore, IKK has been demonstrated to form a complex with IRE1α via TRAF2 and *IRE1α*$^{-/-}$ cells exhibit impaired ER stress-induced NF-κB activation (Hu *et al.*, 2006). Thus, NF-κB activation may be linked with the IRE1 branch of the UPR, linking ER stress with NF-κB activation. Activation of NF-κB has been demonstrated in response to several different cellular stresses including ER stress, UV irradiation and reactive oxygen species (Jiang *et al.*, 2003; Deng *et al.*, 2004; Wu *et al.*, 2004; Jiang & Wek, 2005a; Gloire *et al.*, 2006).

2.3.2.3 *ATF6*

The third ER stress sensor unique to metazoans is ATF6, a type-II membrane protein containing a lumenal sensing domain and a cytosolic bZIP transcription factor domain (Yoshida *et al.*, 1998). Initially, ATF6 was identified as an ERSE-binding transcriptional activator (Hai *et al.*, 1989). Further analysis revealed that, activation of promoter elements required nuclear factor Y (NF-Y) binding in addition to ATF6 binding and that, under stressed conditions, ATF6-ERSE binding requires a cleaved form of ATF6 (Yoshida *et al.*, 1998; Li *et al.*, 2000; Wang *et al.*, 2000; Yoshida *et al.*, 2000; Yoshida *et al.*, 2001b). This cleaved form was identified as the product of N-terminal cleavage and named p50ATF6 (Haze *et al.*, 1999). Although full-length

ATF6 localised to the ER, p50ATF6 was present in the nucleus. Thus, it was predicted that the mechanism behind ATF6 cleavage may be similar to that of the bZIP transcription factor sterol response element binding proteins (SREBPs) since, in response to low cholesterol levels, ER membrane-bound SREBP is transported to the Golgi and cleaved at two sites, releasing the transcription factor domain which is involved in the control of cellular sterol levels (Hua *et al.*, 1996; Sakai *et al.*, 1996). This was confirmed by Ye and colleagues who demonstrated that, under ER stress conditions, ATF6 is transported to the Golgi where it is sequentially processed by Site-1/2 proteases (S1P and S2P), liberating the cytoplasmic transcription factor domain which then translocates to the nucleus to activate the transcription of ER quality control proteins (Ye *et al.*, 2000; Adachi *et al.*, 2008).

The mechanism which regulates the transport of activated ATF6 to the Golgi is not fully understood, however, two conserved putative regulatory domains have been implicated in ER stress-induced ATF6 transport (Ye *et al.*, 2000; Chen *et al.*, 2002). Additionally, recent work has demonstrated that, under non-stressed conditions, the lumenal domain of ATF6 is disulfide bonded and exists in monomeric, dimeric and oligomeric forms, and that ER stress-induced reduction is required but not sufficient for trafficking to the Golgi (Nadanaka *et al.*, 2007). Like IRE1 and PERK, ATF6 activation is controlled by the ER chaperone BiP (Gething, 1999; Shen *et al.*, 2002). However, binding of BiP to the lumenal domain of ATF6 masks the intrinsic Golgi localisation signals GLS1 and GLS2 (Shen *et al.*, 2002). Evidence for this comes from BiP and ATF6 mutation experiments where a) ER stress-induced BiP release was blocked causing the inhibition of translocation and proteolytic processing of ATF6 and b) removal of the BiP binding domain from ATF6 caused constitutive

activation of ATF6 signalling (Shen *et al.*, 2002; Summer & Jarosch, 2002). Thus, the mechanism of negative regulation that BiP exerts on ATF6 may differ to that exerted upon PERK and IRE1 monomers.

However, Shen and colleagues recently demonstrated that BiP binds stably to ATF6 and postulated that BiP-ATF6 dissociation is not a consequence of competition of unfolded proteins for BiP but that ER stress-induced dissociation is a consequence of an active regulatory mechanism (Shen *et al.*, 2005). Perhaps the active reduction of disulfide bonded higher order structures of ATF6 demonstrated by Nadanaka and colleagues is important in the dissociation of BiP from ATF6 (Nadanaka *et al.*, 2007). Since BiP may play a role in modulating the sensitivity of IRE1 to mild stresses (Pincus *et al.*, 2010), it might also be possible that the BiP binding to ATF6 may modulate the activation of ATF6 through other mechanisms, such as direct reduction of ATF6 oligomers. The glycosylation status of ATF6 may also contribute to its activation since, under non-stressed conditions, fully glycosylated ATF6 is retained in the ER by calreticulin binding (Hong *et al.*, 2004). In ER stressed cells however, newly synthesised ATF6 is underglycosylated – presumably due to the folding capacity of the ER being exceeded – preventing the interaction with calreticulin which may, together with BiP dissociation and ATF6 disulfide reduction, serve to regulate the transport of ATF6 to the Golgi.

The product of the G13 gene is a transmembrane bZIP transcription factor with homology to ATF6. Thus, it was named ATF6β (Haze *et al.*, 2001). Unlike the paralog of human IRE1α, IREβ, ATF6β is ubiquitously expressed. Like ATF6α (referred to up until now as ATF6), ATF6β is proteolytically cleaved releasing the

cytosolic bZIP transcription factor domain (Haze *et al.*, 2001). ATF6β can bind to ERSE either as a homodimer or as a heterodimer with ATF6α (Yoshida *et al.*, 2001b). However, unlike ATF6α, ATF6β possesses weak transcriptional activation activity but binds much more stably to promoter elements (Theurauf *et al.*, 2002; Thuerauf *et al.*, 2004; Thuerauf *et al.*, 2007). Thus, ATF6β may act as a transcriptional repressor of ATF6α activity, fine-tuning the strength of ERSE gene activation and cell viability (Theurauf *et al.*, 2007). There is some evidence that full-length ATF6β might regulate ERSE gene activation since underglycosylation of p110ATF6β cannot be proteolytically cleaved, resulting in the loss of its repressor function on p50ATF6α (Guan *et al.*, 2009).

Deletion of ATF6α, although not lethal, compromises the functionality of the secretory pathway during ER stress (Wu *et al.*, 2007). Further evidence supports a role for ATF6α in the ER stress response. In cells deficient in S2P or where S1P activity was inhibited leading to a failure of ATF6 processing, ER stress-induced BiP induction was abolished (Shen *et al.*, 2001; Okada *et al.*, 2003). Additionally, dominant negative forms of ATF6 blocked the induction of BiP reporter genes and RNAi knock-down of ATF6 levels result in reduced induction of many ER stress-inducible genes (Wang *et al.*, 2000; Yoshida *et al.*, 2000; Lee *et al.*, 2003). However, knock-down of ATF6, only in combination with XBP1 loss, reduced the expression of some ER stress-inducible genes and ATF6 knock-down alone did not strongly reduce the induction of BiP (Lee *et al.*, 2003). Thus, it appears that the XBP1 and ATF6 branches of the unfolded protein response exhibit a level of functional redundancy since there is an overlap of transcriptional targets. This is further

exemplified by the fact that *ire1*$^{-/-}$ cells still show comparable levels of *BiP* mRNA induction upon ER stress treatments (Lee *et al.*, 2002). It seems, therefore, that BiP expression can be induced upon ER stress by either the ATF6 or IRE1 branches of UPR.

2.3.2.4 *Other roles of the UPR*

The most well-studied role of the UPR is to respond to the folding demands of the ER, however, many studies have highlighted a role for specific branches of the UPR in the development of tissues and the maturation and differentiation of cell types. XBP1, for example, is highly expressed in developing bone and cartilage cells of the developing skeleton and the exocrine glands – both highly secreting cell types – during mouse embryogenesis (Clauss *et al.*, 1993). The pattern of XBP1 expression in developing skeleton was similar to that of tissue specific genes expressed in osteoclasts, indicating that XBP1 may be involved in the regulation of these genes (Clauss *et al.*, 1996). Further studies have confirmed a role for XBP1 in myogenesis, hepatogenesis and the development of secretory tissues in the mouse embryo and adult mouse models (Masaki *et al.*, 1999; Reimold *et al.*, 2000; Iwawaki *et al.*, 2004; Blais *et al.*, 2005; Lee *et al.*, 2005).

The most well-studied example of the UPR in secretory cell development comes from the differentiation of B lymphocytes into plasma cells. B cell lymphopoesis consists of two phases, one in the bone marrow, which is antigen-independent and one in the periphery, which is antigen-dependent. Differentiation into high-level Ig-secreting plasma cells in the periphery is accompanied by a five-fold expansion of the ER compartment. Studies using LPS to induce differentiation of B lymphocytes

showed that differentiation induces classical UPR target genes such as BiP and Grp94 (Gass *et al.*, 2002; Iwakoshi *et al.*, 2003). Transcription of XBP1 is induced during B cell differentiation, and XBP1 splicing can restore Ig production in XBP1-deficient cells *in vitro* (Reimold *et al.*, 2001; Iwakoshi *et al.*, 2003). It is, therefore, believed that XBP1 promotes differentiation of B cells by regulating the biogenesis and expansion of organelles involved in secretion (Shaffer *et al.*, 2004; Acosta-Alvear *et al.*, 2007; Sriburi *et al.*, 2007; Hu *et al.*, 2009). However, the ability to regulate membrane biosynthesis and organelle biogenesis is most likely not B-cell-specific. Additionally, XBP1 is essential for the regulation of hepatic lipogenesis (Lee *et al.*, 2008b).

Furthermore, IRE1α is required for B cell lymphopoesis, not for ER expansion, but for the downstream activation of genes involved in Ig gene rearrangements (Zhang *et al.*, 2005). ATF6 activation has been observed during LPS-induced B cell differentiation and the overexpression of a dominant negative ATF6α mutant in differentiating B cells resulted in a marked decrease in total IgM release (Gass *et al.*, 2002; Gunn *et al.*, 2004). Enforced expression of constitutively active ATF6α can drive ER expansion, even in the absence of spliced XBP1 (Bommiasamy *et al.*, 2009). However, the role of ATF6 in B cell differentiation is currently unknown. There is evidence that B cell lymphopoesis is a PERK-independent process, since *perk*$^{-/-}$ mice show defects in secretory cells of the pancreas and skeletal system but not in B cell development (Harding *et al.*, 2001; Zhang *et al.*, 2002; Gass *et al.*, 2002; Zhang *et al.*, 2005; Zhang *et al.*, 2006, Gass *et al.*, 2008). Taken together, these studies demonstrate that the UPR provides a crucial physiological role in

professional secretory cell differentiation and suggests the possibility of cross-talk between differentiation signals and the UPR.

2.3.2.5 *Apoptosis vs. Survival*

Mutations in either the IRE1 branch or ATF6 branch of the UPR are fairly well tolerated, but mutations in both branches either prevent the transcriptional activation of some target genes or block development in the case of *C. elegans* (Lee *et al.*, 2002; Lee *et al.*, 2003; Shen *et al.*, 2005). Thus, there is functional redundancy between the IRE1 and ATF6 branches of the UPR. Since these arms of the UPR seem to have overlapping targets, does this mean they can be selectively activated or are temporally distinct? How the UPR coordinates its response to ER stress is still not fully understood, neither are the downstream effects of UPR target gene activation. No single branch of the UPR activates solely proapoptotic or prosurvival pathways. Thus, a paradox exists where all branches of the UPR are activated in response to ER stress but the fate of the cell (i.e. survival or apoptosis) is mutually exclusive.

The transcription of apoptotic mediators such as *CHOP* occurs in response to both acute and chronic ER stress. It has been suggested that cell survival in the face of acute ER stress is governed by the intrinsic instabilities of mRNAs and proteins which promote apoptosis – such as CHOP, ATF4 and GADD34 – and that only chronic ER stress can activate transcription of these cell death mediators at levels which surpass the threshold for apoptosis (Rutkowski *et al.*, 2006). Thus, cells may be able to adapt to low levels of ER stress despite simultaneous activation of both proapoptotic and prosurvival programmes.

As far as the turning point for the promotion of cell death is concerned, much work has focused on CHOP and the phosphorylation of eIF2α. Loss of PERK-mediated eIF2α phosphorylation sensitises the cell to death (Scheuner *et al.*, 2001). CHOP has been implicated in the regulation of phosphorylation of eIF2α since it induces the expression of GADD34, which is responsible for dephosphorylating phosphorylated eIF2α (Marciniak *et al.*, 2004). It has been suggested that, under chronic ER stress conditions, increased levels of CHOP are able to induce the expression of GADD34 which dephosphorylates eIF2α, preventing attenuation of protein synthesis and encouraging the recovery of ER load in stressed cells, which might possibly promote apoptosis (Marciniak *et al.*, 2004). This theory is supported by the fact that $chop^{-/-}$ cells protect against ER stress-mediated cell death. Deletion of CHOP in this instance would lead to reduced GADD34 expression meaning the persistence of phosphorylated eIF2α and continued translational attenuation. Furthermore, salubrinal, a compound shown to attenuate eIF2α dephosphorylation in ER stressed cells, also protects against cell death (Boyce *et al.*, 2005).

Additionally, mitochondria-dependent as well as mitochondria-independent pathways may also contribute to apoptosis. Regulated Ca^{2+} release from the ER into the cytoplasm is conducted by the proapoptotic BCL-2-related proteins BAK and BAX upon ER stress (Scorrano *et al.*, 2003; Zong *et al.*, 2003). This, in turn, regulates caspase activity and promotes cytochrome *c* release from the mitochondria (Boya *et al.*, 2002; Danial & Korsmeyer, 2004). CHOP has also been identified as a promoter of cell death by repression of the apoptosis inhibitor BCL-2 and induction of the proapoptotic BCL-2 family member, BIM (McCullough *et al.*, 2001;

Puthalakath *et al.*, 2007). Furthermore, the IRE1/TRAF2 interaction may promote apoptosis through caspase-12 activation (Yoneda *et al.*, 2001). However, in plasma cell differentiation, CHOP is required for efficient IgM secretion but is dispensable for apoptosis, indicating that CHOP exerts tissue-specific roles which can be independent of apoptosis (Masciarelli *et al.*, 2010).

Since more than one branch of the UPR is associated with proapoptotic activity, it is possible that during long term ER stress induction, there are fluctuations in the activity of certain branches of the ER allowing the predominance of branch-specific signals. These could potentially be mediated by branch-specific sensory mechanisms – in addition to BiP dissociation – of the ER stress sensors. Examples of this may include the ability of ATF6α to be regulated by disulfide bond reduction and glycosylation and the possibility that IRE1 may directly bind unfolded proteins and that BiP may not be the primary determinant for IRE1 activation (Kimata *et al.*, 2004; Hong *et al.*, 2004; Credle *et al.*, 2005; Nadanaka *et al.*, 2006; Zhou *et al.*, 2006; Kimata *et al.*, 2007; Oikawa *et al.*, 2007 Lee *et al.*, 2008a). If these events are required for activation of individual branches of the UPR, it is possible that, over time, signalling by individual branches of the UPR may be attenuated by adaptive processes which promote homeostasis. The predominance of certain UPR branches may then have an influence on cell fate.

Recently, Lin and colleagues developed assays to examine the molecular behaviour of the individual branches of the UPR in human cells in the face of persistent pharmacological stress and discovered that the activity of individual arms of the UPR varied markedly with time after the onset of stress (Lin et al., 2007). They

demonstrated that, after rapid, initial activation of all branches of the UPR, the IRE1 arm selectively attenuated after around eight hours despite the persistence of stress. ATF6 signalling also declined, although after IRE1 attenuation. PERK signalling persisted under prolonged ER stress and was still evident 30 hours after the onset of stress. When activation of the IRE1 branch was selectively prolonged, cells showed increased survival indicating that IRE1 signalling attenuation may be a key step in switching fates from a prosurvival to a proapoptotic strategy. The mechanism by which IRE1 is attenuated is unknown although BAX-inhibitor 1 (BI-1) has been implicated in the negative modulation of IRE1 and possibly ATF6 (Bailly-Maitre *et al.*, 2006; Lisbona *et al.*, 2009). So it seems, initially, when all branches of the UPR are activated, prosurvival outputs outweigh proapoptotic ones, however, when the opportunity to recover from ER stress has passed, the cell may adjust the signalling activity of the IRE1 and ATF6 branches leading to a predominance of proapoptotic outputs. Prolonged activation of the PERK pathway in isolation impairs both cell viability and cell proliferation (Lin *et al.*, 2009). Thus, distinct combinations of individual UPR signalling pathways and the duration of individual UPR branch activity may determine cell fate in response to ER stress. The relationship between the duration of stress and stress severity is further supported by a recent study by Pincus and colleagues who demonstrated that the duration of Ire1 activity in yeast, rather than the maximum amplitude, correlated with the magnitude of the stress (Pincus *et al.*, 2010). This is likely since the Hac1 transcription factor is short lived and so the length of activation determines the duration of UPR target gene induction.

Furthermore, Han and colleagues recently showed that the IRE1 branch of the UPR, rather than promoting solely prosurvival signals, also has a role in the switch to

apoptosis (Han *et al.*, 2009). By using inhibitors and mutant forms of IRE1 in mammalian cells, they decoupled the kinase and RNase activities of IRE1 and showed that activation of RNase activity promotes the degradation of ER-localised mRNAs. This complements the work of Hollien and Weissman who showed that IRE1 is required for the rapid decay of ER-localised mRNA in *D. melanogaster* and in mammalian cells (Hollien & Weissman, 2006; Hollien *et al.*, 2009). However, Han and colleagues showed that prolonged ER stress resulted in the sustained decay of these mRNAs including those secretory proteins involved in protein folding and that sustained RNase activity correlated positively with apoptosis (Han *et al.*, 2009). They postulated that apoptosis is the result of reduced secretory pathway activities due to the sustained decay of ER-localised mRNA. This work is in contrast to Lin and colleagues who attributed only prosurvival activities to IRE1, however it has been noted that the cells used in these experiments were overproducing kinase-dead IRE1 and so did not report the proapoptotic activity seen by Han and colleagues (Lin *et al.*, 2009).

2.3.2.6 *UPR in disease*

Abnormalities in the UPR, as well as conditions which lead to an increase in ER stress due to misfolding, have been linked with a number of different diseases including neurodegeneration, cardiovascular disease, ischemia, bipolar disorder and type I diabetes (Yoshida, 2007; Lin *et al.*, 2008). Here, I will briefly discuss the role of ER stress and the UPR in diabetes mellitus and the neurodegenerative disease Alzheimer’s disease.

Diabetes mellitus (DM) is a disease characterised by an inability to maintain homeostasis of glucose metabolism. In type II diabetes, peripheral tissues develop resistance to insulin as characterised by a failure to respond to normal insulin levels. In response, the pancreatic islet β cells hypersecrete insulin to compensate for insulin resistance and may eventually die (Saltiel & Kahn, 2001). Thus, β cell dysfunction in type II diabetes has been linked with ER stress and the UPR due to the large secretory burden of these cells (Scheuner & Kaufman, 2008). Indeed, overexpression of BiP in β cells subjected to hyperglycemia partially rescues glucose-induced suppression of proinsulin levels and improves glucose-stimulated insulin secretion (Zhang *et al.*, 2008). Type I diabetes, on the other hand, is the result of a primary failure of β cells to secrete sufficient amounts of insulin. This is usually the result of an autoimmune attack on the β cells but can be inherited as a monogenetic disease (Harding & Ron, 2002; Oyadomari *et al.*, 2002a).

A hereditary form of type I diabetes called Wolcott-Rallison syndrome is the result of mutations in the PERK branch of the UPR (Delepine *et al.*, 2000). Similarly, *perk*$^{-/-}$ mice show symptoms characteristic of type I diabetes (Harding *et al.*, 2001; Zhang *et al.*, 2002). In both examples, during post-natal development, there is a loss of β cells, possibly by apoptosis. It has been suggested that the lack of PERK in these cells means they are unable to decrease protein load on the ER in response to high secretory demand (Lin *et al.*, 2008). In line with this, mice expressing non-phosphorylatable eIF2α develop diabetes (Scheuner *et al.*, 2001; Scheuner *et al.*, 2005). However, recent work has demonstrated that PERK is only required during development of β cells and adult mice do not require PERK to maintain glucose

homeostasis suggesting that PERK might have more of an important role in differentiation than survival (Zhang *et al.*, 2006). However, the levels of ER stress in a developing tissue might be significantly higher than in an adult secretory cell. Furthermore, expression of the *WFS1* gene, mutations in which are responsible for Wolfman syndrome (juvenile diabetes), is induced by ER stress and is a PERK-responsive protein that is mostly expressed in the β cells (Inoue *et al.*, 1998; Strom *et al.*, 1998; Fonseca *et al.*, 2005; Ueda *et al.*, 2005).

The Akita mouse model of early-onset/neonatal diabetes contains a missense mutation in the *insulin 2* gene (Wang *et al.*, 1999). Mutant insulin is misfolded and retained in the ER leading to ER stress and possibly subsequent β cell apoptosis (Oyadomari *et al.*, 2002b). Knocking-out CHOP expression in these mice delayed the onset of diabetes further suggesting a role for the UPR. Indeed, UPR target genes are upregulated in the Akita mouse model. BiP, GRP94 and HRD1 are upregulated and both ATF6 and XBP1 are activated in the Akita mouse indicating that the UPR may play a role in ER stress-induced β cell loss (Allen *et al.*, 2004; Nozaki *et al.*, 2004).

The neurodegenerative disease, Alzheimer's disease (AD) is characterised by the formation of cerebral neuritic plaques of amyloid β-peptide (Mattson *et al.*, 1998; Pereira *et al.*, 2004; Lindholm *et al.*, 2006; Zhang & Kaufman, 2006; Roychaudhuri *et al.*, 2008). Studies suggest that disease pathogenesis is the consequence of accumulation of amyloid β-peptide – and possibly tau – in the ER (Schenk *et al.*, 1999; Lee *et al.*, 2001b). Three genes responsible for AD have been identified: amyloid precursor protein (APP), Presenilin1 and Presenilin2. The gene product of

APP is a transmembrane protein, the function of which is currently unknown while the presenilins are essential components of the γ-secretase protease. APP is sequentially cleaved by a β-secretase and γ-secretase which leads to the accumulation of amyloid β-peptide (Selkoe, 2001). The role of amyloid β-peptide accumulation in disease pathogenesis is currently unknown but several reports have implicated the UPR in the cytotoxicity of amyloid β-peptide. Expression of amyloid β-peptide increases ER chaperone levels and activates caspase-12 whereas knock-down of caspase-4 or knock-out of caspase-12 confers resistance to amyloid β-mediated cytotoxicity (Nakagawa *et al.*, 2000; Hitomi *et al.*, 2004; Ferreiro *et al.*, 2006).

The IRE1-ASK1-JNK pathway of the UPR has been implicated in amyloid β-mediated cytotoxicity since $ask1^{-/-}$ neurons are resistant to amyloid β-mediated cell death (Kadowaki *et al.*, 2005). This study also demonstrated that expression of amyloid β in rat PC12 cells selectively activated ASK1 and JNK signalling without activating other outputs from the IRE1 and PERK branches of the UPR. Thus, amyloid β-peptide accumulation might cause the selective activation of the UPR signalling machinery to mediate cytotoxicity. However, this is in conflict with the model of apoptosis discussed earlier which regards IRE1 signalling as short-lived and cytoprotective while PERK signalling is long-lived and after time becomes apoptotic (Lin *et al.*, 2007). More work will be required to elucidate how the UPR is coordinated in amyloid β-peptide-expressing neurons.

2.4 *Ankylosing Spondylitis*

2.4.1 *Ankylosing spondylitis and the spondyloarthropathies*

2.4.1.1 *Clinical features*

ER stress and the UPR have recently been implicated in the pathogenesis of the chronic inflammatory disease ankylosing spondylitis (AS). AS is the major subtype of an inter-related group of chronic rheumatic diseases known as the spondyloarthropathies or the spondyloarthritides (SpA). The clinical features of disease include asymmetrical peripheral oligoarthritis of the lower limbs, inflammatory back pain, enthesitis, psoriasis, anterior uveitis, and chronic inflammatory bowel disease (Braun & Sieper, 2007). Despite all coming under the umbrella of SpA, the disease is divided into five clinical subgroups: ankylosing spondylitis (AS), psoriatic spondyloarthritis (PsA), reactive spondyloarthritis (ReA), spondyloarthritis associated with inflammatory bowel disease (IBD) and undifferentiated spondyloarthritis.

Inflammatory back pain is the most typical presenting symptom of AS characterised by stiffness and pain that is worse in the morning or characterised by long periods of inactivity and is improved with exercise (Sampaio-Barros *et al.*, 2001; Mansour *et al.*, 2006). In addition to lumbosacral pain, patients can experience pain in the gluteal region which is indicative of sacroiliac involvement. Loss of mobility and spinal stiffness at these sites – the lumbosacral region and sacroiliac joints – is explained by spinal inflammation, structural damage, or both (Wanders *et al.*, 2005). Structural changes, such as the formation of syndesmophytes – a bony growth caused by ossification of a ligament – and ankylosis – the fusion of the bones of a joint – are usually caused by osteoproliferation rather than osteodestruction. Peripheral arthritis

affects mainly, but not exclusively, the lower limbs and around a fifth of patients exhibit arthritis in the hip and shoulder joints; which is commonly asymmetrical (Dougados *et al.*, 1991). In addition, around 70% of patients also report neck pain and inflammation in the cervical spine (Maghraoui *et al.*, 2003). Radiographs or magnetic resonance imaging (MRI) of the affected joints show joint space narrowing, erosions with new bone formation, and synovitis (Vinje *et al.*, 1985; Mudlinger *et al.*, 1991; Lambert *et al.*, 2004). Enthesitis – inflammation at the point at which a tendon, ligament or muscle inserts into the bone – is common in AS sufferers at sites such as the Achilles tendon, and both synovial and cartilaginous joints, including those within the spine, and is thought to underlie many of the skeletal manifestations of the disease (Olivieri *et al.*, 1998; Mansour *et al.*, 2006).

Extra-articular manifestations such as anterior inflammation of the eye (uveitis) – seen in up to 40% of patients with AS – and cardiovascular and pulmonary disease can be the earliest manifestations of disease but the incidence of these features tends to rise with increasing disease duration (Mansour *et al.*, 2006). In addition, AS patients can suffer from the inflammatory skin disease (psoriasis) which results in psoriatic nail lesions and swollen finger joints (dactylitis). However, development of concomitant psoriasis and articular inflammation is usually diagnosed as psoriatic arthritis.

Subclinical gut inflammation has been discovered in around a quarter to half of AS patients whereas clinically overt inflammatory bowel disease – which includes Crohn's disease and ulcerative colitis, both of which affect the colon – has been reported to be present in between 5 and 10% of patients with AS (Leirisalo-Repo *et*

al., 1994; de Vlam *et al.*, 2000). Since increased intestinal permeability has been described in AS and IBD, it has been speculated that a disturbed gut/blood barrier could facilitate the interaction between bacteria and the immune system and might initiate or perpetuate disease pathogenesis (Rudwaleit & Baeten, 2006).

2.4.1.2 *Genetics of AS*

A breakthrough in AS and SpA pathogenesis research came in 1973 when the association between disease and the possession of the class I major histocompatibility complex (MHC) allele HLA-B27 was discovered (Brewerton *et al.*, 1973). The MHC class I molecule is involved in the presentation of antigen at the cell surface to T-cells for immune surveillance. HLA-B27 is present in the overwhelming majority of individuals suffering from ankylosing spondylitis (AS) – around 90-95% – and is also associated with reactive arthritis (ReA) and IBD-associated SpA. However, possession of the allele alone is not sufficient to cause onset of AS since only 5% of HLA-B27-positive individuals develop the disease in the absence of family history.

Currently, over 30 HLA-B27 subtypes have been identified. The HLA-B*2705 subtype is considered the ‘parent’ subtype since it is found in all populations and is highly associated with disease. Many of the other subtypes differ from HLA-B*2705, and from each other, by only a few amino acids. Most of the more common subtypes – which include HLA-B*2705, HLA-B*2702, HLA-B*2704 and HLA-B*2707 – are associated with disease whereas HLA-B*2706 and HLA-B*2709 – found in south-east Asia and Sardinia – do no appear to be associated with disease. The molecular differences between HLA-B27 subtypes will be discussed in more detail later.

Recently, two further genetic loci besides HLA-B27 have been associated with AS. The interleukin 23 (IL-23) receptor (IL-23R) and ARTS1/ERAAP were recently identified by SNP analysis (WTCCC, 2007). The IL-23R is involved in the inflammatory response through the promotion of expansion and survival of the IL-17-producing Th17 subset of T-cells suggesting that, in AS patients, these cells might expand under the influence of IL-23 (McGeachy *et al.*, 2009; Melis & Elewaut, 2009). There is also growing evidence that IL-17 is a crucial proinflammatory cytokine since it can act on several cell types including macrophages, fibroblasts, endothelial and epithelial cells to upregulate TNFα, IL-6 and IL-1 (Nakae *et al.*, 2003; Kolls *et al.*, 2004). Aside from IL-17, Th17 cells are also capable of producing TNFα and IL-6 themselves (Cua *et al.*, 2003; Chen *et al.*, 2006). Thus, it is clear that the downstream effects of Th17 expansion may include the production of multiple proinflammatory cytokines.

ARTS1 is an ER-localised aminopeptidase which may trim oligopeptides for antigen presentation (Kastelein *et al.*, 2007). Recently, specific ARTS1 gene polymorphisms have been associated with HLA-B*2705 in AS patients in a Hungarian population (Pazár *et al.*, 2010). Further studies have also linked IL-23R polymorphism with AS (Rahman *et al.*, 2008; Rueda *et al.*, 2008). Additionally, the Th17 subset has been shown to be present at elevated levels in both AS and rheumatoid arthritis (RA) indicating a role for these cells in human inflammatory disease (Shen *et al.*, 2009). Further work, however, is required to understand the associations of IL23R and ARTS1 polymorphisms with disease as well as the impact of polymorphisms on disease pathogenesis. Thus, in addition to HLA-B27, susceptibility to AS may be determined by genes which lie outside the MHC.

2.4.1.3 *Animal models*

In order to understand the pathogenesis of AS, animal models of AS have been developed through the generation of HLA-B27 transgenic rats and mice. Generation of transgenic mice was initially met with disappointment since these animals failed to exhibit inflammatory disease (Kievits *et al.*, 1987). However, further studies revealed that the SpA phenotype could be observed when the endogenous gene for mouse β_2-microglobulin (β_2m) – the accessory molecule which stabilises the MHC class I heavy chain and is necessary for peptide loading and ER egress – was deleted (Khare *et al.*, 1995; Khare *et al.*, 1997; Khare *et al.*, 1998). These transgenic mice have lower cell surface expression of mature HLA-B27 because of the lack of mouse β_2m and may implicate free heavy chains in disease pathogenesis (Khare *et al.*, 1996).

However, the majority of studies have been carried out on the successful and reproducible HLA-B27/human β_2m transgenic rat model developed by Hammer and colleagues (Hammer *et al.*, 1990). This model showed that under certain conditions, overexpression of HLA-B27 was sufficient to induce the development of spontaneous multisystemic inflammation which resembled that of human SpA. This finding was crucial since it was the first piece of evidence that the gene product of HLA-B27 was involved in disease. The phenotype exhibited by B27/hβ_2m transgenic rats included inflammation of the gastrointestinal tract, similar to that of human ulcerative colitis, inflammatory lesions in the skin and nails (resembling psoriasis) and peripheral arthritis involving predominantly the hind limbs. Axial inflammation was rare and ankylosis of the spine – seen in human AS – was not detected (Taurog *et al.*, 1999). However, subsequent studies have demonstrated that further increasing

the expression of transgenic $h\beta_2m$ in B27/$h\beta_2m$ transgenic rats can alter the phenotype and result in more severe arthritis and significant axial disease without affecting the severity of colitis (Tran *et al.*, 2006). Furthermore, expression of the disease phenotype was dependent on the genetic background which implicates the significance of other genes in the development of human SpA.

There is strict correlation between the susceptibility to disease and the level of HLA-B27 transgene expression, which, in turn, is determined by the number of copies of the transgene integrated into the rat genome (Taurog *et al.*, 1993). It was also discovered that a threshold number of copies of the integrated HLA-B27 transgene was required for the disease phenotype. It may also be the case that B27 expression has an impact on human SpA since there is evidence of increased expression of B27 on the cell surface of peripheral blood mononuclear cells (PBMCs) of AS patients compared with normal individuals (Cauli *et al.*, 2002). This difference in expression was not accompanied by general class I upregulation suggesting that high levels of B27 expression may be a contributing factor to disease. The specificity of inflammatory disease for a HLA-B27 transgene was established by showing that HLA-B*0702/$h\beta_2m$ transgenic rats with comparable copy number and expression level to the B27/$h\beta_2m$ disease model does not develop an inflammatory phenotype (Taurog *et al.*, 1999). Taken together, these studies provide direct evidence for a causative role of HLA-B27 in disease.

Other contributing factors to disease in transgenic rats include exposure to bacterial flora. When disease-prone B27/$h\beta_2m$ transgenic rats were reared in sterile conditions, both gut and joint inflammation were completely prevented, whereas

exposure to normal gut flora – particularly *Bacteroides* ssp. – triggered gut inflammation and arthritis (Taurog *et al.*, 1994; Rath *et al.*, 1996). Despite this, the development of skin lesions in these rats was independent of bacterial exposure. Germ-free conditions still allowed for the presentation of antigen, therefore it is possible that non-viable microorganisms may have played a role in the pathogenesis of skin lesions.

In the ANKENT mouse model – which develops ankylosing enthesopathy – the enthesitis and ankylosis phenotype are highly dependent on bacterial flora since mice bred in germ-free conditions are also protected from disease (Rehakova *et al.*, 2000). Thus, enteric bacteria may play a critical role in triggering SpA-like disease in B27/hβ_2m transgenic rats and ankylosis and enthesitis in ANKENT mice. If this is the case in human disease, the requirement for specific gut flora in triggering disease might partially account for the fact that not all B27-expressing individuals develop disease. In addition to this, several gastrointestinal and genito-urinary pathogens including *Campylobacter* ssp., *Chlamydia* ssp., *Salmonella* ssp. and *Shigella* ssp. have been implicated as triggers of HLA-B27-associated reactive arthritis (Colmegna *et al.*, 2004).

2.4.2 *MHC Class I molecule*

As demonstrated by the HLA-B27/hβ_2m transgenic rat, the HLA-B27 gene product plays a key role in the development of AS. Since the presence of pathogens seems to be correlated with disease development, pathogenesis of the disease has been linked with the physiological role of the class I molecule. The role of the MHC class I molecule in the cell is to present antigenic peptide at the cell surface as part of the

innate immune system (Pamer and Cresswell, 1998). Fragments of cellular proteins are bound by MHC class I molecules – expressed on all nucleated cells – in the ER and transported to the cell surface allowing the cell to display a sample of their expressed genes. $CD8^+$ cytotoxic T-cells monitor antigen presented by the MHC class I molecules and are capable of mounting an immune response (including the promotion of cytotoxicity and the production of cytokines such as interferon-γ (IFN-γ) which promote inflammation and stimulate antigen presentation) when foreign antigen – as a result of viral infection for example – occurs. The MHC class I molecule comprises a 45-kD heavy chain comprising an α1, α2 and α3 domain which is non-covalently associated with a 12-kD soluble accessory molecule called β_2m (which has been previously mentioned). Finally, a short 8-10 amino acid peptide is bound in the membrane-distal domains of the heavy chain – known as the peptide binding groove – to form the mature MHC class I molecule. Peptides which are too large for presentation by class I molecules are subsequently trimmed by the gene product of ARTS1/ERAAP, polymorphisms in which are associated with AS (as mentioned previously) (Saric *et al.*, 2002; York *et al.*, 2002). Peptide binding differences between different alleles of MHC class I are the result of extensive polymorphisms, particularly in the amino sequences within the peptide binding groove.

2.4.2.1 *MHC class I folding events in the ER*

The ER is the site of both class I assembly and acquisition of antigen for presentation. The MHC class I molecule must reach its native conformation before receiving high affinity peptides for presentation. The class I heavy chain achieves its correct structure and acquires peptide whilst within the peptide loading complex

(PLC). However, early heavy chain folding events are independent of the PLC. The class I heavy chain is a glycoprotein, and, a short time after cotranslational translocation into the ER, the nascent class I heavy chain is bound by the transmembrane lectin-like chaperone calnexin, which retains the heavy chain in the ER, stabilises class I assembly intermediates and may enhance the association of the class I heavy chain with β_2m (Tector & Salter, 1995; Vassilakos *et al.*, 1996). Calnexin, via its extended arm-like domain, or P-domain, is associated with the substrate-specific thiol-oxidoreductase ERp57 (mentioned previously) which promotes the oxidation of the two highly conserved internal disulfide bonds within the heavy chain (Tector *et al.*, 1997; Lindquist *et al.*, 1998; Morrice & Powis, 1998; Farmery *et al.*, 2000; Dick, 2004; Russell *et al.*, 2004; Kozlov *et al.*, 2006). ERp57 has been shown to require the presence of calnexin or calreticulin to promote disulfide bond formation in glycosylated substrates *in vitro*, however, recent evidence has demonstrated that ERp57 can catalyse heavy chain disulfide bond formation – and substrate specific oxidative folding – in the absence of interactions with calnexin and calreticulin (Zapun *et al.*, 1998; Jessop *et al.*, 2007; Zhang et al., 2009). ERp57 depletion by RNAi delayed heavy chain disulfide bond formation and slowed folding of the α3 domain. Taken together, the data implies that ERp57 is recruited by calnexin and calreticulin to incompletely folded proteins bearing N-linked oligosaccharides but that ERp57 may be capable of recognising substrates directly (Zhang *et al.*, 2006).

Oxidation of the disulfide bond in the α3 domain has been shown to be fast and efficient (Tector *et al.*, 1997). Once this disulfide has been formed, the accessory molecule β_2m binds to the heavy chain, forming a HC- β_2m heterodimer. Evidence

supporting this comes from β_2m-deficient cell lines and *in vitro* studies which showed that in the absence of β_2m, the α3 disulfide but not the α2 disulfide is formed and that β_2m is required for the formation or maintenance of the α2 disulfide (Ribaudo & Margulies 1992; Wang *et al.*, 1994). Newly assembled HC-β_2m heterodimers then enter the PLC in a process typically accompanied by the loss of calnexin-ERp57 and its replacement with calreticulin-ERp57 (Sadasivian *et al.*, 1996). There has been evidence to suggest that complete disulfide oxidation of the heavy chain is required for integration into the PLC (Smith *et al.*, 1995; Tector *et al.*, 1997). Thus, it is possible that the α2 disulfide is unstable and after formation is stabilised by β_2m association and integration into the PLC, which may protect it from reduction (Dick, 2004).

The PLC is a multiprotein complex comprising the HC-β_2m heterodimer, calreticulin, ERp57, the type I transmembrane glycoprotein tapasin and the heterodimeric transmembrane protein transporter associated with antigen presentation (TAP). The tapasin molecule is key in increasing the peptide loading efficiency of the PLC since it acts as a physical bridge between the peptide-receptive HC-β_2m heterodimer and the site of entry of peptides into the ER, i.e. TAP. (Sadasivian *et al.*, 1996; Williams *et al.*, 2002; Momberg & Tan, 2002; Tan *et al.*, 2002). Tapasin also forms a stable disulfide bridge via its Cys^{95} residue with Cys^{57} of ERp57 (Peaper *at al.*, 2005). In addition, ERp57 is also associated with calreticulin in the PLC in the same manner as calnexin (Oliver *et al.*, 1999; Ireland *et al.*, 2008).

The TAP transporter transports peptide fragments into the ER lumen and has a preference for peptides ~8-16 residues in length (Scholz & Tampe, 2009). In *TAP*$^{-/-}$ mice, class I molecules either lack peptide or are bound with low affinity peptides and consequently, $CD8^+$ T-cell responses are impaired (van Kaer *et al.*, 1992). This leads to prolonged association of the HC-β_2m heterodimer with the PLC and the relatively few molecules that reach the cell surface dissociate rapidly (Ljunggren *et al.*, 1990; Baas *et al.*, 1992). Thus, the binding of high affinity peptides is crucial for release from the PLC, ER egress and stable cell surface expression. The absence of tapasin results in a decrease in the stability of TAP as well as failure of the class I molecule to be recruited to the PLC in the proximity of TAP together resulting in decreased peptide transport into the ER lumen, reduced loading of high-affinity peptides and thus, reduced cell surface expression (Ortmann *et al.*, 1997; Garbi *et al.*, 2003).

Several groups have highlighted the importance of tapasin in class I peptide selection in addition to its role as a structural component of the PLC. Studies have demonstrated that tapasin is involved in optimising the peptides class I molecules bind by exchanging abundant low affinity peptides for those which bind more stably in a process known as peptide-editing (Williams *et al.*, 2002; Howarth *et al.*, 2004). Furthermore, a mutant soluble form of tapasin which does not interact with TAP but does bind to class I molecules, presumably via ERp57 and calreticulin, was discovered (Lenher *et al.*, 1998). Despite not being located near the source of peptide, normal peptide loading and cell surface expression of these class I was supported, indicating that tapasin can promote high-affinity peptide binding independent of its ability to recruit class I molecules to TAP. Recent work has

highlighted the importance of ERp57 in this mechanism. The ERp57-tapasin heterodimer was demonstrated to promote efficient loading of high-affinity peptides and stabilise peptide-receptive class I molecules where tapasin alone was ineffective (Wearsch & Cresswell, 2007).

The role of the ERp57-tapasin disulfide bond in peptide loading is not fully understood, however, abolition of the disulfide by mutagenesis results in the loading of low affinity peptides, incomplete oxidation, and the ER egress of unstable class I molecules (Dick *et al.*, 2002). Tobias Dick's group demonstrated the possibility that the ERp57-tapasin conjugate is directly involved in the isomerisation of the class I α2 domain disulfide providing further evidence that the class I heavy chain enters the PLC in a non-fully native conformation. However, ERp57-deficient mice show that ERp57 does not influence the redox state of class I molecules but acts instead as a structural component of the PLC (Garbi *et al.*, 2006). Additionally, ERp57 depletion seems to have no effect on the redox state of the class I heavy chain or peptide loading (Zhang *et al.*, 2006). Mutagenesis experiments to inactivate the two redox-active sites of ERp57 have demonstrated that the enzymatic activity of ERp57 is not required for its functions within the PLC, indicating that it plays a structural role rather than a catalytic one within the PLC (Peaper & Cresswell, 2008; Zhang *et al.*, 2009). These findings have been confirmed by the crystal structure of the ERp57-tapasin conjugate, published recently, which showed that the two active sites of ERp57 are buried in the interaction interface with tapasin (Dong *et al.*, 2009). This is further supported by evidence from Kienast and colleagues, who showed that, in the absence of tapasin, class I heavy chains were subject to rapid α2 disulfide reduction indicating that tapasin restrains the α2-directed redox activity of ERp57 by covalent

sequestration (Kienast *et al.*, 2007). The apparent structural role of ERp57 in class I folding and peptide loading requires further investigation.

The influence of the HC α2 domain isomerisation on peptide loading has been investigated. Recently, PDI was implicated in optimal peptide selection by regulating the oxidation of the heavy chain α2 disulfide (Park *et al.*, 2006). Their data demonstrated that suboptimal peptide binding renders the α2 disulfide more susceptible to PDI-mediated reduction whereas high affinity peptides protect the disulfide from reduction. Since oxidation of the α2 disulfide is necessary for optimal peptide loading, PDI appears to regulate the binding of high-affinity peptides in this regard. Despite this, Kienast and colleagues were unable to detect an interaction of PDI with the PLC (Kienast *et al.*, 2007). Thus, there seems to be a complex relationship between the redox status of the class I heavy chain and the binding of high-affinity peptides, which may involve components outside of the PLC.

Similar to PDI and ERp57, the transmembrane thioredoxin-related protein TMX has also been implicated in oxidative protein folding of MHC class I molecules since it was found in mixed disulfide intermediates with the class I heavy chain (Matsuo *et al.*, 2009). Unlike PDI and ERp57 however, TMX was not essential for normal assembly of the MHC class I molecule since TMX knock-down did not affect class I cell surface expression. However, in this study, TMX prevented the retrotranslocation of misfolded MHC class I heavy chains and, furthermore, TMX-class I HC complexes were promoted when cells were treated with the ER stress inducer tunicamycin. Together, these results indicate that TMX may play a role in

regulating the degradation and refolding of misfolded or incompletely folded proteins in the ER.

Upon acquiring high affinity peptides, class I molecules are released from the PLC, transit through the Golgi and are transported to the cell surface via the exocytic pathway. There is some evidence of PLC components being detected in the ER-Golgi intermediate compartment (ERGIC)/*cis*-Golgi as well as peptide-receptive class I molecules. This calls into question whether peptide-receptive class I molecules remain solely in the ER before acquiring peptide ligand or are recycled back into the ER (Garstka *et al.*, 2007). Fully-folded, peptide-loaded class I molecules then present peptide at the cell surface to $CD8^+$ T-cells and natural killer (NK) cell bearing certain receptor families.

2.4.3 *HLA-B27 and AS*

The features of HLA-B27 which distinguish it from other MHC class I alleles have provided the basis for investigation into the pathogenesis of AS and SpA. These can broadly be separated into two groups relating either to immunological recognition of some form of the HLA-B27 heavy chain or to intracellular events.

2.4.3.1 *Arthritogenic peptide hypothesis*

The first concept arises from the ability of HLA-B27 to present self-peptide to $CD8^+$ T cells. The so-called arthritogenic peptide hypothesis states that disease is a consequence of autoreactivity caused when a specific self-peptide presented by folded HLA-B27/β_2m becomes the target of autoreactive $CD8^+$ T-cells because it resembles foreign peptide derived from either intracellular bacteria or viruses

(Benjamin & Parham, 1990). In this theory, chronic inflammatory disease results from T-cell mediated cytotoxicity. This theory is supported by evidence of HLA-B27-restricted $CD8^+$ T-cell clones with specificity for bacteria which have been detected in the synovium and peripheral blood of AS and ReA patients (Hermann *et al.*, 1993). Additionally, $CD8^+$ T-cells reacting with collagen-derived self-peptides in a B27-dependent manner were detected in the synovium of AS patients (Atagunduz *et al.*, 2005). Aside from this, recurrent structural motifs were discovered in T-cell receptors of B27-specific T-cells from unrelated individuals (May *et al.*, 2002). Despite all of these studies, candidate arthritogenic peptides have not yet been identified. However, a self-peptide derived from the cytoplasmic tail of HLA-B27 and other MHC class I molecules which is constitutively presented by three disease associated subtypes (B*2705, B*2702 and B*2704) but not presented by the non-disease associated subtypes B*2709 or B*2706 was discovered (Ramos *et al.*, 2002). This finding showed great promise since this peptide shows much similarity to an enzyme found in *Chlamydia trachomatis*, a member of a genus of gram-negative bacteria which can trigger ReA. However, attempts to detect presentation of the corresponding chlamydial peptide by HLA-B27 have been unsuccessful (Lopez de Castro, 2006).

Comparing HLA-B27-restricted $CD8^+$ T-cell responses from AS patients with healthy individuals provides another method for identifying candidate peptides. Studies with peripheral blood lymphocytes identified a specific peptide – present in both the HLA-B27 heavy chain and enteric bacteria – which is preferentially recognised by HLA-B27-restricted $CD8^+$ T-cells in AS patients compared with healthy individuals (Schofield *et al.*, 1995; Frauendorf *et al.*, 2003). This finding

supports the idea of cytotoxic T-cells being important in disease pathogenesis but does not provide direct evidence of an arthritogenic peptide since numerous other HLA-A and HLA-B alleles, not associated with AS, also possess this peptide. However, the arthritogenic peptide hypothesis remains a popular theory of pathogenesis since only a small number of polymorphic HLA-B27 subtypes are associated with the disease (Colbert *et al.*, 1994; D'Amato *et al.*, 1995; Lopez-Larrea *et al.*, 1995).

This theory is met by a few further problems. If disease associated subtypes of HLA-B27 present arthritogenic peptides which are unable to be presented by non-disease associated subtypes, there is difficulty in explaining the apparent mutual exclusivity of the peptide repertoire of HLA-B*2705 and HLA-B*1403 despite both being associated with AS (HLA-B*1403 is an example of an AS-associated allotype found in B27-negative individuals in Sub-Saharan populations) (Lopez-Larrea *et al.*, 2002; Merino *et al.*, 2005). Data from the B27 transgenic mouse model demonstrates that SpA-like disease only develops in the absence of β_2m and indicates that stable, fully-formed B27/β_2m/peptide complexes are not required for disease (Khare *et al.*, 1995; Kingsbury *et al.*, 2000). Furthermore, results from B27/hβ_2m transgenic rats where CD8$\alpha\beta^+$ T-cells have been depleted using antibodies, demonstrate that B27/CD8$^+$ T-cells interactions might not be necessary for disease since depletion of these cells does not affect the development of arthritis or gut inflammation (May *et al.*, 2003). Recently, SpA-like disease was detected in CD8$\alpha^{-/-}$ knock-out rats with non-functional CD8$^+$ T-cells, further indicating that classical MHC class I-T-cell interactions are non responsible for disease (Taurog *et al.*, 2009). However, T-cells in general *are* required for inflammatory disease perhaps implicating CD4$^+$ T-cells,

or other cell types bearing CD8 such as macrophages and NK cells, in disease pathogenesis (Breban *et al.*, 1996; Colonna *et al.*, 1999; Smith *et al.*, 2006).

2.4.3.2 *Aberrant* cell *surface heavy chains*

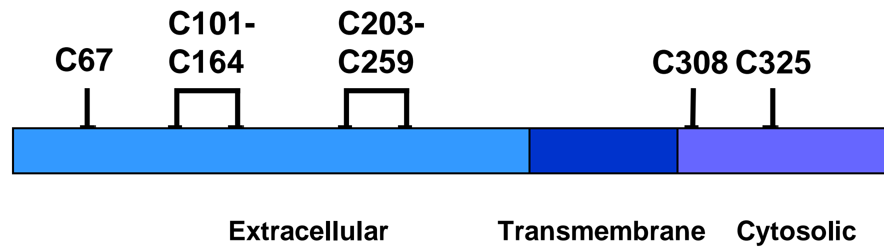
At the end of the last decade, a feature of HLA-B27 unrelated to antigen presentation was described (Allen *et al.*, 1999). Allen and colleagues demonstrated that HLA-B27 formed disulfide-linked heavy chain homodimers *in vitro*. These heavy chain homodimers form without β_2m and are capable of binding peptide. Covalently linked heavy chain homodimers are established via the unpaired cysteine residue that HLA-B27 possesses at position 67 (Fig. 5). The stable homodimers form via an endosome-dependent recycling pathway when fully folded, but unstable, B27/β_2m heterodimers dissociate at the cell surface (Bird *et al.*, 2003). HLA-B27 heavy chain homodimers have been detected at the cell surface of lymphoblastoid cells, rat dendritic cells in the HLA-B27 transgenic rat model and HLA-B27 transfected cells (Allen *et al.*, 1999; Kollnberger *et al.*, 2002; Bird *et al.*, 2003; Kollnberger *et al.*, 2004).

The detection of aberrant forms of HLA-B27 at the cell surface has led to the theory that disease pathogenesis might be the consequence of the modulation of leukocyte function through the specific recognition of these non-canonical versions of HLA-B27. Along with T-cell receptors, mature class I molecules have been shown to bind several other immunomodulatory molecules including a) members of the killer immunoglobulin-like receptor (KIR) family, such as KIR3DL1, which recognises HLA-B27 as well as other HLA class I alleles and b) members of the leukocyte immunoglobulin-like receptor (LIR) (also known as immunoglobulin-like transcripts (ILT)) family (Peruzzi *et al.*, 1996; Andre *et al.*, 2001).

However, further studies on immune receptor recognition have led to the discovery that KIR3DL1, LIRB2 and LIRA1 all recognise the homodimeric form of HLA-B27 in addition to B27/β_2m with bound peptide (Allen *et al.*, 2001; Kollnberger *et al.*, 2002). Furthermore, the KIR3DL2 receptor binds homodimeric B27 but does not bind B27/β_2m heterodimers (Kollnberger *et al.*, 2002). KIR3DL2 does bind HLA-A3 and A11 but is dependent on the specific peptide bound by these class I molecules. On the other hand, KIR3DL1 and KIR3DL2 binding of homodimeric B27 is independent of bound peptide sequence (Chan *et al.*, 2005).

Both KIR3DL1 and KIR3DL2 transmit inhibitory signals through their immunoreceptor tyrosine-based inhibitory motifs and expression of KIR3DL2 has been linked with increased cell survival of T-cells and NK cells (Young *et al.*, 2001). Furthermore, KIR3DL2 is expressed at increased levels in the periphery and synovium of AS patients compared with healthy individuals (Chan *et al.*, 2005). Since development of AS is not associated with the KIR3DL2-binding HLA-A3 and A11 alleles, it is likely that the expansion of KIR3DL2-expressing lymphocytes results from unique properties of the B27 homodimer-KIR3DL2 interaction.

A



B

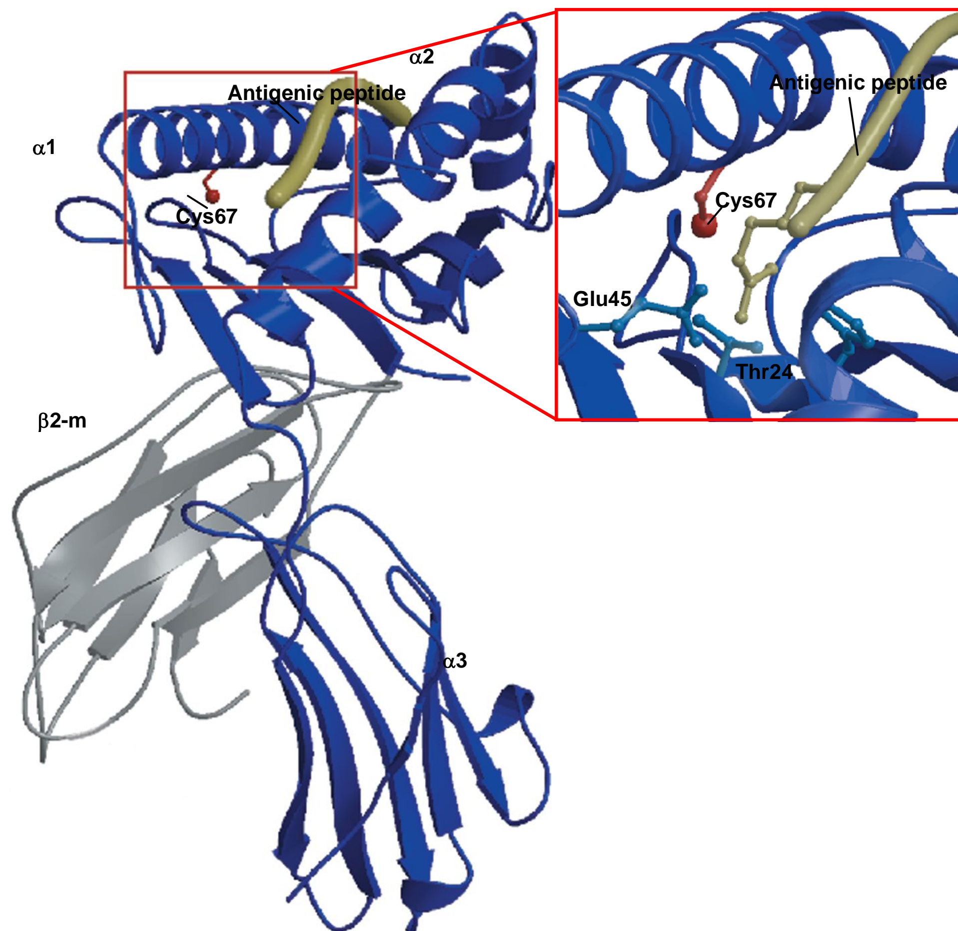


Figure. 5. **The structure of HLA-B27**. A, The position of the disulfide bonds of HLA-B27 are shown as well as the free cysteines in the extracellular and cytosolic domains. B, A ribbon diagram of the extracellular domains of the HLA-B27 heavy chain using the crystal structure coordinates; adapted from Bowness *et al.*, 1999. The α1, α2 and α3 domains are labelled as well as the accessory molecules β_2-microglobulin (β_2m). A close-up view of the B-pocket shows the proximity of the free cysteine at position 67 to the peptide binding groove and to the side chains which are of importance to the B-pocket and P2 anchor region.

Chan and colleagues also demonstrated expansion of $CD4^+$ T-cells in SpA patients (Chan *et al.*, 2005). As mentioned previously, depletion of T-cells but not $CD8\alpha\beta^+$ T-cells prevents SpA-like disease in the transgenic rat model. It has been suggested that $CD4^+$ T-cells might be involved in disease pathogenesis since $CD4^+$ T-cells have been demonstrated to recognise HLA-B27 which is expressed in cells with defective antigen processing pathways – and thus present an array of B27 conformations including homodimers – but not classical B27/β_2m/peptide complexes (Boyle *et al.*, 2001; Boyle *et al.*, 2004). Recently, it has been demonstrated that dendritic cells, from the B27/hβ_2m transgenic rat are dysfunctional since the cell surface expression of mature B27 impairs the formation of an immunological synapse with $CD4^+$ T-cells which may affect the production and/or maintenance of regulatory T-cells, contributing to the expansion of pathogenic $CD4^+$ T-cells (Stagg *et al.*, 1995; Hacquard-Bouder *et al.*, 2007). Taken together, these studies demonstrate that cell surface homodimers of HLA-B27 can interact with several different immune receptors that are found on some cell types – such as NK and $CD4^+$ T-cells – which are expanded during inflammation. Thus, the expression of aberrant forms of HLA-B27 at the cell surface may be important for the pathogenesis of AS.

2.4.3.3 *HLA-B27 misfolding in the ER*

In 1999, Robert Colbert's research group demonstrated that, under normal physiological conditions, HLA-B27 heavy chains were being degraded shortly after synthesis (Mear *et al.*, 1999). ERAD had previously been described as the route for the disposal of misfolded MHC class I molecules (Hughes *et al.*, 1997). Furthermore, this study revealed the importance of the B pocket region of HLA-B27 on folding efficiency. The B pocket, a region of the peptide binding groove which plays a key

role in peptide selection, was thought to be important for the arthritogenic phenotype since it is conserved among B27 subtypes and thus among those subtypes associated with disease (Colbert *et al.*, 1994; Gonzalez-Roces *et al.*, 1997; Lopez de Castro, 1998). Colbert's group demonstrated that amino acids located in the $\alpha 1$ domain, which ultimately form the B pocket, were responsible for the inefficient folding of the B27 heavy chain. This inefficient folding was associated with heavy chain dislocation and degradation by the proteasome indicating that B27 heavy chains were misfolding – defined as an inability to reach a native conformation or preferentially form abnormal conformations – even in the presence of a normal supply of β_2m and peptide.

Further studies have now revealed that HLA-B27 does indeed form abnormal conformations in the ER. Heavy chains spontaneously form misfolded disulfide-linked complexes in the ER and make up a substantial portion (~25%) of newly synthesised heavy chains (Dangoria *et al.*, 2002). However, in this study, the fraction of newly synthesised heavy chains that were being degraded was much less than 25% indicating that heavy chain homodimers are retained in the ER and may still undergo constructive folding events. The ER pool of heavy chain homodimers is distinct from those found at the cell surface since cell surface homodimers are the result of fully folded, β_2m/peptide-associated class I molecules which have become unstable and dissociated, whereas the ER pool of homodimers is the result of an inability to efficiently reach a native conformation.

Intracellular MHC class I heavy chain dimerisation is not unique to HLA-B27 since the mouse class I alleles H-2Ld, H-2Dd and H-2Db have been shown to dimerise

(Capps *et al.*, 1993). However, these mouse alleles form ER dimers in the absence of β_2m whilst ER B27 dimers have been demonstrated to form in the presence of β_2m (Mear *et al.*, 1999). These mouse heavy chains do not possess a free, unpaired cysteine in their extracellular domain and form instead via cytoplasmic tail cysteines. Consistent with studies of homodimer formation at the cell surface, the unpaired cysteine at position 67 – shared by a relatively small number of class I alleles including HLA-B14, -B15, -B38, -B39 and -B73 – was shown to be crucial for homodimer formation in the ER (Kostyu *et al.*, 1997; Dangoria *et al.*, 2002). A mutant form of B27 which folds rapidly and efficiently but does possess an unpaired Cys^{67} does not form homodimers indicating that impaired folding of newly synthesised B27 heavy chains leaves the Cys^{67} residue exposed and therefore prone to the formation of aberrant complexes with other heavy chains. However, substitution of the unpaired cysteine with alanine in disease associated subtypes HLA-B*2704 and B*2705 does not prevent homodimer formation when overexpressed (not in conjunction with increased expression of β_2m) suggesting that these homodimers may form via cytoplasmic tail cysteines (Saleki *et al.*, 2006). This is consistent with homodimer formation in the mouse alleles since heavy chain homodimer formation in these non-Cys^{67}-possessing heavy chains only occurs in the absence of sufficient β_2m.

Further evidence for the role of B27 folding efficiency in homodimer formation comes from Simon Powis' group who showed that introduction of Cys^{67} into rapidly folding HLA-A2 did not lead to homodimer formation indicating that Cys^{67} is not sufficient for homodimer formation (Antoniou *et al.*, 2004). Furthermore, they were able to mimic an HLA-B27 folding phenotype in HLA-A2 by incubating cells at

$26^{o}C$ leading to a decrease in heavy chain folding kinetics and the consequential formation of heavy chain homodimers. Incubation at this temperature also enhanced homodimer formation in HLA-B27 heavy chains. Moreover, the structural cysteine at position 164 also appears to be important for homodimer formation since the *C164S* mutation prevents the formation of B27 homodimers indicating that this cysteine may be involved in interchain disulfides or that the Cys^{101}-Cys^{164} structural disulfide is essential for homodimer formation.

The heavy chain misfolding hypothesis should explain the differential association of HLA-B27 subtypes with disease. Subtypes not associated with AS, B*2706 and B*2709, fold much more efficiently than the disease associated subtypes B*2702, B*2704 and B*2705 which is indicative of a relationship between folding properties and development of disease (Goodall *et al.*, 2006; Lopez de Castro, 2007; Galocha & Lopez de Castro, 2008). However, the B*2707 subtype, which shows association with disease, folds as efficiently as non-AS-associated subtypes. Thus, there appears to be correlation between inefficient heavy chain folding and AS susceptibility except for HLA-B*2707.

Studies with splenocytes from the transgenic rat model of AS also identified B27 disulfide-linked heavy chain homodimers and higher order multimers (Tran *et al.*, 2004). Heavy chain homodimers were identified in the B27/hβ_2m transgenic rat but not in the B7/hβ_2m transgenic rat, which remains healthy, suggesting a possible correlation between heavy chain homodimer formation and disease. Furthermore, in line with previous studies, the Cys^{67} residue was found to be non-essential for homodimer formation although the size and number of high molecular weight

complexes in *C67S* mutant spleen cells was altered compared with wild-type B27 (Dangoria *et al.*, 2002; Tran *et al.*, 2004). B27-*C67S*/hβ_2m transgenic rats do not develop as severe a disease phenotype than wild-type B27/hβ_2m transgenic rats which might suggest that B27 heavy chain homodimers formed in the presence of an unpaired Cys^{67} might be more arthritogenic than homodimers formed via alternative cysteines (Taurog *et al.*, 1999; Tran *et al.*, 2004). However, the transgene copy number in this rat is lower than the wild-type transgenic rat implying that the milder phenotype might be the result of a low copy number of B27 or indeed of hβ_2m which would result in a high ratio of B27 to β_2m. In the study by Tran and colleagues, mentioned earlier, additional hβ_2m was introduced by cross breeding and led to an increase in severity and duration of arthritis but an absence of colitis and gut inflammation. It is interesting to note that in this study B27 heavy chain homodimers were abolished (Tran *et al.*, 2006). These results suggest that 1) the expression of hβ_2m has an influence on B27 heavy chain misfolding and 2) that B27 misfolding and homodimer formation plays a role in gut inflammation but not necessarily in arthritis.

The location of heavy chain homodimers from the transgenic rat was not identified by Tran and colleagues, but, consistent with findings from Dangoria and colleagues, free heavy chains were abundantly immunoprecipitated from B27 transgenic cell lysates with anti-BiP, whereas little BiP was found bound to B7 heavy chains (Dangoria *et al.*, 2002; Tran *et al.*, 2004). These results suggest that disulfide-linked B27 homodimers from the transgenic rat exist as unfolded heavy chains complexed with the ER chaperone BiP. One job of the chaperone BiP is to retain misfolded

proteins in the ER in order to prevent inefficiently folded proteins from exiting the ER into the secretory pathway. Thus, association of BiP with HLA-B27, although a quality control measure, may indeed serve to promote aberrant disulfide formation since misfolding is a consequence of prolonged retention in the ER.

The observation that HLA-B27 misfolds and forms disulfide-linked homodimers and binds stably to the ER chaperone BiP in both transfected cell models and the B27/hβ_2m transgenic rat model, led to the proposal that B27 heavy chains accumulate in the ER causing ER stress and that consequential activation of the UPR might contribute to disease pathogenesis (Mear *et al.*, 1999; Colbert, 2000). This theory was bolstered significantly by a key study in 2005 which demonstrated that, in bone marrow-derived macrophages (BMDM) from the B27/hβ_2m transgenic rat, heavy chain misfolding is associated with activation of the UPR (Turner *et al.*, 2005). B27-expressing cells exhibited overexpression of the UPR target genes *BiP*, *CHOP* and *XBP1* as well as increased *XBP1* splicing.

In line with disease severity in the transgenic rat, UPR activation is dependent on the expression level of HLA-B27. There was no UPR detected in spleen or thymus from premorbid rats – which all express HLA-B27 – suggesting that ongoing UPR activation is not a widespread phenomenon in these animals. However, when cells from these animals are treated with IFN-γ, which induces HLA-B upregulation, UPR activation is observed (Taurog *et al.*, 1993; Turner *et al.*, 2005). However, it is possible that UPR activation is a consequence of IFN-γ-induced upregulation of other gene products. Thus, this study linked, for the first time, increased expression of HLA-B27, misfolding and accumulation of its heavy chain in the ER, induction of

UPR and B27-associated disease. The absence of a UPR in spleen, thymus and macrophages from premorbid rats might be due to many factors including 1) insufficient levels of heavy chains expressed in unstimulated cells 2) these cell types may express different levels of other class I assembly pathway components or 3) these cell types have adapted to chronic misfolding and increased load on the ER.

The role of IFN-γ in HLA-B27 expression and UPR activation has been addressed by several recent investigations. IFN-γ treatment of B27-expressing BMDMs from the B27/hβ_2m transgenic rat is accompanied by increased accumulation of disulfide-linked heavy chain complexes and binding of these complexes to BiP (Turner *et al.*, 2005). However, this does not occur in the B7/hβ_2m transgenic rat. IFN-γ also upregulates components of the class I assembly pathway such as components of the proteasome, TAP, tapasin, β_2m and ARTS1/ERAAP. Thus, B27 misfolding and UPR activation occurs despite an increase in source peptides and HC folding machinery which may indicate that they may be insufficient to prevent misfolding or that one or more of these components may exacerbate heavy chain misfolding (Colbert *et al.*, 2009). It has also been postulated that IFN expression might be regulated by the UPR. Type I IFN and IFN-β are only expressed at low levels in BMDMs from the transgenic rat model or in cells undergoing pharmacological agent-induced ER stress (Lee *et al.*, 2003; Smith *et al.*, 2008). However, when macrophages undergoing a UPR are exposed to ligands for Toll-like receptors such as LPS and dsRNA, robust synergistic IFN-β production occurs (Smith *et al.*, 2008). This synergistic effect seems to require the spliced version of XBP1 and suggests a fundamental relationship between ER stress and innate immune signalling. Furthermore, HLA-

B27 homodimers have been detected in dendritic cells upon cell activation by PMA/ionomycin and peripheral blood monocyte-derived dendritic cells upon stimulation of TLR4 by LPS further indicating a link between immune cell activation and/or maturation and B27 homodimer formation (Santos *et al.*, 2008).

The various theories of pathogenesis under consideration are not mutually exclusive despite any single theory being able to explain the pathogenesis of HLA-B27-associated inflammatory disease yet. However, since ER stress and UPR activation can be pathogenic (Oyadomari *et al.*, 2002a) and B27-driven UPR activation has been demonstrated in activated immune cells (Santos *et al.*, 2008), it is likely that a UPR driven by HLA-B27 misfolding has the potential to adversely affect immune function and contribute to inflammatory disease. Moreover, if HLA-B27 misfolding can mediate inflammation, it seems that a prior event – such as interferon-mediated stimulation of B27 expression – is required to exacerbate the accumulation of misfolded heavy chains. Furthermore, the contribution of T-cell and leukocyte receptor recognition of B27 homodimers and the involvement of other genetic factors such as polymorphisms in ARTS1 and IL-23R must also not be overlooked.

2.5 *Thesis aims*

Activation of the UPR is implicated in a number of non-inflammatory and inflammatory diseases including ankylosing spondylitis. Activation of the UPR in AS is associated with the misfolding of the MHC class I heavy chain HLA-B27. The main objectives of this project were:

- To use pharmacological agents to induce ER stress in different cell types to investigate the activation of different branches of the UPR.
- To determine if there are cell-specific differences in the activation of the UPR in response to different forms of ER stress.
- To investigate the consequences of UPR activation on cell survival in different cell types.
- To examine the misfolding of HLA-B7 and HLA-B27 in different cell types and investigate the activation of the UPR in these cells.
- To examine the impact of the tyrosine kinase inhibitor genistein on UPR target gene expression.

3. MATERIALS AND METHODS

3. Materials and Methods

3.1 *Cell Lines, Tissues and Antibodies*

Human cervical carcinoma HeLa cells (a gift from J. Neefjes, NKI, Netherlands) were maintained in minimum essential medium (MEM) (22561-021; Gibco, Invitrogen), human fibrosarcoma HT-1080 cells (a gift from J. Neefjes, NKI, Netherlands) were maintained in Dulbecco's modified Eagle's medium (D-MEM) (21063-029; Gibco, Invitrogen). Both cell lines were free from mycoplasma as determined by the EZ-PCR mycoplasma test kit which detects a conserved, mycoplasma-specific 16S rRNA gene region by PCR methods (Geneflow, UK). Human lymphoblastoid, WEWAK1, JESTHOM, HOM-2 (all from ECACC, UK) and the human monocyte THP1 cells (a gift from J. Robinson, Newcastle-upon-Tyne University, UK) were maintained in RPMI 1640 (21875-034; Gibco, Invitrogen). Immortalised AS patient lymphocytes (a gift from J Goodall, Cambridge University, UK) maintained in RPMI 1640 (21875-034; Gibco, Invitrogen). These cells were generated by Goodall and colleagues from peripheral blood mononuclear cells by incubation with the supernatant of the Epstein-Barr virus (EBV)-producting cell line B95.8 at 37°C for 1 hour. Cells were then cultured in RPMI (Life Technologies Ltd, UK), supplemented with 5 % (v/v) FCS (HI FCS; Life Technologies Ltd, UK), 10mM HEPES (Sigma), 2 mM glutamine (Sigma), 100 μg ml^{-1} streptomycin sulphate BP (Evans Medical Ltd, UK) and 100 U/ml benzylpenicillin sodium BP (Glaxo, UK) in the presence of 1 μg ml^{-1} cyclosporin A. The cells were passaged at weekly intervals with fresh medium supplemented with cyclosporin A until the cell line was established, at which time the use of cyclosporin A was discontinued.

Untransfected rat thymoma C58 cells and C58 cells stably transfected with HLA-A2, HLA-B27, and a HLA-B27.H114D.D116Y mutant (a gift from A. Antoniou, University College London, UK) were maintained in RPMI 1640 (21875-034; Gibco, Invitrogen). Stable transfectants were generated by electroporation and selected in R10 medium (RPMI 1640 plus 10 % (v/v) FCS) plus 1 mg/ml G418. Vectors for stable transfection, clone selection and clone characterisation were not disclosed. Media for stable transfectants was supplemented with 1 mg/ml G418 (Sigma-Aldrich) for selection. All cell lines were supplemented with 8 % (v/v) foetal calf serum (FCS) (10106-151; Sigma-Aldrich), 2 mM Glutamax (A12860-01; Invitrogen), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (15140-122; Invitrogen) and propagated at 37°C under 5 % CO_2. Sterile cell culture passage took place every 2-5 days depended on cell density. Spent medium was removed from cells in 10 cm^2 cell culture dishes. The cells were then washed with 10 ml warm (37°C) PBS and trypsinised with 0.25 ml 1x trysin-EDTA (Invitrogen). Cell culture dishes were placed in the 37°C incubator for 5 mins or until the cells had loosened from the dish. Cells were then resuspended in 10 ml warm fresh media. One ml of this suspension was added to 9 ml warm fresh media and plated into a new cell culture dish and placed in the incubator to allow the cells to adhere.

The polyclonal rabbit antisera against PDI (Benham *et al.*, 2000), has been described. The mouse monoclonal (mAb) antibody HA-7 (H9658; Sigma-Aldrich) and the anti-myc mAb 9B11 (#2276; Cell Signalling) are both commercially available. The conformation-specific mAb W6/32 (Parham *et al.*, 1979) and the heavy chain-specific mAb HC10 recognising free B and C allele heavy chains (Stam *et al.*, 1986) were gifts from J. Neefjes, Netherlands Cancer Institute, The Netherlands. The rabbit

polyclonal antisera against the C-terminus of GRP78/BiP (H-129) (sc-13968), the rabbit polyclonal antisera against human ATF6α (H-280) (sc-22799), the rabbit polyclonal antisera raised against the N-terminus of PERK (H-300) (sc-13073), the rabbit polyclonal antisera raised against human IRE1α (H-190) (sc-20790) and the rabbit polyclonal antisera raised against mouse XBP1 (M-186) (sc-7160) are commercially available (Santa Cruz Biotechnology). The rabbit polyclonal antisera against the phosphorylated Ser51 of eukaryotic initiation factor 2 alpha (eIF2α) (#9721), the mouse monoclonal antisera against a broad range of tyrosine-phosphorylated proteins (P-Tyr-100) (#9411) and the rabbit polyclonal antisera against GRP94 (#2104) are all commercially available (Cell Signalling Technology). The mAb Jol 2, which recognises nuclear lamins A and C, was a gift from R. A. Quinlan, University of Durham, UK. The polyclonal sheep antisera against the human glycoprotein TGN46 is commercially available (AHP500; AbD Serotec).

Table 2 – Antibody concentrations

Antibody	Dilution
HC10 (mAb)	1:200-1:300 (WB)
anti-PDI (pAb)	1:1000 (WB), 1:400 (IF)
anti-HA (mAb)	1:2000 (WB, IF)
anti-myc (mAb)	1:4000 (WB)
W6/32 (mAb)	1:200-1:300 (WB)
anti-BiP (pAb)	1:200 (WB)
anti-ATF6α (pAb)	1:200 (WB), 1:100 (IF)
anti-PERK (pAb)	1:200 (WB)
anti-IRE1α (pAb)	1:200 (WB)
anti-XBP1 (pAb)	1:200 (WB)
anti-eIF2α (Ser51) (pAb)	1:1000 (WB)
anti-P-Tyr-100 (mAb)	1:1000 (WB)
anti-Grp94 (pAb)	1:1000 (WB)
Jol2 (mAb)	1:100-1:200 (WB), 1:100 (IF)
anti-TGN46	1:200 (IF)

3.2 *Constructs*

The pcDNA3-HLA-B2705 construct (confirmed by DNA sequencing) was a gift from J. Neefjes, NKI, Netherlands. This plasmid features an intermediate-early cytomegalovirus (CMV) promoter which drives high-level expression of the transgene in a variety of mammalian cell lines and a bovine growth hormone (BGH) polyadenylation signal providing transcriptional termination. The plasmid also features an ampicillin resistance gene allowing bacterial selection and a neomycin resistance gene allowing mammalian selection and an SV40 replication origin. The pCGN-ATF6α-HA construct contains an N-terminal influenza virus haemagglutinin (HA) epitope tag (SSYPYDVPDYASLGGPSR) (described in Chen *et al.*, 2002 and Tanaka & Herr, 1990) and was a gift from R. Prywes, Columbia University, New York, USA. This plasmid features a human CMV promoter, an HSV tk gene 5' untranslated leader and initiation codon, rabbit β-globulin gene splicing and polyadenylation signals and the replication origin of SV40.

3.3 *Transfection*

Plasmid DNA for transfection was transformed in DH5α *E. coli* bacteria, amplified using the mini- or maxi-prep kits (Qiagen) and purified by isopropanol precipitation. Plasmid DNA concentration was determined by spectrophotometry and diluted to 1 μg/μl. Two-3 days pre-transfection, cells were grown in 6 cm dishes to 80% confluency. Cells were then transfected with the appropriate construct using either the transfection reagent lipofectamine 2000 (11668-019; Invitrogen) or Fugene HD transfection reagent (04709705001; Roche). For lipofectamine 2000 transfection, cells were initially washed with 2 ml Hank's balanced salt solution (HBSS) (140 mg

l^{-1} $CaCl_2$, 100 mg l^{-1} $MgCl_2$-$6H_2O$, 140 mg l^{-1} $MgSO_4$-$7H_2O$, 400 mg l^{-1} KCl, 60 mg l^{-1} KH_2PO_4, 350 mg l^{-1} $NaHCO_3$, 8000 mg l^{-1} NaCl, 48 mg l^{-1} Na_2HPO_4 and 1000 mg l^{-1} Dextrose) (14025-050; Gibco, Invitrogen) then 2 ml serum-free optiMEM (11058-021; Gibco, Invitrogen). 2.5 μl lipofectamine 2000 reagent was diluted in 1 ml warm optiMEM and allowed to mix for 5 minutes. Separately, 1 μg of plasmid DNA was added to 1 ml optiMEM before being added to the lipofectamine/optiMEM mix. The 2 ml optiMEM/lipofectamine/plasmid DNA preparation was allowed to couple for 20 minutes before being added to the cells for 6 hours at 37°C. Post-incubation, cells were washed with 2 ml warm optiMEM then 2 ml warm HBSS and replaced with 2 ml fresh complete medium. Cells were then lysed 24 hours post-transfection. For Fugene HD transfection, cells were initially washed with 2 ml warm PBS and 3.5 ml fresh complete medium was added. 2.1 μg of plasmid DNA was incubated with 5.25 μl Fugene reagent in 175 μl serum-free optiMEM for 10 minutes before being added drop-wise to the cells containing fresh medium. Cells were incubated at 37°C for 24 hours before being washed twice in 2 ml warm PBS for lysis. Mock transfections were subject to all wash and incubation steps but did not receive plasmid DNA or transfection reagent.

3.4 *Cell treatments*

HeLa and HT1080 cells were split from confluent 10 cm dishes into 6 cm dishes and grown for 2-3 days prior to treatment. To induce the UPR, media was supplemented with 10 mM DTT, 1 μg ml^{-1} tunicamycin, 2 μM A23187 or 2 μM thapsigargin for the times stated. The chemicals were obtained from Sigma-Aldrich. Treatments were staggered in order to achieve the desired course of treatment, washed with PBS and

subjected to MNT or RIPA lysis or lysis in tri reagent for RNA analysis (see section 3.5 below). For experiments involving a change of media (low glucose (1000 mg/l) DMEM (D2429; Sigma-Aldrich), serum withdrawal), cells were washed twice with warm PBS before being replaced with fresh media for the times indicated. For serum withdrawal, cells were grown in the presence of serum for two days, before the media was replaced with media lacking FCS for 6 hours in the presence or absence of ER stress inducers. For the genistein (Sigma-Aldrich) experiments, a 35 mM stock was made in DMSO (Sigma-Aldrich) and supplied to the cells at 140 μM for the stated times. A vehicle control was included.

3.5 *Cell lysis*

Post-transfection and -treated cells were lysed for protein analysis by SDS-PAGE at 4 ^{o}C in either 300 μl MNT lysis buffer (1 % (v/v) Triton X-100, 30 mM Tris-HCl, 100 mM NaCl, 20 mM MES, pH 7.4) supplemented with 20 mM *N*-ethylmaleimide (NEM) (Sigma-Aldrich) and 10 μg ml^{-1} protease inhibitors chymostatin, leupeptin, antipain and pepstatin A (Sigma-Aldrich) or 300 μl RIPA lysis buffer (1% (v/v) Triton X-100, 50 mM Tris HCl pH8, 150 mM NaCl, 0.5% (w/v) Na-deoxycholate, 0.1% (w/v) SDS) supplemented with 20 mM NEM, 10 μg ml^{-1} protease inhibitor cocktail (containing chymostatin, leupeptin, antipain and pepstatin A) and 1% phosphatase inhibitor cocktail 1 (v/v) (Sigma-Aldrich). RIPA lysates were DNase treated with benzonase (30 U/ml) (Novagen) for 60 minutes on ice. Alternatively, cells were lysed in 1 ml/10 cm^2 TRI-reagent (T9424; Sigma-Aldrich) for RNA isolation according to the manufacturer’s protocol and subsequent RT-PCR.

3.6 *RT-PCR*

The concentration of extracted RNA was determined by spectrophotometry using an eppendorf biophotometer and diluted to 50 ng/µl. 50 ng total cell RNA was subjected to reverse transcriptase polymerase chain reaction (RT-PCR) using the AccessQuick RT-PCR kit (A1702; Promega). Primers for RT-PCR can be seen in Table 3. Primer sets were designed across intron-exon boundries. The RT-PCR cycle used was: 1h 45 ^{o}C, 2 mins 94 ^{o}C, (30 sec 94 ^{o}C, 1 min 60 ^{o}C, 1 min 72 ^{o}C) 30x, 5 min 72 ^{o}C, 4 ^{o}C for ever. XBP1 cDNA was then subjected to *PstI* digest (see below). All cDNA were either ran on 1% (w/v) agarose gel (*XBP1* before *PstI* digest) or ran on 2% (w/v) agarose gel (after *PstI* digest) at 100 mV for ~50 mins before exposure to UV light at 365 nm.

3.7 *PstI digestion*

Amplified XBP1 cDNA was subjected to *PstI* (MBI/Fermentas) digest to detect the presence of spliced mRNA. 12.5 µl RT-PCR product was digested using 1 µl (10 U) *PstI* in a total volume of 15 µl at 37°C for 2 h. cDNA was extracted from the restriction digest using a PCR purification kit (Qiagen) according to the manufacturer's instructions. Purified cDNA was run on 2% (w/v) ethidium bromide agarose gel and visualized by UV light at 365 nm.

Table 3 – RT-PCR Primers

mRNA Target	Product Size (nt)	Forward/ Reverse	Primers
ATF4	760	Forward	CCTCGATTCCAGCAAAGCACCGC
		Reverse	GCTCCTATTTGGAGAGCCCCTGG
ATF6α	890	Forward	TGATGCCTTGGGAGTCAGAC
		Reverse	GTGTCAGAGAACCAGAGGCT
BiP	1092	Forward	AGAGCTGTGCAGAAACTCCGGC
		Reverse	CCTCTTCACCAGTTGGGGGAGG
CHOP	389	Forward	GCTGGAAGCCTGGTATGAGGACC
		Reverse	CGCTCGATTTCCTGCTTGAGC
XBP1	544	Forward	GAAACTGAAAAACAGAGTAGCAGC
		Reverse	GCTTCCAGCTTGGCTGATG
β-actin	823	Forward	CCACACCTTCTACAATGAGC
		Reverse	ACTCCTGCTTGCTGATCCAC

3.8 *PNGase digestion*

Post-nuclear lysates were denatured using 10x denaturing buffer (5% (w/v) SDS, 0.4 M DTT) (New England Biolabs Inc.) and boiled at 95 ^{o}C for 10 minutes. 1000 U of PNGase were added to the lysates together with 10x G7 reaction buffer and 1% (v/v) NP-40 and incubated at 37°C for 2 hours before being taken into sample buffer for loading on an SDS-PAGE gel.

3.9 *SDS-PAGE*

Samples were prepared for SDS-PAGE separation by the addition of 2x Laemmli loading buffer (1 M Tris pH 6.8, 20% (w/v) SDS, 5% (v/v) glycerol, 0.01% (w/v) bromophemol blue) in the presence or absence of 50 mM dithiothreitol (DTT) (Sigma-Aldrich), boiled at 95 ^{o}C for 5 min at 95 ^{o}C and spun down at 4 ^{o}C. All Blue Precision Plus protein standard (250-10 kD) (#161-0373; BioRad) was used as a protein marker. This marker contained ten protein bands at 10 kD, 15 kD, 20 kD, 25 kD, 37 kD, 50 kD, 75 kD, 100 kD, 150 kD and 250 kD. Samples were run on 8-10 % SDS-PAGE gels (Running gel: 8-10% (v/v) Acrylamide (Sigma-Aldrich), 0.375 M Tris pH 8.8, 0.1% (w/v) SDS (Sigma-Aldrich), 0.1% (w/v) Ammonium persulfate (APS) (Sigma-Aldrich) and 0.04% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich). Stacking: 5% (v/v) Acrylamide, 0.125 M Tris pH 6.8, 0.1% (w/v) SDS, 0.075% (w/v) APS and 0.075% (v/v) TEMED) for ~50 min at 15 mV in 1x Tris-Glycine SDS buffer (Sigma-Aldrich) in order to resolve all proteins. The SDS-PAGE gel was cast using BioRad casting apparatus. Initially, the running gel was made up, cast and left for 40 mins with a layer of distilled water on top to prevent drying out. The water was drawn out using blotting paper and the stacking gel was made and cast on top. A 15-well comb was immediately placed in the

stacking gel. This was left to set for 30 mins before the gels were removed from the apparatus and placed in a BioRad running tank. Running buffer was added to the tank and prior to loading the gel, the combs were removed. Once the gel had run, PVDF transfer membranes (Millipore) were pre-moistened for transfer in methanol and then in 1x transfer buffer (190 mM glycine, 25 mM Tris, 20% (v/v) methanol). Gels were then wet transferred in 1x transfer buffer for 2 h at 30 mV. Membranes were then blocked in 8 % (w/v) milk in PBST (100 ml dm^{-3} PBS, 0.1% (v/v) Tween 20 (Sigma-Aldrich)) or TBST (10 mM Tris pH 8, 150 mM NaCl, 0.1% (v/v) Tween 20) for 60 min.

3.10 *Ponceau S staining*

Post-transfer PVDF membranes are washed in PBS and immersed in Ponceau S staining solution (P7170; Sigma-Aldrich) for 5 minutes. Membranes are then washed with distilled water to visualise bands and remove background. Membranes can either be dried at this point or further washed with distilled water for 5 minutes (or 0.1 M NaOH for 10-30 seconds) to remove the remaining stain allowing immuno-detection.

3.11 *Western Blotting*

Membranes were incubated with the appropriate primary antibody dilution (see Table 2) in 8% (w/v) milk in PBST or 5% (w/v) BSA (A9418; Sigma-Aldrich) in TBST (Cell Signalling antibodies only) for 60 minutes before being washed three times for five minutes with PBST or TBST. Membranes were then incubated with the corresponding secondary antibody (DAKO) at 1:3000 in 8% (w/v) milk in PBST or TBST for 60 min then subsequently washed three times for five minutes with

PBST or TBST. Proteins were then visualised with 200 μl enhanced chemiluminescence fluid (Amersham). The solution was prepared from the two individual solutions and incubated at RT for 1 min. The solution was applied to air-dried membranes for 1 min on Saran wrap before removing the excess. Membranes were then wrapped in Saran wrap to prevent the membrane drying out and luminescent stickers were applied to the Saran wrap to aid visualisation in the dark room and to enable alignment of the film with the membrane after exposure. The membrane was then exposed to chemiluminescent film (Kodak) for a range of exposures and developed using an X-ray developer machine.

3.12 *Glycine stripping*

To remove antibodies after Western blotting, membranes were rehydrated in PBST and stripped with glycine stripping buffer (15 g/l Glycine, 0.05 % (v/v) Tween pH 2.5) for 5-10 minutes, washes in PBST for 1 min, stripped again for 5-10 minutes with glycine stripping buffer then washed with PBST for 1 min before blocking in 8% (w/v) milk in PBST for 60 minutes.

3.13 *Immunofluorescence*

Cells were grown on 100% ethanol-sterilised cover slips (VWR) in 6 cm dishes 3 days prior to transfection. Cells on the cover slips were either transfected and/or treated as normal before being washed in the dish with 4 ml warm PBS (Gibco, Invitrogen). Cover slips were then removed from the dish and were either then fixed in 4 ml -20 ^{o}C methanol (Fisher Scientific) for 10 minutes or paraformaldehyde-fixed in 1 ml 4% (w/v) paraformaldehyde (VWR) in a 6 well dish for 10 minutes at room temperature and then placed in 1 ml 10% (v/v) Triton X-100 (T8787; Sigma-Aldrich)

for 5 minutes at room temperature in a 6 well dish. Cover slips are then washed in 4 ml room temperature PBS. Then, individual cover slips are blocked with 75 µl 0.2% (w/v) BSA at room temperature for 30 minutes before a PBS wash. Cover slips are incubated with 50 µl of the appropriate primary antibody dilution (in 0.2 % (w/v) BSA in PBS) at room temperature for 45 minutes. After washing in PBS, cover slips are incubated in the dark with 75 µl of either swine anti-rabbit tetramethyl rhodamine isomer R (TRITC)-conjugated (R0156; DAKO) or donkey anti-mouse Alexa Fluor 488 (A21202; Invitrogen) secondary antibody (in 0.2 % (w/v) BSA in PBS) for 30 minutes. Cover slips are washed with PBS and stained with 75 µl 1x DAPI for 5 minutes in the dark before washing with PBS. Finally, cover slips are mounted on slides using hard-setting Vectashield (Vector Laboratories) and analysed by confocal microscopy.

3.14 *Cell viability assay*

Cells were grown and treated with ER stress inducers in 6 cm dishes for the times stated. Cells were washed in PBS, tyrpsinised and diluted in fresh medium. 50 µl of cells were serial diluted into a cell culture-treated 96-well plate, made up to a volume of 200 µl with fresh media and grown overnight. Media was then removed; cells were washed in PBS before being fixed in 50 µl -20 ^{o}C methanol for 10 minutes. Cells were then washed in PBS, stained with 50 µl 1x DAPI in PBS for 10 minutes in the dark and washed in PBS again. Cells nuclei were imaged on a Zeiss Axiovert 200M microscope using the Image Associates' (IMAS) Axiovision 4.7 program. At least three internal repeats were done for each dilution. Cell number was determined using Image J.

4. RESULTS

4. 1 Induction of the UPR by pharmacological agents

4.1.1 *Introduction*

The UPR is a multifaceted system which monitors the folding capacity of the ER and coordinates appropriate transcriptional and translational responses to perturbations in ER homeostasis. So-called ER stress can be caused by a number of perturbations including the accumulation of unfolded or misfolded proteins – which could be caused by elevated secretory protein synthesis or mutant protein production – disturbances in Ca^{2+} storage, changes in redox status, altered glycosylation, hypoxia, ischemia and nutrient deprivation. Commonly, pharmacological agents are used to induce ER stress conditions in the cell, exploiting the sensitivity of the ER to various perturbations.

4.1.1.1 *Tunicamycin*

Tunicamycin, a drug first identified and isolated from *Streptomyces lysosuperificus*, was initially studied for its antibiotic/antiviral effects but is now frequently employed to induce ER stress in cells by using commercially available tunicamycin which is a mixture of similar compounds (Takatsuki *et al.*, 1971; Back *et al.*, 2005). Structurally, tunicamycin is composed of uracil, a fatty acid and two glycosidically linked sugars – N-acetylglucosamine and tunicamine (Fig. 6). Tunicamycin inhibits the first step in the lipid-linked oligosaccharide pathway; the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P by GlcNAc phosphotransferase (GPT) to form dolichyl-PP-GlcNAc (Tkacz & Lampen, 1975).

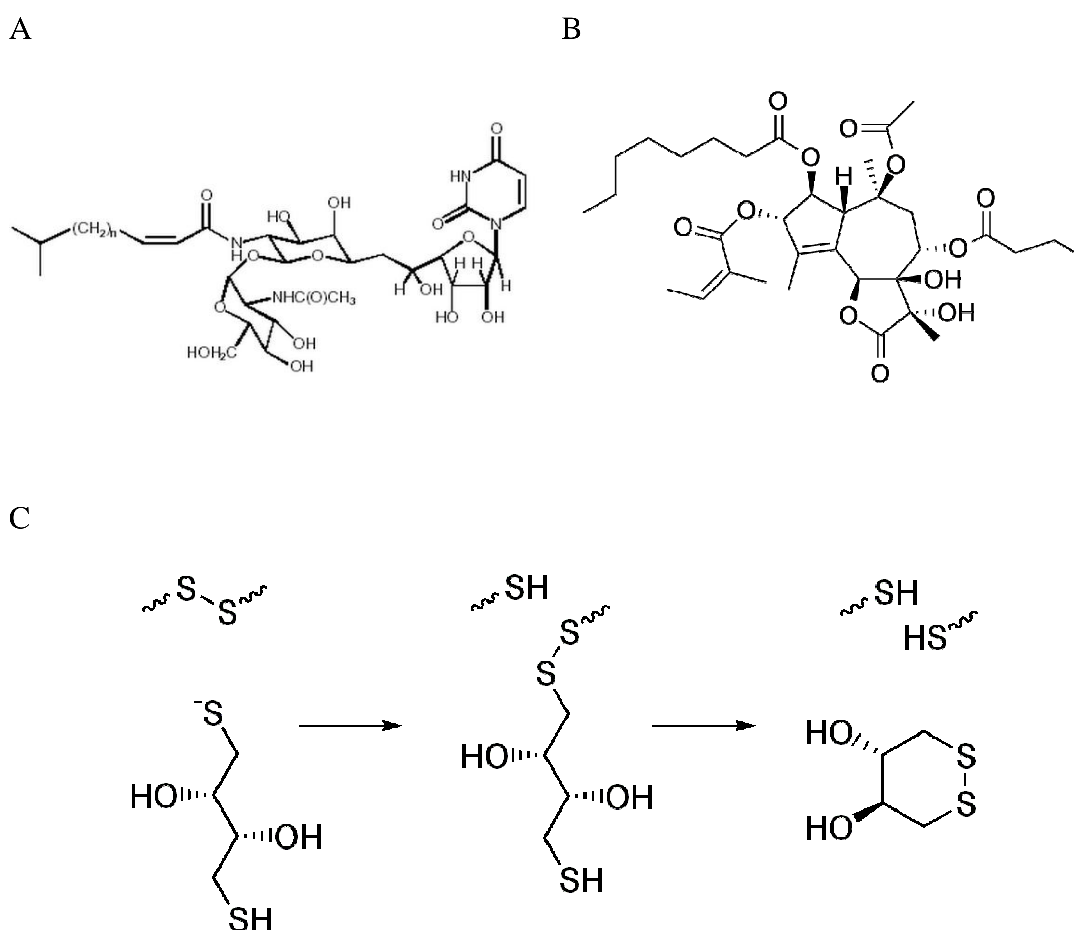


Figure 6. **The ER stress-inducing agents tunicamycin, thapsigargin and dithiothreitol.** A, The GlcNAc phosphotransferase (GPT) inhibitor tunicamycin PubChem CID: 6433557. B, The calcium ATPase pump inhibitor thapsigargin PubChem CID: 446378. C, The action of the reducing agent dithiothreitol (DTT) which reduces disulfide bonds PubChem CID: 19001.

Inhibition of the transfer of phosphorylated N-acetylglucosamine to dolichyl-P prevents N-linked glycosylation of proteins in the ER. If proteins fail to become fully glycosylated, their folding becomes inefficient since interaction with resident ER proteins, such as lectins, may be abolished and the protein becomes more hydrophilic. Inefficient folding of non- or under-glycosylated proteins can lead to their accumulation in the ER and saturation of the protein folding machinery. Thus, tunicamycin is a good inducer of the UPR since it triggers an increase in the protein folding demand.

4.1.1.2 *Disruption of cellular* Ca^{2+} *levels*

The ER is the cell's principle Ca^{2+} store and contains a high concentration of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps which regulate cytosolic Ca^{2+} levels. Thapsigargin is another commonly-used inducer of the UPR and exerts its ER stress-inducing activity by inhibiting intracellular Ca^{2+} ATPase pumps (Campbell *et al.*, 1991; Lytton *et al.*, 1991). SERCA pump inhibition leads to depletion of ER Ca^{2+} levels and causes an increase in cytosolic Ca^{2+} due to passive leakage out of the ER (Thastrup *et al.*, 1990). Extracellular free Ca^{2+} also influxes into the cytosol due to changes in plasma membrane permeability (Parekh *et al.*, 1993). Perturbation in cellular Ca^{2+} levels leads to induction of the ER stress response and prolonged treatment leads to UPR-dependent and -independent apoptosis (Nakagawa & Yuan, 2000; Rao *et al.*, 2001). Thapsigargin is a sesquiterpene lactone originally isolated from *Thapsia garganica* (Rasmussen *et al.*, 1978) (Fig. 6). Additionally, ionophores such as A23187 (calcimycin) and ionomycin, act as mobile carriers of divalent Ca^{2+} ions – and other cations –

elevating the cytosolic Ca^{2+} concentration and depleting ER Ca^{2+} levels which results in the disruption of ER function (Dedkova *et al.*, 2000; Rao *et al.*, 2004).

4.1.1.3 *DTT and glucose starvation*

The ER is the site of disulfide bond formation and harbours a redox environment which is favourable for oxidation. Perturbations in the redox environment of the ER can lead to reduced protein folding efficiency and retention of proteins in the ER (Jämsä *et al.*, 1994). Dithiothreitol (DTT) – also known as Cleland's reagent – is a potent reducing agent which has high propensity to form a six-membered ring with an internal disulfide bond (Ruegg & Rudinger, 1977) (Fig. 6). Thus, DTT is used to reduce disulfide bonds in the ER causing the accumulation of reduced proteins and consequential ER stress. DTT is also used post-lysis to reduce proteins for gel electrophoresis.

Glucose starvation was one of the first inducers of the UPR to be studied (Shiu *et al.*, 1977). Withdrawal of glucose from the growth media most likely results in a decrease in the amount of oligosaccharide intermediates used for protein N-glycosylation as well as depleting ATP, which is required for protein folding. Prevention of glycosylation can lead to changes in protein folding efficiency and the accumulation of unfolded/misfolded proteins in the ER and activation of the UPR (Lee, 2001; Yoneda *et al.*, 2001; Nadanaka *et al.*, 2006).

4.1.2 *Results*

4.1.2.1 *HeLa and HT1080 both upregulate BiP in response to ER stress induction*

In order to understand how different cell types coordinate the unfolded protein response, I initially studied the effects of a range of pharmacological agents, known to induce ER stress, in model established cell lines. The immortalised HeLa cell line, derived from Henrietta Lacks in 1951, is a human cervical carcinoma cell line used widely in research while HT1080 is an immortalised human cell line derived from fibrosarcoma cells established in 1974 (Von *et al.*, 1954; Rasheed *et al.*, 1974). These cell lines were chosen primarily for their practicality since they grow rapidly, can be successfully transfected and have been used in our laboratory for many years. Furthermore, there is a raft of published experimental data pertaining to both these cell lines which allows for effective comparison of results.

Immunoglobulin-binding protein, or BiP, negatively regulates the UPR by binding to the lumenal domain of the three ER stress transducers IRE1, ATF6 and PERK. Detection of the amount of free BiP is important in assessing the protein folding capacity of the ER, although ER stress sensors may be able to detect unfolded protein directly. However, in response to reduced amounts of free BiP, the UPR is induced (Shamu *et al.*, 1994; Liu *et al.*, 2003; Kimata *et al.*, 2007). Accordingly, one of the major targets of UPR induction is to increase BiP expression, which serves to restore ER homeostasis by increasing the folding capacity of the ER (Lee *et al.*, 2003).

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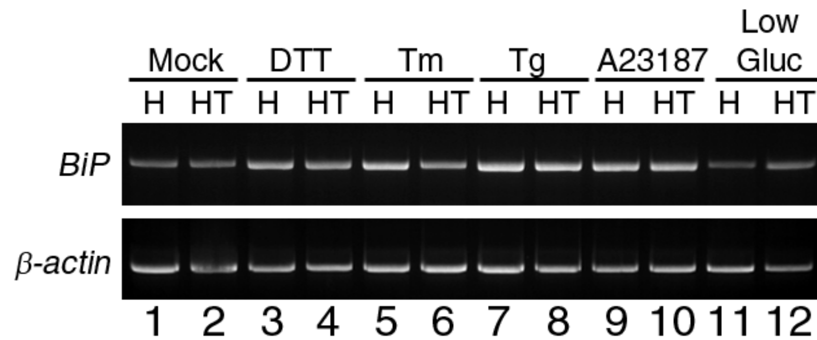

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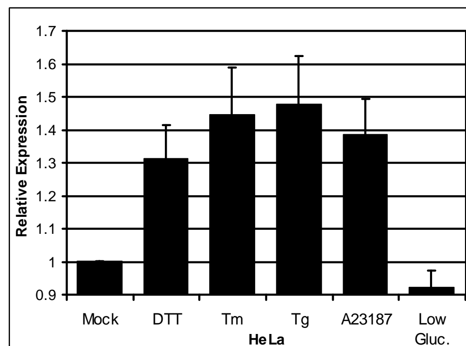

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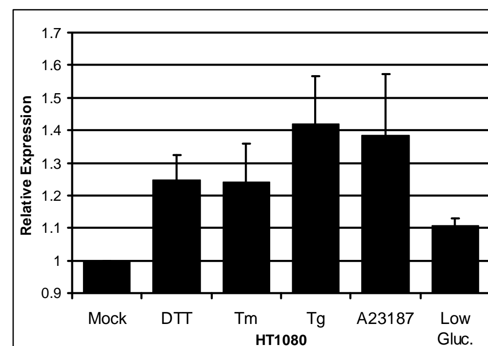

Figure 7. ***BiP* expression in HeLa and HT1080 cells in response to ER stress-inducing treatment.** A, HeLa (H) and HT1080 (HT) cells were subjected to cell treatment with the pharmacological agents dithiotheritol (DTT), tunicamycin (Tm), thapsigargin (Tg), A23187, low glucose (Low Gluc.) or Mock-treated (-) for 6 hours. Cells were lysed with TRIreagent and subjected to RT-PCR using primers designed against intron-exon boundaries of *BiP* and *β-actin*. B and C, Graphical representation of the relative expression of *BiP* in response to the indicated ER stress inducers. BiP expression was normalised to *β-actin* and is the average of at least 2 independent RT-PCR experiments

HeLa and HT1080 cells were treated with 10 mM DTT, 1 μg/ml tunicamycin, 2 μM thapsigargin, 2 μM A23187 or mock treated, as suggested by previous literature (Duskin & Mahoney, 1982; Pagani et al., 2000). Additionally, mild glucose deprivation was also studied by replacing cell media with low glucose (1000 mg/L) D-MEM. Cells were treated for 6 hours under these conditions before being washed, lysed and subject to RNA extraction. *BiP* and *β-actin* expression were analysed by reverse transcriptase polymerase chain reaction (RT-PCR) and visualised by agarose gel electrophoresis (Fig. 7A).

Steady-state expression of *BiP* in both HeLa and HT1080 cells was similar under mock treatment, however, under treatment of the reducing agent DTT, *BiP* expression was upregulated (Fig. 7A, compare lanes 3 and 4 with lanes 1 and 2). Treatment with tunicamycin also induced upregulation of *BiP* in both cell lines, although to a lesser extent in HT1080. Treatment of HeLa and HT1080 with thapsigargin and A23187, which promote Ca^{2+} efflux from the ER, significantly induced the expression of *BiP* (Fig. 7A, lanes 7-10). Mild glucose deprivation had little or no effect on *BiP* expression since *BiP* levels in Fig. 7A, lanes 11-12, were comparable to mock treatment in high glucose medium. Glucose starvation can induce *BiP* upregulation (Nadanaka *et al.*, 2006); however this occurs when cells are cultured in glucose-free medium. Conversely, exposure of cells to 1000 mg/L glucose (low glucose) for 6 hours did not lead to upregulation of *BiP* in HeLa cells (Fig. 7A, lanes 11). Treatment of HT1080 cells in low glucose seemed to slightly induce expression of *BiP* in HT1080 cells (Fig. 7C), however, since *BiP* expression was normalised to *β-actin* levels, the weak *β-actin* control for this sample may be misleading. *β-actin* is not a secretory pathway substrate and does not require

glycosylation. It is therefore unlikely that weak *β-actin* expression in this sample is due to low levels of glucose in the medium.

Relative expression of *BiP* in HeLa and HT1080 is illustrated in Figure 7B and 7C. *BiP* induction was assessed by calculating the relative density of bands from independent RT-PCR experiments in which cells were exposed to ER stress inducers. Thus, these results show that when HeLa and HT1080 cells are treated with the same concentration of tunicamycin, expression of *BiP* occurs more strongly in HeLa than in HT1080 cells. This is despite the fact that treatments with DTT, thapsigargin and A23187 demonstrate very similar effects in both cell lines.

4.1.2.2 *Induction of XBP1 splicing by ER stress inducers in HeLa and HT1080*

In order to determine if the UPR was responsible for an increase in the transcription of *BiP*, I examined the IRE1 pathway. The main target of IRE1 RNase activity is *XBP1* mRNA. I used *XBP1* splicing as a readout for IRE1-mediated UPR signalling. Since the IRE1-mediated removal of a 26-base intron from *XBP1* mRNA eliminates a novel *PstI* site, I differentiated between the two versions of *XBP1* by digestion of *XBP1* cDNA by *PstI*. This assay was first demonstrated by Calfon and colleagues and is illustrated in Figure 8 (Calfon *et al.*, 2002). HeLa and HT1080 cells were again treated with ER stress inducers for 6 hours and analysed by RT-PCR using primers against *XBP1* and *β-actin*. *XBP1* cDNA was then digested with *PstI* restriction enzyme and digestion reaction components were removed using the PCR purification kit from Qiagen. Spliced *XBP1*, (*XBP1* (S)), was differentiated from unspliced *XBP1*, (*XBP1* (U)), since it was resistant to *PstI*

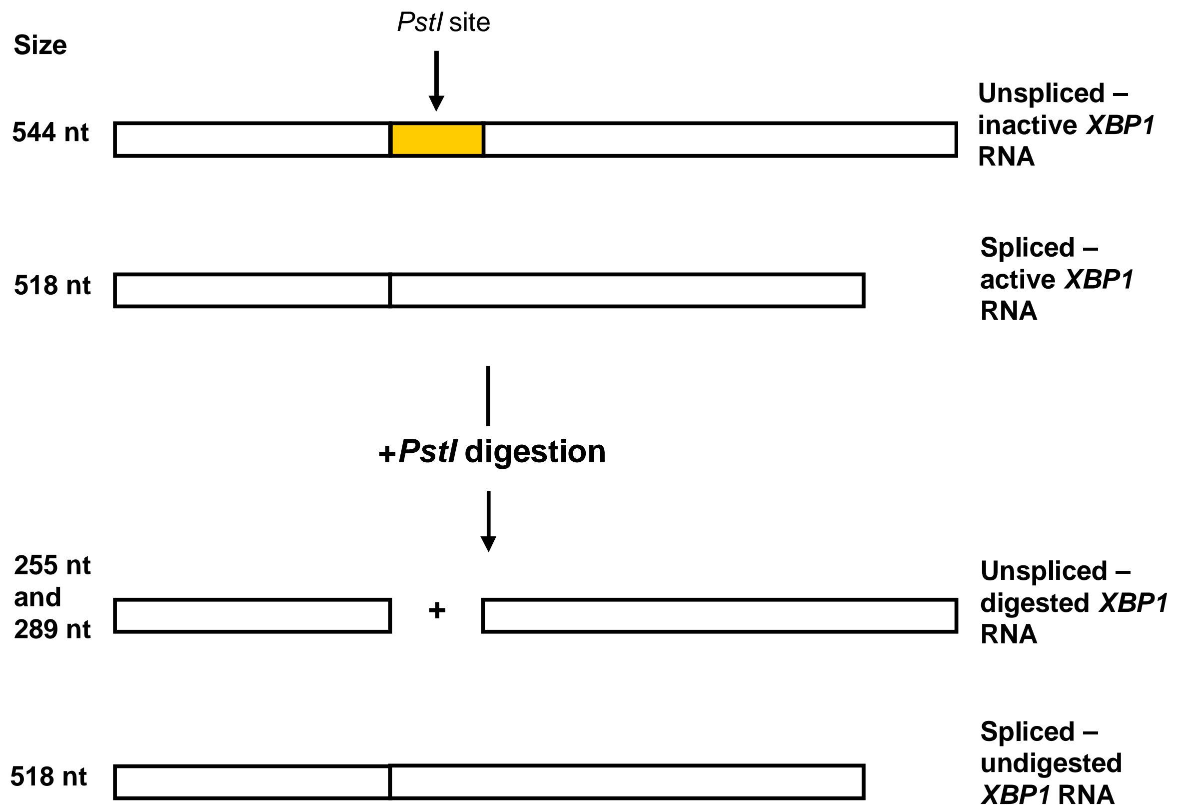

Figure 8. ***PstI* digestion as a technique for analysing *XBP1* processing.** When inactive *XBP1* mRNA is spliced, a 26 nucleotide intron is removed containing a unique *PstI* site. Upon digestion of inactive *XBP1* RNA, two fragments are generated of 255 and 289 nucleotides (nt) due to the presence of the *PstI* site. Activated (spliced) RNA however is not digested by the restriction enzyme due to its removal by IRE1, and thus migrates as a single 518 nt fragment.

digestion and thus migrated at a slower rate than the unprocessed product on a 2% (w/v) agarose gel. Without treatment, *XBP1* mRNA was expressed at low levels and was sensitive to *PstI* digestion in both HeLa and HT1080. The fast migrating doublet represents the 255- and 289-nucleotide digestion products of *PstI* (Figure 9A, lanes 1 and 7). Thus, in non-stressed cells, *XBP1* exists as an unspliced, inactive transcript.

Upon treatment with the ER stress inducers DTT, tunicamycin, thapsigargin and A23187, *XBP1* cDNA became resistant to *PstI* digestion and the 518-nucleotide cDNA migrated more slowly than *PstI*-sensitive *XBP1* (Figure 9A, lanes 2-5 and 8, 10-11). *PstI*-resistant *XBP1* represents the spliced version of *XBP1* lacking the *PstI*-site-containing intron present in unspliced *XBP1*. Thus, when ER stress is induced by DTT, tunicamycin, thapsigargin and A23187 in HeLa and HT1080 cells, *XBP1* undergoes splicing, presumably by IRE1. The level of *XBP1* splicing in HeLa and HT1080 is dependent on which ER stress inducer is used. DTT, thapsigargin and A23187 induced robust splicing of *XBP1*. This is in line with the induction of *BiP* which was most robust with thapsigargin treatment and was strongly induced by A23187 in both cell lines (Figure 7A).

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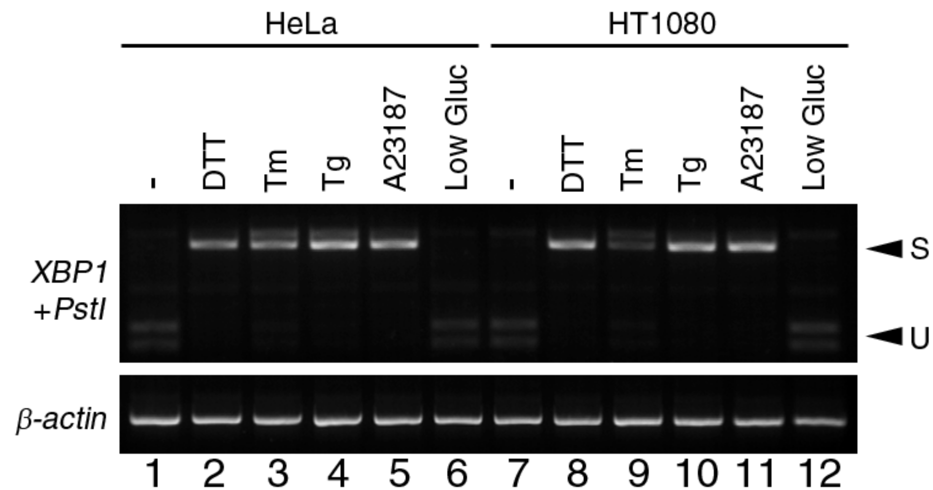

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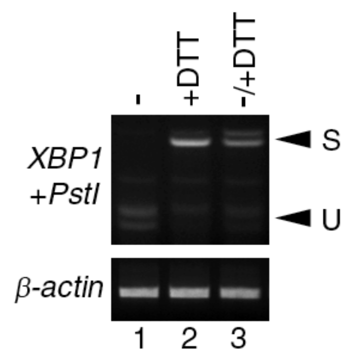

Figure 9. ***XBP1* is spliced in response to a range of ER stress-inducing agents.** A, Both HeLa and HT1080 cells were subjected to 6 hours treatment with the pharmacological agents indicated. *XBP1* RT-PCR products were digested with *PstI* restriction enzyme and cleaned up using a PCR purification kit. The eluate was then run on a 2% gel to resolve the spliced (S) and unspliced (S) version of *XBP1*. Primers against *β-actin* were used as a control. B, To identify the nature of the 518-nt+ band observed in A, mRNA from Mock (-), and DTT (+DTT) as well as a mixture of mRNA from Mock and DTT-treated cells (-/+DTT) was subjected to *XBP1* RT-PCR. Like A, RT-PCR product was digested with *PstI* and cleaned up before being run on a 2% agarose gel. *β-actin* was used as a control.

Treatment with tunicamycin induced robust splicing of *XBP1* in HeLa cells but was considerably less strong in HT1080 cells (Figure 9A, compare lanes 3 and 9). Additionally, tunicamycin-induced *XBP1* (S) migrated as a doublet with a more slowly migrating band in addition to the 518-nucleotide band (which represents spliced *XBP1* cDNA). Since this slower migrating band was only present in samples which contain partially spliced *XBP1* and not fully spliced or fully unspliced *XBP1* (Figure 9A, 9B and data not shown), I investigated whether or not this band was the product of a hybrid between spliced and unspliced *XBP1*, which was resistant to *PstI* digestion. Since DTT induced complete splicing of *XBP1* in HeLa cells, I *PstI*-digested the RT-PCR products of untreated, DTT-treated and a mixture of untreated and DTT-treated RNA (Figure 9B).

The 518+ band was not detectable in either the untreated or DTT-treated samples, however, when a mixture of unspliced and spliced *XBP1* – from the untreated and DTT-treated samples – was analysed, it contained both the 518-nucleotide band – representing spliced *XBP1* – and the 518+ band (Figure 9B, compare lane 3 with lanes 1-2). Thus, the more slowly migrating band in these samples may be a *PstI*-resistant hybrid of unspliced and spliced *XBP1*. This finding is supported by the work of Shang and Lehrman who identified a similar hybrid of *XBP1*, detectable with mild stress induction (Shang & Lehrman, 2004).

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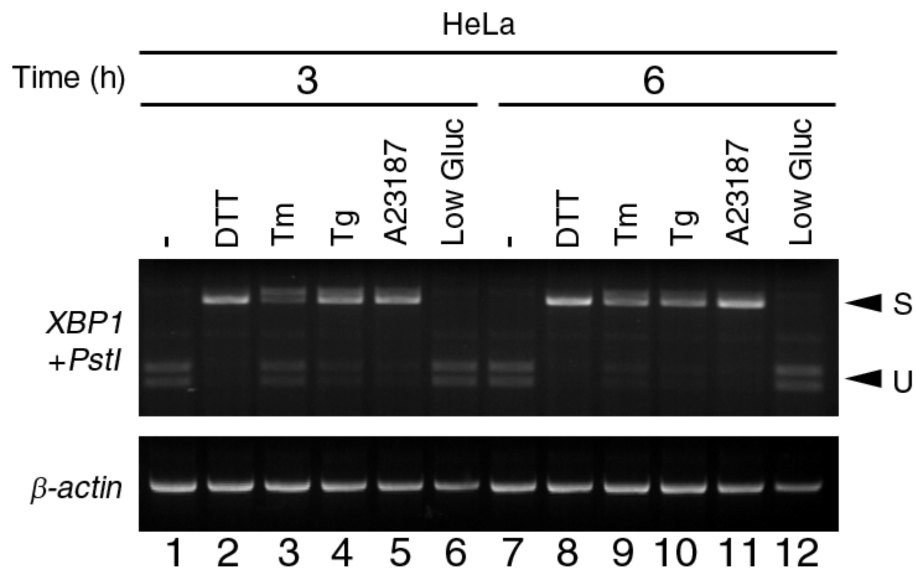

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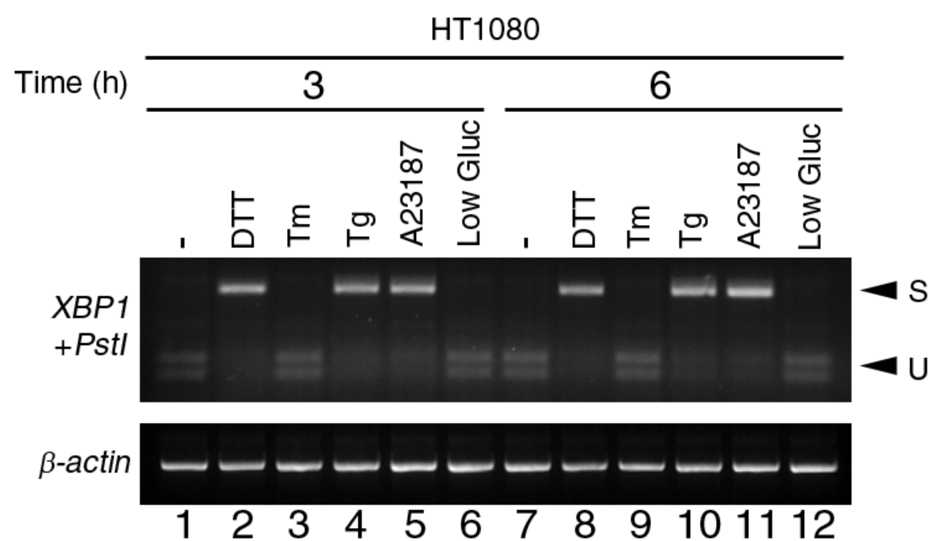

Figure 10. **HT1080 cells inefficiently process *XBP1* in response to tunicamycin treatment.** A, HeLa cells were treated with a range of ER stress inducers for either 3 or 6 hours. RT-PCR for XBP1 and subsequent *PstI* digestion and clean up was carried out. *β-actin* was used as a control. B, As in A, but treatments were carried out on HT1080 cells.

In order to determine if the IRE1 branch of the UPR responds to some types of ER stress in a shorter time frame, I studied *XBP1* splicing in HeLa and HT1080 which had been treated with ER stress-inducing agents for 0, 3 and 6 hours. In HeLa cells, treatment with DTT, tunicamycin, thapsigargin and A23187 strongly induced *XBP1* splicing after 6 hours treatment, as seen previously (compare Fig. 10A, lanes 8-11 with Fig. 9A, lanes 2-5). With a shorter treatment of 3 hours, the four stress-inducing agents all induced *XBP1* splicing, albeit slightly less robustly than with a 6 hour treatment. Both mock treatment and growing cells in low glucose medium for either 3 or 6 hours resulted in no *XBP1* splicing in HeLa cells. These results suggest that HeLa cells can trigger *XBP1* splicing within 3 hours of treatment with the ER stress inducers DTT, tunicamycin, thapsigargin and A23187.

In HT1080 cells, a 6 hour treatment with DTT, thapsigargin and A23187 resulted in *XBP1* splicing similar to that seen previously (compare Fig. 10B, lanes 8, 10-11 with Fig. 9A, lanes 8, 10-11). However, in this experiment, tunicamycin treatment for 6 hours resulted in low levels of *XBP1* splicing, consistent with the previous experiment (compare Fig. 10B, lane 9 with Fig. 9A, lane 9). The slight difference in *XBP1* splicing levels in these two experiments is most likely due to variation in cell density. A shorter 3 hour treatment of HT1080 cells resulted in robust splicing of *XBP1* with DTT, thapsigargin and A23187 treatment (Fig. 10B, lanes 2, 4-5). Treatment with tunicamycin for 3 hours did not induce *XBP1* splicing, indicating that the IRE1-mediated response to tunicamycin, in HT1080 cells, does not peak in a shorter time frame (Fig. 10B, lane 3). Unlike HeLa cells, the levels of DTT-, thapsigargin- and A23187-induced *XBP1* splicing in HT1080, after 3 hours, is very similar to that after 6 hours, possibly indicating that the RNase activity of IRE1 is

maximal by 3 hours treatment under these experimental conditions. Similar to HeLa cells, untreated HT1080 and HT1080 cells exposed to low glucose conditions for either 3 or 6 hours did not induce *XBP1* splicing.

Since *XBP1* splicing has been shown to diminish in HEK 293 cells between four and eight hours of continuous treatment with tunicamycin at 5 μg/ml (Lin *et al.*, 2007), I performed a tunicamycin time-course treatment on both HeLa and HT1080 cells to investigate the possibility that tunicamycin-induced *XBP1* splicing attenuates more quickly in HT1080 cells than in HeLa cells (Fig. 11A). Tunicamycin-induced *XBP1* splicing in HeLa cells is evident at the two hour time point; increasing after 3 hours and again with 6 hours treatment (Fig. 11A, lanes 1-5). Accordingly, *BiP* expression in HeLa cells started to increase within two hours of exposure to tunicamycin. *XBP1* splicing in HT1080, on the other hand, was not induced by tunicamycin treatment throughout the range of the time-course suggesting that the lack of tunicamycin-induced *XBP1* splicing at the two time points seen previously (6 and 3 hours) was not due to rapid attenuation of IRE1-mediated signalling in this case (Fig. 11A, lanes 6-10). Interestingly, *BiP* expression in HT1080 remained at levels similar to untreated HT1080 throughout the time-course. Since *BiP* expression can be induced in response to ER stress in the absence of IRE1 (Lee *et al.*, 2002), tunicamycin treatment should still induce *BiP* upregulation through ATF6 activation (Lee *et al.*, 2003). These results suggest that there are differences in the way tunicamycin stress is sensed by different cell types.

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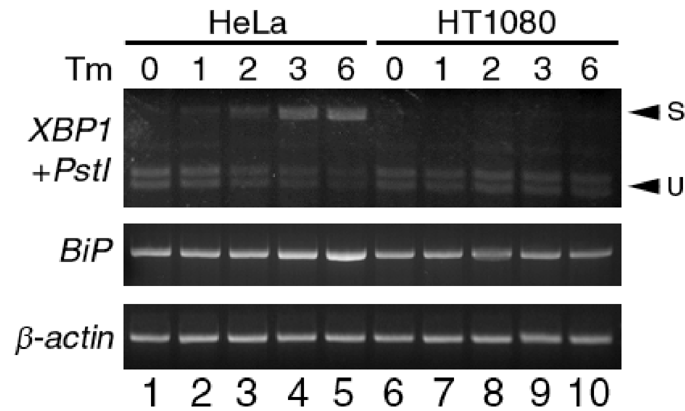

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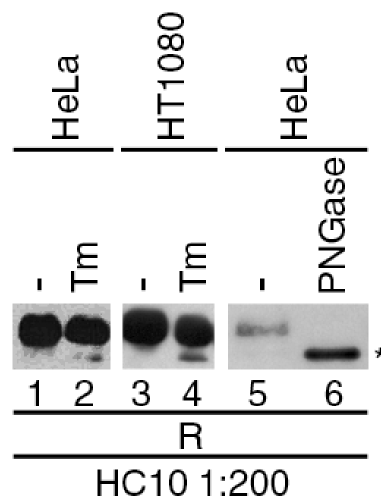

Figure 11. **HT1080 cells exposed to tunicamycin do not process *XBP1*, but tunicamycin prevents glycosylation of MHC class I heavy chains.** A, HeLa and HT1080 cells were treated with tunicamycin (Tm) for the indicated time. *XBP1* RT-PCR and subsequent *PstI* digestion was carried out in addition to *BiP* and *β-actin* RT-PCR. B, HeLa and HT1080 cells were treated with tunicamycin (Tm) for 6 hours. Cells were then lysed in MNT buffer and HeLa cell lysates were treated or not treated with PNGase. Lysates were then subjected to reducing SDS-PAGE and subsequent Western blotting with the monoclonal antibody (mAb) HC10. The monomeric MHC class I heavy chains were detected in addition to the unglycosylated version (*). The PNGase treated lysate acts as a control for complete deglycosylation.

To rule out cell-specific permeability to tunicamycin being a factor in diminished levels of *XBP1* splicing in HT1080, I treated both HeLa and HT1080 cells with tunicamycin for 6 hours and analysed the effect on glycosylation. The MHC class I heavy chain (cI HC) contains an N-linked glycosylation site with an attached oligosaccharide at Asn^{86} in the $\alpha 1$ domain (Parham *et al.*, 1977; Parham, 1996). Analysis of the glycosylation status of the cI HC was carried out by Western blotting with the pan-cI HC monoclonal antibody HC10 (Fig. 11B). 6 hour treatment with tunicamycin resulted in the appearance of an unglycosylated version of the cI HC in both HeLa and HT1080 indicating that tunicamycin is effective in preventing glycosylation in both cell lines within the time scale used to detect UPR induction. Identification of the unglycosylated version of the cI HC was confirmed by PNGase treatment of HeLa cell lysates (Fig. 11B, lane 6). Taken together, these results suggest that the level of IRE1-mediated splicing of *XBP1* is dependent on the duration of cell treatment and that there are cell-specific differences in *XBP1* splicing in response to exposure to tunicamycin.

4.1.2.3 *The downstream UPR target CHOP is differentially induced by distinct ER stress inducers*

I have demonstrated that UPR activation – at the level of *XBP1* splicing – is differentially sensitive to tunicamycin exposure in HeLa and HT1080 cells. IRE1-mediated *XBP1* splicing is an upstream event in UPR signalling. In order to investigate the downstream effects of ER stress induction by a range of ER stress inducers, the expression of the proapoptotic transcription factor *CHOP*, which is induced downstream of PERK activation, and the expression of the PERK-branch specific bZiP transcription factor ATF4, was examined.

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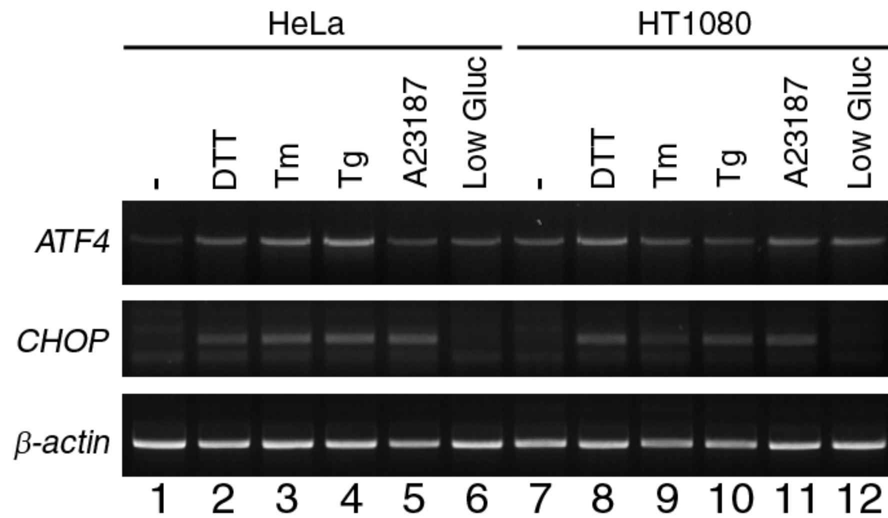

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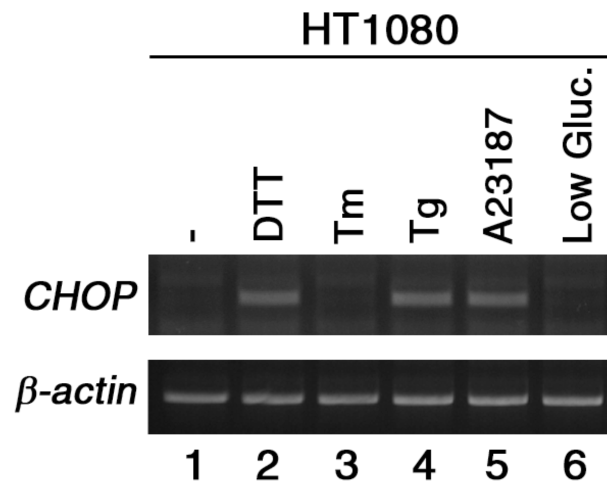


Figure 12. **Expression of *CHOP* is differentially induced by distinct pharmacological agents.** A, HeLa and HT1080 cells were treated with the indicated ER stress-inducers for 6 hours. A *ATF4* and *CHOP* RT-PCR was carried out on extracted mRNA from these treatments. *β-actin* was used as a control. B, HT0180 cells were treated with the indicated ER stress inducers for 3 hours. Subsequent *CHOP* RT-PCR on mRNA from these treatments was carried out.

HeLa and HT1080 cells were treated for 6 hours with the ER stress inducers DTT, tunicamycin, thapsigargin and A23187 as well as being exposed to low glucose conditions or untreated. RNA from these cells was subjected to RT-PCR with primers against *ATF4*, *CHOP* and *β-actin* (Fig. 12A). In HeLa cells, DTT, tunicamycin and thapsigargin treatment induced robust expression of *ATF4* mRNA, with thapsigargin treatment being the most effective (Fig. 12A, lanes 2-4). Levels of *ATF4* expression after A23187 treatment or exposure to low glucose conditions were only slightly elevated over the untreated control (Fig. 12A, lanes 5-6).
Analysis of *CHOP* levels in HeLa revealed that upon treatment with DTT, tunicamycin, thapsigargin and A23187, *CHOP* expression was induced. When cells were untreated or exposed to low glucose media, *CHOP* was not expressed, as expected. (Fig. 12A, lanes 2-5). In HT1080 cells however, strong induction of *CHOP* occurred when cells were treated with DTT, thapsigargin and A23187 but not mock- or low glucose-treated cells.

In HT1080 cells, ATF4 expression was upregulated in response to DTT and mildly elevated in response to A23187 treatment and with exposure to low glucose medium. This is contrary to HeLa cells which did not upregulate ATF4 in response to A23187 but did elevate ATF4 levels strongly with thapsigargin treatment (Fig. 10A, compare lanes 8-12 with lanes 2-6). ATF4 is primarily regulated at the translational level but there is evidence to suggest that ATF4 can be regulated at the transcriptional level (Dey *et al.*, 2010). ATF4 protein levels are regulated by eIF2α phosphorylation, however, these data indicate that HeLa and HT1080 can regulate *ATF4* mRNA levels differently in response to the same types of stress. HT1080 cells treated with tunicamycin exhibited limited *CHOP* induction (Fig. 12A, lane 9) indicating that an

impaired response to tunicamycin-induced ER stress is not limited to the IRE1 branch of the UPR but may also involve the PERK branch. Thus, there are differences in *CHOP* induction between HeLa and HT1080 cells in response to tunicamycin.

In order to investigate the likelihood that tunicamycin can induce rapid *CHOP* induction which subsided by the 6 hour time point, I analysed *CHOP* expression in HT1080 cells after 3 hours exposure to tunicamycin (Fig 12B). Similar to a 6 hour treatment, *CHOP* expression was extremely low when compared to induction by DTT, thapsigargin and A23187 (Fig 12B, compare lane 3 with lanes 2 and 4-5). In fact, tunicamycin-induced expression of *CHOP* was comparable to mock-treated samples, indicating that at this 3 hour time point, *CHOP* induction does not occur in response to tunicamycin.

4.1.2.4 *ATF6α can form higher order structures which are influenced by DTT and tunicamycin treatment*

ATF6α is a membrane-tethered transcription factor which, after proteolytic liberation, directly influences transcription without any known downstream effectors. To further understand the coordination of the UPR in response to ER stress, I studied the mechanisms of activation of ATF6α. Since ATF6α protein is expressed at low levels in HeLa cells (Thuerauf *et al.*, 2002) and due to the fact that I was unable to detect p90 ATF6α using our α-ATF6α antibody (Fig. 13A and data not shown), I used a liposome-mediated cell transfection approach to introduce a tagged version of ATF6α into the cell.

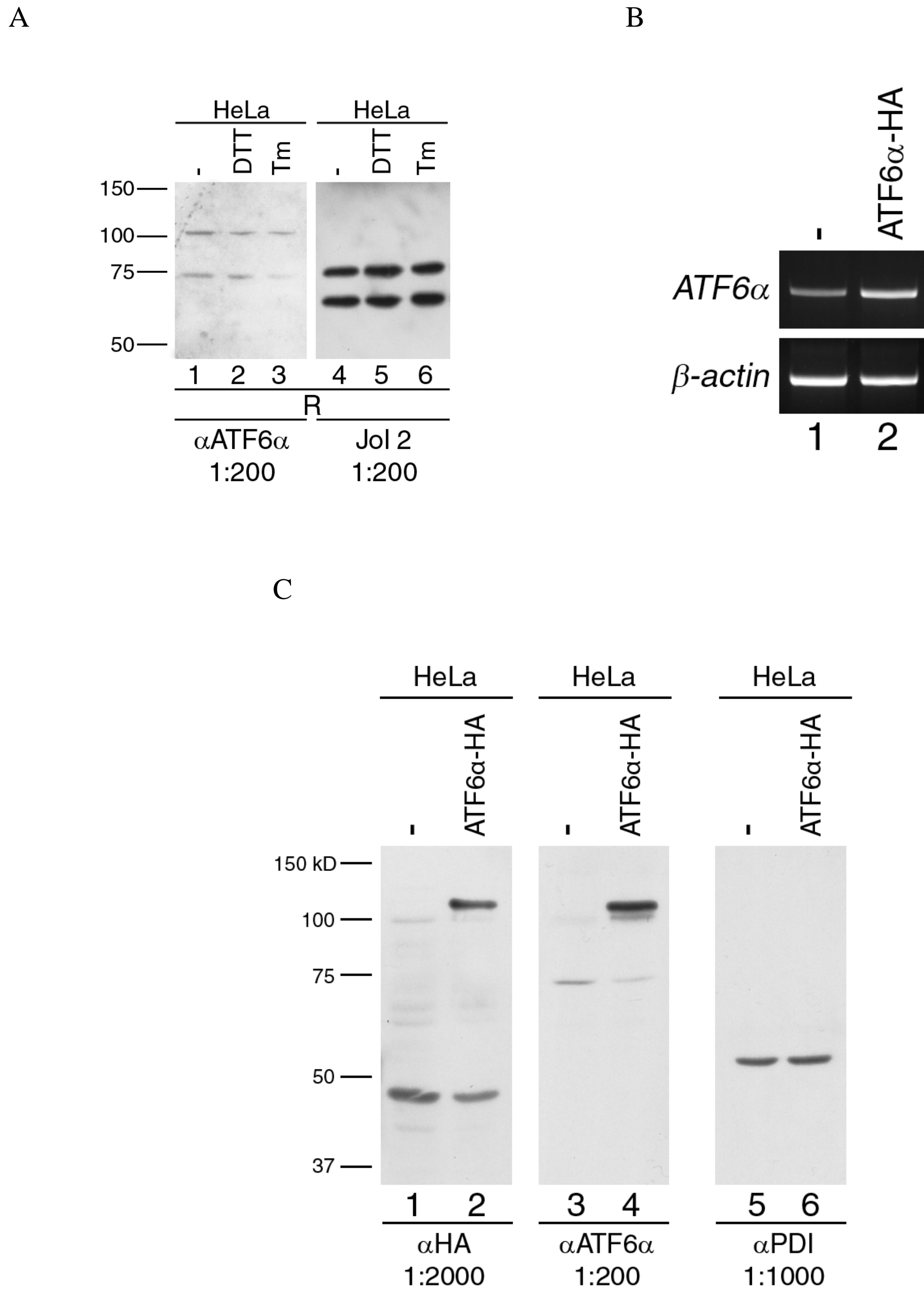


Figure 13. **ATF6α-HA transfection in HeLa cells leads to increased ATF6α transcript and protein.** A, No p90 ATF6α was detected using the αATF6α antibody either in the absence or presence of ER stress. The anti-lamin A and C antibody Jol 2 was used as a control. B, HeLa cells were subjected to liposome-mediated transfection of HA-tagged ATF6α. mRNA from Mock (-) and ATF6α-HA-transfected (ATF6α-HA) was subjected to *ATF6α* RT-PCR. *β-actin* was used as a control. C, MNT lysates from ATF6α-HA transfected HeLa cells were analysed by αHA, αATF6α and αPDI immunoblotting after separation by SDS-PAGE. Protein marker sizes are shown on the left of the image.

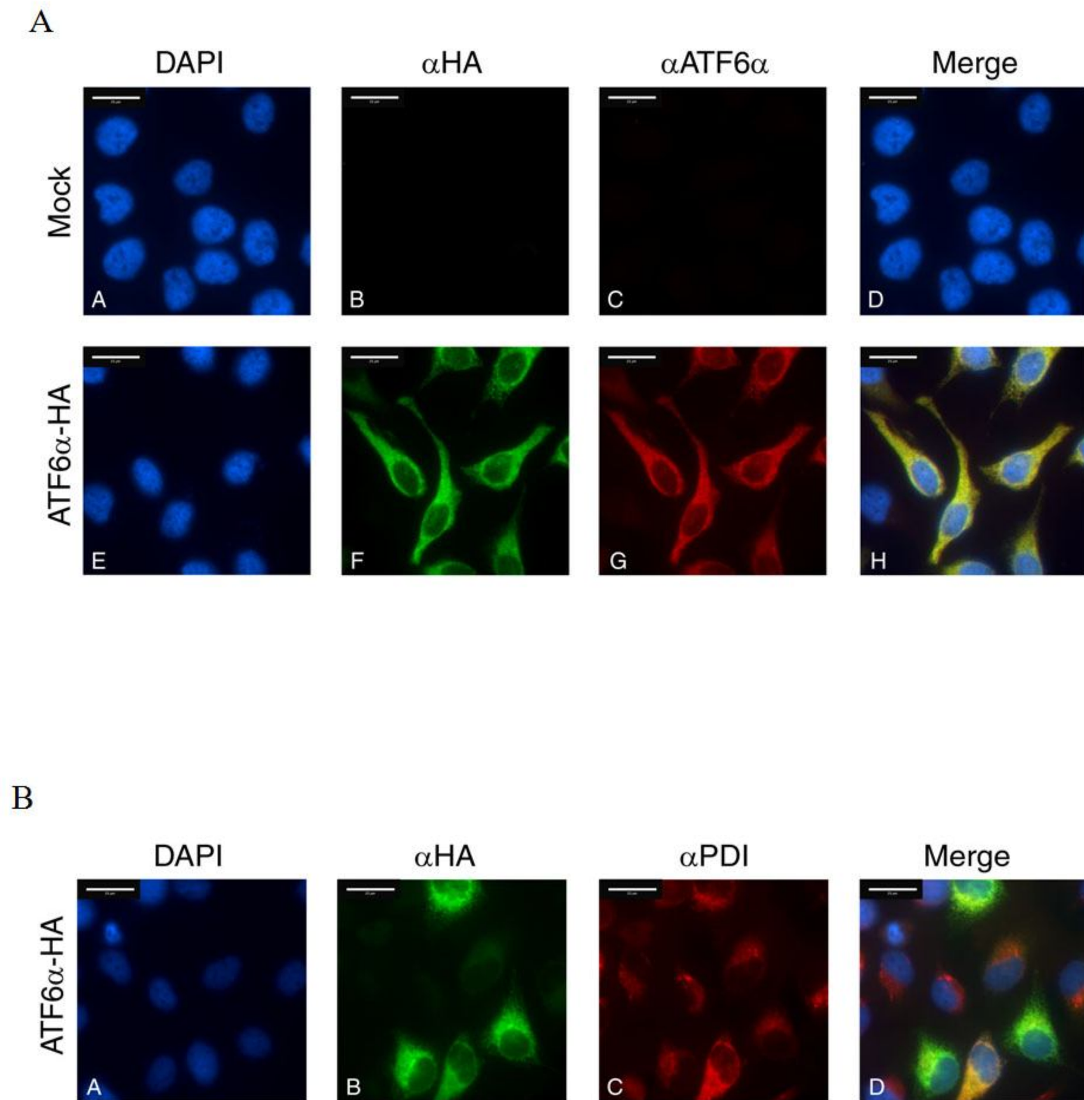


Figure 14. **Transfected ATF6α-HA localises to the ER.** A, Mock and ATF6α-HA transfected HeLa cells were stained with DAPI and antibodies against the HA tag (αHA) and ATF6α (αATF6α) and then fluorescence-conjugated secondary antibodies. A merge of DAPI, αHA and αATF6α (panels D and H) show colocalisation. B, ATF6α-HA transfected HeLa cells were stained with αHA and αPDI and stained with secondary antibodies as in A. The merge in panel D shows colocalisation of HA-tagged ATF6α with the ER resident protein PDI. Scale bars are 25 μm.

HeLa cells were either mock transfected or transfected with an N-terminal HA-tagged version of full length p90 ATF6α (ATF6α-HA) using the transfection reagent Lipofectamine 2000. Expression of ATF6α was monitored by RT-PCR using primers against ATF6α and revealed that transfection of ATF6α-HA resulted in an increase in transcript (Fig. 13B, lane 2).

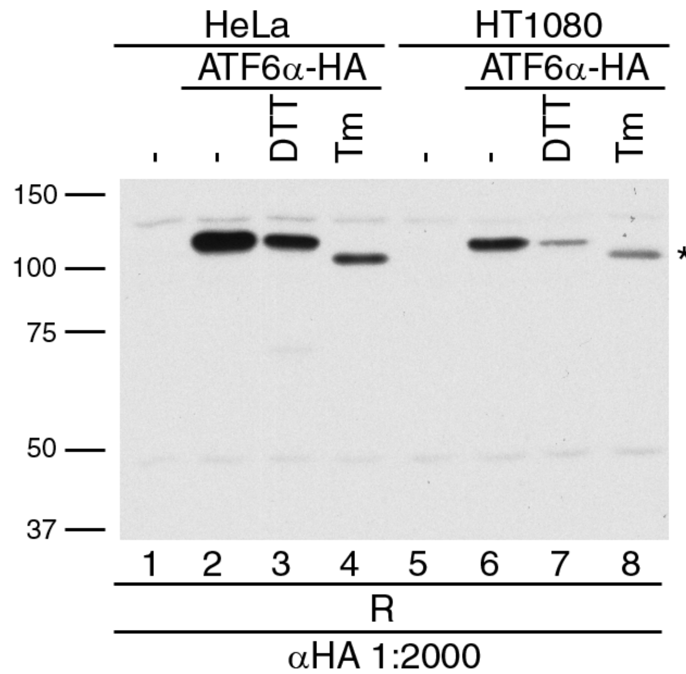
ATF6α protein levels were monitored by SDS-PAGE and Western blotting under reducing conditions. The HA tagged ATF6α was detected by the α-HA monoclonal antibody indicating that transfection had been successful (Fig. 13C, lane 2). This 90 kD protein migrated more slowly through the gel (between 110-120 kD) because of the additional HA tag. The α-HA antibody detected background bands present in both the mock and transfected lysates of HeLa cells at ~48 kD and ~100 kD. Additionally, ATF6α-HA was detected using the commercially available α-ATF6α polyclonal antibody. This antibody was raised against amino acids 31-310 of human ATF6α and recognised the tagged version of ATF6α also (Fig. 13C, lane 4). Neither the α-HA or the α-ATF6alpha antibodies detected the p50 fragment of transfected ATF6α-HA. This is not a surprise because these cells received no exogenous stress treatment other than transfection-mediated ER stress. Protein levels were controlled for using the polyclonal α-PDI antibody (Fig. 13C, lanes 5-6).

Expression of ATF6α-HA was further confirmed by immunofluorescence (Fig. 14). Mock transfected HeLa cells were negative when probed with both the α-HA and the α-ATF6α primary antibodies and secondary donkey anti-mouse Alexa Fluor 488-conjugated and swine anti-rabbit TRITC-conjugated antibodies respectively (Fig.

14A, panels B-C). Upon transfection of ATF6α-HA, both the α-HA and α-ATF6α antibodies detected the HA-tagged ATF6α. In some experiments, transfection efficiency was near 100% as evidenced by the detection of ATF6α in every cell – determined by DAPI staining (Fig. 14A, panel H). In order to determine the localisation of ATF6α-HA, cells were antibody-stained with the α-HA and α-PDI primary antibodies which revealed that ATF6α-HA colocalised with the ER resident protein PDI indicating that ATF6α-HA was present in the ER. This was detected in several images taken from two independent staining experiments.

I next studied how ATF6α responded to ER stress treatment in both HeLa and HT1080 cells. Once again, ATF6α-HA was introduced into cells by transfection prior to a 6 hour treatment with the reducing agent, DTT or the glycosylation inhibitor, tunicamycin. Lysates were then analysed by reducing and non-reducing SDS-PAGE and subsequent Western blotting (Fig. 15). Transfection of ATF6α-HA was successful in both HeLa and HT1080 cells and ATF6α-HA was detected using the α-HA antibody (Fig. 15A, lanes 2 and 6). Under reducing conditions, full length ATF6α was detected after treatment with both DTT and tunicamycin but at lower levels than mock-treated cells (Fig. 15A, compare lanes 3-4 with lane 2 and lanes 7-8 with lane 6). Additionally, treatment with tunicamycin resulted in a shift in migration (*) of the full length ATF6α-HA protein in both cell lines (Fig. 15A, lanes 4 and 8). This shift represents the unglycosylated version of ATF6α and was presumably a direct consequence of the action of tunicamycin. ATF6α-HA existed in a completely

A



B

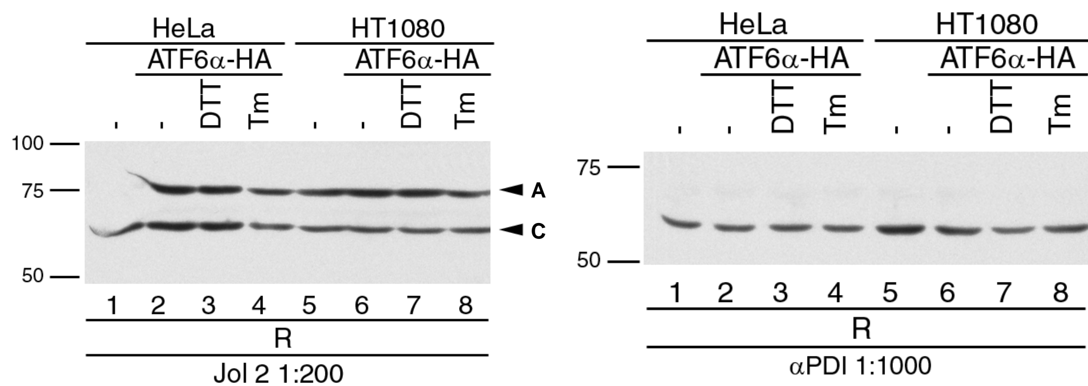


Figure 15. **p50 ATF6α-HA is not detected upon treatment with DTT or tunicamycin but the amount of p90 ATF6α-HA is diminished.** A, αHA immunoblot of mock and ATF6α-HA transfected HeLa and HT1080 RIPA buffer lysates under reducing (R) conditions. Cells were either untreated (-) or treated with DTT or tunicamycin (Tm). p90 ATF6α and the unglycosylated form of p90 ATF6α (*) were detected. B, Reducing Jol 2 (against lamin A and lamin C) and αPDI immunoblots. These act as controls for nuclear and ER fraction lysis respectively.

unglycosylated form in both cell lines. This is consistent with data from other groups which suggest that p90 ATF6α is rapidly turned over and has a half life of ~2 hours (Haze *et al.*, 1999). This data also provides further evidence that tunicamycin is efficient in inhibiting glycosylation in both HeLa and HT1080 cells.

In the transfected lysates, even in presence of an ER stress inducer, I was unable to detect a p50 fragment representing the liberated transcription factor portion of ATF6α. A very minor band at ~70kD was detected in response to DTT treatment in HeLa cells transfected with ATF6α-HA, which may represent the tagged p50 fragment (Fig. 15A, lane 3 and unpublished data). Detection of the p50 fragment with the α-HA antibody should be possible since the HA-tag is located in the liberated transcription factor portion of ATF6α. Accordingly, the decrease in detectable full length p90 ATF6α after DTT and tunicamycin treatment may be an indirect indication that ATF6α is being proteolytically cleaved since the α-HA antibody will not recognise the C-terminal anchored portion of ATF6α. However, a decrease in steady-state p90 ATF6α may be attributable to translational attenuation. In addition, the lysis buffer used was able to solubilise both the ER and nuclear fraction, as indicated by Western blotting for the nuclear lamins A and C using the monoclonal antibody Jol 2 in addition to the α-PDI control (Fig. 15B).

Our inability to detect the liberated p50 transcription factor portion of ATF6α is not without precedence. p50 ATF6α occurs in very low amounts and its appearance is thought to be transient since it is likely to be degraded quickly (Yoshida *et al.*, 1998;

Haze *et al.*, 1999). Furthermore, detection of p50 using the HA-tagged version of ATF6α has proven troublesome to other groups (Li *et al.*, 2000).

Interestingly, analysis of ATF6α-HA transfection and DTT/tunicamycin treatment by non-reducing SDS-PAGE and α-HA immunoblotting revealed higher order structures of ATF6α. Upon transfection of ATF6α-HA in HeLa and HT1080, ATF6α could be detected in monomeric, dimeric (~250 kD) and oligomeric (>250 kD) forms (Fig. 16). In both cases, the majority of ATF6α existed as an oligomer. This is consistent with the findings of Nadanaka and colleagues who reported the formation of dimeric and oligomeric ATF6α in addition to monomeric ATF6α in unstressed cells (Nadanaka *et al.*, 2007). Furthermore, they showed that these higher order structures were formed via intermolecular disulfide bridges between two conserved cysteines in the lumenal domain of ATF6α.

The relative magnitude of forms of ATF6α can be influenced by treatment with the ER stress inducers DTT and tunicamycin. Treatment with DTT for 6 hours post-transfection resulted in the majority of ATF6α existing in monomeric form with vastly diminished oligomeric ATF6α in both HeLa and HT1080 (Fig. 16, lanes 3 and 7). This is not a surprise since the higher order structures of ATF6α are likely to be disulfide-linked – as suggested by Nadanaka and colleagues. The addition of a reducing agent to the media thus resulted in the reduction of intermolecular disulfides in ATF6α. In addition, treatment with DTT in both cell lines resulted in a decrease in dimeric ATF6α, presumably also a consequence of DTT-mediated reduction (Fig. 16, lanes 3 and 7).

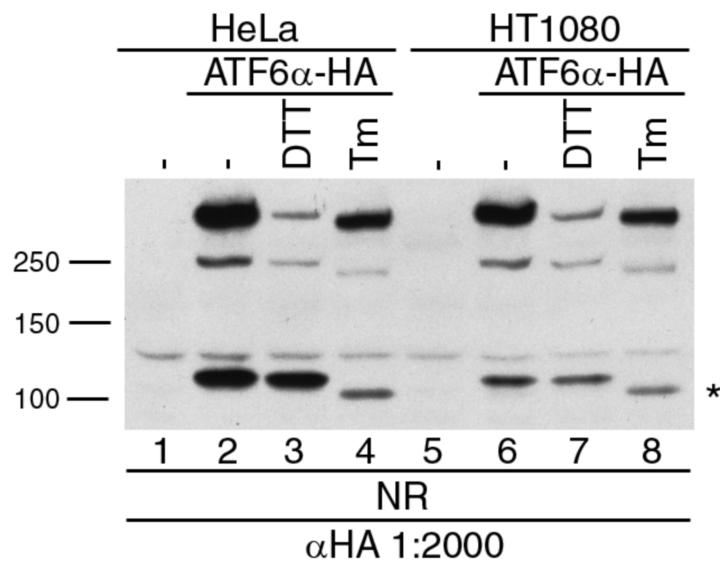


Figure 16. **The oligomerisation status of ATF6α is influenced by the ER stress inducers DTT and tunicamycin.** A non-reducing αHA immunoblot of ATF6α-HA transfected HeLa and HT1080 cells which have been either mock treated (-) or treated with DTT or tunicamycin (Tm). p90 ATF6α and the unglycosylated version of p90 ATF6α (*) are detectable. In addition, higher order dimers and oligomers were also detected.

Treatment with tunicamycin did not decrease the oligomeric pool of ATF6α as significantly as DTT but overall, the oligomeric as well as the dimeric form of ATF6α was diminished in comparison to the untreated cells (Fig. 16, compare lanes 4 and 8 with lanes 2 and 6). The monomeric pool of ATF6α was also diminished in response to tunicamycin treatment. Reduction of ATF6α upon treatment with tunicamycin is unlikely to be a direct consequence of tunicamycin activity. Rather, as suggested by Nadanaka and colleagues, reduction may be carried out by one of the many ER-localised oxidoreductases (Nadanaka *et al.*, 2007). ATF6α oligomerisation in response to tunicamycin was similar in both HeLa and HT1080 cells, indicating that the drug had a similar effect on this aspect of the UPR. Thus, both DTT and tunicamycin can influence the redox status of higher order structures of ATF6α. These forms of ATF6α, therefore, may be differentially sensitive to different forms of ER stress and add an extra layer of regulation to the activation of the ATF6 branch of the UPR.

4.1.3 *Discussion*

The UPR works to either promote the survival of cells undergoing ER stress, or, if subjected to prolonged stress, facilitate apoptosis. In order to understand how the UPR arrives at these 'decisions', we must first understand how the UPR coordinates its distinct pathways in response to ER stress and how those pathways are regulated. Here, I provide evidence that cell types respond in different ways to distinct forms of ER stress but also that these responses vary between cell lines. I report that, despite initiating a robust stress response to other forms of pharmacologically-induced ER stress such as thapsigargin and A23187, HT1080 does not respond as strongly to tunicamycin-induced ER stress. Treatment of HT1080 cells with the glycosylation inhibitor tunicamycin only weakly promoted the splicing of *XBP1*, a key step in the IRE1 branch of the UPR (Fig. 9A and 10B). This was in contrast to HeLa cells where tunicamycin-induced *XBP1* splicing was strong.

Additionally, *CHOP* expression was not induced in tunicamycin-treated HT1080 cells (Fig. 12A and 12B). Again, this was in contrast to HeLa cells where *CHOP* expression was strongly induced upon tunicamycin treatment. Moreover, *CHOP* was strongly induced upon treatment with DTT, thapsigargin and A23187 in both HeLa and HT1080 cells in line with studies showing that *CHOP* is induced by a variety of ER stress inducers (Price & Calderwood, 1992; Carlson *et al.*, 1993; Halleck *et al.*, 1997). In HT1080 cells a 3 hour treatment with the stress inducers DTT, thapsigargin and A23187 resulted in robust *CHOP* induction demonstrating that both *CHOP* induction and *XBP1* splicing occur with a 3 hour treatment under our experimental conditions. Limited induction of *CHOP* in response to tunicamycin may indicate that the PERK branch of the UPR is not activated. Further experiments, such as analysis

of eIF2α phosphorylation or ATF4 protein levels, will help determine if HT1080 cells are activating the PERK branch of the UPR in response to tunicamycin. However, low levels of tunicamycin-induced *CHOP* is not necessarily evidence of a lack of PERK signalling since the IRE1 branch of the UPR has been implicated in *CHOP* induction (Wang *et al.*, 1998). The mechanism by which IRE1 mediates *CHOP* expression is currently unknown although it has been postulated that downstream signalling of IRE1 may lead to modifications in the ER function which might impact on *CHOP* expression (Wang *et al.*, 1998). However, *XBP1* is dispensable for *CHOP* expression, implying that IRE1 may contribute to *CHOP* induction via a mechanism other than *XBP1* (Lee *et al.*, 2003). ATF6 is also thought to have a role in *CHOP* induction since a dominant negative of ATF6 blocks the induction of *CHOP* by ER stress (Yoshida *et al.*, 2000). The regulation of *CHOP* is illustrated in Figure 17.

The consequences of limited *CHOP* induction in HT1080 cells will be explored further in chapter 4.3; however, since *CHOP* is regarded as a proapoptotic component of the UPR, inhibition of *CHOP* induction could be associated with increased survival. Moreover, deletion of *CHOP* protects cells from the lethal consequences of ER stress (Zinszner *et al.*, 1998; Oyadomari *et al.*, 2002b; Pennuto *et al.*, 2008) indicating that the regulation of *CHOP* expression may directly influence cell fate decisions. Taken together, these results show that HeLa and HT1080 cells respond to tunicamycin-induced ER stress in different ways despite responding similarly to other forms of ER stress.

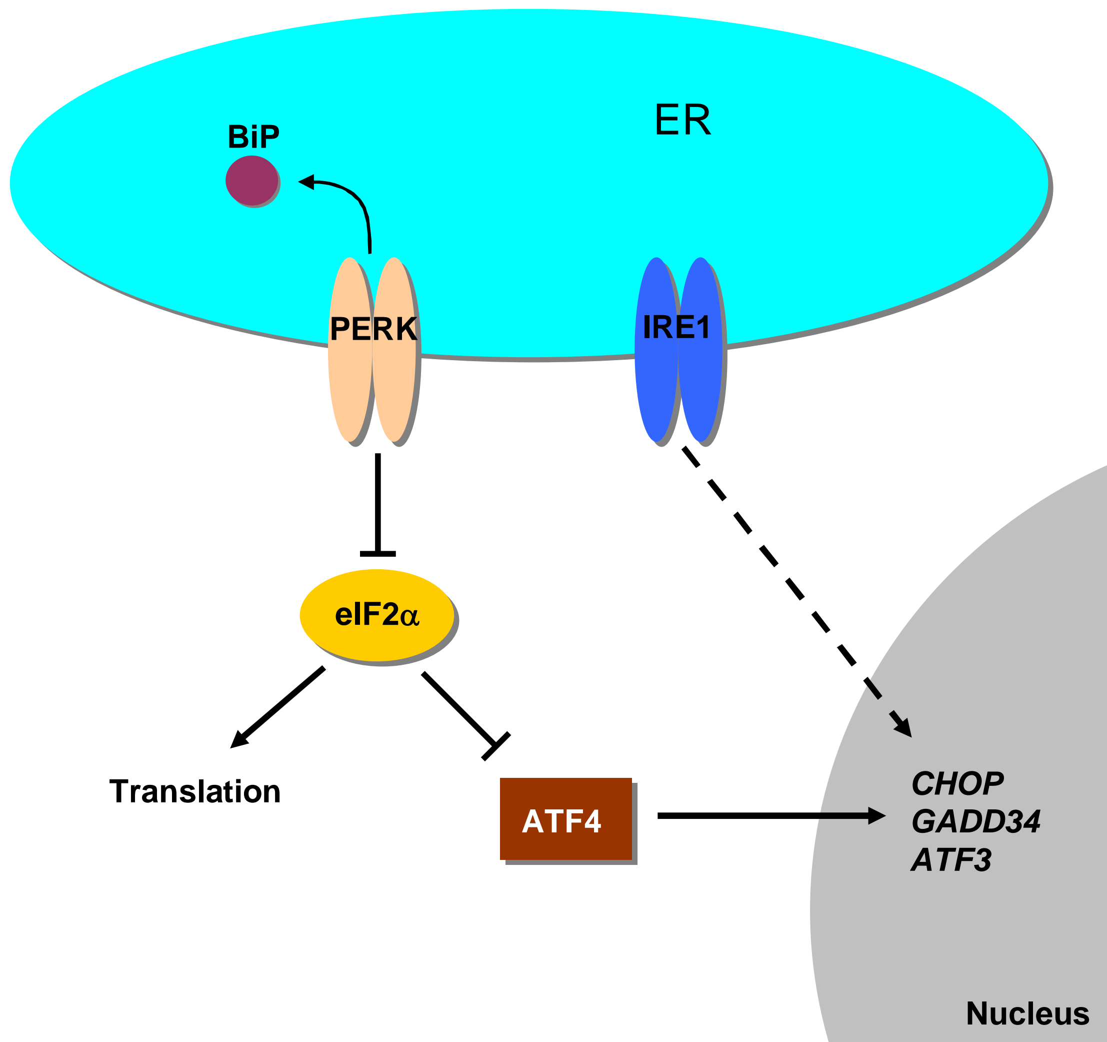


Figure 17. **The regulation of CHOP expression.** ER stress results in PERK activation by titration of BiP from its lumenal domain. As a consequence, eIF2α, which promotes protein translation, is inhibited via phosphorylation by PERK. This then prevents eIF2α inhibition of ATF4 translation, allowing the translation of ATF4 and other mRNAs containing multiple upstream open reading frames (ORFs). ATF4 then moves to the nucleus to upregulate target genes such as *CHOP*, *GADD34* and *ATF3*. Since the induction of *CHOP* was limited in tunicamycin-treated HT1080 cells, it is possible that PERK, or events downstream of PERK, are not activated. Furthermore, since IRE1 has been implicated in *CHOP* activation, limited *CHOP* induction could be a consequence of a lack of IRE1 signalling, as evidenced by low levels of *XBP1* splicing.

Initially, it was thought that tunicamycin was not entering HT1080 cells as efficiently as HeLa cells. However, Western blotting for the glycoproteins MHC class I heavy chain and ATF6α post-tunicamycin treatment revealed that the inhibition of glycosylation was comparable in both cell lines (Fig. 11B and 15A). The possibility exists that HT1080 cells may have a slightly higher concentration of GlcNAc-1-P transferase and treatment with tunicamycin at the concentration used might not inhibit the whole pool of the enzyme. However, this is unlikely since short-lived p90 ATF6α is completely unglycosylated after 6 hours tunicamycin treatment in HT1080 cells (Fig. 15A). If tunicamycin was not in excess, one would expect – in a protein with high turnover such as ATF6α – both a glycosylated and unglycosylated pool of p90 ATF6α.

Despite limited activation of the IRE1 and PERK branches of the UPR – inferred from *XBP1* splicing and *CHOP* induction – in tunicamycin-treated HT1080 cells, *BiP* expression was upregulated in response to this treatment (Fig. 7). However, *BiP* expression may not directly equate to an increase in BiP protein level and will, therefore, require further investigation. Despite this, it is possible that *BiP* mRNA upregulation in this case is mediated though ATF6α. I was unable to detect activation of ATF6α in response to DTT and tunicamycin; most likely due to the transience and low abundance of p50 ATF6α (Yoshida *et al.*, 1998; Haze *et al.*, 1999). However, I did note that detection of the reduced form of HA-tagged ATF6α, using the α-HA monoclonal antibody, was diminished upon treatment with DTT and tunicamycin (Fig. 15A). Since the α-HA antibody does not recognise the remaining C-terminal product after proteolysis, the lower ATF6α levels in these treatments

might be attributed to the loss of full length p90 ATF6α, possibly due to proteolytic cleavage.

I also demonstrate that ATF6α forms higher order structures when overexpressed in unstressed cells (Fig. 14). Nadanaka and colleagues reported this phenomenon in Chinese hamster ovary (CHO) cells and I now report similar findings in both HeLa and HT1080 cells. Nadanaka and colleagues showed that dimeric and oligomeric disulfide bonded- ATF6α is reduced in response to ER stress. Our data also supports this finding. Nadanaka and colleagues reported a decrease in monomeric p90 ATF6α after treatment with DTT, tunicamycin and thapsigargin. This is correlated with the appearance of p50 ATF6α, supporting our theory that a diminished p90 ATF6α levels after treatment might be the consequence of proteolytic cleavage. In line with this finding, I report diminished monomeric p90 ATF6α in our non-reducing gel upon DTT, and especially, tunicamycin treatment. Since monomeric ATF6α was detected in the Golgi upon ER stress and the reduced monomer is a better substrate for site 1-protease activity, it has been suggested that reduction of ATF6α is necessary for Golgi localisation and efficient cleavage (Nadanaka *et al.*, 2007). However, reduction of ATF6α is not sufficient for activation since it has been shown by Nadanaka and colleagues that BiP binding retains ATF6α in the ER.

Together, these results demonstrate that there are intrinsic differences in way the UPR responds to distinct forms of ER stress. Regulation at the level of transcription is one way the cell differentiates between different forms of ER stress. This may have implications for inflammatory diseases such as ankylosing spondylitis, since in

this disease, only certain cell types exhibit a UPR. Additionally, the regulation of higher order structures of ATF6 may play a role in differentiating between different forms of ER stress. Both IRE1 and PERK have been reported to oligomerise to regulate their function (Bertolotti *et al.*, 2000; Korennykh *et al.*, 2009). However, ATF6α appears to deoligomerise in response to ER stress whereas both IRE1 and PERK oligomerise demonstrating another difference in the way the separate branches of the UPR are regulated.

4. 2 HLA-B27 misfolding and the unfolded protein response

4.2.1 *Introduction*

Tuner and colleagues' key paper in 2005 provided the first evidence that HLA-B27 misfolding is associated with UPR activation in bone marrow-derived cells from the B27/hβ_2m transgenic rat (Turner *et al.*, 2005). Differential expression of *BiP* and *CHOP* was not observed in whole spleen or thymus, or in bone marrow macrophages from 4-wk-old premorbid animals. However, transcripts for both *BiP* and *CHOP* were elevated in bone marrow macrophages from 10-wk-old B27/hβ_2m transgenic rats exhibiting inflammatory disease. Furthermore, *XBP1* splicing, a key step in the IRE1-mediated branch of the UPR, was increased by ~5 fold and relative expression of *XBP1* was increased by ~2.6 fold in 10-wk-old B27/hβ_2m transgenic rats. Thus, B27 misfolding, determined by SDS-PAGE using the HC10 antibody which recognises free class I heavy chains, is associated with the increase transcription of UPR target genes as well as an increase in proximal UPR signalling events. This was further supported by microarray data which showed that bone marrow macrophages from the transgenic rat exhibited a 'UPR signature' as determined by treatment with tunicamycin (Turner *et al.*, 2005). Furthermore, expression of *BiP*, *CHOP*, *XBP1* and *XBP1* splicing increase when bone marrow macrophages from B27-expressing 4-wk-old premorbid rat cells are exposed to IFN-γ. This is likely to be due to the ~3 fold increase in HLA-B27 transcript levels since IFN-γ treatment of wild-type cells does not alter *BiP*, *CHOP*, *XBP1* expression or *XBP1* splicing.

In addition, this study confirmed the increased expression of IFN-γ in the colon of the transgenic rat which had been suggested previously (Taurog *et al.*, 1999). Moreover, increased expression of IFN-γ is associated with increased expression of *BiP* and *CHOP* in the distal colon of the B27/hβ_2m transgenic rat. However, differences in *BiP* and *CHOP* expression in transgenic and wild-type tissue were less pronounced than the same comparison in macrophages, indicating that high levels of UPR activity might be limited to cells with high heavy chain expression. Recently, defects in *XBP1* have been linked with inflammation of intestinal epithelium in mice (Kaser *et al.*, 2008). Deficiency in *XBP1* induces ER stress and this led to a heightened proinflammatory response of the epithelium to known inducers of IBD. Furthermore, increased *BiP* expression has recently been documented in IBD patients suggesting that ER stress – and associated UPR activity – might be an integral component of gut inflammation (Shkoda *et al.*, 2008; Heazlewood *et al.*, 2008). However, the contribution of the UPR to gut inflammation requires further study.

Recently, the IL-17-producting subset of T-cells, the Th17 cells, have been implicated in a number of autoimmune diseases including SpA. Polymorphisms in the IL-23 receptor, typical expressed on Th17 cells, have been associated with the development of AS and IBD (Duerr *et al.*, 2006; WTCC, 2007; Rahman *et al.*, 2008; Rueda *et al.*, 2008). The mechanism by which HLA-B27 misfolding and UPR induction are linked to IL-23 induction was demonstrated recently (DeLay *et al.*, 2009). This study, using B27/hβ_2m transgenic rats, revealed that IL-23 was synergistically upregulated by LPS in macrophages undergoing a UPR induced by either pharmacological agents or by HLA-B27 misfolding. This study also verified that IL-23 levels were also elevated in

the colon of B27/hβ_2m transgenic rats exhibiting gut inflammation, further supporting the finding that IL-23R polymorphisms are associated with IBD development. It has been suggested that in the gut, low level immune responses to bacterial colonisation might result in increased levels of type I and/or type II interferons leading to upregulation in MHC class I heavy chain expression – and so in HLA-B27-expressing cells, activation of the UPR. Macrophages would then become sensitised to TLR agonists, such as LPS, pushing them towards increased production of IL-23 and IFN-β. IL-23 would then drive IL-17 production through committed Th17 cells (Colbert *et al.*, 2009). Whether or not the Th17 lineage of T-cells is important for the arthritic phenotype in transgenic rats or indeed in human SpA patients will require further work.

Taken together, recently published results demonstrate the importance of HLA-B27 heavy misfolding in UPR induction and the possible influence that ER stress and UPR induction might have on driving inflammatory disease in animal models of AS. Despite this, the level of UPR induction and UPR target gene activation in cells expressing misfolded B27 heavy chains is still not fully understood. Thus, the aim of this chapter is to move towards a better understanding of the contribution of B27 misfolding/misoxidation in UPR induction, to help understand the influence of heavy chain homodimer formation in inflammation and identify targets for therapy.

4.2.2 *Results*

4.2.2.1 *Transient transfection of HLA-B7 and HLA-B27 into HeLa and HT1080 cells causes misoxidation of MHC class I heavy chains*

In order to study the effect of HLA-B27 misoxidation in HeLa and HT1080 cells, I transiently transfected HLA-B7 – a class I heavy chain similar to HLA-B27 (Fig. 18) but not associated with AS – or the HLA-B27 MHC class I heavy chain into these adherent cell lines. Transfected cells were lysed in the presence of the alkylating agent NEM (to trap any free disulfides and prevent post-lysis disulfide bond formation) and analysed by SDS-PAGE. Immunoblotting with the pan-class I monoclonal antibody HC10 revealed that both untransfected HeLa and HT1080 cells endogenously express MHC class I heavy chains (Fig. 19A, lanes 1 and 4). Under non-reducing conditions, these heavy chains exist as monomers representing free heavy chains (Fig. 19A, lanes 7 and 10). In transfected cells, the expression of MHC class I molecules increased, as expected (Fig. 19A, lanes 2-3 and 5-6).

Under non-reducing conditions, both HLA-B7 and HLA-B27 were resolved as a ladder of high molecular weight complexes in addition to the monomeric class I heavy chain (cI HC) (Fig. 19A, lanes 8-9 and 11-12). The ladder of bands seen as a consequence of HLA-B7 transfection was similar in both HeLa and HT1080 but dissimilar to that of HLA-B27 (Fig. 19A, compare lanes 8 and 11 with lanes 9 and 12). Likewise, transient transfection of HLA-B27 led to a similar make-up of high molecular weight complexes in HeLa and HT1080 cells.

```
                       *        20         *        40         *
B2705 : GSHSMRYFHTSVSRPGRGEPRFITVGYVDDTLFVRFDSDAASPREEPRAP
B0702 : GSHSMRYFYTSVSRPGRGEPRFISVGYVDDTQFVRFDSDAASPREEPRAP

                      60         *        80         *       100
B2705 : WIEQEGPEYWDRETQICKAKAQTDREDLRTLLRYYNQSEAGSHTLQNMYG
B0702 : WIEQEGPEYWDRNTQIYKAQAQTDRESLRNLRGYYNQSEAGSHTLQSMYG

                       *       120         *       140         *
B2705 : CDVGPDGRLLRGYHQDAYDGKDYIALNEDLSSWTAADTAAQITQRKWEAA
B0702 : CDVGPDGRLLRGHDQYAYDGKDYIALNEDLRSWTAADTAAQITQRKWEAA

                     160         *       180         *       200
B2705 : RVAEQLRAYLEGECVEWLRRYLENGKETLQRADPPKTHVTHHPISDHEAT
B0702 : REAEQRRAYLEGECVEWLRRYLENGKDKLERADPPKTHVTHHPISDHEAT

                       *       220         *       240         *
B2705 : LRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVP
B0702 : LRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVP

                     260         *       280         *       300
B2705 : SGEEQRYTCHVQHEGLPKPLTLRWEPSSQSTVPIVGIVAGLAVLAVVVIG
B0702 : SGEEQRYTCHVQHEGLPKPLTLRWEPSSQSTVPIVGIVAGLAVLAVVVIG

                       *       320         *
B2705 : AVVAAVMCRRKSSGGKGGSYSQAACSDSAQGSDVSLTA : 338
B0702 : AVVAAVMCRRKSSGGKGGSYSQAACSDSAQGSDVSLTA : 338
```

Figure 18. **Protein sequence alignment of HLA-B7 and HLA-B27.** The amino acid sequence of the HLA-B7 and HLA-B27 heavy chain after removal of the 24-amino acid signal peptide. Cysteine residues are highlighted in red. Note the additional cysteine residue at position 67. The transmembrane region is highlighted in blue.

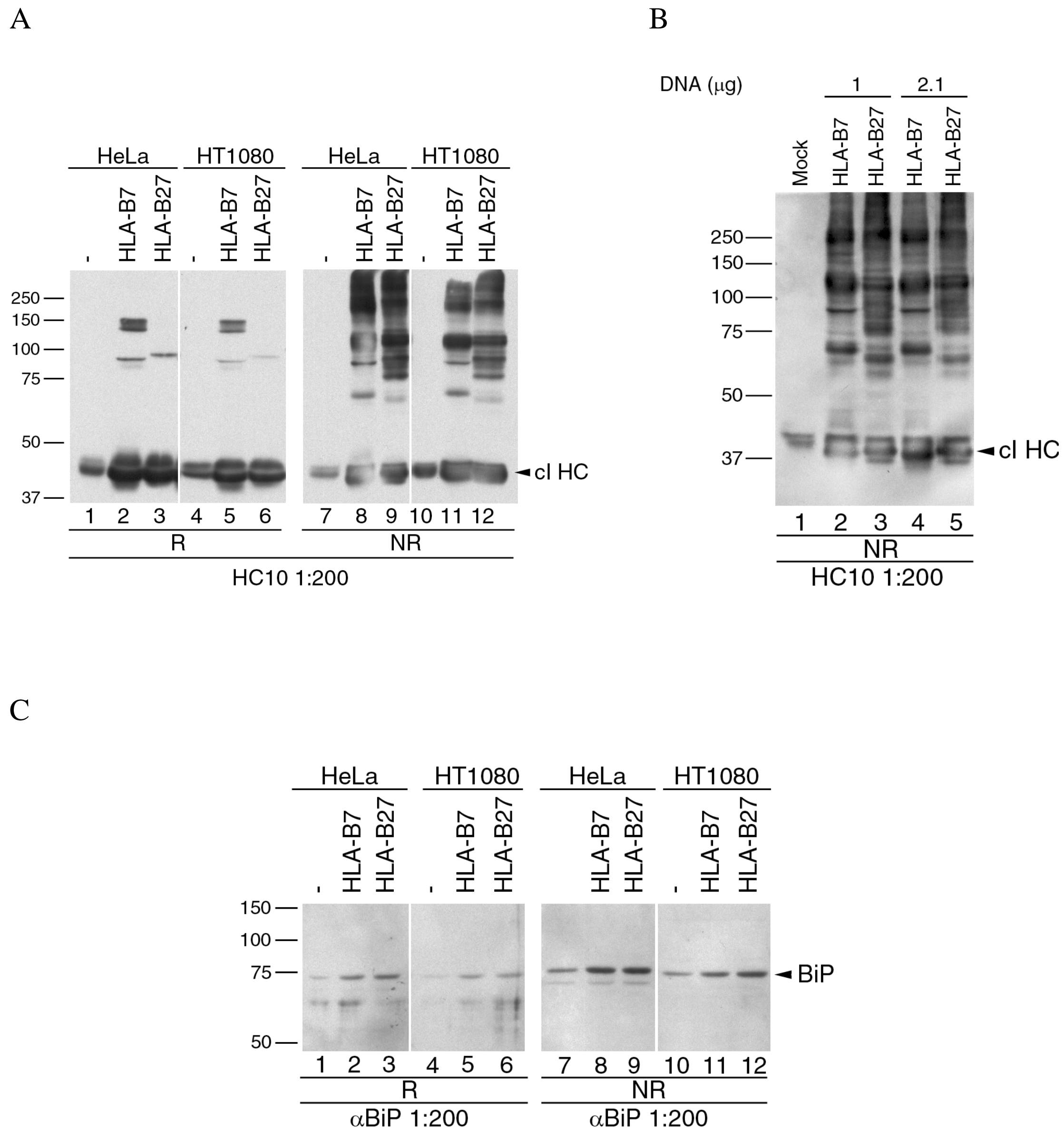


Figure 19. **Transient transfection of HLA-B7 and HLA-B27 causes misoxidation in HeLa and HT1080 cells which is accompanied by an increase in BiP expression.** A, HeLa and HT1080 cells were either mock (-) or HLA-B7- or HLA-B27-transfected using the transfection reagent Fugene. Lysates were separated by reducing (R) and non-reducing (NR) SDS-PAGE and immunoblotted with HC10. B, An HC10 non-reducing blot of HeLa cells transfected with either 1 μg or 2.1 μg HLA-B7 or HLA-B27 DNA. C, As in A, but the lysates were separated by non-reducing (NR) SDS-PAGE and immunoblotted with anti-BiP antibody H-129 (αBiP).

In HeLa cells, the formation of high molecular weight complexes did not depend on the amount of DNA transfected, since increasing the plasmid concentration to 2.1 μg/dish resulted in a very similar distribution of heavy chain complexes in a non-reducing blot (Fig. 19B).

Upon the reduction of these samples with DTT, the majority of the high molecular weight complexes, observed in the non-reduced samples, resolved as a monomer (Fig. 19A, compare lanes 2-3 and 5-6 with lanes 8-9 and 11-12). Thus, transient transfection of HLA-B7 and HLA-B27 induced the disulfide dependent intermolecular misoxidation of MHC class I heavy chains in both HeLa and HT1080 cells. Misoxidation of cI HC was most likely due to overexpression in the absence of sufficient β_2m. This is consistent with other published work from our group which has shown that overexpression of wild-type and cysteine mutant forms of HLA-B*2705 in HeLa cells causes misoxidation of heavy chains and the formation of disulfide-dependent high molecular weight complexes (Saleki *et al.*, 2006).

HC10-reactive and DTT-resistant bands were resolved at ~90-150 kD in the reducing gel of HLA-B7- and HLA-B27-transfected HeLa and HT1080 cells (Fig. 19A, lanes 2-3 and 5-6). These samples were boiled prior to loading and could either be heavy chain dimers that are resistant to reduction by DTT or a non-covalent interaction with the ER chaperone BiP which remains stable under reducing conditions (Saleki *et al.*, 2006). Equally, the bands could be a combination of these two phenomena. The appearance of these bands was reproducible but varied between experiments. Since BiP has been shown to associate with HLA-B27 when overexpressed (Saleki *et al.*,

2006), I immunoblotted the same lysates with the anti-BiP antibody H-129 under non-reducing conditions (Fig. 19C). BiP was resolved as a monomer at 78 kD but could not be detected at higher molecular weights in our experiments. The boiling- and DTT-resistant high molecular weight complexes observed in the reducing gel in Figure 19A did not correspond to either the molecular weight of BiP (78 kD) or the combined molecular weight of BiP and the cI heavy chain together, further indicating that these bands might not be BiP-cI HC conjugates. Mass spectrometric analysis of HLA-B7 and HLA-B27 co-associating proteins in these lysates is needed to verify the identity of components of the high molecular weight complexes.

The number and size of the HC complexes were dependent upon the heavy chain. HLA-B7 expression led to the formation of three distinct boiling- and DTT-resistant distinct bands while expression of HLA-B27 only led to the formation of one high molecular weight band (Fig. 19A, lanes 2-3 and 5-6). Interaction of cI HCs with ER-resident proteins and peptide loading complex components has been shown to be dependent on the 116 residue (Turnquist *et al.*, 2000). Turnquist and colleagues showed that TAP interactions – as well as tapasin and calreticulin interactions – were improved in the HLA-B7 (*Y116D*) mutant over wild-type HLA-B7. Furthermore, natural subtypes of HLA-B15, which differ in residue identity at position 116, also showed disparity in ER-resident protein-interaction. Given that HLA-B7 possesses an tyrosine residue and HLA-B27 possesses an aspartic acid residue at position 116, the differences in the DTT-resistant band make-up between HLA-B7 and HLA-B27 may be, in part, due to the amino acid at this position.

It has also been reported that polymorphisms at position 116 in HLA-B27 influence conformational homodimer formation (Blanco-Gelaz *et al.*, 2009). Thus, the differences in high molecular weight complexes between HLA-B7 and HLA-B27 seen in the non-reducing blot in Figure 19A might be attributed to the sequence differences at this residue between the two molecules.

The transient expression of both HLA-B7 and HLA-B27 in HeLa and HT1080 cells increased protein levels of BiP (Fig. 19C). It must be noted, however, that this experiment did not have a loading control. Since misoxidation of cI HC is likely to have an impact on ER homeostasis, an increase in BiP expression may be attributed to the activation of the UPR.

4.2.2.2 *Misoxidation of HLA-B7 and HLA-B27 upregulates BiP and induces XBP1 splicing*

In order to verify the effect of HLA-B7 and HLA-B27 misoxidation on BiP expression, and to further study the effect of HC misoxidation on the UPR, I used RT-PCR to examine both *BiP* mRNA levels and the splicing of constitutively-expressed *XBP1* (Fig. 20). RNA was extracted from cells transfected with HLA-B7 or HLA-B27 and was subjected to RT-PCR using primers against *XBP1*, *BiP* and *β-actin*. The *XBP1* RT-PCR product was digested to differentiate between the spliced and unspliced version (See Results Chapter 4.1). Transient transfection of both HLA-B7 and HLA-B27 induced splicing of *XBP1* whereas mock transfection did not induce *XBP1* splicing. This occurred in both HeLa and HT1080 cells (Fig. 20, compare lanes 2-3 with lane 1 and lanes 5-6 with lane 4). There was no apparent difference in the

level of *XBP1* splicing. Additionally, at the mRNA level, *BiP* expression was upregulated as a consequence of HLA-B7 and HLA-B27 transfection (Fig. 20). This supports the data in Figure 19C which shows upregulation of BiP in response to HLA-B transfection at the protein level. HC misoxidation-induced *XBP1* splicing was not as much as that induced by DTT treatment of HeLa and HT1080 cells (Fig. 20, compare B with A), however, induction of *BiP* expression was robust when compared to that induced by DTT. Thus, misoxidation of MHC class I heavy chains triggers *XBP1* splicing and *BiP* upregulation in HeLa and HT1080 cells.

When *CHOP* expression was analysed in HLA-B27-transfected HT1080 cells – which trigger *XBP1* splicing and upregulate *BiP* – it was found that *CHOP* was not induced (Fig. 20C, lane 3). DTT treatment of these cells, however, initiated *CHOP* expression, demonstrating that HT1080 cells were capable of inducing *CHOP* upon ER stress. Consequently, while HC induced-misoxidation was able to trigger processing of *XBP1* and *BiP* upregulation, it was unable to promote the expression of the apoptotic transcription factor *CHOP* which may have consequences for cell survival.

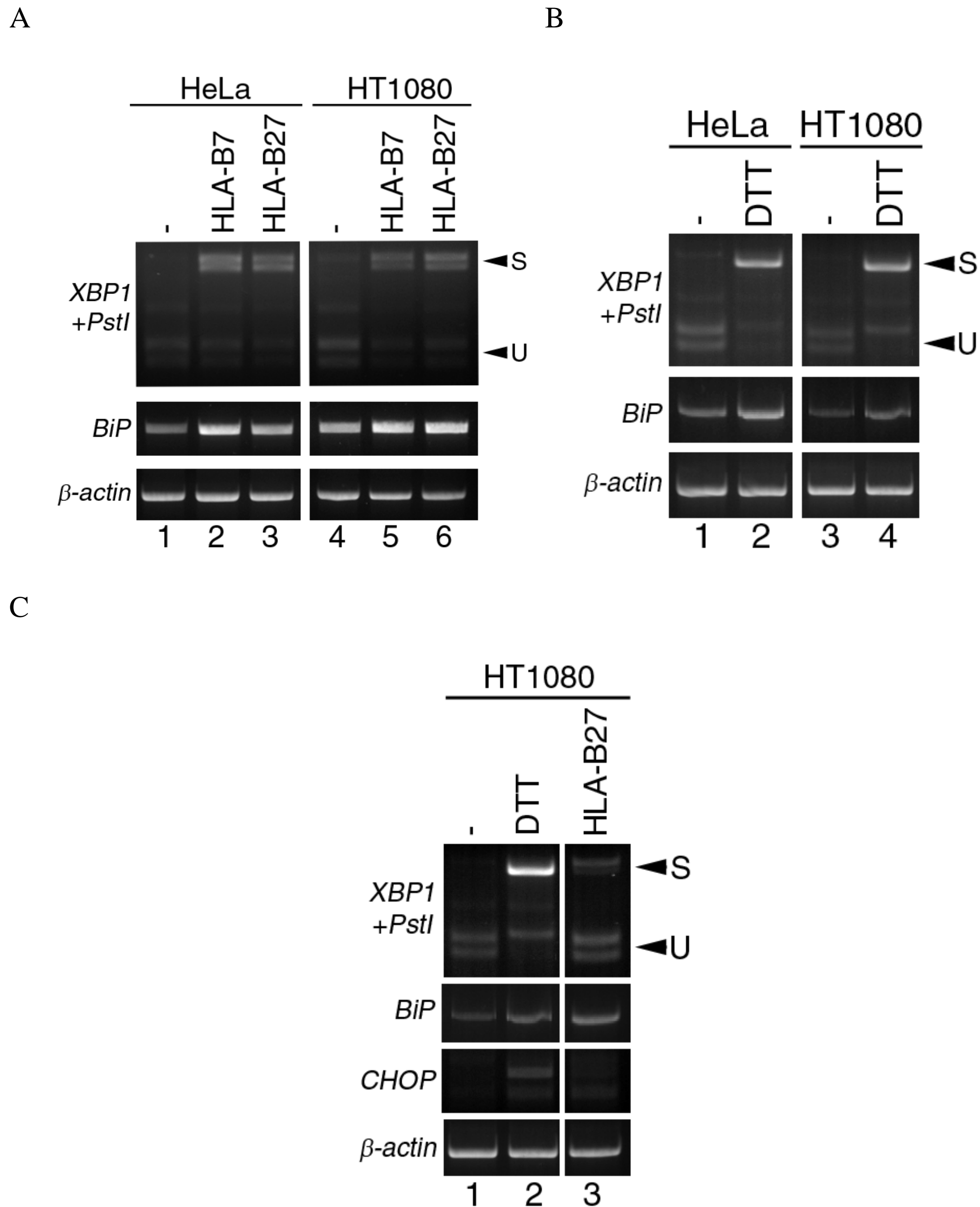


Figure 20. **Misoxidation of HLA-B7 and HLA-B27 upregulates *BiP* and induces *XBP1* splicing.** A, mRNA from mock (-), HLA-B7 and HLA-B27 transfected HeLa and HT1080 cells were subjected to RT-PCR for *XBP1*, *BiP* and *β-actin*. The *XBP1* product was digested with *PstI* and run on a 2% agarose gel to differentiate between the spliced (S) and unspliced (U) forms. B, As in A but cells were either mock treated (-) or treated with dithiothreitol (DTT) for 6 hours prior to lysis.

4.2.2.3 *Rapamycin treatment of HLA-B27-expressing cells*

One of the mechanisms the cell uses to degrade ER protein is autophagy. This is a catabolic process which degrades and recycles cytosolic or aggregated proteins and cellular organelles by delivering them to an autolysosome. Recently, autophagy has been shown to have a role in ER protein quality control and DTT and tunicamycin have can effectively induce autophagy via Atg1 kinase activity (Berger *et al.*, 2006; Kruse *et al.*, 2006; Kamimoto *et al.*, 2006; Yorimitsu *et al.*, 2006; Fujita *et al.*, 2007; Ishida *et al.*, 2009). Ishida and colleagues showed that the cell initiates autophagic mechanisms in response to the accumulation of cytotoxic aggregates of procollagen and that autophagy confers a cytoprotective role on these cells (Ishida *et al.*, 2009). The clearance of protein aggregates from the ER has implications for ER homeostasis and consequently, cell survival. The role of autophagy is therefore relevant to our understanding of diseases which have components of aberrant protein formation in the ER. I was therefore interested in the role of autophagy in cI HC misoxidation and the consequences of autophagy for the UPR.

The lipophilic antibiotic rapamycin induces autophagy by inhibiting the mammalian target of rapamycin (mTOR). Rapamycin binds to the intracellular receptor FK506 binding protein (FKBP12), forming a rapamycin-FKBP12 complex which binds directly to mTOR, a protein kinase involved in regulating the cell cycle (Heitman *et al.*, 1991; Lorenz & Heitman, 1995). In yeast, mTOR inhibition by rapamycin leads to Atg1 kinase activity which leads to the recruitment of multiple Atg proteins and the initiation of autophagosome formation (Cheong *et al.*, 2008; Kawamata *et al.*, 2008). Rapamycin treatment has been widely used to induce macroautophagy and, more

recently, to counter aggregate formation (Ravikumar *et al.*, 2002; Berger *et al.*, 2006; Ishida *et al.*, 2009).

Initially, HeLa cells were transfected with or without the cI HC HLA-B27. Six hours prior to lysis, cells were either treated or non-treated with rapamycin at 10 µg/ml – the concentration used by Ishida and colleagues – for 6 hours. Cells were then lysed and analysed either by SDS-PAGE and Western blotting or by RT-PCR (Fig. 21). Transfection with HLA-B27 was successful since a ladder of misoxidised heavy chains was detected after transfection in HeLa cells (Fig. 21, lanes 3-4). This is consistent with previous observations (Fig. 19A). Treatment of non-transfected as well as HLA-B27-transfected cells had no effect on the detectable level of heavy chain monomers. The ladder of misoxidised, high-molecular weight aggregates of HLA-B27 heavy chains was not affected by rapamycin treatment (Fig. 21, compare lane 4 with lane 3). It could be that the concentration of rapamycin, despite being sufficient for aggregate removal in mouse embryonic fibroblast cells, is not high enough to promote autophagy in HeLa cells. Alternatively, it may indicate that autophagy doesn't promote removal of disulfide bonded HC complexes in HeLa cells. Further experiments are required to address this issue.

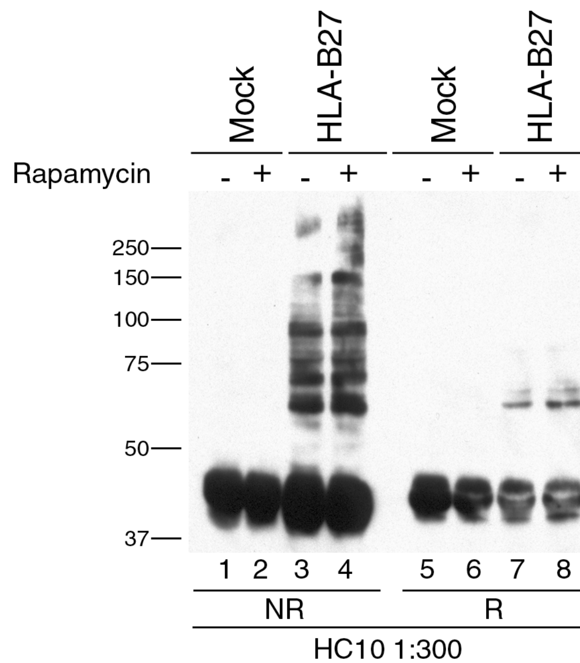


Figure 21. **The effect of rapamycin on misoxidised MHC cI heavy chains.** A, HeLa cells were either non-transfected (mock) or transfected with HLA-B27. Six hours prior to lysis, cells were treated with 10 μg/ml rapamycin to induce autophagy. Cells were lysed and analysed under non-reducing (NR) and reducing (R) conditions with the pan class-I HC antibody HC10.

4.2.2.4 *XBP1 processing in HLA-B27 stable transfectants*

Since overexpression of non-disease associated MHC cI heavy chains by transient transfection leads to different misoxidation patterns (Fig. 19A), it is likely that a high expression level of heavy chains in the absence of β_2-microglobulin is not solely responsible for oxidative misfolding. Therefore, I asked whether cells that were stably transfected with MHC class I heavy chains activated a UPR and if mutations thought to improve the folding kinetics of HLA-B27 had an effect on UPR induction.Fussell and colleagues recently showed that a single amino acid substitution in HLA-B27 led to an increase in resistance to DTT-induced reduction of HC monomers (Fussell *et al.*, 2008). In their experiment, C58 rat thymoma cells were stably transfected with a mutant form of HLA-B27 containing an amino acid substitution of Asp for His at position 114. This B27.H114D mutant was more susceptible to reduction by the reducing agent DTT.

Susceptibility to reduction is dependent on cysteine residue exposure. Therefore, forms of HLA-B27 that are resistant to reduction exist as fully folded, compact class I molecules with their cysteine residues buried. On the other hand, forms of HLA-B27 which are sensitive to reduction are poorly folded. Conformations with exposed cysteines could result from low-affinity peptide binding or slow folding kinetics (Williams *et al.*, 2002; Antoniou *et al.*, 2004). Thus, mutating a key structural residue in the F-pocket (p114) of the peptide-binding groove – which determines the requirement for the accessory molecules tapasin – can result in an improvement in folding and can lead to a more compact heavy chain, as indicated by resistance to reduction (Williams *et al.*, 2002; Antoniou *et al.*, 2004). Thus, in addition to

monitoring *XBP1* processing in HLA-B27 stable transfectants, I wished to examine the effect of improved folding of the B27.H114D.D116Y mutant over wild-type B27.

C58 cells stably expressing HLA-A*0210, HLA-B*2705 and HLA-B*2705.H114D.D116Y were given as a gift from Antony Antoniou. The cells were cultured in medium containing 1 mg/ml G418 to maintain the resistant cells. Cells were either non-treated or treated with DTT or tunicamycin then lysed and RNA-extracted before undergoing RT-PCR analysis for *XBP1* splicing and *β-actin* levels as a control (Fig. 22). Untransfected C58 cells exhibited negligible levels of *XBP1* splicing without ER stress inducer-treatment (Fig. 22, lane 1). Untreated C58.A2, C58.B27 and C58.B27.H114D.D116Y cells also exhibited low levels of *XBP1* splicing. Thus, stable transfection of MHC cI heavy chains in C58 cells did not induce splicing of *XBP1*. The H114D.D116Y mutation did not cause an HLA-B27 induction of *XBP1* splicing under these experimental conditions. Each cell line responded to DTT and tunicamycin indicating that these cell lines are capable of initiating *XBP1* splicing in response to ER stress. However, transfection of A2, B27 and B27.H114D.D116Y all caused an increase in the ratio of spliced:non spliced *XBP1* in response to tunicamycin when compared to the untransfected C58 cells. (Fig. 22, compare *XBP1* (S):*XBP1* (U) in lanes 6,9,12 with lane 3). Thus, tunicamycin did not have as strong an effect in C58 cells which have been subjected to stable transfection with MHC class I heavy chains compared to untransfected C58 cells. This may suggest that chronic overexpression of MHC class I molecules "pre-sensitises" cells to some secondary stress events.

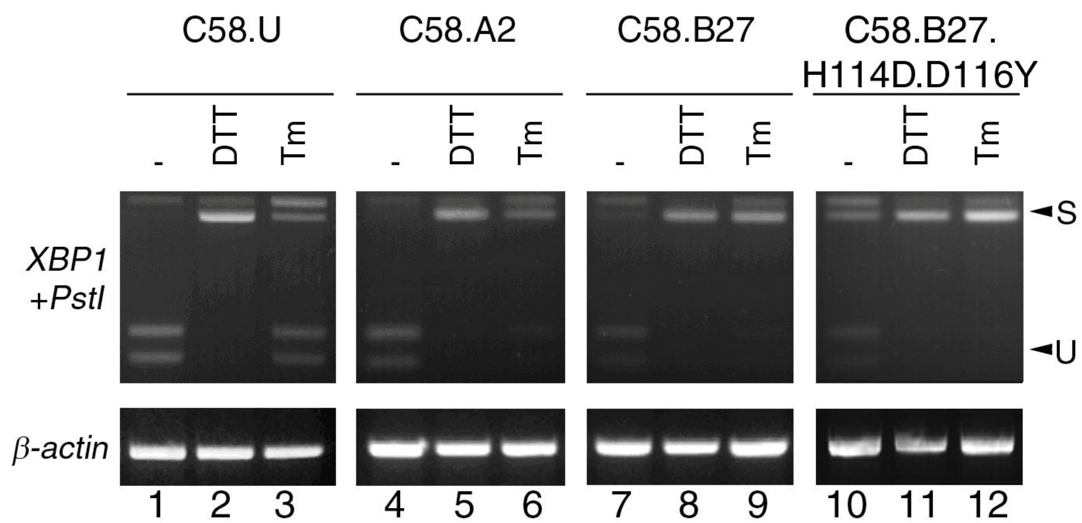


Figure 22. ***XBP1* processing in C58 stable transfectants.** Stable transfectants were maintained in RPMI at 10% FCS with 1 mg/ml G418. Lysates of each cell line were subjected to RNA extraction and subsequent RT-PCR analysis for *XBP1* and *β-actin*. Samples were then run on a 1 or 2% agarose gel.

4.2.2.5 *Lymphocytes from SpA patients exhibit HLA-B27 misoxidation*

Analysis of cells from patients suffering from AS gives us an insight into the physiological condition of cells involved in disease. Oxidative misfolding has been demonstrated in splenocytes from HLA-B27/hβ_2-microglobulin transgenic (B27-Tg) rats, which spontaneously develop an inflammatory disease similar to human AS (Turner *et al.*, 2005). HLA-B27 heavy chains from these cells formed disulfide-linked complexes in the ER, with prolonged BiP binding and inefficient folding.

I received a selection of immortalised lymphocytes taken from spondyloarthropathy patients expressing the HLA-B27 allele as well as healthy controls (kind gift from J. Goodall, Cambridge). To ask whether these immune cells also exhibited oxidative misfolding, I analysed the lysates from these cell populations by Western blotting.

Alkylated proteins from lysates were separated by SDS-PAGE under reducing and non-reducing conditions to disrupt or preserve any disulfide-linked complexes. Heavy chains were detected using the monoclonal antibody HC10 (Fig. 23). Under reducing conditions, cI HCs from a healthy individual not expressing HLA-B27 migrated as a single band (Fig. 23, lane 5). HLA-B27-positive lymphocytes from reactive and psoriatic arthritis patients exhibited stronger and more diffuse HC bands, which may be due to higher MHC class I expression. An additional faster-migrating band below is likely to represent the unglycosylated form of the HC (Fig. 23, lanes 7-8).

Analysis of the non-reducing gel revealed that HLA-B27-negative lymphocytes from a healthy individual migrated as a single monomeric HC. In contrast, a healthy

individual possessing the HLA-B27 allele exhibited a high molecular weight complex in addition to the monomeric form of HC. It is likely that this is a disulfide-linked complex since reduction of this sample disrupted the slower-migrating band.

The HCs of HLA-B27-positive patients presenting disease also exhibited disulfide-linked complexes (Fig. 23, lanes 3-4) unlike the HCs in the HLA-B27 negative cell line (Fig. 23, lane 1) and the HCs in untransfected HT1080 and HeLa cells (Fig 19A). Thus, in these individuals, possession of the HLA-B27 allele is associated with MHC class I "misoxidation". However, whether MHC class I molecules in these cells are truly misfolded requires further investigation. Rather more dimers were detected in the diseased patients (Fig. 23, lanes 3 and 4), although the presence of HC dimers in the healthy patient suggests that dimers per se are not a causal link with AS. However, it remains possible that the dimers are not B27-B27 homodimers, or that dimers are a precursor of disease, with other events being required for disease to occur. These samples only represent a population of cells from an individual and there is likely to be variation in HC oxidation dependent on cell type, HLA-B27 subtype and severity of disease. However, these data indicate that B27 misoxidation can occur in cells from a variety of genetic backgrounds.

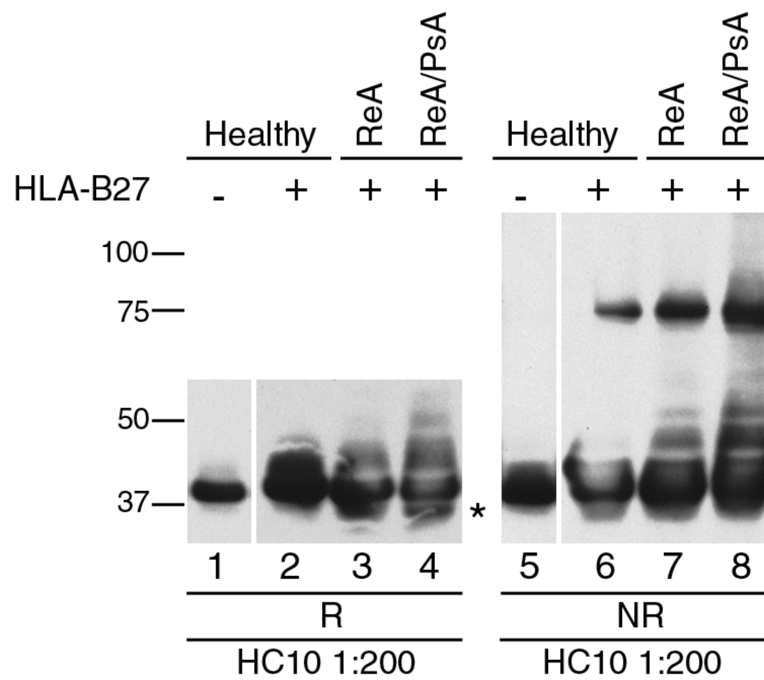


Figure 23. **HLA-B27 misfolding in AS patient lymphocytes.** Immortalised lymphocytes were lysed and analysed under non-reducing (NR) and reducing (R) conditions and subjected to Western blotting with HC10. HLA-B27-positive (+) and negative (-) cells are indicated. A population of unglycosylated cI HCs are also indicated (*).

4.2.3 *Discussion*

High expression levels of HLA-B27 influence disease susceptibility in the B27-Tg rat (Taurog *et al.*, 1993). However, the transgene copy number and expression level of the HLA-B7/hβ_2-microglobulin transgenic rat – which does not develop disease – is similar to that of the B27-Tg rat, indicating that a disease phenotype is not simply an artefact of high HLA-B expression but represents an HLA-B27-specific effect (Hammer *et al.*, 1990; Taurog *et al.*, 1999).

Previously, in work which led to the completion of my Masters thesis, I showed that HLA-B27 misoxidises when transfected into HeLa cells (Lemin, A. J. MSc Thesis 2007). Here, I provide evidence that overexpression of HLA-B27 and the non-disease associated HC HLA-B7 by transient transfection in HeLa and HT1080 cells is sufficient to induce oxidative misfolding (Fig. 19A). Transient transfection of HLA-B heavy chains may therefore be a useful model for analysing how misfolded membrane proteins in general are handled by the ERAD and UPR systems. A model for how the cell might respond to heavy chain misfolding is proposed in Figure 24. This illustrates the interplay between ERAD, the peptide loading complex and the UPR in HC misfolding. HLA-B27 misoxidation in the B27-Tg rat has been linked with activation of the UPR (Turner *et al.*, 2005; Turner *et al.*, 2007). I studied the IRE1-mediated splicing of *XBP1* – a key step in the IRE1 branch of the UPR – in cells exhibiting misoxidation of cI HCs to determine if HC misoxidation is correlated with UPR

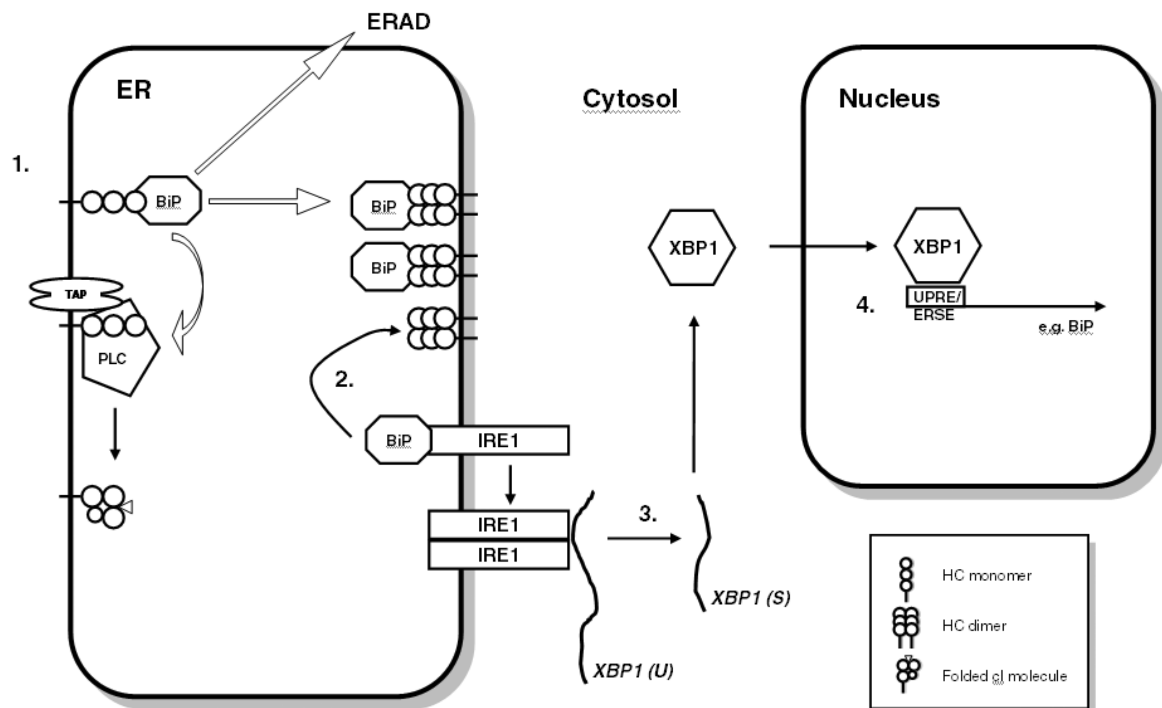


Figure 24. **The consequences of heavy chain misfolding for the ER.** Newly synthesised MHC class I heavy chains (cI HC) enter the ER and are bound by the ER chaperone BiP (1.). In order to reach a fully formed state, the heavy chain must bind the ER lectin calnexin (not shown) and enter into the peptide loading complex (PLC) where it will acquire peptide and β_2-microglobulin. Alternatively, HCs terminally bound by BiP may be actively removed by ER-associated degradation. If misfolded HCs are not removed by ERAD they may form HC dimers or oligomers which accumulate in the ER. One of the responses to the accumulation of misfolded HCs is the release of BiP by IRE1 – which binds HCs to prevent aggregation (2.). This in turn causes activation of the RNase activity of IRE1 which results in the splicing of full length XBP1 (*XBP1 (S)*) (3.). XBP1 then enters the nucleus and recognises specific promoter elements upregulating target genes, such as *BiP*, in order to combat HC accumulation. TAP = Transporter associated with antigen processing. UPRE = Unfolded protein response element. ERSE = ER stress response element.

induction. I demonstrate that cells transiently transfected with both HLA-B27 and HLA-B7 exhibit moderate *XBP1* splicing and increased transcript levels of *BiP* as well as an increase in BiP protein levels (Fig. 20A and 19B). Although, in order to state that the increase in BiP protein levels observed here are relevant, a more quantitative approach, utilising a loading control, will be required.

Consistent with the observation of B27 upregulation in the macrophages of B27-Tg rats, the magnitude of BiP upregulation and *XBP1* splicing correlated with HC expression level (data not shown) (Turner *et al.*, 2007). This was demonstrated in both HeLa and HT1080 cells indicating that this response may be conserved between different cell lines. *XBP1* splicing was more sturdy in HeLa and HT1080 with DTT treatment than HC misoxidation. This is expected since DTT will have a global reducing effect whereas the effect of HC misoxidation on the ER as a whole will be more limited. Thus, in the transient transfection model, *XBP1* splicing and *BiP* upregulation is correlated with cI HC misoxidation and is independent of heavy chain disease association. Therefore, it is likely that UPR induction in this regard is a consequence of overexpression-induced misoxidation – in the absence of a concomitant increase in β_2m expression.

Further analysis of HT1080 transiently transfected with HLA-B27 showed that despite moderate *XBP1* splicing and *BiP* upregulation, *CHOP* was not induced in these cells (Fig. 20C). I have previously shown that HT1080 cells do not induce *CHOP* expression in response to tunicamycin treatment (Fig. 10). However, tunicamycin treatment only induces very mild levels of *XBP1* splicing in these cells.

The consequences of limited *CHOP* induction may include increased cell survival since *CHOP* has been demonstrated to promote apoptosis (Oyadomari & Mori, 2004). Increased survival of B27-expressing pro-inflammatory cells could have implications for disease pathogenesis including increased cytokine production and exacerbation of the inflammatory response. Whether or not attenuated *CHOP* expression in response to HC misoxidation is demonstrable for other cell types – including more physiologically relevant cells types such as the professional antigen presenting cells like macrophages and dendritic cells – remains to be seen.

C58 cells stably expressing HLA-B27 had negligible levels of *XBP1* processing; comparable to background *XBP1* splicing in untransfected C58 cells (Fig. 22). Non-disease associated HLA-A2-expressing C58.A2 cells also showed extremely low levels of *XBP1* splicing. Since I have observed that the level of *XBP1* splicing is correlated with HC expression levels, it is possible that these stably transfected cell lines do not express HCs at as high a level as transiently transfected cells. Alternatively, long-term expression of cI HCs in stable transfectants may have led to adaptation and the attenuation of IRE1-mediated UPR signalling. The IRE1 branch of the UPR has been demonstrated to selectively attenuate during persistent ER stress whereas PERK signalling continued during this time (Lin *et al.*, 2007). However, Lin and colleagues demonstrated that persistent IRE1 signalling promoted cell survival and suggested that the attenuation of IRE1 signalling may represent a switch in fate from prosurvival to proapoptotic pathways. Analysis of PERK and ATF6 signalling in C58 stable transfectants would help determine if attenuation of UPR signalling is occurring in these cells.

The oxidative status of transfected cI HCs can tell us much about the way HCs behave when expressed at various concentrations. However, analysis of lymphocytes from SpA patients – and healthy individuals – can tell us about the physiological status of the cells after disease is triggered. I demonstrate that lymphocyte populations from HLA-B27-expressing individuals exhibit some degree of oxidative misfolding of cI HCs. This finding was independent of disease presentation since lymphocytes from the healthy B27-expressing individual exhibited HC misoxidation. This suggests that oxidative misfolding may be a feature of HLA-B27 expression in these individuals. Since fewer than 5% of B27-expressing individuals develop disease – in the absence of a family history - it is clear that the genetic susceptibility to pathogenesis is complex.

The consequence of HC misoxidation in the patient cells shown in Figure 23 is unknown but previous work from our group determined that the HLA-B27-expressing lymphocytes WEWAK1, HOM-2 and JESTHOM cells may display elevated levels of baseline *XBP1* splicing which may indicate that HC misfolding in the patient cells presented here may be associated with XBP1 splicing (Lemin *et al.*, 2007). The presence of unglycosylated HCs in lymphocyte populations from the individuals presenting ReA or PsA may further indicate that HCs undergo inefficient folding events in the ER. Moreover, inappropriately oxidised HCs could be caused by free HC homodimerisation at the cell surface of B27-expressing cells. Further work will be required to identify the molecular basis for the differences in HLA-B heavy chain oxidative misfolding between patients presenting disease and healthy individuals.

In addition, I explored the relationship between HC misoxidation and UPR activation by manipulating the degradation of HC aggregates through the promotion of autophagy. The effect of rapamycin, an inhibitor of mTOR, on HeLa cells transiently transfected with HLA-B27 was minimal, indicating that either autophagy was insufficiently activated in HeLa cells or that autophagy is not involved in removing cI HC aggregates from the ER. It should be noted that the conclusions from this experiment are limited since no positive control for the detection of autophagy induction is included. An appropriate control for the induction of autophagy is the conversion of microtubule-associated protein 1 LC3 from the free version (LC3-I) to the membrane-bound (LC3-II) version (Kabeya *et al.*, 2000). LC3 is a mammalian homologue of yeast ATG8, an ubiquitin-like protein which is involved in vesicle formation during macroautophagy. Therefore, further work is required to elucidate the role of autophagy in cI HC removal.

Together, our results indicate that HC misoxidation – evident in HLA-B27-expressing cells – and promoted by high expression levels in the absence of β_2m, contributes to the activation of *XBP1* processing and *BiP* upregulation. Future work on the physiological significance of ER stress signals and how UPR target genes promote and maintain the inflammatory response is needed. In addition, the role of oxidative stress and ROS production in UPR signalling and inflammation also requires further study.

4. 3 Context dependent activation of the unfolded protein response

4.3.1 *Introduction*

Protein tyrosine kinase signalling cascades play an important role in the regulation of cell proliferation, cell differentiation and signalling processes in the immune system, including the activation of inflammatory cells (Page *et al.*, 2009; Lemmon & Schlessinger, 2010). Consequently, tyrosine kinase inhibitors have been considered for their potential anti-inflammatory activity (Sahu & August, 2009; Cohen & Fleischmann, 2010). Recent work – using the tyrosine kinase inhibitor genistein – has linked tyrosine kinase signalling with the UPR (Hong *et al.*, 2005). This is of special interest when considering inflammatory diseases such as ankylosing spondylitis, as it may provide novel targets for therapy.

The transcriptional activation of the stress-response gene *grp78/BiP* is induced by a number of well documented pharmacological agents which disrupt ER function (see General Introduction and Results Chapter 4.1). In mammals, tyrosine- and serine/threonine kinases were demonstrated to be important for *grp78* transactivation since treatment with the potent tyrosine kinase inhibitor genistein is able to reduce thapsigargin-induced induction of *grp78* mRNA levels whereas the serine-threonine kinase phosphatase inhibitor okadaic acid enhances thapsigargin induction of *grp78* (Price *et al.*, 1992; Cao *et al.*, 1996). The mechanism by which genistein regulates the gene expression of *grp78* was addressed in a key paper by Zhou and Lee in 1998

(Zhou & Lee, 1998). They demonstrated that genistein specifically inhibits the induction of HSP70 and GRP78 through interference of the CCAAT binding factor (CBF)/NF-Y and the proximal CCAAT site – part of the ER stress response element (ERSE) – of the *hsp70* and *grp78* promoters. However, the mechanism by which the DNA binding property of CBF/NF-Y is regulated by genistein is unknown.

Furthermore, the recently identified multifunctional transcription factor TFII-I has been implicated in the regulation of GRP78 and as a potential target of genistein-mediated stress response gene suppression (Hong *et al.*, 2005). TFII-I demonstrates enhanced binding to the GC-rich motif in the ERSE of the *grp78* promoter following thapsigargin treatment and stimulates the ERSE-binding activity of ATF6 (Parker *et al.*, 2001; Yoshida *et al.*, 2001b). The activity of TFII-I is regulated by phosphorylation at serine/threonine and tyrosine residues and it has been demonstrated that the tyrosine kinase c-Src – activated by thapsigargin-induced ER stress – is required for the phosphorylation of tyrosine residues at positions 248 and 611 (Novina *et al.*, 1998; Cheriyath *et al.*, 2002; Hong *et al.*, 2005). Since tyrosine phosphorylation is not required for the DNA binding property of TFII-I, it is possible that phosphorylation of these residues regulates the formation of transcription factor complexes which, in turn, regulates the stability of these complexes at the promoter (Novina *et al.*, 1998; Hong *et al.*, 2005). A role for TFII-I tyrosine phosphorylation in the regulation of the stress response is illustrated by the evidence that a dominant negative mutant of c-Src attenuates thapsigargin stress induction of the *grp78* promoter whereas overexpression of c-Src activates the *grp78* promoter (Hong *et al.*, 2005). Since TFII-I undergoes considerable tyrosine phosphorylation after ER stress

and tyrosine phosphorylation is required for activity, coupled with the fact that genistein treatment of thapsigargin stressed cells suppresses *grp78* induction, it has been suggested that TFII-I is the target for the inhibitive effect of genistein on the ER stress response. Thus, TFII-I is an ER stress-regulated transcription factor which links a tyrosine kinase pathway with UPR activity.

The induction of glucose regulated proteins – and specifically GRP78 – has been linked with resistance to apoptotic cell death; the maintenance of cell viability under stressed conditions; survival during development as well as the formation of tumours and resistance to cell mediated toxicity in cancerous cells (Sugawara *et al.*, 1993; McCormick *et al.*, 1997; Dong *et al.*, 2004; Luo *et al.*, 2006; Lee, 2007). Thus, the induction of GRP78 is a double-edged sword, providing an adaptive, prosurvival function in stressed cells but also affording dangerous tumorigenic or neoplastic cell resistance to apoptosis. Levels of GRP78, and indeed GRP94, are elevated – and correlated with malignancy – in several cancer cell lines and solid tumours and human cancer biopsies (Fernandez *et al.*, 2000; Lee *et al.*, 2001). Therefore, targeted suppression of GRP induction or function in cancer cells might offer a novel approach to cancer therapy. Indeed, suppression of GRP78 induction by overexpressing antisense GRP78 mRNA inhibits tumour progression and increases cytotoxicity (Koong *et al.*, 1994; Jamora *et al.*, 1996).

Selective targeting of genistein to leukaemia cells results in tyrosine kinase inhibition and apoptotic cell death (Uckun *et al.*, 1995). In addition, administration of genistein to neonatal rats affords prevention against the development of mammary tumours

following carcinogen treatment later in life (Lamartiniere *et al.*, 1995). Moreover, genistein is considered a potential anti-inflammatory agent since genistein treatment confers a cytoprotective effect on an IL-1β- and IFN-γ-treated rat pancreatic β-cell line, thus preventing cytokine-mediated cytotoxicity (Kim *et al.*, 2007).

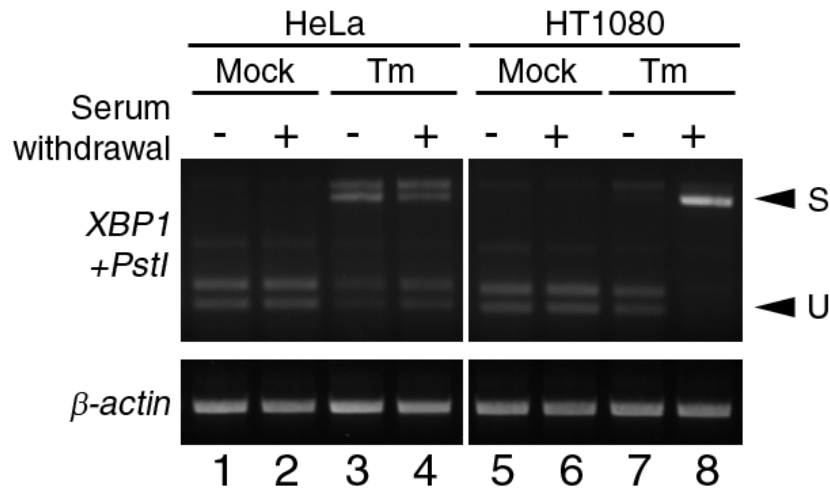
This chapter aims to investigate the role of genistein on the pharmacologically-induced ER stress response as well as examining the effect of serum withdrawal on cell viability of HeLa and HT1080 cells.

4.3.2 *Results*

4.3.2.1 *Serum withdrawal promotes XBP1 processing by tunicamycin in HT1080 cells.*

Previously, I have shown that HT1080 cells are relatively insensitive to tunicamycin despite exhibiting *XBP1* splicing in response to other stimuli (see Chapter 4.2). In order to determine if this was a general defect in ER to cytoplasm signalling, I subjected HT1080 and HeLa cells to treatments with tunicamycin in the presence or absence of foetal calf serum and analysed *XBP1* splicing (Fig. 25A). Serum withdrawal is known to activate several intracellular signalling pathways including those linked with UPR activation (Allsopp *et al.*, 2000; Kilic *et al.*, 2002). Cells were grown in complete media and then replaced with either serum-free medium or complete medium in the presence or absence of tunicamycin for 6 hours before subsequent lysis and analysis. Both HeLa and HT1080 cells did not initiate *XBP1* splicing when mock-treated even in the absence of serum (Fig. 25A, lanes 1-2 and 5-6). Tunicamycin treatment of HeLa cells resulted in *XBP1* splicing, as expected, but was not further augmented by the absence of serum. HT1080 cells exhibited negligible *XBP1* processing in response to tunicamycin, as seen previously (Fig. 9A, 10B and 11A) but, unlike HeLa cells, HT1080 displayed extensive splicing in the absence of serum. Thus, in HT1080 cells, serum withdrawal in the presence but not in the absence of tunicamycin leads to activation of the UPR and splicing of *XBP1* (Fig. 25A, lane 8).

A



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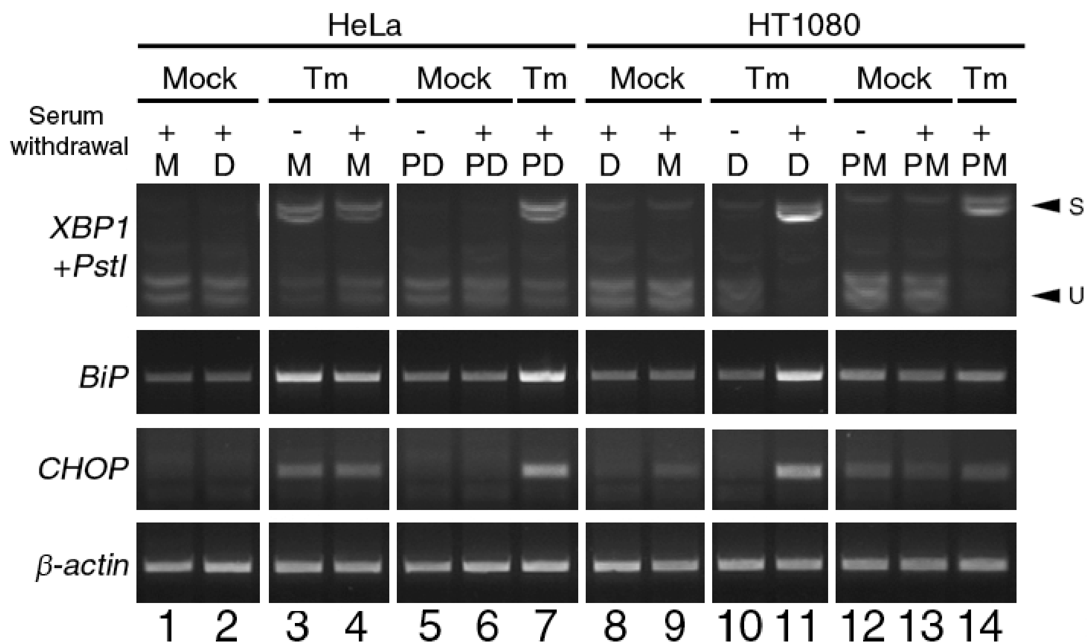


Figure 25. **Serum withdrawal in the presence of tunicamycin promotes *XBP1* splicing and *BiP* and *CHOP* upregulation in HT1080.** A, HeLa and HT1080 cells were either mock- or tunicamycin (Tm)-treated either in the presence of FCS (-) or in the absence of FCS (+) before being subjected to RT-PCR for *XBP1* to resolve the spliced (S) and unspliced (U) versions. *β-actin* is shown as a positive control. B, As in A, but cells were either treated in MEM (M), DMEM (D) or in DMEM preconditioned in HT1080 cells overnight (PD) or in MEM preconditioned in HeLa cells overnight (PM). In addition, RNA was used as a template to detect the expression of *BiP* and *CHOP*.

The data from Figure 25A suggests that, in HT1080, *XBP1* splicing in response to tunicamycin might be regulated by a signal which originates from outside the ER. One possible source of this signal might be a secreted factor that is released into the medium which might either have an activatory or inhibitory role in signalling. To address this, I subjected each cell line to the environment of the other cell line by swapping the growth media. Cell media were preconditioned either by HeLa cells (destined for HT1080 cells) or HT1080 cells (destined for HeLa cells) over night in the presence or absence of serum. After preconditioning, the filtered media was swapped over onto the opposite cell line and cells were subsequently cultured with or without tunicamycin for 6 hours. The cells were then analysed by RT-PCR for *XBP1* splicing, *BiP*, *CHOP* and *β-actin* expression (Fig. 25B). When HeLa cells were cultured in the absence of serum either in their native medium (MEM) or DMEM in the absence of tunicamycin, no *XBP1* splicing or *CHOP* induction was observed (Fig. 25B, lanes 1 and 2). This was also the case for mock-treated HeLa cells cultured for the 6 hour period in preconditioned DMEM either in the presence or absence of serum (Fig. 25B, lanes 5 and 6). DMEM is a modified version of MEM which contains an increased concentration of vitamins, amino acids and glucose. It should be noted that HT1080 cells cultured in MEM alone did not initiate *XBP1* splicing and were therefore unlikely to be sensitive to the decrease in glucose concentration or amino acid availability. This is consistent with previous experiments which showed that both HeLa and HT1080 cells do not initiate a UPR when exposed to diminished glucose conditions (Fig. 10 and 12).

As seen previously, when HeLa cells were grown in MEM in the presence or absence of serum, *XBP1* splicing is induced upon tunicamycin treatment. This is accompanied by upregulation of *BiP* and *CHOP* induction (Fig. 25B, lanes 3 and 4). Serum withdrawal, in this case, has a negligible effect on *BiP* and *CHOP* induction. In addition, HeLa cells cultured in preconditioned DMEM lacking serum (from HT1080 cells) in the presence of tunicamycin induced *XBP1* splicing and *BiP* and *CHOP* induction (Fig. 25B, lane 7). The level of *XBP1* splicing in these cells was comparable to that induced by tunicamycin treatment in serum-negative or -positive MEM. Thus, preconditioning the media in the environment of HT1080 cells does not lead to potentiation of *XBP1* splicing in non-tunicamycin-treated HeLa cells (Fig. 25B, lane 6) and, in the presence of tunicamycin, does not augment further *XBP1* splicing (Fig. 25B, lane 7).

As seen previously, when HT1080 cells were treated with tunicamycin in the presence of serum, *XBP1* splicing was not activated (Fig. 25B, lane 10). Tunicamycin treatment also failed to induce *CHOP* expression or upregulate *BiP* expression beyond the level induced by mock treatment (Fig. 25B, compare lane 10 with lanes 8 and 9). Tunicamycin treatment in the absence of serum however, induced robust *XBP1* splicing which was accompanied by strong upregulation of *BiP* and induction of *CHOP*. Culturing of HT1080 cells in serum-withdrawn and non-serum withdrawn preconditioned media from HeLa cells in the absence of tunicamycin did not induce *XBP1* splicing or *BiP* or *CHOP* induction. When HT1080 cells were cultured in serum-withdrawn, preconditioned media in the presence of tunicamycin, however, *XBP1* splicing was induced although not as strongly as with tunicamycin treatment in

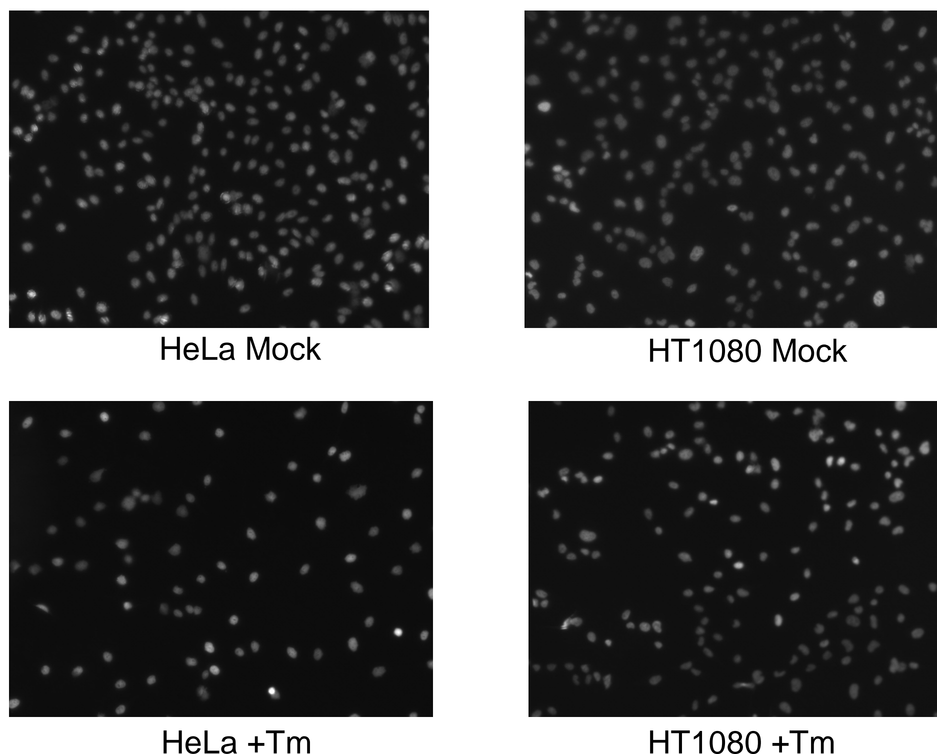
serum withdrawn DMEM (Fig. 25B compares lane 14 with lane 11). Surprisingly, *BiP* and *CHOP* expression were not strongly induced under these conditions. Thus, although the potentiation of the stress response in HeLa cells is largely unaffected by preconditioning of the media in HT1080 cells, when HT1080 cells are treated with tunicamycin in preconditioned, serum-starved HeLa medium, *XBP1* splicing and *BiP* and *CHOP* induction is attenuated. This may be due to some factor that HeLa is secreting which might regulate downstream UPR target gene induction. This factor might be viewed as 'prosurvival' since culturing of HT1080 cells in this medium prevents strong induction of the proapoptotic factor *CHOP*. Furthermore, HeLa may suffer from more severe nutrient deprivation than HT1080 cells or elevated production of toxic mertabolic waste products by HeLa cells. However, further work will be required to determine if there are any associated differences in cell survival between tunicamycin-treated, serum-starved HT1080 cells grown in DMEM or in preconditioned media from HeLa cells.

4.3.2.2 *The magnitude of tunicamycin-induced UPR induction correlates with cell viability in HeLa and HT1080 cells.*

The induction of the transcription factor CHOP is associated with the promotion of apoptosis (Zinszner *et al.*, 1998; McCullough *et al.*, 2001; Oyadomari et al., 2002b; Ma *et al.*, 2002). However, many other components of the UPR influence cell viability and cell fate (McCormick *et al.*, 1997; Scheuner *et al.*, 2001; Yoneda *et al.*, 2001; Scorrano *et al.*, 2003; Zong *et al.*, 2003; Rutkowski *et al.*, 2006; Lin *et al.*, 2009).

In the presence of serum, HT1080 cells do not induce *CHOP* in response to tunicamycin treatment (Fig. 25B, lane 10). This suggests that under these conditions, HT1080 cells would be resistant to cell death. In order to determine this, I examined the viability of HeLa and HT1080 by reseeding cells after exposure to tunicamycin in the presence or absence of serum. Cells were seeded in 6 cm dishes and left to grow to about 80% confluency (~5 x 10^5 cells) before being treated. After treatment, cells were trypsinised, and resuspended in the same volume of fresh medium before being diluted by a factor of 4 into the 96-well plates. The cells were then left to grow overnight before being washed, fixed and DAPI stained, in order to identify cell nuclei. The number of viable cells was then determined by photographing the wells under a fluorescence microscope and analysing the images with Image J software. An example of the images obtained from DAPI staining cells treated with tunicamycin can be seen in Figure 26A. The number of cells in untreated HeLa and HT1080 wells was similar (Fig. 26A). However, the number of viable HeLa cells, which had previously undergone tunicamycin treatment, was less when compared to the same treatment in HT1080 cells. This is represented in Figure 26B which shows that tunicamycin-treated HeLa cells were comparatively less viable than HT1080 cells compared to untreated (Fig. 26B).

A



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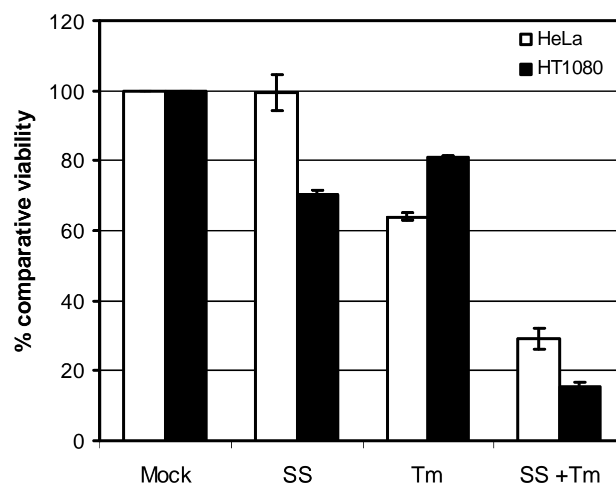


Figure 26. **Tunicamycin-treated HT1080 cells remain comparatively resistant to cell death.** A, After 6 hours tunicamycin treatment, HeLa and HT1080 cells were reseeded on a 96-well plate and left to grow overnight. Cells were then washed and stained with DAPI to visualise nuclei. B, HeLa and HT1080 cells were subjected to mock or tunicamycin (Tm) treatment either in the presence or absence (SS) of FCS for 6 hours. The cells were then trypsinised, reseeded in 96-well plate and grown overnight. Cells were then methanol fixed and DAPI stained before being photographed. Cell number was then determined and an average of 3 wells was calculated. The error bars refer to the results from at least 2 individual experiments.

Serum starvation of HeLa cells did not affect cell viability whilst serum starvation of HT1080 cells decreased HT1080 viability by ~30% (Fig. 26). The error bars are the standard deviation of at least two separate experiments, the data from each individual experiment being the average of four or more duplicates. Tunicamycin treatment of HeLa cells decreased cell viability by over a third whilst similar treatment of HT1080 cells only reduced viability by ~20%. Under these conditions, HeLa cells initiate *XBP1* splicing, *BiP* upregulation and *CHOP* induction whereas HT1080 cells do not. Thus, this crucial difference in UPR induction may be responsible for HT1080 cells being ~20% less sensitive than HeLa cells to tunicamycin-induced cell death.

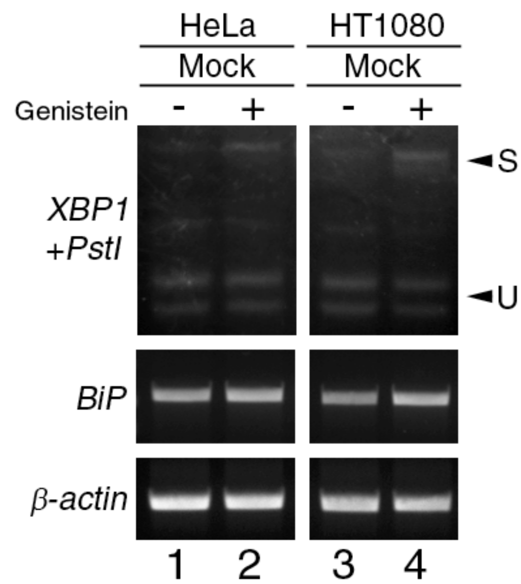
When cells were serum starved concomitantly with tunicamycin treatment, cell viability, in both cell lines, was reduced further, to under a third (Fig. 26). However, despite identical cell treatments, viability between cell lines differed with HT1080 cell viability at almost half that of HeLa cells. Since serum-starved, tunicamycin-treated HT1080 cells exhibit stronger induction of *CHOP* and *XBP1* splicing in comparison to HeLa cells, these results indicate that cell viability may be in part determined by the level of UPR signalling. However, since serum withdrawal of tunicamycin-treated HeLa cells decreased viability without any apparent effect on UPR induction over tunicamycin-only-treated HeLa cells, it is likely that other mechanisms such as the IRE1/TRAF2 pathway and mitochondria-dependent pathways influence cell viability also (Yoneda *et al.*, 2001; Boya *et al.*, 2002; Danial & Korsmeyer, 2004).

4.3.2.3 *Genistein suppresses the expression of BiP and XBP1 in HeLa cells undergoing different forms of ER stress.*

Our observation that HT1080 cells induced *XBP1* splicing in response to serum withdrawal concomitant with tunicamycin treatment lead us to investigate whether tyrosine kinases might be involved in *XBP1* activation since growth factor receptors have been shown to have intrinsic protein tyrosine kinase activity (Pawson, 2004). Initially, I treated HeLa and HT1080 cells with genistein and analysed *XBP1* processing, and BiP expression in order to confirm that there is no associated UPR induction with general tyrosine kinase inhibition (Fig. 27A).

As seen previously, serum-starved HeLa cells induced *XBP1* splicing and *BiP* induction upon tunicamycin treatment (Fig. 27B, lane 1 and Fig. 25B, lane 3). As expected, when HeLa and HT1080 cells were treated with genistein, *XBP1* processing and *BiP* expression levels were similar to those obtained by mock-treatment. Next, HeLa and HT1080 cells which were subjected to serum starvation and tunicamycin treatment were simultaneously treated with genistein – which specifically inhibits protein tyrosine kinases. When these cells were simultaneously treated with genistein, *XBP1* splicing was attenuated and *BiP* levels decreased (Fig. 27B, lane 2). This is in line with published data demonstrating that genistein inhibits UPR-driven transcription of *BiP/GRP78* in mammalian cells exposed to thapsigargin (Cao *et al.*, 1995).

A



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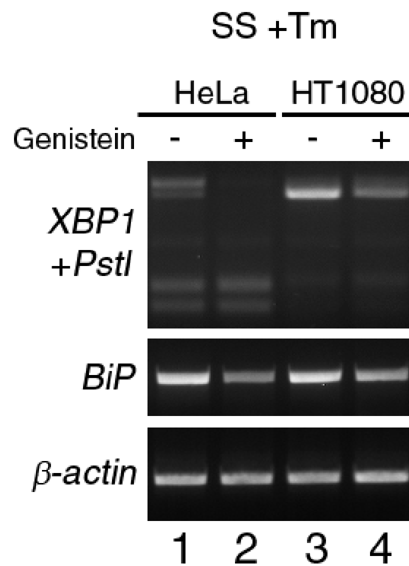


Figure 27. **Genistein suppresses *BiP* expression in serum-starved HeLa and HT1080 cells exposed to tunicamycin.** A, HeLa and HT1080 cells were either mock treated (-) or exposed to 140 µM genistein (+) for 6 hours in the absence of any ER stress inducer (Mock). B, Serum-starved (SS) HeLa and HT1080 cells were treated with tunicamycin (Tm) for 6 hours either in the absence (-) or presence (+) of genistein.

Genistein treatment of serum-starved, tunicamycin-treated HT1080 cells resulted in a decrease in – but not an abolition of – *XBP1* splicing and *BiP* expression (Fig. 27B, lane 4). Taken together, these results indicate that genistein treatment is able to suppress tunicamycin-induced UPR target gene upregulation and proximal UPR signalling events in serum-starved HeLa and HT1080 cells.

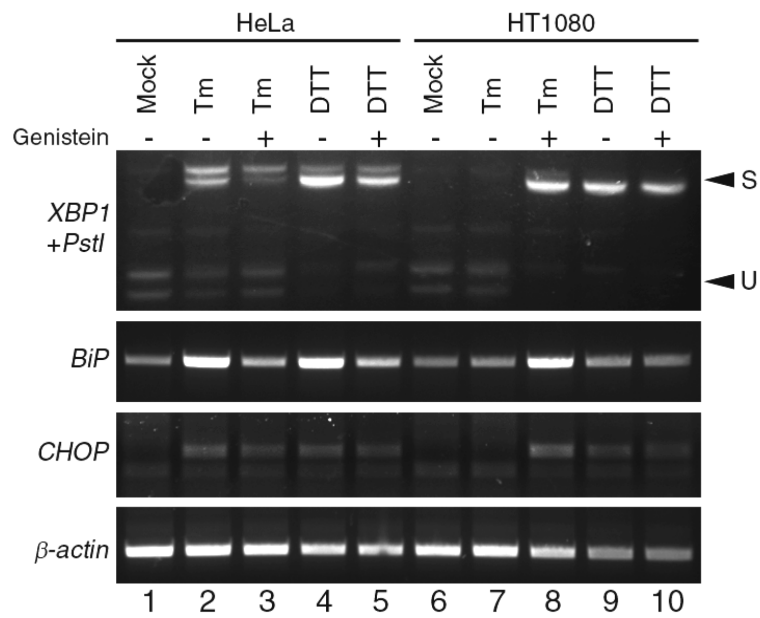
In order to determine if this effect was limited to tunicamycin-induced ER stress, I investigated the effect of genistein on HeLa and HT1080 cells treated with DTT and compared this with tunicamycin. These experiments were conducted in the presence of serum in order to examine the effect of genistein on UPR regulation under normal serum conditions. In addition to *XBP1* splicing and *BiP* expression, *CHOP* expression was also monitored under these conditions. As previously reported, tunicamycin treatment of HeLa cells resulted in splicing of *XBP1* and upregulation of *BiP* and *CHOP* whereas in HT1080 cells, tunicamycin treatment showed no effect when compared to mock treatment (Fig. 28A, compare lane 2 with lane 1 and lane 7 with lane 6). DTT treatment of HeLa cells also resulted in *XBP1* splicing and *BiP* and *CHOP* upregulation. In HT1080 cells, DTT treatment resulted in *XBP1* splicing, *CHOP* upregulation and limited *BiP* upregulation. When tunicamycin- and DTT-treated HeLa cells were subjected to genistein treatment, *BiP* levels were downregulated but *CHOP* levels remained constant. In addition, *XBP1* expression was decreased although splicing remained at a similar level. These results indicate that, in HeLa cells, genistein can suppress the UPR-driven transcription of both *BiP* and *XBP1* but does not inhibit *XBP1* splicing. Furthermore, genistein exerted this effect when the UPR was initiated by a number of different mechanisms such as prevention

of glycosylation by tunicamycin and global disulfide reduction by DTT in addition to depletion of ER Ca^{2+} caused by thapsigargin shown by other groups.

Treatment with genistein in tunicamycin- and DTT-treated HT1080 cells, on the other hand, led to contrasting results. Genistein treatment of HT1080 cells promoted, rather than prevented *XBP1* splicing as well as *BiP* and *CHOP* upregulation by tunicamycin in the presence of serum (Fig. 28A, compare lane 8 with lane 7). Fig. 28A provides further evidence that tunicamycin is bio-available to HT1080 cells, since in the presence of serum and genistein, tunicamycin can activate *XBP1* splicing. These results are in contrast with genistein treatment of tunicamycin-treated HT1080 cells in the absence of serum, in which genistein downregulates *XBP1* and *BiP* expression, confirming that genistein is bio-active at this concentration (Fig. 27B, lane 4).

Under serum-starved, tunicamycin-treated only conditions, *XBP1* splicing and *BiP* were upregulated in HT1080 cells (Fig. 27B, lane 3). This was in contrast to tunicamycin treatment in the presence of serum which did not promote *XBP1* splicing or *BiP* upregulation (Fig. 28A, lane 7). In HT1080 cells exposed to DTT, however, genistein treatment did not lead to changes in *XBP1*, *BiP* or *CHOP* expression (Fig. 27C, compares lane 10 with lane 9). Together these results indicate that under normal serum conditions, genistein treatment of HT1080 cells can promote *XBP1* splicing and *BiP* and *CHOP* upregulation with concomitant tunicamycin but not DTT treatment.

A



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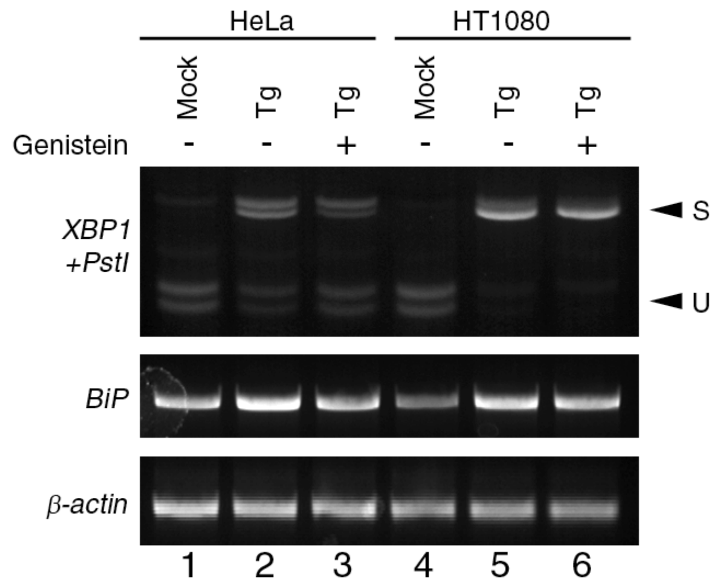


Figure 28. **Genistein suppresses transcriptional activation of *XBP1* and *BiP* induced by a range of ER stress inducers.** A, HeLa and HT1080 cells were mock-, tunicamycin (Tm)- or dithiothreitol (DTT)-treated either in the absence (-) or presence (+) of genistein for 6 hours. B, HeLa and HT1080 cells were either mock- or thapsigargin (Tg)-treated in the absence (-) or presence (+) of genistein for 6 hours.

To further investigate how genistein regulates UPR-driven transcription, I studied thapsigargin-induced *XBP1* splicing and *BiP* upregulation in HeLa and HT1080 cells. Thapsigargin-induced ER stress has been used previously to investigate the transcriptional regulation activity of genistein (Zhou & Lee, 1998). Treatment of both HeLa and HT1080 cells with thapsigargin resulted in increased expression of *BiP* and induction of *XBP1* splicing (Fig. 28B, lanes 2 and 5). However, whereas concomitant treatment of thapsigargin and genistein decreased both *XBP1* and *BiP* expression in HeLa cells, there was no further effect on expression levels of *XBP1* and *BiP* upon treatment of genistein in HT1080 cells. These results further strengthen our conjecture that expression of *XBP1* – in addition to *BiP* – is also suppressed by genistein in HeLa cells and that this mechanism is independent of the type of stress experienced by the cell. These results also indicate that the synergistic effect of genistein and tunicamycin treatment demonstrated in HT1080 cells (Fig. 28A, lane 8) is specific to tunicamycin.

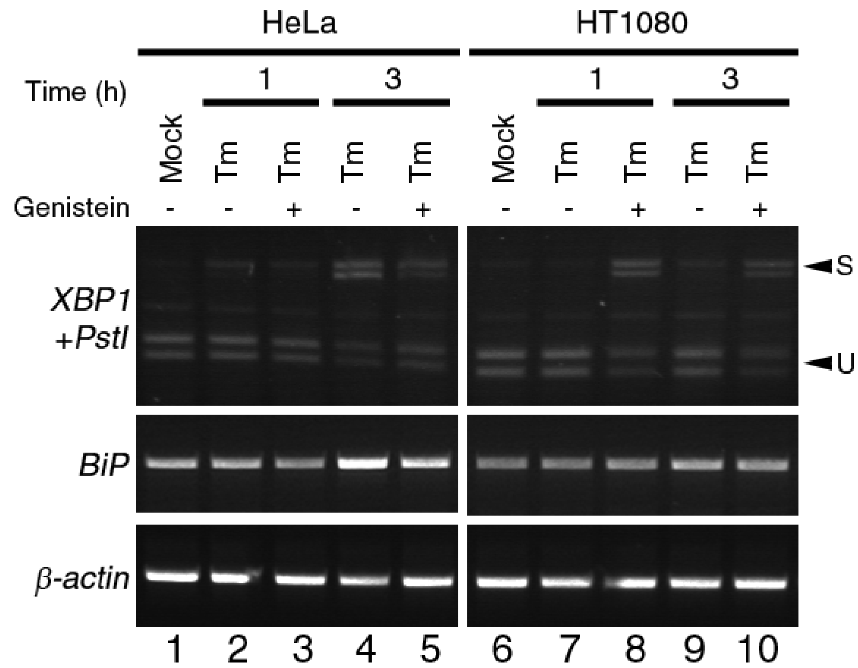
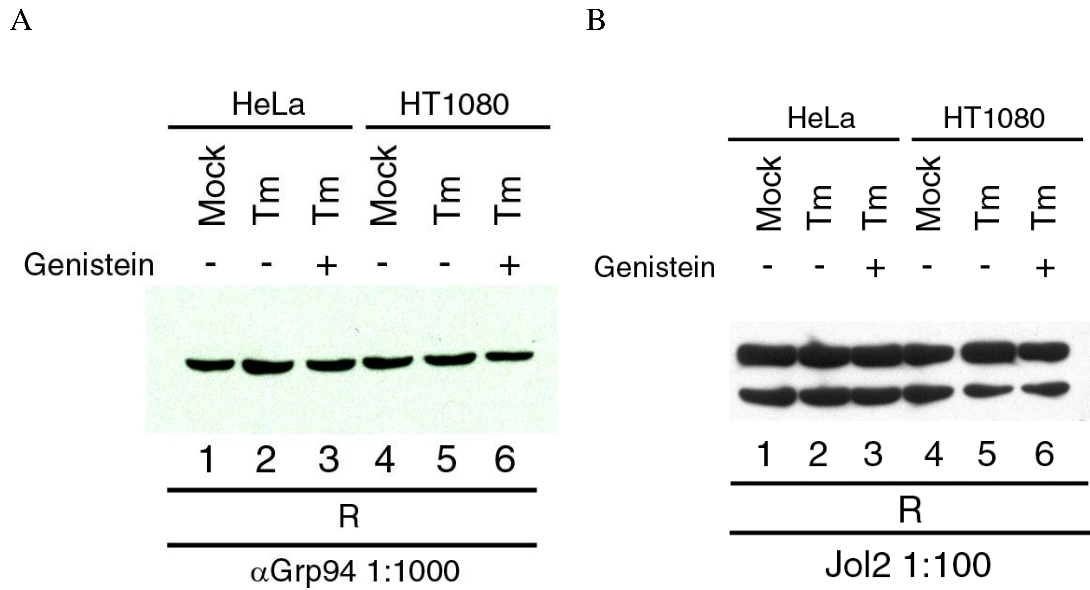


Figure 29. **Genistein can suppress *XBP1* and *BiP* transcriptional activity or promote *XBP1* splicing at early time points.** HeLa and HT1080 cells were mock- or tunicamycin (Tm)-treated in the absence (-) or presence (+) of genistein for either 1 or 3 hours.



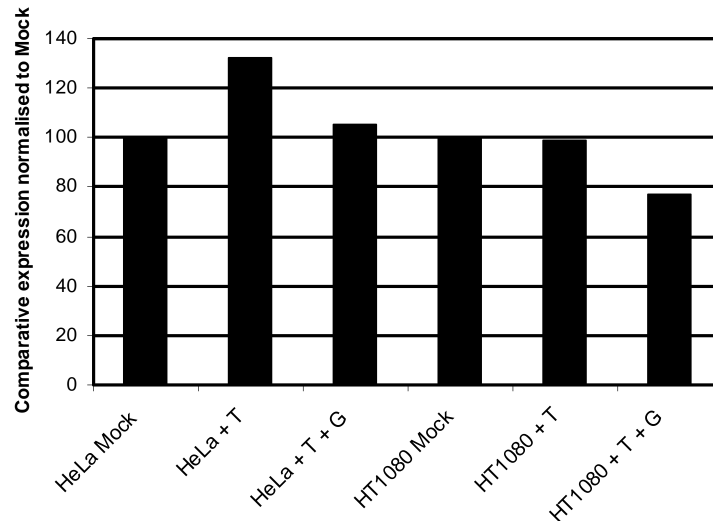


Figure 30. **The UPR-driven protein expression of GRP94 is suppressed by genistein in HeLa cells.** A, HeLa and HT1080 cells were either mock- or tunicamycin (Tm)-treated in absence (-) or presence (+) of genistein for 6 hours. Cells were then lysed and subjected to SDS-PAGE and Western blotting using the αGrp94 antibody. B, A Jol 2 control. C, GRP94 expression levels from A, calculated by optical density of each band using TINA.

Our experimental approach thus far was to treat cells with genistein for the duration of ER stress exposure; usually 6 hours. However, the effects of genistein on HeLa and HT1080 cells can be seen as early as 3 hours (Fig. 29). HeLa cells treated with tunicamycin for 3 hours induce *XBP1* splicing and upregulate *BiP*. However, genistein can suppress this effect at the 3 hour time point (Fig. 29, compare lane 5 with lane 4). The synergistic effect of tunicamycin and genistein in promoting *XBP1* splicing in HT1080 cells can be detected as early as 1 hour (Fig. 29, lane 8). However, a 3 hour treatment with tunicamycin and genistein is insufficient to upregulate *BiP* expression in HT1080 cells – although a 6 hour treatment is sufficient (Fig. 28A, lane 8). Thus, the synergistic induction of *BiP* by tunicamycin and genistein is in HT1080 cells is slower than the inhibition of tunicamycin-induced *BiP* expression by genistein in HeLa cells.

Since, in response to stress in mammalian cells, *BiP* expression is coordinately induced at the transcription level with *grp94* (Liu & Lee, 1991), I investigated the effect of genistein treatment on GRP94 protein levels (Fig. 30). HeLa and HT1080 cells were subjected to tunicamycin treatment only, or tunicamycin and genistein treatment together. Tunicamycin treatment resulted in a mild increase in GRP94 levels in HeLa cells at steady state (Fig. 30A, compare lanes 1 and 2).

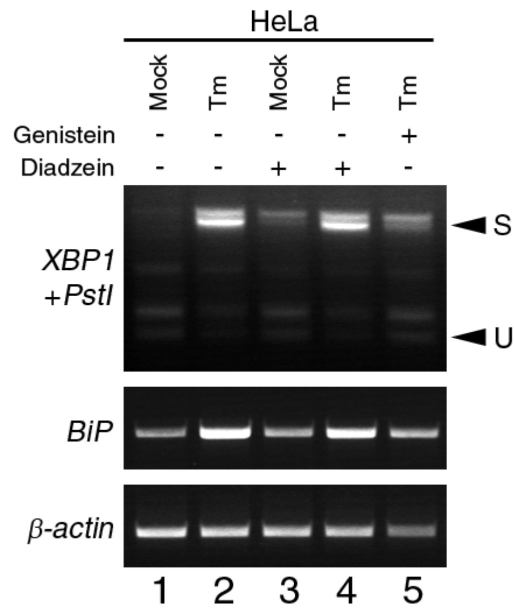
In the presence of genistein, protein levels of GRP94 in HeLa were decreased to near mock-treated levels (Fig. 30A, lane 3 and Fig. 30C). Conversely, for HT1080, tunicamycin treatment did not measurably induce GRP94 (Fig. 30A, compare lane 5 with lane 4 and Fig. 30C). This behaviour is contrary to that of *BiP* which is

transcriptionally induced – albeit less robustly than in HeLa – in tunicamycin-induced HT1080 cells (Fig. 7). Although I have not analysed the induction of GRP94 message levels, this result provides further evidence of HT1080 cells being unable to launch a strong tunicamycin-induced UPR. Genistein treatment of tunicamycin-treated HT1080 cells, however, resulted in a small decrease in GRP94 protein levels. Taken together, these results indicate that genistein may be able to suppress UPR-dependent and -independent *grp94* expression, but further work will be required to clarify this.

4.3.2.4 *Genistein regulates the transcriptional activation of BiP and XBP1.*

Since tyrosine phosphorylation has been implicated in the regulation of transcription factor complex formation (Novina *et al.*, 1998; Hong *et al.*, 2005), I investigated whether the tyrosine kinase inhibitor activity of genistein might be responsible for its ability to suppress ER stress target gene induction. I studied the tyrosine kinase inhibitor activity of genistein by comparing its ability to regulate transcription with the isoflavonoid diadzein. Diadzein is a phytoestrogen antioxidant which is also found in dietary soy products (Wiseman & Duffy, 2001). It is a structural analogue of genistein, however, it lacks the tyrosine kinase inhibitor activity (Akiyama *et al.*, 1987).

A



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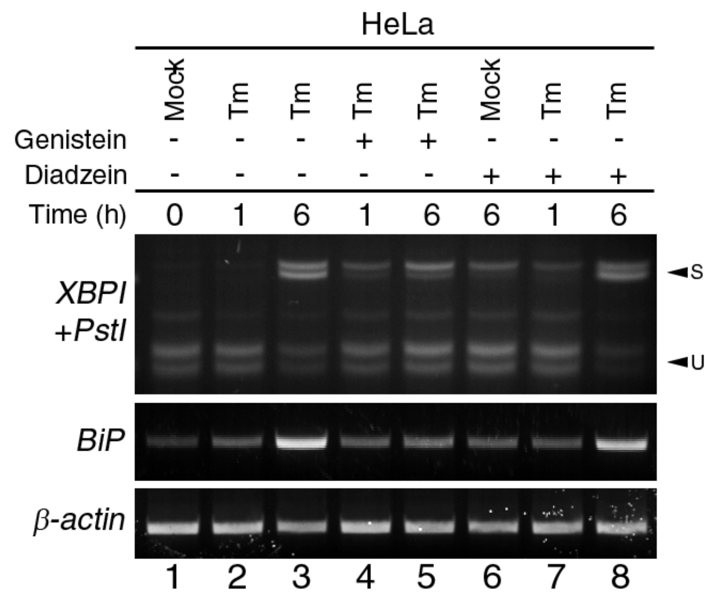

Figure 31. **Diadzein does not suppress the UPR-driven transcriptional activity of *XBP1* or *BiP* in HeLa cells.** A, HeLa cells were either mock- or tunicamycin (Tm)-treated in the absence (-) or presence (+) of either genistein or diadzein for 6 hours. B, As in A but cells were treated for either 1 or 6 hours.

HeLa cells were either mock- or tunicamycin-treated in the absence or presence of genistein or diadzein for the duration of the 6 hour treatment. When HeLa cells were subjected to diadzein treatment only, there was minimal *XBP1* splicing and *BiP* induction when compared to non-treated cells (Fig. 31A, compare lane 3 with lane 1). When tunicamycin-treated HeLa cells were exposed to diadzein, *XBP1* splicing as well as the expression level of *XBP1* and *BiP* remained comparable to tunicamycin-only treatment (Fig. 31A, compare lane 4 with lane 2). As previously seen, exposure of tunicamycin-treated HeLa cell to genistein resulted in decreased *XBP1* and *BiP* expression. Since the effects of genistein on tunicamycin-treated cells can be demonstrated at early time points (Fig. 29), I investigated the effects of diadzein on tunicamycin-induced *XBP1* and *BiP* expression in HeLa cells at both 1 hour and 6 hours in order to rule out any early inhibitory effects on transcription (Fig. 31B).

Analysis of concomitant diadzein and tunicamycin treatment after 1 hour revealed similar expression levels of *XBP1* and *BiP* compared to diadzein-only treatment (Fig. 31B, compare lane 7 with lane 6). Taken together, these results indicate that diadzein has no effect on tunicamycin-induced *BiP* and *XBP1* expression with short- or long-term treatment in HeLa cells. Thus, it is probable that the tyrosine kinase inhibitory activity of genistein confers an ability to regulate UPR-driven transcriptional activation of *BiP* and *XBP1*. These results are supported by published data which suggest that tyrosine phosphorylation is essential for efficient transcriptional activation of *BiP*, at least in HeLa cells.

4.3.2.5 *Genistein treatment does not strongly suppress MHC class I misoxidation-induced XBP1 or BiP expression.*

I have shown that *XBP1* and *BiP* expression can be attenuated with genistein treatment in the face of pharmacologically induced ER stress. An interesting question arising from these observations is whether ER protein misfolding/misoxidation is influenced by genistein. Since I am interested in how the UPR is coordinated in response to misoxidation, I investigated whether genistein treatment could suppress *XBP1* and *BiP* expression induced by HLA-B27 misoxidation. HLA-B27 was transiently transfected into HeLa cells and treated with or without genistein 6 hours prior to lysis. Lysates were analysed for heavy chain misoxidation by HC10 Western blotting under reducing and non-reducing conditions and for *XBP1* and *BiP* expression by RT-PCR (Fig. 32). Transfection of the HLA-B27 heavy chain resulted in extensive misoxidation and the formation of high molecular weight complexes in the non-reducing HC10 blot (Fig. 32A, lane 2). Post-transfection treatment with genistein did not appreciably affect heavy chain misoxidation (Fig. 32A, lane 3).

Analysis of *XBP1* and *BiP* expression levels revealed that genistein treatment decreases misoxidation-induced *XBP1* expression only very mildly whereas *BiP* expression was not suppressed by genistein under these conditions. Whereas previously I had treated cells with genistein for the duration of the stress period (usually 6 hours), this procedure only allowed the presence of genistein several hours into the stress period.

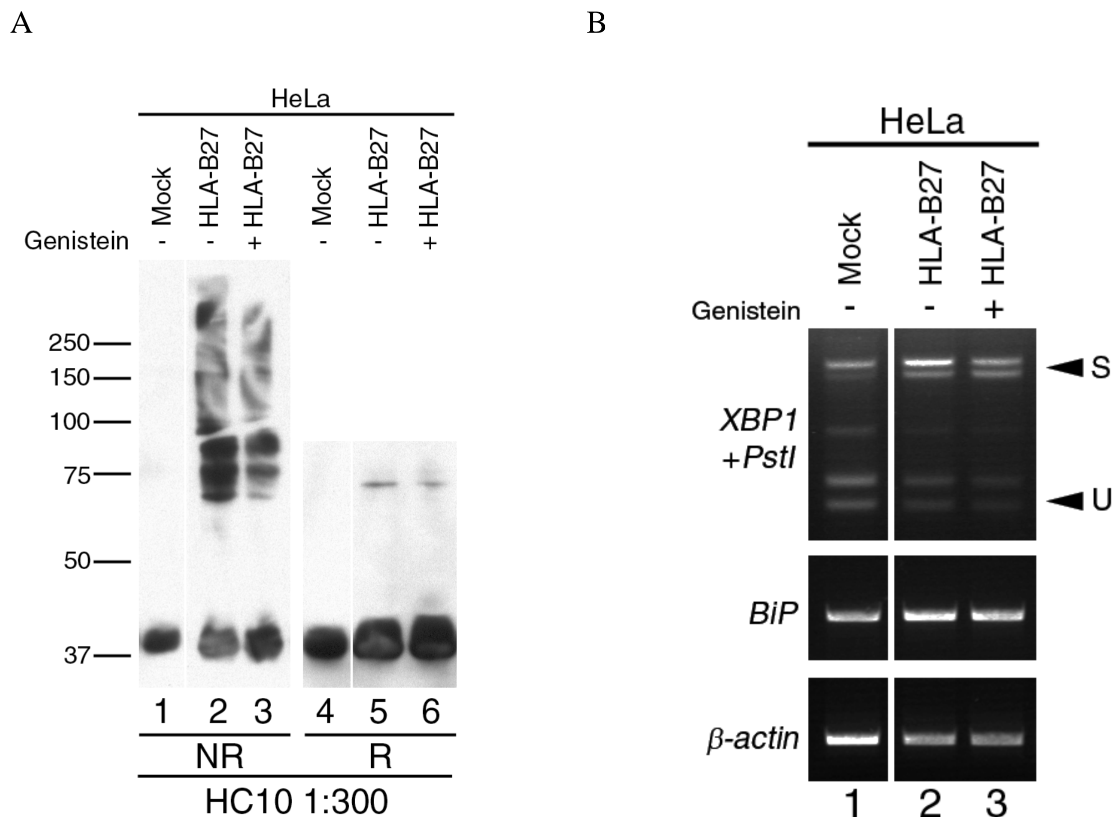

Figure 32**. Genistein does not suppress MHC class I heavy chain misoxidation-induced transcriptional activation of *XBP1* or *BiP* in HeLa cells.** A, HeLa cells were either mock transfected or transfected with HLA-B27 (HLA-B27), grown overnight and then either treated without (-) or with (+) genistein for 6 hours. Cells were then lysed and analysed reducing (R) and non-reducing (NR) SDS-PAGE and subsequent Western blotting with the anti-MHC class I HC antibody HC10. B, As in A but cells were analysed by RT-PCR for XBP1 splicing and BiP and β-actin expression.

Prolonged ER stress induced by protein misfolding may trigger a UPR response that is fundamentally distinct – in so much as it may utilise different branches of the UPR – from short term ER stress. Thus, genistein might be unable to suppress mechanisms of *XBP1* and *BiP* upregulation that arise from prolonged ER stress. Note that *BiP* levels and *XBP1* splicing was mildly activated in the mock treated cells of this experiment (Fig. 32B, lane 1), and that untransfected cells will also be present in the transfected dish. Thus, this experiment may under-represent the extent of stress signalling in response to ER protein misoxidation. Overexpression of heavy chains by transient transfection may not mimic physiological stress, in which case genistein treatment might still be able to suppress UPR-driven transcriptional activation in cells from the HLA-B27 transgenic rat model for example (Tran *et al.*, 2004). However, there are likely to be tissue- and cell-specific differences in UPR-driven transcriptional activation, so the ability of genistein to suppress transcriptional activity may vary dependent on cell types. Further work will be required to determine if genistein can attenuate UPR-driven upregulation of *XBP1* and *BiP* in animal model systems and in the clinic.

4.3.3 *Discussion*

I demonstrate that genistein can suppress UPR-driven upregulation of *BiP* in response to tunicamycin, thapsigargin and DTT treatment but not MHC class I heavy chain misoxidation by transient transfection (Figs. 27, 29 and 32). Thus, I provide evidence of the transcriptional regulation activity of genistein in response to various types of ER stress. However, genistein treatment does not downregulate basal *BiP* expression (Fig. 27A). This is in line with published work which shows that genistein has no effect on basal *BiP* promoter activity (Cao *et al.*, 1995; Zhou & Lee, 1998).

The isoflavonoid genistein is a potent inhibitor of tyrosine kinase activity but does not inhibit the activity of serine and threonine kinases (Akiyama *et al.*, 1987). Although originally isolated in *Pseudomonas* sp., genistein, as well as other isoflavonoids such as diadzein, is present in large concentrations in soy products – such as tofu – which is a fundamental part of the Chinese and Japanese diet. Studies using an immunoconjugate of genistein and the monoclonal antibody B43 – which targets genistein to the B-cell-specific receptor CD19 – demonstrated that, in B-cell precursor (BCP) leukaemia cells, genistein selectively inhibited CD19-associated tyrosine kinases leading to rapid apoptotic death of these cells (Uckun *et al.*, 1995). Thus, it was postulated that protein tyrosine kinase inhibitors may have therapeutic potential in the treatment of haematological malignancies. Since then, tyrosine kinase inhibitors such has erlotinib, which specifically targets the epidermal growth factor receptor (EGFR) tyrosine kinase, have been developed for the treatment of non-small cell lung cancer (Perez-Solder *et al.*, 2004; Amann *et al.*, 2005).

Furthermore, genistein has been shown to downregulate cytokine-induced signal transduction events and, through its protein kinase inhibitor activity, impairs humoral immunity by blocking B-cell receptor mediated signalling (Lane *et al.*, 1991; Kim *et al.*, 2007). The tyrosine kinase inhibitor activity of genistein also downregulates the expression of the ER chaperone *grp78/BiP* which has implications for cell survival, and may therefore have therapeutic implications particularly for tumorigenic cells. Fazlul Sarkar's group showed that genistein can inhibit NF-κB activation in prostate cancer cells through Akt signalling, which is important in controlling the balance between cell survival and apoptosis (Davis *et al.*, 1999; Li & Sarkar, 2002; Song *et al.*, 2005; Banerjee *et al.*, 2008). This indicates that genistein might regulate cell survival via the NF-κB and Akt signalling pathways. Since NF-κB is implicated in inflammatory diseases such as inflammatory bowel disease, a component of spondyloarthritis (Tak & Firestein, 2001; Chang *et al.*, 2009), these findings may provide an approach to therapy for inflammatory disease. The preferential survival of certain cell types may contribute to inflammatory disease, since if certain cell types, such as cells which express high levels of IFN-γ, exhibit increased survival in the face of ER stress, this might lead to an increase in cytokine signalling and be a contributing factor to chronic inflammation.

I demonstrate that *XBP1* expression, but not *XBP1* splicing, might also be regulated by genistein treatment. UPR-driven expression of *XBP1* was consistently low when cells were exposed to genistein (Figs. 27B, 4, 5 and 7). *XBP1* mRNA expression is induced in response to ER stress by ATF6 (Yoshida *et al.*, 2000; Yoshida *et al.*, 2001a). However, ATF6-mediated stimulation of the *grp78* promoter activity is

resistant to genistein treatment suggesting that ATF6 does not contribute to genistein-mediated suppression of transcriptional activity (Li *et al.*, 2000). The transcription factor TFII-I, which is a downstream target of genistein tyrosine kinase inhibitor activity, interacts with ATF6 (Parker *et al.*, 2001). Since tyrosine phosphorylation of TFII-I is essential for transcription factor complex formation, genistein treatment might prevent TFII-I-ATF6 complex formation leading to suppression of ATF6 UPR target genes. Moreover, there is some evidence to suggest that UPR-driven upregulation of *XBP1* may occur by ATF6-independent mechanisms (Lee *et al.*, 2003). Alternatively, UPR-driven ATF6 expression might be suppressed by genistein which would then have consequences for *XBP1* induction. Further work will be required to verify if UPR-driven *XBP1* induction can be suppressed by genistein and the mechanism that underlies its transcriptional regulation. Additionally, our results also suggest that protein levels of GRP94, an ER chaperone which is coordinately induced with *BiP*, may also be downregulated by genistein (Fig. 30).

Studies by Amy Lee's group have highlighted the importance of c-Src-mediated phosphorylation of TFII-I in *BiP* expression and suggested that c-Src is a potential target of genistein tyrosine kinase inhibitor activity (Parker *et al.*, 2001; Hong *et al.*, 2005). However, Bruton's tyrosine kinase (BTK) – which is important in B-cell maturation – and Janus kinase 2 (JAK2) also directly phosphorylate tyrosine residues on TFII-I. Our studies comparing a structural analogue of genistein, diadzein, also point to tyrosine kinase inhibition as important for the attenuation of *BiP* expression. Diadzein, like genistein, is a phytoestrogen but lacks its tyrosine kinase inhibitor activity. I demonstrate that diadzein has no effect on tunicamycin-driven *BiP* or *XBP1*

upregulation either at short (1 hour) or long (6 hour) time points (Fig. 31). This is contrary to genistein treatment, which suppresses tunicamycin-induced *XBP1* and *BiP* upregulation. Thus, this finding supports the hypothesis that the tyrosine kinase inhibitor activity of genistein is responsible for suppression of *grp78* transcriptional activation although further work is required to analyse the phosphorylation of TFII-I and determine the tyrosine kinase targets of genistein.

Our studies reveal that different cell types can respond to the same stimuli in diverse ways. I show that cell viability is reduced in HT1080 cells to a lesser extent than HeLa cells in response to tunicamycin treatment (Fig. 26). This finding was consistent although further investigation into cell viability using other methods such as crystal violet staining would further validate this finding. Furthermore, I establish that genistein can differentially activate *XBP1* in a cell specific manner. Whereas exposure of tunicamycin-treated HeLa cells to genistein suppresses transcriptional activation of *BiP*, the same treatment in HT1080 cells potentiates *BiP* expression and *XBP1* splicing (Fig. 28A and 5). This synergistic effect was only observed with tunicamycin treatment. Furthermore, DTT- and thapsigargin-induced *XBP1* splicing and *BiP* expression was not suppressed by genistein in HT1080 cells. However, genistein is capable of suppressing UPR-driven transcriptional activation in serum-starved HT1080 cells exposed to tunicamycin (Fig. 27B) indicating that the mechanism by which genistein attenuates transcriptional activation in HeLa cells may be functional in HT1080 cells. A summary of cell-specific differences in UPR regulation can be seen in Figure 33.

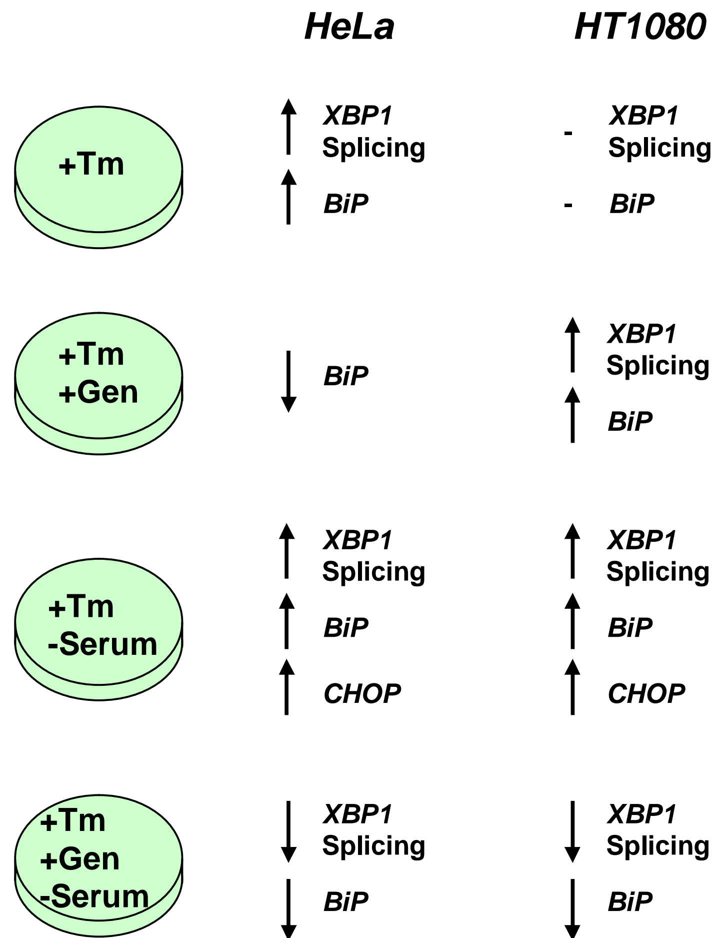

Figure 33. **Cell-specific differences in UPR regulation between HeLa and HT1080.** Each individual treatment is indicated on the left. Cells were treated with tunicamycin (Tm), genistein (Gen) or/and serum starved. The corresponding outcome in HeLa and HT1080 is indicated on the right compared to the previous treatment. An increase in expression, or activation in the case of *XBP1* splicing, is represented by and up-arrow. A decrease is represented by a down-arrow and no effect is represented by a dash. All outcomes are based on data from this chapter or Results Chapter 4.1.

Unusually, HT1080 cells, when treated with tunicamycin only, do not initiate *XBP1* splicing or strongly upregulate *BiP* or *CHOP* (Figs. 25, 28A, 29 and Results Chapter 1). Initially, this was thought to be the consequence of tunicamycin being less available to the cell, but the observation that tunicamycin can activate *XBP1* splicing in the presence of genistein in HT1080 cells and that tunicamycin treatment can prevent glycosylation of MHC class I heavy chains demonstrates that tunicamycin is bio-available to HT1080 cells (Figs. 28A and 29 and Fig. 9B). Genistein is unlikely to activate tunicamycin import to or prevent tunicamycin export from HT1080 cell since vesicle assays have shown that genistein stimulates the ATPase activity of multi-drug resistance protein (MRP) transporters (Hooijberg et al., 1997). Therefore, if MRPs were involved in tunicamycin transport in HT1080, genistein should decrease, rather than increase, its bioavailability.

I also demonstrate that the weak response to tunicamycin in HT1080 cells can be overcome by serum starvation and that HT1080 cells treated with tunicamycin are relatively resistant to cell death (Fig. 25 and 26). Therefore, I propose that there is a molecular regulator of *XBP1* activity in HT1080 cells. A candidate protein which may play a role in regulating *XBP1* activity and the response to stress-inducing stimuli is the apoptotic regulator in the membrane of the endoplasmic reticulum (ARMER)/ARL6IP1, which protects HT1080 cells from apoptosis when exposed to a variety of stimuli which can cause ER stress (Pettersson *et al.*, 2000; Lui *et al.*, 2003). ARMER has been shown to protect cells from tunicamycin-induced apoptosis by modulating caspase-9 activity. ARMER interacts with ADP-ribosylation-like factor-6 (ARL6), a member of the Ras superfamily, which interacts with a component of the

SEC61 channel, SEC61β (Ingley *et al.*, 1999). Moreover, ARMER is a direct target of C/EBPα, which possesses strong apoptotic activity; is involved in the regulation of cell growth and is controlled by a complex network or pathways which differ in different cell types (Wang *et al.*, 2008). Thus, a possible role for regulation of *XBP1* and the UPR by the ARMER/C/EBPα pathway deserves further exploration.

The results of our studies have particular relevance to inflammatory disease which is becoming increasingly linked with UPR activation. The effect of genistein on the immune system is cell type-dependent (Sakai & Togiso, 2008). Our results suggest that these differences may be linked to the control of *XBP1*, or of *BiP* induction, by genistein. This work provides an explanation for differences in tunicamycin responsiveness between cell lines, and provides a framework for identifying novel regulators of the UPR.

5. FINAL DISCUSSION

5. Final Discussion

Recently ankylosing spondylitis has been linked to the misfolding of the MHC class I heavy chain HLA-B27 (Turner *et al.*, 2005). Increased expression levels of HLA-B27, induced by IFN-γ, in the B27/hβ_2m transgenic rat result in activation of the UPR and an associated increase in disulfide linked B27 complexes (Turner *et al.*, 2007). I show that overexpression of HLA-B27 by transient transfection leads to the formation of disulfide linked B27 complexes and associated activation of *XBP1* and upregulation of *BiP* (Fig. 19 and 20). This is relevant to AS since *XBP1* activation has been linked with intestinal inflammation, a key component of AS. Furthermore, I demonstrate that different cell types are differentially sensitive to certain forms of ER stress which may impact upon cell survival and death (Fig. 10, 12, 26 and 28). I show that while tunicamycin treatment of HeLa cells leads to *XBP1* splicing and induction of the proapoptotic component *CHOP*, tunicamycin treatment of HT1080 cells does not (Figs. 9-12). This is important in the context of AS since apoptosis of certain synovial cells, such as synovial fibroblasts, can lead to tissue damage and may contribute to disease pathogenesis (Yang *et al.*, 2007). Furthermore, soluble tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is upregulated in AS patients indicating that apoptosis may be important in disease (Yang *et al.*, 2007). Thus, the work in results chapter 2 has implications for inflammatory disease and AS.

5.1 Coordination of the UPR in response to diverse ER stimuli

The unfolded protein response (UPR) is a multifaceted system for relieving ER stress and restoring ER homeostasis. The three branches of the UPR were originally thought to be activated in the same manner, by titration of BiP away from the lumenal domains of the three stress inducers, but recent studies have determined that certain UPR branches might be subject to further regulation, such as disulfide reduction in ATF6, or have additional activities related to activation, such as IRE1-mediated mRNA degradation, IRE1 clustering and mRNA targeting to IRE1 (Hollien & Weissman, 2006; Nadanaka *et al.*, 2006; Nadanaka *et al.*, 2007; Aragon *et al.*, 2009; Korennykh *et al.*, 2009; Schindler *et al.*, 2009; Yanagitani *et al.*, 2009; Kohno, 2010). Furthermore, direct binding of IRE1 to unfolded proteins has been suggested to be necessary for activation, which may allow the discrimination of certain conformations (Credle *et al.*, 2005; Kimata *et al.*, 2007; Pincus *et al.*, 2010). Thus, the individual branches of the UPR, aside from having distinct downstream effects – although some functional redundancy does exist – might also be activated by distinct mechanisms in addition to the common mechanism of negative regulation by BiP.

In the first results chapter, I analysed the induction of the UPR using pharmacological agents. For the ATF6 branch of the UPR I demonstrate that ATF6α can form higher order structures and, in addition, show that upon treatment with DTT and tunicamycin, the ratio of oligomeric and dimeric to monomeric ATF6α is changed leading to an increase in the monomer pool of ATF6α (Fig 16). Tunicamycin is less efficient in inducing ATF6α oligomer/dimer reduction than DTT, suggesting stimuli-

specific regulation of ATF6α. DTT is likely to reduce disulfide-bound ATF6α directly, but this begs the question of how tunicamycin treatment induces ATF6α reduction. Could ATF6α reduction be caused by PDI, the most abundant oxidoreductase in the ER? Since PDI can act as a chaperone, in a situation where misfolded proteins have accumulated in the ER one might expect there to be less free PDI available since it will be found bound to substrates. One theory is that the increase in protein folding machinery brought about by UPR activation could increase the concentration of potential reductants in the ER which may possibly favour ATF6α reduction. However, increased free oxidoreductases might initiate the restoration of ER homeostasis and so continued UPR signalling through ATF6α would be counter intuitive. Thus, further work, such as pulse-chase experiments to study deoligomerisation over time, or analysis of ATF6α cysteine mutants will be required to determine the significance of ATF6α reduction in activation of the ATF6 branch of the UPR. Further studies to identify potential molecules involved in the reduction of ATF6α are also required. Our results are supported by those of Nadanaka and colleagues who showed that under non-stressed conditions ATF6 exists as disulfide-bonded dimers and oligomers in addition to the monomeric form (Nadanaka *et al.*, 2007).

Our results show that in response to different ER stress-inducing stimuli, cells upregulate the UPR target gene *BiP* but not all to the same degree (Fig. 7). Using pharmacological agents which prevent glycosylation (tunicamycin) and cause perturbation in ER Ca^{2+} levels (thapsigargin) led to the biggest increase in *BiP*

expression (Fig. 7). However, processing of *XBP1*, which also exhibited diverse levels of activation dependent on stimuli, did not show the same pattern since disruption of disulfides with DTT caused the most robust splicing of *XBP1* (Figs. 9 and 10). Thus, different ER-stress-inducing stimuli may induce distinct patterns of UPR activity at both the activatory (*XBP1* splicing) and downstream transcriptional activity (*BiP* expression) level. Although *BiP* upregulation is a target of *XBP1*(S), the discrepancy in activation pattern between *BiP* upregulation and *XBP1* splicing is most likely to be due to the influence of p50ATF6, which also upregulates *BiP*.

The work of Lin and colleagues took steps towards understanding how the UPR is coordinated in response to stress and how coordination of the UPR branches contributes to cell survival (Lin *et al.*, 2007; Lin *et al.*, 2009). They showed that there is temporal regulation of UPR signalling by demonstrating that *XBP1* splicing levels decline after long periods of prolonged ER stress whereas PERK signalling is sustained over time. They suggest that this may sensitise the cell to apoptosis after chronic ER stress resulting in the enhancement of PERK-dependent *CHOP* expression and a decrease in IRE1-mediated prosurvival signalling through XBP1. Lin and colleagues showed that, in HEK293 cells, *XBP1* splicing is detectable at 4 hours post treatment (Lin *et al.*, 2007). This is consistent with our work since I demonstrate that, in HeLa cells, *XBP1* splicing starts to occur between 3 and 6 hours (Fig. 11A).

Here, I also show that different cell types respond differently to distinct forms of ER stress. I report that the HT1080 fibrosarcoma cell line, despite upregulating *BiP*, *CHOP* and *XBP1* splicing in response to treatment with DTT, thapsigargin and

A23187, are selectively resistant to UPR induction by tunicamycin (Figs. 9-12). Thus, HT1080 cells do not upregulate *CHOP* or activate *XBP1* splicing in response to tunicamycin. I demonstrate that the weak response to tunicamycin in HT1080 cells can be overcome by serum starvation (Fig. 25). However, tunicamycin-treated HT1080 did upregulate *BiP* to levels comparative to that induced by DTT (Fig. 7). Although on other occasions I have detected less robust BiP induction in response to tunicamycin (Fig. 11A). Investigating the activity of tunicamycin in HT1080 cells revealed that tunicamycin was preventing glycosylation indicating that it was being taken up by the cells (Fig. 11B and 15). Furthermore, I show that HT1080 cells are relatively resistant to cell death indicating that increased cell survival is linked with low level UPR activity (Fig. 26). The proapoptotic UPR component *CHOP* is not induced in tunicamycin-treated HT1080 cells. Since the deletion of *CHOP* protects cells from the lethal consequences of ER stress, it is possible that failure to induce *CHOP* in HT1080 cells is responsible for increased cell survival (Zinszner *et al.*, 1998; Oyadomari *et al.*, 2002; Pennuto *et al.*, 2008). However, since IRE-mediated JNK activity has been linked with apoptosis, further work is needed to verify if the IRE1-JNK pathway similarly fails to activate in HT1080 cells (Urano *et al.*, 2000; Nishitoh *et al.*, 2002). This is important in the context of inflammation, which may be caused in part by the preferential survival of cells that promote inflammation.

5.2 MHC class I heavy chain misfolding-driven UPR activation

Recently HLA-B27 misfolding has been linked with activation of the UPR in the B27/hβ_2m animal model of AS (Turner *et al.*, 2005). These rats express high levels of class I heavy chain due to a high copy number of the B27/hβ_2m transgene. High

expression levels of B27 have been linked with disease in the transgenic rat, mouse and in human SpA sufferers (Taurog *et al.*, 1993; Khare *et al.*, 1995; Khare *et al.*, 1997; Cauli *et al.*, 2002). Thus, I used transient transfection to overexpress different MHC class I heavy chains in HeLa cells to determine the level of misfolding and investigate the comparative level of UPR induction induced from disease associated and non-disease-associated heavy chains (Ch. 4.2).

I demonstrate that overexpression of both HLA-B7 and HLA-B27 – in the absence of increased expression of β_2m – in HeLa cells is sufficient to induce misfolding/misoxidation defined by the presence of disulfide-linked, high molecular weight complexes (Fig. 19). Misfolding of both heavy chains was sufficient to induce upregulation of BiP protein levels as well as *XBP1* splicing and increased transcription of *BiP* (Fig. 19 and 20). The levels of B27-driven UPR induction was lower than those induced by pharmacological agents. This has been described in the literature relating to the transgenic rat model where UPR associated with B27 misfolding is less than that observed with tunicamycin treatment (Turner *et al.*, 2005). This is not surprising since misfolding of a single protein species is unlikely to lead to as much cellular disruption as the global effect of disulfide reduction (as with DTT) or prevention of glycosylation (as with tunicamycin). Thus, in this transient transfection model, *XBP1* splicing and *BiP* upregulation is correlated with cI HC misoxidation and is independent of heavy chain disease association In contrast, stable transfection of HLA-B27 and HLA-A2 in C58 cells did not result in *XBP1* splicing levels beyond background levels (Fig. 22). Since I have observed that the level of *XBP1* splicing is correlated with HC expression levels, it is possible that these stably transfected cell

lines do not express HCs at as high a level as transiently transfected cells, although further work will be required to clarify this. However, a lack of *XBP1* splicing in these cells could indicate that they have adapted and attenuated the IRE1 prosurvival arm of the UPR. Studying the UPR activity of these cells from initial transfection through to becoming established stable transfectants would give an insight into how coordination of the UPR occurs in cells undergoing prolonged ER stress.

XBP1 activation is becoming increasingly linked with inflammatory disease *in vivo* and especially intestinal inflammation (Kaser & Blumberg, 2010). Conditional deletion of *XBP1* in the intestinal epithelium of mice leads to the spontaneous development of intestinal inflammation showing great similarity to IBD (Kaser *et al.*, 2008). Indeed, hypomorphic *XBP1*, that is *XBP1* with reduced gene activity, actually results in ER stress determined by upregulation of *BiP*. Thus, it seems that intestinal inflammation, a key component of SpA and of inflammatory disease in the B27/hβ_2m transgenic rat model, is linked with ER stress activation and *XBP1* activity.

Consistent with our novel findings that HT1080 cells do not induce *CHOP* expression in response to tunicamycin treatment (Fig. 12), B27 misfolding did not induce *CHOP* expression either. Given that B27 misfolding induces a milder UPR than tunicamycin treatment in the transgenic rat, it is not a surprise that HT1080 cells, which are unresponsive to tunicamycin, do not induce *CHOP* in response to B27 misfolding.

Activation of NF-κB, a master regulator of inflammation, has long been associated with ER dysfunction and ER stress (Pahl, 1999; Deng *et al.*, 2004; Zhang & Kaufman,

2008). The contribution of NF-κB signalling to inflammatory disease is currently unknown although the expression of HLA-B27 markedly enhances NF-κB activation and TNFα secretion in monocyte cells in response to LPS, suggesting that B27 may modulate NF-κB activation in response to infection (Penttinen *et al.*, 2002). However, gene expression analysis of bone marrow macrophages from the B27/hβ_2m transgenic rat model did not reveal a strong NF-κB-dependent transcriptional response (Turner *et al.*, 2005). Turner and colleagues postulated that under prolonged IFN-γ stimulation, a more robust UPR with NF-κB activation might be observed. Thus, the contribution of NF-κB to inflammatory disease is worthy of further exploration. Levels of the inhibitor of NF-κB, IκB as well as immunoprecipitation studies to reveal the extent of IκB-bound NF-κB in our transient transfection model might give an insight into the consequences of B27 misfolding on NF-κB activation.

5.3 The integration of genistein activity with the UPR

Genistein is an isoflavone with tyrosine kinase inhibitory activity. It has previously been demonstrated to regulate ER-stress induced *BiP* upregulation through interference with transcription factor complex assembly and interaction with the *BiP* promoter. I demonstrate that genistein can suppress tunicamycin- and DTT-induced *BiP* upregulation in HeLa cells (Fig. 28). Consistent with this observation, thapsigargin-induced *BiP/Grp78* upregulation is suppressed by treatment with genistein in Chinese hamster ovary cells (Cao *et al.*, 1995). Furthermore, I establish that ER stress-driven expression of *XBP1* but not *CHOP* is also suppressed by genistein in HeLa cells (Fig. 28). Thus, I provide evidence of the transcriptional

regulation activity of genistein in response to various types of ER stress. *XBP1* expression is driven by ATF6, yet ATF6-mediated stimulation of the *Grp78* promoter activity is resistant to genistein treatment suggesting that genistein activity regulates the activity of more than one branch of the UPR (Lee *et al.*, 2000; Yoshida *et al.*, 2000; Yoshida *et al.*, 2001a). Further work is required to verify if genistein is able to suppress upregulation of *XBP1* or indeed other UPR target genes.

Genistein activity appears to be cell specific, since genistein can differentially activate *XBP1* in HT1080 cells exposed to tunicamycin (Fig. 28 and 29). This further highlights the diversity of activity of tunicamycin-driven UPR in HT1080 and HeLa cells. Genistein does not suppress thapsigargin-induced *BiP* upregulation – or indeed *XBP1* upregulation – in HT1080 cells (Fig. 28). Unsurprisingly, *BiP* expression is not attenuated in genistein- and tunicamycin-treated HT1080 cells since tunicamycin does not induce *BiP* upregulation in HT1080 cells in the absence of genistein. Rather, genistein treatment in the presence of tunicamycin – and not DTT or thapsigargin – promotes *XBP1* splicing in HT1080 cells (Fig. 28).

Genistein does suppress UPR-driven transcriptional activation in serum-starved HT1080 cells exposed to tunicamycin (Fig. 27). Thus, the mechanism by which genistein attenuates transcriptional activation in HeLa cells may be functional in HT1080 cells. Therefore, I propose the presence of a molecular regulator of *XBP1* in activity in HT1080 cells. One candidate is the apoptotic regulator ARMER which protects HT1080 cells from apoptosis when exposed to various stimuli including tunicamycin. Gene silencing of ARMER in HT1080 cells combined with analysis of

UPR induction in response to a wider variety of ER stress inducers, including those which disrupt the glycosylation machinery, would provide an insight into the potential influence of ARMER on HT1080 UPR induction and determine if a failure to respond to certain stimuli is dependent on the nature of that stimulus. Furthermore, recent work has highlighted HSP72 as a regulator of ER stress-induced apoptosis (Gupta *et al.*, 2010). HSP72 protected PC12 cells from ER-stress induced apoptosis by increasing the amplitude of IRE1-XBP1 signalling via a direct interaction, thus promoting survival. Although I do not observe increased *XBP1* signalling in HT1080 cells exposed to tunicamycin, HSP72 has been reported to inhibit *CHOP* and TNFα-induced apoptosis (Gotoh *et al.*, 2004). Therefore, diminished UPR signalling in HT1080 cells might be the consequence of direct modulation of ER efferent signalling.

The pathogenesis of AS is likely to be a complex interaction of innate and adaptive immune responses. The influence of HLA-B27 misfolding on disease pathogenesis is irrefutable; however, it is likely that other mechanisms such as $CD4^+$ T-cell recognition of cell surface homodimers may influence disease. Recent analysis of genetic susceptibility to AS has highlighted the IL-23 receptor. Furthermore, *IL-23R* polymorphisms have been implicated in Crohn's disease and other inflammatory phenotypes (Duerr *et al.*, 2006; Cargill *et al.*, 2007). Rather than contributing to T-cell differentiation, IL-23 favours the expansion and maintenance of Th17 cells suggesting that, in SpA patients, these cells might expand under the influence of IL-23 (McGeachy *et al.*, 2009; Melis & Elewaut, 2009). There is also growing evidence that IL-17 is a crucial proinflammatory cytokine since it can act on several cell types

including macrophages, fibroblasts, endothelial and epithelial cells to upregulate TNFα, IL-6 and IL-1 (Nakae *et al.*, 2003; Kolls *et al.*, 2004). Aside from IL-17, Th17 cells are also capable of producing TNFα and IL-6 themselves (Cua *et al.*, 2003; Chen *et al.*, 2006). Thus, it is clear that the downstream effects of Th17 expansion may include the production of multiple proinflammatory cytokines. The influence of HLA-B27 misfolding on IL-23 production, Th17 cell expansion and IL-17 production may provide novel insights into the pathogenesis of AS and other inflammatory diseases associated with a misfolding phenotype.

6. REFERENCES

6. References

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