



Investigation of the OS-9/Grp94 interaction and its role in Endoplasmic Reticulum-Associated Degradation (ERAD)

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Declaration

I declare that this thesis has never been submitted, either in the same or a different form, to this or any other university, for a degree or any other qualification. All of the work presented in this thesis is the result of my own work unless otherwise stated and was carried out while I was a graduate student at the Ludwig Institute for Cancer Research, University of Oxford, under the supervision of Dr. John Christianson.

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Stephanie M Jones, Merton College, DPhil in Clinical Medicine, Trinity 2014

Abstract

Proteins that misfold or are improperly assembled in the endoplasmic reticulum (ER) are exported to the cytosol to be degraded via the ubiquitin-proteasome pathway in a process termed ER-associated degradation (ERAD). The ER resident lectin OS-9 occupies a central role, linking recognition to the delivery of aberrant proteins to the membrane-embedded SEL1L/Hrd1 ubiquitin-ligase complex. Additionally, OS-9 interacts with Grp94, the ER-resident Hsp90 family member, in a manner that is mutually exclusive of its interaction with SEL1L. To further characterise the complexes formed by OS-9 with SEL1L and Grp94, an extensive series of OS-9 truncations/deletions were generated and the resulting co-precipitation profiles compared. It was found that OS-9 interacts with Grp94 in the ER lumen and involves the C-terminal domain of OS-9. More precisely, a 65 a. a. region (OS-9₄₄₃₋₅₀₇) is necessary for OS-9/Grp94 binding, which was designated the 94BR (Grp94 binding region). Naturally occurring point mutations and alternative splice variants within this region were compromised for binding to Grp94 but not SEL1L or substrate, indicating a highly specific interaction. A melanoma cell line derived from a lymph node metastasis and homozygous for a somatic OS-9 mutation (P446L) exhibited an increased rate of growth when compared to a parental counterpart with one wild type allele, suggesting that disruption of the OS-9:Grp94 interaction may confer an activity that favours tumour progression. These studies point toward functional consequences when OS-9 partitioning between Grp94 and SEL1L is altered.

Imscrúdú ar an OS-9 idirghníomhaíocht Grp94 agus a ról i ER-Associated Degradation (ERAD)

Stepháníe Nic Sheoin, Coláiste Merton, DPhil i leigheas cliniciúil, Trinity 2014

Abstract

Nuair a bhíonn próitéiní lúbtha nó curtha le chéile go mícheart sa líontán ionphlasmach (LI), seoltar chuig an gcíteasol iad ionas go ndíghrádófar iad trí bhealach ubiquitin-proteasome i bpróiseas darb ainm ER-Associated Degradation (ERAD). Bíonn ról lárnach ag leictín OS-9 (de chuid LI) sa mhéid is go nasctar aitheantas le seachadadh phróitéiní iomrallacha go coimpléasc ubiquitin-liogáise SEL1L/Hrd1. Chomh maith leis sin, gníomhaíonn OS-9 le Grp94 (ball de chuid Hsp90) ar nós atá comheisiatach i gcomparáid lena gníomhú le SEL1L. Ionas go léireofaí na coimpléisc a rinne OS-9 le SEL1L agus le Grp94, gineadh sraith fhairsing de theascadh/scriosadh OS-9 agus rinneadh comparáid idir próifíilí na gcomhdheascán a tháinig aisti. Fuarthas amach go ngníomhaíonn OS-9 le Grp94 sa lúman LI agus go bhfuil baint ag an bpróiseas seo le fearann C-teirminéalach an OS-9. Tá réigiún 65 a. a. (OS-9₄₄₃₋₅₀₇) de dhíth chun OS-9/Grp94 a nascadh, agus tugadh 94BR (réigiún nasctha Grp94) air. Athchóiríodh ionadú péire bunaidh agus athraithigh spladhsáilte a bhí sa réigiún seo de bharr nascadh le Grp94 ach ní le SEL1L nó lena fhoshraith. Léirítear, mar sin, gur sainghníomh atá ann. Méadaíodh ráta fáis cheall-líne mheileanóma a bhí gafa ó mheiteastáis nóid limfe agus a bhí homaisigeach i gcomhair shóchán sómatach OS-9 (P446L). Ba é seo i gcomparáid le ráta fáis thuistemhacasadhail a bhfuil ailléil amháin aici. Tugtar le tuiscint,

mar sin, go gcabhraítear le siad teacht chun cinn nuair a chuirtear isteach ar ghníomh OS-9:Grp94. Léiríonn na staidéir seo gur torthaí feidhmiúla atá ann nuair a athchóirítear deighilt OS-9 idir Grp94 agus SEL1L.

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LIST OF ABBREVIATIONS

Å	Ångström
a. a.	Amino acid
AAA	ATPases associated with diverse cellular activities
AAT	Alpha-1 antitrypsin
Amp	Ampicillin
AP	Affinity precipitation
APS	Ammonium persulphate
ATP	Adenosine-5'-triphosphate
BiP	Binding immunoglobulin protein
CFTR	Cystic fibrosis transmembrane conductance regulator
CFSE	Carboxyfluorescein succinimidyl ester
CHX	Cycloheximide
CNX	Calnexin
CPY	Carboxypeptidase Y
CPVL	Carboxypeptidase vitellogenic-like protein
CQ	Chloroquine
C-terminal	Carboxy terminal
CRT	Calreticulin
ddH₂O	Double distilled water
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
ECL	Enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERQC	ER quality control
EndoH	Endoglycosidase H
FCS	Foetal calf serum
FBS	Foetal bovine serum
GI	Glucosidase I
GII	Glucosidase II
GFP	Green fluorescent protein
GlcNAc	N-acetylglucosamine
Grp78/BiP	Binding immunoglobulin protein
Grp94	Heat shock protein 90 kDa beta member 1
GST	Glutathione S-transferase
GT	Glucosyltransferase
HEK293	Human embryonic kidney 293
HIF	Hypoxia-inducible factor
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA

Hrd1	E3 ubiquitin-protein ligase synoviolin
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IF	Immunofluorescence
IP₃R	Inositol 1,4,5-triphosphate receptor
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IRE1	Inositol requiring enzyme-1
KDa	Kilodalton
LC3	Microtubule-associated protein 1A/1B-light chain 3
Man	Mannose
MEFs	Mouse embryonic fibroblast cells
MHC	Major histocompatibility complex
MRH	Mannose 6-phosphate receptor homology
NEM	N-ethylmaleimide
NHK	Alpha-1 antitrypsin, null Hong Kong variant
ORF	Open-reading frame
OS-9	Amplified in osteosarcoma 9
OST	Oligosaccharyl transferase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PERK	Protein Kinase RNA (PKR) – like kinase
PMSF	Phenylmethylsulfonyl fluoride

PPI	Peptidyl prolyl isomerase
QC	Quick change
QR-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEL1L	Suppressor of lin-12-like protein
SRP	Signal recognition particle
SS	Signal sequence
TA	Tail-anchored proteins
TEMED	N,N,N',N'-tetramethyl-ethane-1,2-diamine
TMD	Transmembrane domain
Tris	Tris (hydroxymethyl aminomethane)
TX-100	Triton X-100
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
UV	Ultraviolet
WB	Western blot
XBP1	X-box binding protein-1

CHAPTER ONE

Introduction

1.1 The endoplasmic reticulum

The endoplasmic reticulum (ER) is a multifaceted, highly organised organelle that is present in the cells of all eukaryotic organisms, within which it plays vital roles in numerous cellular processes, acting as both a calcium reservoir and regulating the production of lipids and cholesterol (Berridge 2002). However, it is in the folding and assembly of transmembrane and secretory proteins that the ER is particularly adept. In all cell types of all living organisms, proteins are the key factors that execute and maintain the processes necessary for life. Transcription, translation, catabolic and anabolic metabolism, and respiration are only a few of the cellular processes that depend upon correctly processed and folded proteins for their function and maintenance. The ER luminal environment is highly specialised to the task of protein folding, where it is rendered oxidising by the high ratio of oxidised to reduced glutathione, promoting the formation of disulphide bonds. In addition, it contains a large set of resident molecular chaperones dedicated to facilitating the maturation and processing of proteins that traverse the secretory pathway (Hwang, Sinskey *et al.* 1992).

The ER is a highly dynamic organelle; yet its complex functions can be significantly influenced by an array of parameters both inside the cell and in its microenvironment. The availability of oxygen (hypoxia) or glucose (hypoglycaemia), hyperthermia (extreme temperature elevation), acidosis, calcium levels, the redox milieu, and other factors can all impact and disturb proper function. Therefore, the ability of the ER to adapt its capacity to manage

synthetic, metabolic, and other adverse conditions is of paramount importance for the cell. To ensure the correct folding and assembly of proteins, the ER employs a number of safeguards to optimise the processing of newly synthesised secretory proteins and also dispose of defective ones.

1.2 ER membrane translocation

Approximately one third of all newly synthesised proteins in eukaryotes are targeted to the secretory pathway, with the ER being the first membrane compartment that nascent polypeptides encounter (Ghaemmaghami, Huh *et al.* 2003). Within the lumen, the sequestered oxidising environment and resident chaperones promote the folding and processing of proteins to the native three-dimensional structures required for their proper function. Targeting to the ER is mediated by a hydrophobic signal peptide (approximately 17 - 35 residues) at the N-terminus of secretory proteins or by a transmembrane domain (Walter, Gilmore *et al.* 1982). The signal recognition particle (SRP), a ribonucleoprotein complex, co-translationally binds signal sequences as they emerge from the ribosome, temporally stalling translation and targeting the ribosome-nascent chain complex to the ER via the SRP receptor (Wild *et al.* 2004). Subsequently, proteins are translocated into the ER lumen or integrated into the ER membrane through a channel formed by the heterotrimeric Sec61 complex (Sec61 α , Sec61 β and Sec61 γ) (Johnson and van Waes 1999). The Sec61 translocon forms an aqueous, ion-conducting channel (Wirth, Jung *et al.* 2003) that is structurally dynamic, with an estimated maximum dimension of ~ 15 Å (Breyton,

Haase *et al.* 2002). Once on the ER luminal side of the membrane, the signal-peptide is cleaved co-translationally by the signal peptidase (Lemberg and Martoglio 2004).

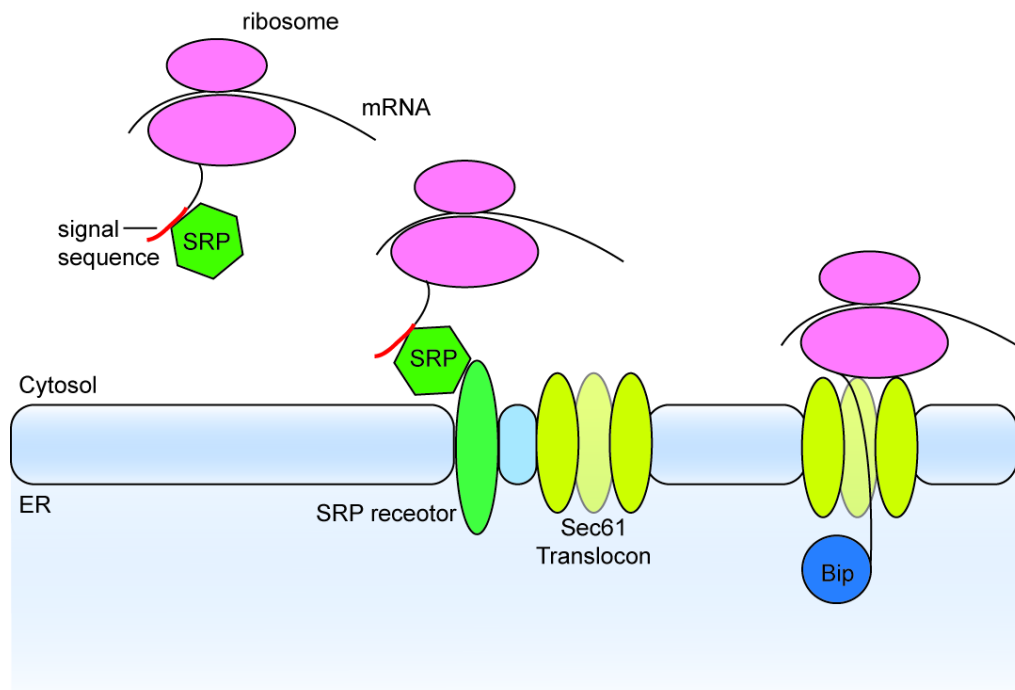


Figure 1.1: ER membrane translocation

Binding of SRP to the signal sequence of a nascent polypeptide as it emerges from the ribosome. Targeting to the Sec61 translocon complex through interaction with the SRP receptor, followed by translocation of the nascent polypeptide into the ER via the translocon pore, where it is stabilised through interaction with ER resident chaperones.

A small sub-set of proteins require post-translational insertion as opposed to co-translational insertion. Tail-anchored proteins (TA) contain a single transmembrane domain (TMD) at their C-terminus which upon termination of translation is yet to emerge from the ribosomal tunnel (accommodates ~40 a.a.), thus recognition would be required post-translationally (Beilharz, Egan *et al.* 2003). TA proteins represent ~3-5 % of all membrane proteins, are found in all

cellular membranes and have been shown to have roles in vesicular trafficking, apoptosis and protein degradation (Beilharz, Egan *et al.* 2003; Kalbfleisch, Cambon *et al.* 2007; Kriechbaumer, Shaw *et al.* 2009).

In budding yeast, TA protein insertion is mediated by the Get complex (Get1, Get2, Get3) (Schuldiner, Metz *et al.* 2008) (Figure 1.2). The crystal structure revealed Get3 to be a symmetric homodimer, with each monomer containing an ATPase domain and an α -helical domain. In the presence of ATP, the helical domains associate to form a hydrophobic groove that recognises and binds the TMD of a TA protein (Bozkurt, Stjepanovic *et al.* 2009; Hu, Li *et al.* 2009; Mateja, Szlachcic *et al.* 2009; Suloway, Chartron *et al.* 2009; Yamagata, Mimura *et al.* 2010). Get1 and Get2 are multi-spanning ER membrane proteins that interact with Get3 through their cytosolic domains (Mariappan, Mateja *et al.* 2011; Wang, Whynot *et al.* 2011). Additionally, Get1 orchestrates substrate release and Get2 directs the complex to the ER membrane (Mariappan, Mateja *et al.* 2011; Stefer, Reitz *et al.* 2011; Wang, Whynot *et al.* 2011). Get3 does not associate with the ribosome directly. Instead, small Glu-rich tetratricopeptide repeat-containing 2 (Sgt2) binds the TMDs of TA proteins as they emerge from the ribosomal exit tunnel (Chang, Chuang *et al.* 2010). The TA protein is subsequently passed to Get3 with the assistance of the scaffolding proteins Get4 and Get5, with Get4 recruiting Get3, and Get5 recruiting Sgt2 respectively (Copic, Dorrington *et al.* 2009; Jonikas, Collins *et al.* 2009; Chartron, Suloway *et al.* 2010).

In mammals, TRC35 (homologous to Get4) and UBL4A (homologous to Get5) are in complex with BAG6, which is predicted to be bound to SGTA (Sgt2) (Winnefeld, Grewenig *et al.* 2006; Leznicki, Clancy *et al.* 2010; Mariappan, Mateja *et al.* 2011). The TA-protein's insertion into the ER lipid bilayer requires TMD-recognition complex protein of 40 kDA (TRC40) (Stefanovic and Hegde 2007), which is composed of Trp-rich protein (WRB) and mediates targeting to the ER membrane (Vilardi, Lorenz *et al.* 2011), and calcium-modulating cyclophilin ligand (CAML) which is additionally required for membrane insertion (Yamamoto and Sakisaka 2012).

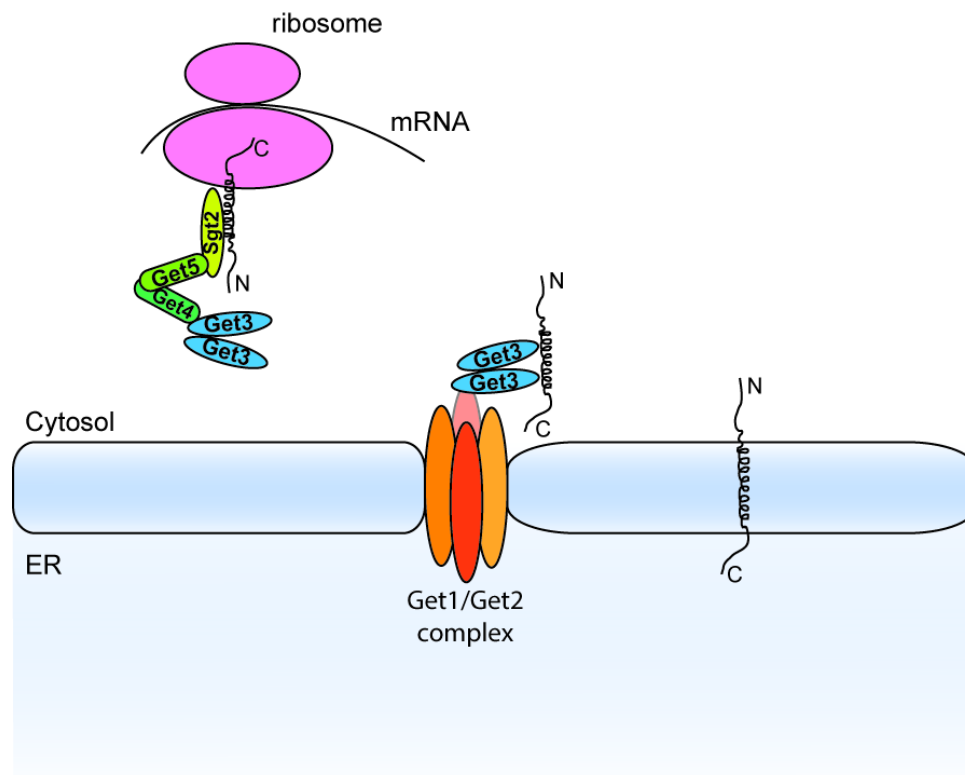


Figure 1.2: ER membrane post-translational insertion

In yeast, the transmembrane domain (TMD) of tail anchored (TA) proteins is bound by Sgt2 and targeted to Get3 through the assistance of Get4/Get5. The TA protein-Get3 complex binds to the ER membrane heterodimer Get1/Get2, mediating TA protein membrane insertion.

1.3 Chaperone assisted folding

The final 3D native conformation of a polypeptide is encoded within its linear sequence. This coding is often sufficient for correct folding and indeed, unassisted folding of isolated proteins has been studied *in vitro* (Anfinsen 1973; Dobson and Karplus 1999). However, the environment within living cells is highly complex and extremely crowded. This macromolecular crowding has several consequences; the most prominent being the increased possibility for aggregation. Exposure of hydrophobic surfaces on nascent chains, particularly during their early synthesis, can render them prone to aggregation. As correct protein folding is an essential process for protein function in all organisms, cells have evolved a plethora of sophisticated mechanisms to ensure that proper protein folding occurs and to prevent protein misfolding.

Molecular chaperones are composed of several classes of proteins performing diverse functions ranging from *de novo* protein folding and translocation across organellar membranes, to the prevention of aggregation, refolding of un/misfolded proteins, disaggregation of small aggregates, and the targeting of proteins for degradation (De Los Rios, Ben-Zvi *et al.* 2006; Hinault, Ben-Zvi *et al.* 2006; Powers, Clarke *et al.* 2009; Hartl 2011; Halperin, Jung *et al.* 2014). The following sections will discuss some of the prominent family members.

1.3.1 Heat shock protein homologues

Many chaperones were first discovered in studies of the cellular response to thermal stress, and are hence referred to as Heat Shock Proteins (Hsp). Categorised by their molecular weights, Hsp chaperone families include Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100. Although denoted by a similar prefix, each family acts uniquely to aid protein folding and can vary dramatically in mechanism. Many are up-regulated upon different stresses such as temperature, ethanol, oxidising stress and an accumulation of unfolded proteins (Richter, Haslbeck *et al.* 2010). Two of the most prominent ER chaperones are members of the Hsp70 and Hsp90 families BiP and Grp94, respectively.

The luminal Hsp70 chaperone BiP (heavy chain binding protein, also known as GRP78 (glucose-regulated protein 78)) preferentially binds exposed hydrophobic patches of newly synthesised proteins (Haas and Wabl 1983; Hendershot 2004) and catalyses the ATP-coupled folding and refolding of substrates (Flynn, Pohl *et al.* 1991; Blond-Elguindi, Cwirla *et al.* 1993). BiP contains conserved ATPase and peptide-binding domains, as well as the highly conserved Lys-Asp-Glu-Leu (KDEL) sequence in its C-terminus, rendering it an ER-resident chaperone (described in detail in section 1.4). Similar to other Hsp70 family members, the substrate binding affinity of BiP is regulated by the ATPase cycle (Ma and Hendershot 2004; Schroder and Kaufman 2005). When ATP is bound, BiP has a low peptide affinity open conformation, while ADP converts BiP into a high-affinity closed state conformation. Thus, alternation of ATP-hydrolysis and ADP to ATP exchange results in cycles of substrate binding

and release (Mayer, Reinstein *et al.* 2003). BiP also plays a crucial role in the unfolded protein response (UPR), described in detail later in this chapter.

Grp94 (Glucose-regulated protein 94), also known as endoplasmin, CaBP4, ERp99, Hsp108 and gp96, is the ER-resident Hsp90 paralogue (Argon and Simen 1999; Nicchitta, Carrick *et al.* 2004). Similar to other resident ER luminal proteins, Grp94 bears an N-terminal signal sequence and a C-terminal KDEL, ER retention/retrieval motif. In contrast to its cytoplasmic counterpart (Hsp90), which performs essential functions in the cytoplasm of cells from all kingdoms of life, Grp94 is present only in metazoans. However, it is the most abundant ER-resident protein, constituting 5-10 % of the ER luminal contents (Koch, Smith *et al.* 1986). Reflective of the lengthy list of synonyms attributed to Grp94, it has been independently discovered under a wide range of experimental paradigms. It was initially identified in response to glucose starvation (Grp94) (Shiu, Pouyssegur *et al.* 1977) and later as a tumour rejection antigen (gp96) (Srivastava, DeLeo *et al.* 1986). It was subsequently classified as the most abundant Ca^{2+} binding protein in the ER lumen (endoplasmin or CaBP4) (Koch, Smith *et al.* 1986; Van, Peter *et al.* 1989), as induced in differentiating B lymphocytes (ERp99) (Lewis, Mazzaella *et al.* 1985), and as a stress response protein involved in steroid induction (Hsp108) (Kulomaa, Weigel *et al.* 1986; Sargan, Tsai *et al.* 1986). Grp94 has high calcium binding affinity and functions as an important calcium buffer (Van, Peter *et al.* 1989), but has been shown to interact *in vitro* with immunoglobulin chains (Melnick, Aviel *et al.* 1992), MHC class II molecules (Schaiff, Hruska *et al.* 1992), thyroglobulin

(Kuznetsov, Chen *et al.* 1994), erbB2 (Chavany, Mimnaugh *et al.* 1996), a herpes virus glycoprotein (Ramakrishnan, Tugizov *et al.* 1995), apolipoprotein B (Linnik and Herscovitz 1998), collagen (Ferreira, Norris *et al.* 1994), protein C (Katsumi, Senda *et al.* 1996) and BSDL (Bruneau, Lombardo *et al.* 1998). Physiological substrates for Grp94 have not been confirmed, although a role in the assembly and maturation of toll-like receptors has been suggested (Yang, Liu *et al.* 2007; Liu and Li 2008). Despite the limited client set currently identified for Grp94, its importance is highlighted by embryonic lethality in Grp94 mouse knockouts, with development arresting at gestation day 7, before gastrulation and formation of the mesodermal tissues (Wanderling, Simen *et al.* 2007). Conversely, it is not essential in cell culture as isolated MEFs grow normally, albeit more slowly (Randow and Seed 2001; Wanderling, Simen *et al.* 2007), do not have a global deficiency in surface receptor expression (Randow and Seed 2001), and are still capable of differentiating (Wanderling, Simen *et al.* 2007). Intriguingly, it was recently shown that Grp94 knockdown (via siRNA) led to the inhibition of cell proliferation, migration and metastasis in two aggressive breast cancer cell lines (Dejeans, Glorieux *et al.* 2012) and decreased the proliferation rate of multiple myeloma cells (Hua, White-Gilbertson *et al.* 2013).

Grp94, like other Hsp90 family members, exists as an obligate homodimer, with each subunit consisting of four domains (Figure 1.3), the N-terminal domain (NTD) contains a nucleotide binding site (Wearsch, Voglino *et al.* 1998; Schulte, Akinaga *et al.* 1999), a peptide binding site (Vogen, Gidalevitz *et al.* 2002; Gidalevitz, Biswas *et al.* 2004) and unmapped sites responsible for

interaction with dendritic cells during antigen presentation (Biswas, Sriram *et al.* 2006). The linker domain (LD), a charged region between the N and middle domains (MD), required for binding of both nucleotide and calcium (Biswas, Ostrovsky *et al.* 2007). The MD interacts with the NTD (Ali, Roe *et al.* 2006) and the C-terminal domain (CTD) mediating the dimerisation of Grp94 (Wearsch and Nicchitta 1996). The first ~50 a. a. of mature Grp94 are distinct from the N-terminus of Hsp90 in both length and sequence however they are followed by highly homologous sequences throughout the NTD, including the contact residues within the binding pocket and those that catalyse ATP hydrolysis (Richter, Moser *et al.* 2006; Marzec, Eletto *et al.* 2012). Although controversial for some time, ATPase activity has been demonstrated for Grp94 (Dollins, Immormino *et al.* 2005). While the rate of hydrolysis is comparable to that of its cytosolic homologues (Dollins, Warren *et al.* 2007; Frey, Leskovar *et al.* 2007), ATP binding is 5-10 fold less in Grp94. The NTD also binds nucleotides and two small molecule inhibitors (geldanamycin and radicicol) in a pocket opposite the peptide-binding site (Vogen, Gidalevitz *et al.* 2002; Gidalevitz, Biswas *et al.* 2004)

Like other Hsps, Grp94 is induced by the accumulation of misfolded proteins (Kozutsumi, Segal *et al.* 1988) but, unlike HSP90 α and β , is not up-regulated by high temperature or other stresses unique to the cytosol (Subject and Shyy 1986). However, all types of stresses that affect the ER machinery result in Grp94's induction; perturbations of calcium or redox homeostasis,

inhibition of glycosylation, hypoxia, glucose levels etc. (Subjeck and Shyy 1986; Spindler, Crew *et al.* 1990; Lee 1992).

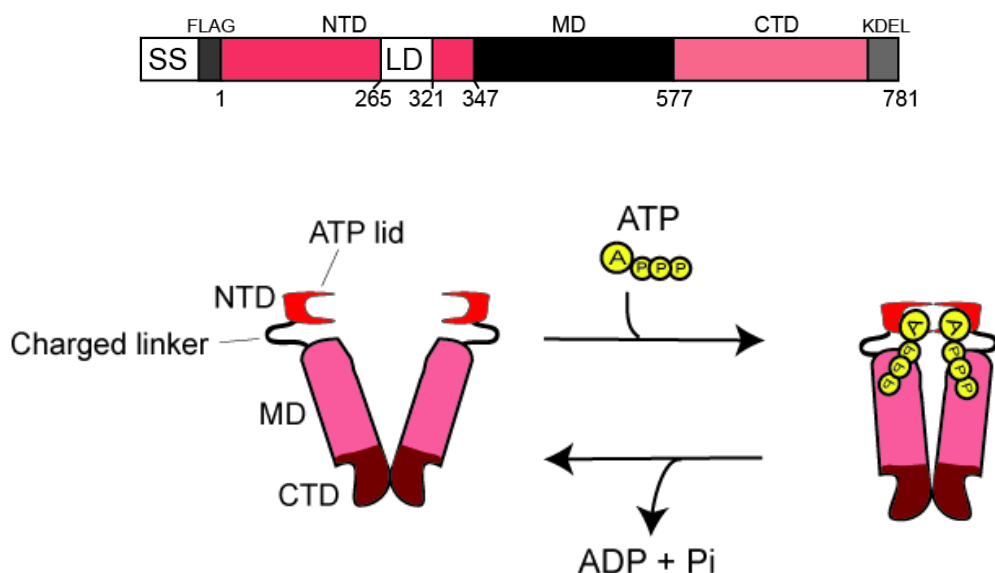


Figure 1.3: Domain structure and conformational cycle of Hsp90

Hsp90 consists of three domains: an N-terminal domain (NTD); a middle domain (MD) and a C-terminal domain (CTD). Nucleotide-free Hsp90 is in an open state with its C-terminal domains constitutively dimerised. On binding of ATP, a conformational change is induced resulting in the closed state of Hsp90. After ATP is hydrolysed, the N-domains dissociate, release ADP and Pi, and Hsp90 returns to the open conformation.

1.3.2 Protein disulphide isomerase (PDI)

The ER contains a sequestered oxidising environment, with a ratio of reduced (GSH) to oxidised (GSSG) glutathione of 3:1, in comparison to the highly reducing cytosol (GSH:GSSG ratio ~100:1) (Hwang, Sinskey *et al.* 1992), rendering it an ideal environment for the formation of the disulphide bonds essential for the maturation of a large majority of proteins. The oxidation, reduction and isomerisation of disulphide bonds are catalysed by protein

disulphide isomerases (PDIs), with members of the enzymatic family defined by the number and arrangement of thioredoxin-like domains (TLDs) and the sequences of the catalytic active site CXXC motifs (Freedman, Klappa *et al.* 2002; Ellgaard and Ruddock 2005). At least 19 PDI family member proteins have been identified in the ER of mammalian cells (Appenzeller-Herzog and Ellgaard 2008), with the most abundant family member being PDI, a highly conserved and soluble 56 kDa protein comprising 2 % of total ER protein (Yoshimori, Semba *et al.* 1990; Laboissiere, Sturley *et al.* 1995). PDI consists of four TLDs: two non-catalytic TLDs denoted b and b', and two catalytically active TLDs termed a and a', with a 19 a. a. linker region between the b' and a' domains and a C-terminal acidic extension (Ellgaard and Ruddock 2005). To facilitate disulphide bond formation in folding intermediates, the cysteines in the catalytically active domains of PDI are present in the oxidised state, forming mixed disulphides with the substrate and leaving the reaction in the reduced state (Ellgaard and Ruddock 2005; Hebert and Molinari 2007). In addition to its role as an oxidase, reductase and isomerase, PDI has been reported to function as a molecular chaperone, preventing the aggregation and the promoting folding of D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a protein with no disulphide bonds (Cai, Wang *et al.* 1994; Quan, Fan *et al.* 1995; Hebert and Molinari 2007).

A second well characterised member of the PDI family is ERp57, with four thioredoxin-like domains, also labelled a, b, b' and a', but missing the acidic C-terminal domain observed in PDI. The enzymatic activity of ERp57 is

mediated through its two active site CXXC motifs residing within the α and α' domains, with a more reducing redox potential compared to PDI. ERp57 interacts with the two lectin chaperones calnexin and calreticulin (described in detail later in section 1.3.4), and thereby promotes the oxidative folding of newly synthesised glycoproteins (Oliver, van der Wal *et al.* 1997).

1.3.3 Peptidyl prolyl *cis-trans* isomerase

The *cis-trans* isomerisation of prolyl-peptide bonds is a rate-determining step in the *in vitro* refolding of some proteins (Fischer, Berger *et al.* 1989). Peptidyl-prolyl isomerases (PPIs) catalyse the isomerisation of *cis* and *trans* peptide bonds on the N-terminal side of proline residues (Zapun, Jakob *et al.* 1999). PPIs are classified into three families based on their binding affinities to specific inhibitors, namely parvulins, cyclophilins (CyPs), and FK506-binding proteins (FKBPs). In the ER, members of the latter two families have been identified, CyB and up to six FKBPs. CyB has been described in association with several ER chaperones and is involved in the co-operation with Hsp47 in the folding of procollagen (Smith, Ferreira *et al.* 1995) and has also been identified as a HIV-dependency factor (Brass, Dykxhoorn *et al.* 2008) that binds HIV Gag and HIV capsid protein p24 (Sokolskaja, Olivari *et al.* 2010). Although the refolding of denatured proteins is significantly enhanced in the presence of CyPs and FKBPs *in vitro*, and has been shown to be induced by the unfolded protein response, their actual contribution to protein maturation in living cells remains

poorly understood, since yeast lacking all PPI enzymes are still viable (Travers, Patil *et al.* 2000).

Table 1.1: Selection of prominent ER-resident folding factors

Family	Mammals	Yeast	Function
Hsp100	TorsinA		Chaperone
Hsp90	Grp94		Chaperone
Hsp70	BiP/Grp78	Kar2p	Chaperone
Hsp40	Erdj 1-5	Sec63p, Scdp, Jem1p	Cofactors for Hsp70
GrpE-like	BAP	Sls1p	Cofactors for Hsp70
Lectins	CNX	Cne1	Glycoprotein-dedicated-chaperone
	CRT		
	EDEMs 1, 2, 3	Htm1p	Glycoprotein-degradation
	OS-9, XTP3-B	Yos9	Glycoprotein-degradation
Glycan-processing enzymes	α -Glucosidase I	Gls1p	N-glycan trimming
	α -Glucosidase II	Gls2p	N-glycan trimming
	α -Glucosidase III	Mns1p	N-glycan trimming
	UGGT	Kre5p	Folding sensor
	ER mannosidases		Removes terminal mannoses
PDI	PDI	Pdi1p	Thiol oxidoreductase
	ERp57		Thiol oxidoreductase
	ERp72		Thiol oxidoreductase
Cyp	CypB	Cpr5p	Peptidyl-prolyl isomerase
FKBP	FKBP13	Fpr2p	Peptidyl-prolyl isomerase

1.3.4 N-linked glycosylation

Upon ER entry through the Sec61 channel, the majority of nascent polypeptides are modified by the addition of one or more N-linked oligosaccharide (Figure 1.4) (Apweiler, Hermjakob *et al.* 1999). N-linked glycan, a 14-member carbohydrate comprised of three glucoses, nine mannoses and

two *N*-acetylglucosamines ($\text{Glu}_3\text{Man}_9\text{GlcNAc}_2$), is pre-assembled on the cytoplasmic side of the ER on the dolichol-linked oligosaccharide donor. This structure is subsequently “flipped” to the ER luminal surface and covalently attached *en bloc* to the newly formed polypeptide chains on asparagine residues in Asn-X-Ser/Thr motifs (X designates any residue except proline) by the oligosaccharyl transferase (OST) complex (Kornfeld and Kornfeld 1985; Popov, Tam *et al.* 1997). While in the ER, *N*-linked glycans are trimmed and can reflect the folding state of a protein as it is processed (Figure 1.5).

A newly attached oligosaccharide is rapidly processed by removal of the terminal glucose moiety by ER glucosidases I. Glucosidase I activity has been shown to occur as soon as the glycan has cleared a distance of 72 residues from the ribosome P-site. Since OST activity requires the same distance, this indicates that the processing can occur immediately after the *N*-glycan has been transferred to the nascent polypeptide chain (Jakob, Burda *et al.* 1998; Deprez, Gautschi *et al.* 2005). Subsequent processing by glucosidase II (GII), results in the removal of the second glucose (M, Figure 1.4) residue, generating the monoglycosylated form (Glu_1Man_9) that is recognised by the ER chaperones calnexin (CNX), a type I membrane protein, and its soluble paralogue calreticulin (CRT) (Ou, Cameron *et al.* 1993; Hammond, Braakman *et al.* 1994; Hebert, Foellmer *et al.* 1995; Spiro, Zhu *et al.* 1996). CNX and CRT prevent aggregation of the polypeptides and promote oxidative folding via association with the protein disulphide isomerase-like protein ERp57 (Oliver, van der Wal *et al.* 1997). ERp57 catalyses the formation of disulphide bonds, a rate limiting reaction of

polypeptide maturation, by acting as an oxidising agent. Additionally, ER retention signals on both chaperones ensure their retention and, consequently, the retention of the immature protein within the ER (Rajagopalan, Xu *et al.* 1994).

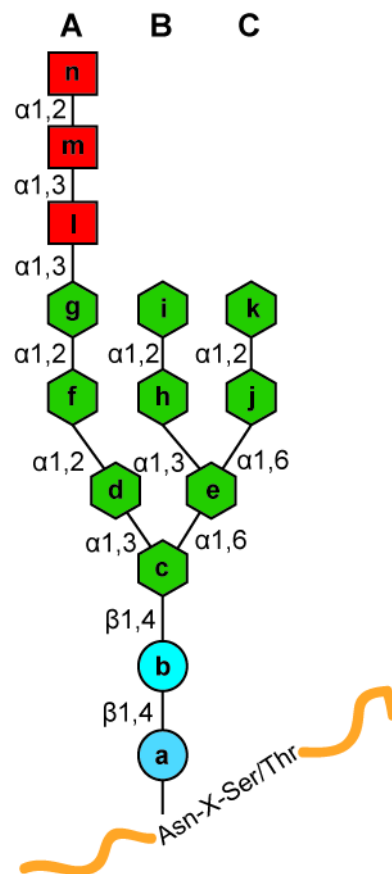


Figure 1.4: Structure of the N-linked $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ oligosaccharide

The N-linked core oligosaccharide is composed of two N-acetylglucosamines (Blue circles), nine mannoses (green hexagons) and three glucoses (red squares). The sugars are covalently linked via $\alpha 1,2$; $\alpha 1,3$; $\alpha 1,6$; or $\beta 1,4$ linkages, yielding the $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ glycan containing three branches (A, B, and C). The letters represent the order in which the sugars are added during the synthesis of the oligosaccharide.

CNX/CRT binding is dependent on the presence of the terminal glucose which, upon its cleavage by GII, signifies the release of the Man_9 -linked proteins from the cycle. Removal of the second glucose (M in Figure 1.4) by GII is

relatively rapid, however removal of the third and final glucose (L in Figure 1.4) is slower due to it being differently oriented to glucose (M), thus requiring GII to transiently separate and reposition (Petrescu, Butters *et al.* 1997). This lag time in the enzymatic activity of GII may be exploited by CNX/CRT to associate with the monoglucosylated intermediate (Deprez, Gautschi *et al.* 2005).

Glycoproteins may fold after a single association with a lectin chaperone (Hebert and Molinari 2007). However, if a polypeptide fails to reach its native confirmation the terminal glucose can be re-attached by UDP-glucose: glycoprotein glucosyl transferase (UGGT), allowing re-entry into the CNX/CRT cycle until folding is completed (Hammond, Braakman *et al.* 1994; Hebert, Foellmer *et al.* 1995). Biochemical studies using purified UGGT have demonstrated that UGGT recognises surface-exposed hydrophobic regions on maturing proteins that are associated with non-native or unassembled proteins (Sousa and Parodi 1995; Trombetta and Helenius 2000; Keith, Parodi *et al.* 2005). In comparison to UGGT and glucosidase II, α -mannosidase I has a relatively slow catalytic rate allowing it to act as a “timer” to retrieve polypeptides from futilely cycling through the CNX/CRT cycle. Removal of the terminal mannose from the A branch of the *N*-glycan (mannose G in Figure 1.4), which is the acceptor for UGGT, irreversibly prevents re-glycosylation and thus elimination of the terminally misfolded protein from interaction with CNX/CRT

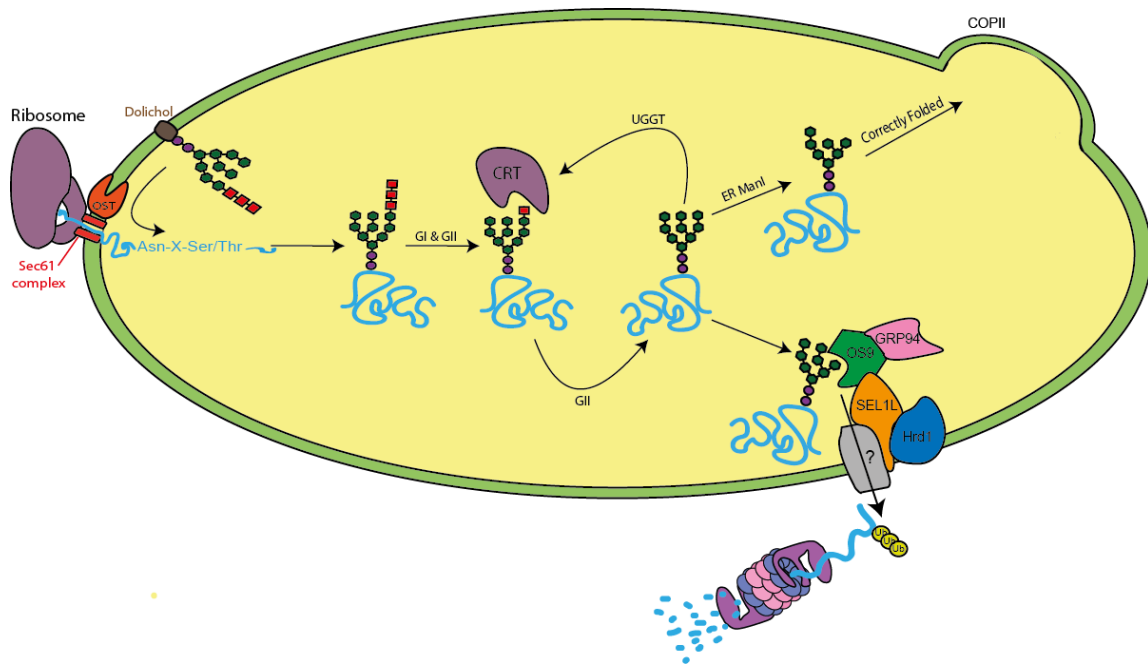


Figure 1.5: Processing and quality control of *N*-glycosylated proteins in the ER

The *N*-glycans ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) are conjugated co-translationally to the incoming nascent chain by the Oligosaccharyltransferase (OST) complex. The carbohydrate moiety is then trimmed by glucosidase I and II (GI and GII) to a mono-glucosylated state and enters the calnexin (CNX) or calreticulin (CRT) folding cycle. Trimming of the last glucose residue by GII promotes the release of substrate from CNX/CRT. If the CNX/CRT-released substrate is not properly folded, it may be re-glucosylated to the mono-glucosylated form by UDP-glucose: glycoprotein glucosyl transferase (UGGT), enabling the substrate to rebind CNX/CRT and undergo another series of folding attempts. If the protein eventually reaches its native conformation, it becomes trimmed by ER mannosidases I and/or II (Man I and Man II) to obtain a high-mannose glycan conformation that is packed into COPII vesicles for secretion towards the Golgi. The misfolded glycoproteins that enter in and out of the CNX/CRT folding cycle ultimately become trimmed by ER mannosidase I (Man I) or EDEM 1-3 to a $\text{Man}_{5-7}\text{GlcNAc}_2$, which is recognised by various ER-resident chaperones/folding enzymes (e.g. OS-9) that deliver the misfolded protein to the dislocation and ubiquitination machinery for transfer across the ER membrane and subsequent degradation by the 26S proteasome.

(Helenius 1994; Cabral, Liu *et al.* 2001; Hosokawa, Tremblay *et al.* 2003; Wu, Swulius *et al.* 2003; Lederkremer and Glickman 2005) targeting it instead for degradation (Section 1.6). The importance of this cycle is underscored by the demonstration that if CNX/CRT function is impaired (e.g. through inhibition of glucosidases), folding is more rapid but frequently incorrect, leading to increased aggregation and the loss of quality control of incompletely folded substrates (Jackson, Cohen-Doyle *et al.* 1994; Hebert, Foellmer *et al.* 1996; Vassilakos, Cohen-Doyle *et al.* 1996; Molinari, Eriksson *et al.* 2004). In addition to providing a “glycan code”, glycans confer increased solubility and it is believed that some glycans exert their effect on folding through limiting the conformational space of denatured polypeptide chains, whereas others induce the local formation of secondary structure (O'Connor and Imperiali 1996).

1.4 Retention of ER-resident proteins within the ER

Anterograde trafficking through the secretory pathway is essential for cargo proteins. However, of equal importance is the retention of ER-resident enzymes and chaperones within the ER, to ensure their continued availability to cope with the continuous load of substrate proteins entering the ER. The KDEL retrieval motif and the KDEL receptor (Erd2) work together to provide retrograde movement preventing ER-residents from travelling further along the secretory pathway. The KDEL motif is located at the C-terminus and is both necessary and sufficient to confer ER-retention. Deletion of the KDEL sequence results in *bona-fide* ER-resident proteins being no longer retained in the ER, while

conversely artificial incorporation of the sequence into the C-terminus of non-ER resident proteins results in their retention (Munro and Pelham 1987; Pelham 1989). The motif is charged and consequently affected by pH, a property central to the retention mechanism – the KDEL receptor binds the KDEL ligand under acidic conditions and is released in neutral conditions (Wilson, Lewis *et al.* 1993). Non-invasive pH measurements within individual compartments of HeLa cells indicate an ER luminal pH of 7.1 (Kim, Johannes *et al.* 1998), while the Golgi is more acidic (Anderson and Pathak 1985), thus a pH gradient is created (Wu, Grabe *et al.* 2001). The KDEL receptor therefore binds its ligand in the relatively acidic Golgi prior to association with the retrograde transport machinery (Aoe, Lee *et al.* 1998) and is directed back towards the ER. Once returned to the ER, the neutral pH induces dissociation and the receptor returns to the Golgi in an anterograde fashion.

Some variability around this sequence is found among resident ER proteins. In analogy to the KDEL-motif, type I ER transmembrane proteins often contain a KKXX (di-lysine) sequence at their C-termini to ensure ER-localisation (Nilsson, Jackson *et al.* 1989; Jackson, Nilsson *et al.* 1993). The di-lysine motif interacts with the coatamer complex present in COPI-coated vesicles (Cosson and Letourneur 1994) and so provides a method to return ER-resident proteins to the ER. Similarly, Type II integral membrane proteins, with luminal C-termini, are thought to be retrieved and retained within the ER by an N-terminal di-arginine motif (Schutze, Peterson *et al.* 1994). Calnexin is an example of a type I protein retained by a C-terminal di-arginine motif (Rajagopalan, Xu *et al.* 1994).

Despite these mechanisms, 60-70 % of the known ER-localised proteins lack a validated retention signal (Scott, Thomas *et al.* 2004), suggesting that additional strategies are employed to reduce the “leakage” of ER residents. In particular, the dense, network-like structure created through interactions between chaperones and enzymes, both with each other and with substrates, may provide a means to retard unwanted trafficking (Tatu and Helenius 1997; Meunier, Usherwood *et al.* 2002).

1.5 ER quality control (ERQC)

To ensure secretory pathway fidelity, proteins are subjected to the scrutiny of ER quality control (ERQC) prior to anterograde trafficking to the Golgi. Transcriptional errors, translational errors or genetic mutations can all lead to failures in protein maturation. Moreover, protein folding can be an extremely intricate and error-prone process and, even under physiological conditions, some proteins have an intrinsically low folding efficiency. One example is the cystic fibrosis transmembrane conductance regulator (CFTR), which has an estimated yield of folded protein of approximately 25 % (Ward and Kopito 1994). The situation is further complicated by the high protein concentration in the ER lumen, calculated to be in excess of 300 mg/ml, which presumably restricts protein mobility and would be expected to potentiate the risk of inappropriate protein interactions and aggregations (Ellis 2001; Kleizen and Braakman 2004). Collectively, these aspects underlie the need for and illustrate the complex task of ERQC.

1.6 ER-associated degradation (ERAD)

By recognising and retaining potentially harmful protein species, ERQC performs a crucial surveillance activity that safeguards cellular homeostasis. However, the accumulation of unfolded, misfolded and unassembled subunits of oligomeric complexes in the ER poses an equally dangerous risk. These aberrant polypeptides may form toxic aggregates, crowding the ER, siphoning away ER chaperones from their functional counterparts, inducing ER stress and thus impairing the ER's capacity to assist the maturation of newly synthesised secretory proteins. To maintain continuous flow through the ER and mitigate the accumulation of potentially toxic aggregates, terminally misfolded proteins are selectively recognised and directed for destruction by the ubiquitin-proteasome system (UPS), in a process termed endoplasmic reticulum-associated degradation (ERAD) (McCracken and Brodsky 1996; Kopito 1997).

Degradation of ER proteins was first observed for the human T-cell receptor subunit α (TCR α), which was demonstrated to degrade prior to trafficking to the Golgi in a lysosome independent manner (Lippincott-Schwartz, Bonifacino *et al.* 1988). The mechanism of ERAD can be broadly divided into four stages (Figure 1.6): (1) substrate recognition; (2) dislocation (integral membrane proteins) or retro-translocation (secreted proteins) across the ER membrane to the cytosol; (3) ubiquitination, which targets the polypeptide for; (4) degradation via the 26S proteasome, an ATP-dependent protease complex located in the cytoplasm (Hershko and Ciechanover 1998). Spatial separation of misfolded polypeptides in the ER lumen from the cytoplasmic UPS necessitates

that they be exported across the lipid bilayer in order to be degraded. The nature of this retro-translocation machinery remains undefined, however, a number of protein complexes have been suggested as possible channels including the Sec61 α translocon complex (Plempner, Bohmler *et al.* 1997; Zhou and Schekman 1999), the derlin-1 complex (Lilley and Ploegh 2004; Ye, Shibata *et al.* 2004) and the ubiquitin ligase Hrd1 (Carvalho, Stanley *et al.* 2010).

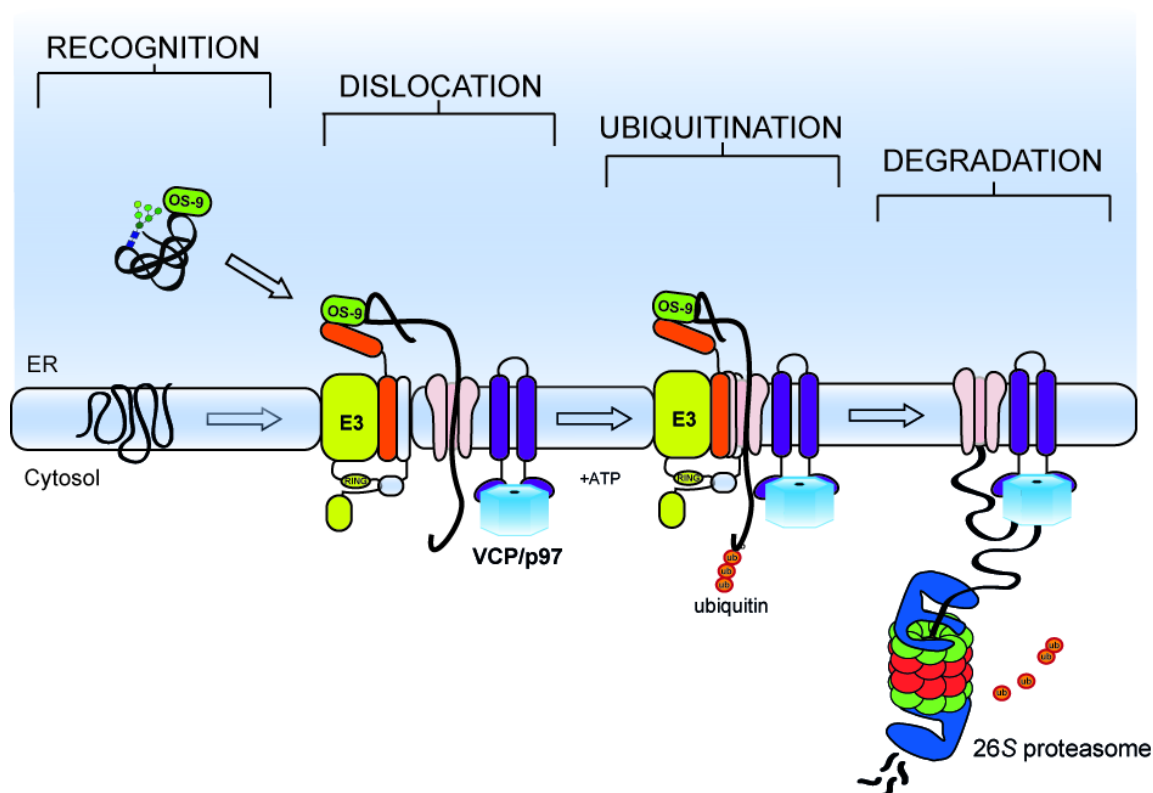


Figure 1.6: Stages of ERAD

The mechanism of ERAD can be broadly divided into four stages: (1) substrate recognition; (2) dislocation (integral membrane proteins) or retro-translocation (secreted proteins) across the ER membrane to the cytosol; (3) ubiquitination, which targets the polypeptide for; (4) degradation via the 26S proteasome, an ATP-dependent protease complex located in the cytoplasm.

The covalent modification of proteins with ubiquitin polymers is necessary for targeting to and recognition by the 26S proteasome (Varshavsky 1997, Hershko and Ciechanover 1998). Three distinct enzymes are required to attach ubiquitin to proteins: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-carrier or conjugating proteins), and E3 (ubiquitin-protein ligase), which recognise a specific protein substrate and catalyse the transfer of activated ubiquitin to the client protein (Bonifacino and Weissman 1998). Polyubiquitin chains bind to the surface of the 19S cap of the 26S proteasome, delivering the substrate for unfolding, de-ubiquitination and finally translocation into the lumen of the 20S core particle for degradation (Heinemeyer, Kleinschmidt *et al.* 1991; Baumeister, Walz *et al.* 1998; Pickart 2000; Thrower, Hoffman *et al.* 2000).

1.7 ERAD substrates

Proteins that fail to correctly fold or are synthesised in stoichiometric excess of partners are destined for degradation via ERAD. However, in addition to aberrant proteins, the ERAD pathway is utilised in the physiological regulation of the levels of some proteins and is exploited by viruses that hijack the degradation machinery, redirecting biosynthetic precursors of normal cell surface proteins towards destruction. The following examples of each of the four classes of ERAD substrates are by no means exhaustive, but serve to reflect the vast range of diverse and distinct substrates selected by the ERAD machinery for degradation.

1.7.1 Terminally misfolded proteins

Cystic fibrosis (CF) is a severe hereditary disease resulting from mutations to the cystic fibrosis transmembrane conductance regulator (CFTR), a 168 kDa multi-domain chloride channel that belongs to the adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily (Kerem, Rommens *et al.* 1989; Riordan, Rommens *et al.* 1989; Rommens, Iannuzzi *et al.* 1989; Smith and Welsh 1993). Disruption in electrolyte and fluid movement regulation across epithelial cell layers leads to mucus membrane thickening within the airways impairing lung functionality, and providing the perfect environment for bacterial infection. Additionally, most patients are pancreatic insufficient, and almost all males are infertile due to obstruction of the vas deferens (Reeves, Molloy *et al.* 2012). In excess of 1,900 different CFTR gene mutations have been reported (<http://www.genet.sickkids.on.ca/cftr/app>) that result in a CF phenotype, however, approximately 90 % of all mutant CFTR alleles bear the $\Delta F508$ mutation. This mutant is functionally active (Dalemans, Barbry *et al.* 1991; Haws, Nepomuceno *et al.* 1996) but constitutively degraded, resulting in the loss of protein expression on the cell surface (Cheng, Gregory *et al.* 1990). Proteasome inhibitors prevent CFTR $\Delta F508$ degradation but cannot rescue its maturation (Ward, Omura *et al.* 1995), owing to its aggregation in the cytosol (Johnston, Ward *et al.* 1998). However, it should also be noted that even wild type CFTR is inefficiently processed, with the majority degraded in the ER (Cheng, Gregory *et al.* 1990; Yang, Janich *et al.* 1993; Marshall, Fang *et al.*

1994). Pulse-chase studies indicate that approximately 25 % of wild type protein actually reaches the cell surface (Ward and Kopito 1994; Amaral 2005).

α -1 antitrypsin (AAT) is a serine protease inhibitor produced principally in liver cells. Secreted into the circulation, it primarily provides anti-protease protection in the lungs through the neutralisation of the serine protease neutrophil elastase (Elliott, Stein *et al.* 1996). Several disease-causing mutations have been identified for the AAT gene (DeMeo, Carey *et al.* 2004), with the most predominant being the Z mutation (ZAAT), which differs from the normal M variant by a single glutamic acid to lysine substitution at position 342 (Glu342Lys). This single nucleotide polymorphism alters the reactive centre loop of the protein enabling it to interact with a second ZAAT molecule, ultimately resulting in large polymer aggregation (McCracken, Kruse *et al.* 1989; Mahadeva, Dafforn *et al.* 2002). A second naturally occurring mutant, the Null Hong Kong (NHK) variant (which will be employed in chapter four of this thesis), was cloned and characterised by Sifers *et al.* from an individual exhibiting a total absence of immunoreactive α 1-AT in their serum (Sifers, Brashears-Macatee *et al.* 1988). A TC dinucleotide deletion within the codon for Leu³¹⁸ in exon IV results in a frame-shift mutation that generates a premature termination codon at residue 334, upstream of the active inhibitory site. The mutant gene is expressed as a truncated protein of 45 kDa, which is retained within the ER (Sifers, Brashears-Macatee *et al.* 1988). Subsequently, NHK and its non-glycosylated counterparts NHK-QQQ and NHK-AAA have been employed as model substrates in numerous studies delineating the components and

processes which compose ERAD (Hosokawa, Tremblay *et al.* 2003; Hirao, Natsuka *et al.* 2006; Olivari, Cali *et al.* 2006; Hosokawa, You *et al.* 2007; Christianson, Shaler *et al.* 2008; Yamaguchi, Hu *et al.* 2010).

1.7.2 Unassembled subunits of protein complexes

Subunit assembly/maturation efficiency in the ER of many large oligomeric receptors/ion channels is surprisingly inefficient, with only 20 to 40 % reaching maturity (Merlie and Lindstrom 1983, Schmidt and Catterall 1986, Ward and Kopito 1994). Examples of oligomeric complexes with rapidly degraded constituent subunits include GABA_A receptors (Gorrie, Vallis *et al.* 1997), voltage-gated Na⁺ channels (Schmidt and Catterall 1986), CFTR (Cheng, Gregory *et al.* 1990; Jensen, Loo *et al.* 1995), connexin 32 (VanSlyke, Deschenes *et al.* 2000) and ENaC (Valentijn, Fyfe *et al.* 1998).

The muscle-type acetylcholine receptor (AChR) is a heterooligomeric ion channel composed of five individual subunits (α , β , γ/ϵ , δ), whose abundance in the plasma membrane is limited by the assembly of individual subunits into mature pentameric receptors (Merlie and Lindstrom 1983). In vertebrate muscle cells, only 30 % of the synthesised α subunits finally assemble into a mature pentameters (Merlie, Sebbane *et al.* 1983), with the unassembled subunits being degraded through ERAD (Christianson and Green 2004).

1.7.3 Physiologically regulated proteins

Control of protein expression is a facet that is often attributed to the level of mRNA transcribed and translated. However, for a number of proteins, secretion from the ER and thus expressed protein level can be regulated by degradation through the ERAD pathway, offering an additional and more rapidly responsive layer of regulation. In particular, ERAD is involved in the regulated breakdown of key enzymes of the mevalonate pathway. HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase is required for the reduction of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate, a critical step in cholesterol biosynthesis (Gil, Faust *et al.* 1985; Webster, Tiwari *et al.* 2003). In a classical feedback loop, high sterol levels promote the binding of INSig-1 to HMG-CoA reductase, resulting in the recruitment of the E3 ubiquitin ligase gp78 and subsequent degradation of HMG-CoA. While under low sterol levels, gp78 targets INSig-1 for proteasomal degradation, protecting HMG-CoA reductase and promoting cholesterol synthesis (Chin, Gil *et al.* 1985; Lee, Lin *et al.* 2006).

Apolipoprotein B-100 (ApoB-100) is a major component in very low density lipoproteins (VLDL) that is overproduced in metabolic disorders such as type II diabetes, increasing the risk of atherosclerosis (Grundy 1987; Tyroler, Haynes *et al.* 1987; Davignon and Little 1988; Levy, Thibault *et al.* 1988). Due to the hydrophobic nature of apoB, it requires lipidation for its stability, without which (for example when cholesterol or lipid levels are low) it fails to correctly fold and is degraded (Yeung, Chen *et al.* 1996; Benoist and Grand-Perret 1997; Liang, Wu *et al.* 1998). Studies in HepG2 cells and primary hepatocytes show

that between 40-60 % (Borchardt and Davis 1987), and up to 80 % (Bostrom, Wettsten *et al.* 1986) of newly synthesised apoB is degraded.

The inositol 1,4,5-triphosphate receptor (IP₃R) forms Ca²⁺ channels in the ER membrane and mediates calcium release from the ER following activation by the co-agonists IP₃ and Ca²⁺ (Bosanac, Michikawa *et al.* 2004; Parker, Gergely *et al.* 2004; Patterson, Boehning *et al.* 2004). However, chronic activation of IP₃ receptors leads to their degradation by the ubiquitin-proteasome pathway (Wojcikiewicz, Tobin *et al.* 1994; Wojcikiewicz and He 1995). Upon their activation, IP₃R tetramers undergo a conformational change that opens the Ca²⁺ channel but also triggers the association of the ER membrane proteins SPFH1 and SPFH2 (erlin 1 and 2), targeting the receptors for ubiquitination and subsequent degradation (Pearce, Wang *et al.* 2007; Pearce, Wormer *et al.* 2009; Wang, Pearce *et al.* 2009).

1.7.4 Viral hijacking

A number of viruses have evolved strategies to further their propagation through exploitation of components of the ERAD machinery. This may be achieved either through aiding in the maturation of the virus or in the degradation of host components, resulting in the down-regulation or subversion of the immune response. For example, Human cytomegalovirus (HCMV) US11 and US2 proteins, through their binding of BiP, selectively target and induce dislocation of major histocompatibility complex (MHC) class I heavy chains,

resulting in their rapid degradation by way of the ERAD machinery, assisting the virus in immune evasion (Jones, Hanson *et al.* 1995; Wiertz, Jones *et al.* 1996; Jones and Sun 1997; Tortorella, Story *et al.* 1998; Shamu, Story *et al.* 1999).

Human immunodeficiency virus type 1 (HIV-1) has also been shown to manipulate the immune system through ERAD (Willey, Maldarelli *et al.* 1992). It triggers the degradation of the otherwise stable cell surface receptor CD4. CD4 is responsible for the uptake of viral particles by cells through binding to the viral envelope (Env) protein (McClure, Sattentau *et al.* 1987). Additionally, CD4 participates in T-cell activation (and consequently antibody production, phagocytic cell function and cytotoxic T-cell responses) through binding to both the T-cell receptor and MHC class II molecules on antigen-presenting cells (Tubo and Jenkins 2014). The HIV-1 accessory protein Vpu is a low molecular mass protein with a single transmembrane domain that inserts into the ER. Vpu binds to the cellular CD4 protein in the ER and recruits the human F-box protein β TrCP, targeting CD4 for degradation via the ubiquitin-proteasome pathway (Willey, Maldarelli *et al.* 1992; Bour, Geleziunas *et al.* 1995; Schubert, Anton *et al.* 1998; Belaidouni, Marchal *et al.* 2007; Malim and Emerman 2008; Nomaguchi, Fujita *et al.* 2008). The resulting reduction in cell surface CD4 promotes HIV-1 infection by preventing re-infection, facilitating virus release by avoiding Env-CD4 interactions during their trafficking to the cell surface, and minimising adaptive immune responses (Lanzavecchia, Roosnek *et al.* 1988; Willey, Maldarelli *et al.* 1992; Arganaraz, Schindler *et al.* 2003).

1.8 Substrate recognition

This study will predominantly focus on the substrate recognition aspect of the ERAD mechanism. The ER trafficks a diverse range of proteins that must be precisely folded to their correct 3-dimensional shape and, in many cases, assembled into oligomeric complexes. Since all proteins entering the secretory pathway have the potential to become misfolded, the ERAD substrate recognition machinery must be able to identify a vast assortment of substrates, ranging from large and complex proteins such as CFTR, to relatively small soluble proteins like transthyretin (TTR) (Sorgjerd, Ghafouri *et al.* 2006). In addition, ERAD must correctly distinguish within the concentrated protein environment, non-native states from native proteins and folding intermediates. Exposure of hydrophobic regions or structural elements typically buried within a natively folded protein, unpaired cysteines or the exhibition of partially trimmed *N*-linked oligosaccharides are some of the ways in which misfolded proteins distinguish themselves, and thus represent facets that may be exploited by ERAD chaperones in the identification of misfolded proteins (Ellgaard, Molinari *et al.* 1999; Ellgaard and Helenius 2003; Hegde and Ploegh 2010). However, these features are also present at intermediate folding states, making the ability to differentiate between the two both complex and of the utmost importance.

1.8.1 The role of *N*-glycans in recognition

As introduced in section 1.3.4, the majority of nascent polypeptides entering the ER are modified through the addition of *N*-linked oligosaccharides (Figure 1.4). Recognition and subsequent processing through the CNX/CRT cycle results in either: 1) a correctly folding protein competent for packaging into COPII vesicles and subsequent progression through the secretory pathway; or 2) a terminally misfolded glycoprotein that is removed from the CNX/CRT cycle through the action of α -mannosidase I upon the terminal mannose of the A branch (Figure 1.5). Trimming of the A-branch mannose is crucial for protein disposal, with pharmacological inhibition (with 1-deoxymannojirimycin and kifunensine) of the α 1,2-mannosidases shown to prevent glycoprotein degradation (Su, Stoller *et al.* 1993; Marcus and Perlmutter 2000; Tokunaga, Brostrom *et al.* 2000; Ruddock and Molinari 2006). The catalytic action of α -mannosidase I is relatively slow in comparison to UGGT and glucosidase II, acting as a “molecular timer” permitting proteins a number of folding attempts before condemnation to ERAD (Helenius 1994; Cabral, Liu *et al.* 2001; Lederkremer and Glickman 2005). This mechanism allows the selective elimination of proteins that have failed to mature properly even after extensive folding attempts, but also, facilitates the wide array of folding kinetics presented by the large range of secretory proteins transiting the ER.

Three ER degradation-enhancing α -mannosidase-like proteins (EDEMs) have been identified in mammals and are thought to participate in the mannose trimming of ERAD substrates. EDEM1 was first discovered as a mammalian

orthologue to the yeast Htm1p protein that contained a mannosidase-like domain, but was believed to lack mannosidase activity (Hosokawa, Wada *et al.* 2001). The overexpression of EDEM1 accelerates the turnover of glycosylated ERAD substrates, whereas its knock-down stabilises them (Hosokawa, Tremblay *et al.* 2010; Ron, Shenkman *et al.* 2011; Shenkman, Groisman *et al.* 2013; Ninagawa, Okada *et al.* 2014). However, mutations to the EDEM1 mannosidase-like domain retain their ability to accelerate ERAD (Olivari, Cali *et al.* 2006; Hosomi, Tanabe *et al.* 2010; Ninagawa, Okada *et al.* 2014), suggesting a function independent of its α 1,2-mannosidase activity, perhaps as a lectin for substrate delivery (Cormier, Tamura *et al.* 2009). Concurrently, EDEM1 has been demonstrated to bind SEL1L, the HRD1 ubiquitin ligase adaptor protein (Saeed, Suzuki *et al.* 2011; Bernasconi, Galli *et al.* 2012). EDEM2 shares sequence similarity with Class I mannosidases similar to EDEM1, and has been shown to interact with the misfolded AT variant NHK, with its overexpression accelerating ERAD (Mast, Diekman *et al.* 2005). Until very recently, EDEM2 was considered to lack enzymatic activity. However, results from the Mori lab suggest that it catalyses the first mannose trimming step from M9 to M8B. Further trimming catalysed by EDEM3, and to a lesser extent EDEM1, results in the generation of an “oligosaccharide degradation signal” (GlcNAc₂Man₅-Man₇) that is recognised by specialised lectins OS-9 and XTP3-B (Hirao, Natsuka *et al.* 2006; Ninagawa, Okada *et al.* 2014). Intriguingly, it was recently shown that EDEM1 and EDEM2 bind ricin A chains, but bind with reduced efficiency to mutants of reduced hydrophobicity (Sokolowska, Walchli *et al.* 2011; Slominska-

Wojewodzka, Pawlik *et al.* 2014), suggesting the possibility of bipartite recognition of not just sugar structure but also protein structure. Concomitantly, the Chen lab demonstrated that EDEM2 was required for ERAD of both glycosylated and non-glycosylated SHH (Sonic Hedgehog), while EDEM3 was only necessary for glycosylated SHH and EDEM1 was dispensable for both (Tang, Huang *et al.* 2014).

The importance of *N*-glycosylation to substrate recognition is highlighted by treatment of cells with tunicamycin, an inhibitor of *N*-glycosylation, results in the accumulation of misfolded proteins in the ER and UPR induction (Okuda-Shimizu and Hendershot 2007). Furthermore, glycoprotein degradation is prevented when processing of *N*-glycans is blocked through the inhibition of α 1,2-mannosidases with kifunensine (Su, Stoller *et al.* 1993). Studies in *S. cerevisiae* have revealed that the ERAD model substrate CPY* (the G255R mutant of the *N*-glycosylated vacuolar protease, carboxypeptidase Y) does not correctly fold, it is retained in the ER and rapidly degraded via ERAD (Finger, Knop *et al.* 1993). However, a non-glycosylated version of CPY* is degraded with slower kinetics (Knop, Hauser *et al.* 1996; Gauss, Jarosch *et al.* 2006; Benitez, Stolz *et al.* 2011).

1.8.2 Yos9p

Yos9p was identified in a *Saccharomyces cerevisiae* genetic screen to identify components that participate in glycoprotein turnover (Buschhorn, Kostova *et al.* 2004). It contains both an N-terminal signal sequence and a C-

terminal HDEL peptide that deliver and retain Yos9p in the ER (Buschhorn, Kostova *et al.* 2004; Bhamidipati, Denic *et al.* 2005; Szathmary, Biemann *et al.* 2005). Yos9p binds to the misfolded variant of carboxypeptidase Y, CPY* (a classical ERAD substrate) but does not interact with wild type CPY (Bhamidipati, Denic *et al.* 2005). This substrate recognition is thought to be a consequence of the presence of trimmed glycans on the surface of the polypeptide, which are recognised through the Mannose-6-phosphate receptor homology (MRH) domain of Yos9p, a domain that shares significant homology with a region of the mannose-6-phosphate receptor (MPR) (Munro 2001; Bhamidipati, Denic *et al.* 2005; Kim, Spear *et al.* 2005). MPRs are well characterised as receptors that sort lysosomal proteins through the recognition of *N*-glycans (Dahms and Kornfeld 1989; Ghosh, Dahms *et al.* 2003). Indeed, mutation of the MRH domain of Yos9p was shown to suppress CPY* turnover (Bhamidipati, Denic *et al.* 2005). However, as Yos9p has also been shown to associate with non-glycosylated proteins, it appears to possess both carbohydrate and protein-protein selection processes through a bipartite mechanism of recognition (Bhamidipati, Denic *et al.* 2005; Denic, Quan *et al.* 2006). Additionally, Yos9p binds to the ER-resident glycoprotein Hrd3p (Carvalho, Goder *et al.* 2006; Denic, Quan *et al.* 2006; Gauss, Jarosch *et al.* 2006), which forms a complex that stabilises the membrane-embedded ubiquitin ligase Hrd1p/Der3p (Gardner, Swarbrick *et al.* 2000). These interactions reflect a dual nature for Yos9 to bind both ERAD substrates and the ubiquitin ligase machinery.

1.8.3 Mammalian orthologues of Yos9p: OS-9 & XTP3-B

The mammalian orthologues of Yos9p, OS-9 and XTP3-B/Erlectin, share approximately 15 % identity with Yos9p and approximately 23 % identity with each other (Christianson, Shaler *et al.* 2008). Both OS-9 and XTP3-B are upregulated by the unfolded protein response (UPR) (described in detail in section 1.12.1), specifically through Ire1/Xbp-1 activation (Bernasconi, Pertel *et al.* 2008; Hosokawa, Wada *et al.* 2008). Similar to Yos9p, both contain a region (or regions in the case of XTP3-B) with homology to MPR, namely MRH, which have been implicated in their ability to identify and bind to misfolded substrates in a glycan-dependent manner.

XTP3-B is a soluble ER protein, with two MRH domains and two spliced variants (Hosokawa, Wada *et al.* 2008). The shorter variant has a small segment of the linker that connects the two MRH domains omitted. The C-terminal MRH domain appears to be involved in substrate recognition as it is required for glycan-dependent binding to NHK (Yamaguchi, Hu *et al.* 2010). Over-expression of XTP3-B was demonstrated to retard the degradation of NHK, and also the non-glycosylated NHK- QQQ (Hosokawa, Wada *et al.* 2008). However, knockdown of XTP3-B does not affect the degradation of NHK, suggestive of redundant cellular factors being involved in its turnover (Christianson, Shaler *et al.* 2008; Hosokawa, Wada *et al.* 2008). Similar to Yos9p, association of XTP3-B with a ubiquitin ligase complex, Hrd1-SEL1L ubiquitin ligase complex, has been observed (Christianson, Shaler *et al.* 2008; Hosokawa, Wada *et al.* 2008). This interaction has been demonstrated to occur through the SEL1L adaptor protein

and not directly through Hrd1, but of particular note is the observation that the long form, and not the short form, of XTP3-B is capable of association with SEL1L (Christianson, Shaler *et al.* 2008; Hosokawa, Wada *et al.* 2008), suggesting that differential splicing of XTP3-B may produce differentially functioning isoforms.

OS-9, located on chromosome 12q13 q15 and containing 15 exons spanning 30.4 kB, was identified as a gene frequently amplified and over-expressed in human sarcoma (Su, Trent *et al.* 1994). Four OS-9 isoforms result from alternative gene splicing (OS-9.1-4) in which either entire (ex. 13, 55 a. a.) or partial exons (ex. 11b, 15 a. a.) are excised to yield putative protein isoforms of unique lengths (667, 612, 597 and 652 a. a., respectively), with all isoforms conserving essential features such as the signal sequence that directs OS-9 to the ER and MRH domain (Kimura, Nakazawa *et al.* 1997). A number of earlier studies localised OS-9 to the cytosol (Litovchick, Friedmann *et al.* 2002; Baek, Mahon *et al.* 2005; Wang, Fu *et al.* 2007), which suggested that OS-9 was not the functional orthologue of Yos9p. However, Christianson *et al.* demonstrated that OS-9 (and XTP3-B) was in fact an ER-resident lectin (Christianson, Shaler *et al.* 2008). Degradation of several misfolded glycoproteins is partially mitigated by OS-9 knockdown (Bernasconi, Pertel *et al.* 2008; Christianson, Shaler *et al.* 2008; Hosokawa, Kamiya *et al.* 2009) but interestingly not all, indicating that functional redundancy with XTP3-B may be present in the system (Christianson, Shaler *et al.* 2008; Hosokawa, Kamiya *et al.* 2009; Bernasconi, Galli *et al.* 2010; Fujimori, Kamiya *et al.* 2013). Transcription of OS-9, and in particular OS-9.2, is

upregulated by ER stress as part of the UPR (Bernasconi, Pertel *et al.* 2008; Alcock and Swanton 2009; Christianson, Olzmann *et al.* 2012), likely through 3 ERSE sites in the 5' UTR (Eletto, Maganty *et al.* 2012). Similar to Yos9p and XTP3-B, OS-9 occupies a position between the recognition of misfolded substrates and their ubiquitination through interaction with the Hrd1-SEL1L ubiquitin ligase (Christianson, Shaler *et al.* 2008).

Analogous to Yos9p, the ability of OS-9 to recognise and interact with misfolded glycoproteins is thought to be supplied by its MRH domain. As mentioned, the best characterised function of the MPRs is their ability to direct the delivery of ~60 different newly synthesised soluble lysosomal enzymes bearing mannose-6-phosphate on their *N*-linked oligosaccharides to the lysosome (Dahms and Kornfeld 1989; Ghosh, Dahms *et al.* 2003). Therefore, OS-9 (and XTP3-B) MRH may function in a similar manner. The recombinant human OS-9 MRH domain was found to associate preferentially with glycans missing C-arm mannose residues (Figure 1.4). Indeed, the MRH domain of OS-9 (and Yos9) is sufficient to selectively bind ER-derived, high mannose oligosaccharide forms with exposed $\alpha_{1,6}$ mannose moieties. Using frontal affinity chromatography, both OS-9 (Hosokawa, Kamiya *et al.* 2009) and Yos9p (Quan, Kamiya *et al.* 2008) MRH domains were shown to specifically recognise *N*-linked glycans trimmed of terminal branch C mannoses. Further trimming of A and B branch terminal mannoses to Man7 and below (to Man5) also bound OS-9, while glucosylated forms bound poorly. A co-crystal structure of OS-9 MRH with pentamannose identified tandem tryptophan residues in the MRH domain (WW)

that are essential for coordinating the recognition of exposed $\alpha_{1,6}$ mannose moieties observed on ERAD substrates (Satoh, Chen *et al.* 2010). The $\alpha_{1,6}$ mannose (<Man8) forms recognised arise from the activity of the ER-resident mannosidases EDEM1-3 and ERMnl.

It is attractive to visualise the binding of OS-9 to substrates through its MRH domain, however, this role of the MRH domain has been disputed. Christianson *et al.* reported that the MRH domain is not required for the interaction and recognition of the ERAD substrate, but rather for interaction with SEL1L (Christianson, Shaler *et al.* 2008). More recently, this apparent discrepancy was resolved when OS-9 was shown to be capable of binding both SEL1L and ERAD substrate *N*-glycans, through a tryptophan/tryptophan (WW) motif present in the MRH domain of OS-9 (Satoh, Chen *et al.* 2010). In this model, simultaneous binding of OS-9 to both SEL1L and ERAD substrates may be possible if OS-9 is homotrimeric, resulting in an oligomer with multiple MRH domains available for binding (Satoh, Chen *et al.* 2010).

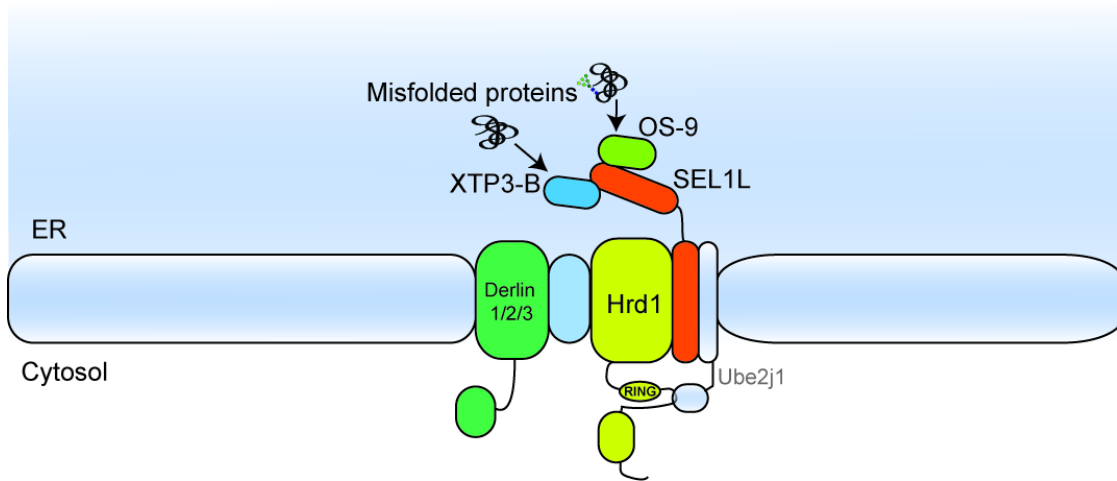


Figure 1.7: Components of the Hrd1/SEL1L ubiquitin ligase complex

Recognition of misfolded proteins by the lectins OS-9 and XTP3-B results in their targeting to the Hrd1 ubiquitin ligase complex. OS-9 and XTP3-B interact with Hrd1 through its adapter protein SEL1L.

1.9 Substrate dislocation/retrotranslocation

Once identified, ERAD substrates are destined for degradation by the 26S proteasome. However, initial differential compartmentalisation of the ERAD substrate and the 26S proteasome, in the ER and cytosol respectively, presents a substantial physical barrier in the form of a lipid bilayer, which the substrate is required to traverse. The identity of the retrotranslocation channel(s)/dislocon(s) remains controversial and are, as of yet, undefined. However, several candidates have been suggested, including the Derlin family of proteins (Derlin 1-3) (Lilley and Ploegh 2004; Ye, Shibata *et al.* 2004), signal peptide peptidase (Loureiro, Lilley *et al.* 2006), the Sec61 translocon complex (Plemper, Bohmler *et al.* 1997; Zhou and Schekman 1999; Scott and Schekman 2008) and the E3 ubiquitin ligase Hrd1 (Carvalho, Stanley *et al.* 2010).

As previously discussed, Sec61 provides a portal of entry to the ER for nascent polypeptides through the formation of an aqueous, ion-conducting channel (Wirth, Jung *et al.* 2003). As such it is an attractive translocon candidate. However, it is mechanistically complicated by the need to delineate the protein import and export processes. There is a wealth of evidence in favour of this model: yeast strains possessing mutations in Sec61p (the yeast orthologue of Sec61) have been described that support the efficient translocation of proteins out of the ER (Pilon, Schekman *et al.* 1997; Plemper, Bohmler *et al.* 1997; Scott and Schekman 2008; Wheeler and Gekakis 2012). The inactivation or depletion of Sec61 prevents dislocation of the A1 subunit of cholera toxin into the cytosol (Schmitz, Herrgen *et al.* 2000), and a number of ERAD substrates have been observed to associate with Sec61 during late stages of quality control (Wiertz, Tortorella *et al.* 1996; Pilon, Schekman *et al.* 1997; Plemper, Bohmler *et al.* 1997). However, crystallographic studies of the archaeal homologue (SecY) suggest too small a pore size (~ 3 Å) to accommodate folded protein structures, which may reach in excess of 30 Å, thus making the Sec61 mode of operation conceptually problematic (Van den Berg, Clemons *et al.* 2004).

Human cytomegalovirus (US2/US11)-induced dislocation of class I heavy chains has been reported to depend on the Derlin proteins, rather than Sec61 (Lilley and Ploegh 2004; Ye, Shibata *et al.* 2004). The Derlin family, composed of Derlin-1, Derlin-2 and Derlin-3, are multi-spanning membrane proteins. Their overexpression has been shown to accelerate, and conversely their knockdown

shown to retard, the degradation of some ERAD substrates (Lilley and Ploegh 2004; Ye, Shibata *et al.* 2004; Oda, Okada *et al.* 2006). They interact with a variety of ERAD factors in and across the ER membrane, including luminal chaperones, cytosolic p97 and membrane-associated E3 ubiquitin ligases (Lilley and Ploegh 2004; Ye, Shibata *et al.* 2004; Katiyar, Joshi *et al.* 2005; Schulze, Standera *et al.* 2005; Ye 2005; Wang, Ye *et al.* 2006; Younger, Chen *et al.* 2006; Wang, Hua *et al.* 2008). Additionally, the Derlins possess the ability to form homo-oligomers and hetero-oligomers with one another, suggesting the potential formation of a channel that may act as a retrotranslocon (Lilley and Ploegh 2004; Lilley and Ploegh 2005; Ye 2005). Recently, it has been suggested that Derlin-1, in its capacity as a rhomboid pseudoprotease (shares homology with the rhomboid family but is catalytically inactive), may play a role in substrate dislocation (Greenblatt, Olzmann *et al.* 2012). The rhomboids are a well-conserved family of intramembrane serine proteases that are unrelated to classical serine proteases. Their active site is buried within the plane of the membrane, and they cleave substrates in or near transmembrane domains (Urban 2006; Freeman 2008; Urban and Dickey 2011). Derlin-1's rhomboid-homologous domain is necessary for dislocation of the model ERAD substrate NHK (Greenblatt, Olzmann *et al.* 2011). Additionally, evolutionarily conserved rhomboid protease RHBDL4 performs cleavage of membrane proteins in an ERAD-dependent manner (Fleig, Bergbold *et al.* 2012; Greenblatt, Olzmann *et al.* 2012).

The third and final candidates are the E3 ubiquitin ligases. In mammalian cells, the family of E3 ligases involved in ERAD has expanded dramatically in recent years, with currently 26 family members identified (Neutzner, Neutzner *et al.* 2011). In yeast, the two principal ERAD E3 ligases, Hrd1 (hydroxymethylglutaryl reductase degradation protein) and Doa10, both have large multi-spanning membrane domains (Hrd1: 6 spans, Doa10: 14 spans) (Sato, Schulz *et al.* 2009; Kreft and Hochstrasser 2011). The Hrd1 transmembrane domain has a high proportion of hydrophilic residues, as might be expected to line a peptide-transporting pore (Sato, Schulz *et al.* 2009) and indeed, mutating selected residues of the yeast ubiquitin ligase Hrd1p to hydrophobic residues specifically impairs the degradation of membrane-bound substrates (Sato, Schulz *et al.* 2009). It has also been demonstrated that excess Hrd1p is able to carry out ERAD without the need of other Hrd1p complex components (Gardner, Swarbrick *et al.* 2000; Carvalho, Stanley *et al.* 2010) and that its oligomerisation, induced by Usa1, is required for the degradation of luminal ERAD substrates but is dispensable for the targeting of soluble substrates (Horn, Hanna *et al.* 2009; Carvalho, Stanley *et al.* 2010).

1.10 Substrate ubiquitination

Ubiquitin is a 76 a. a. polypeptide conjugated to proteins through a multistep enzymatic cascade (Glickman and Ciechanover 2002) (Figure 1.8). The conjugation of ubiquitin to substrates begins with the ATP-dependent activation of ubiquitin by the formation of a thiol-ester linkage between its C-

terminal glycine and an active site cysteine residue present within the ubiquitin-activating enzyme (E1) (Figure 1.8). Ubiquitin is then transferred to a cysteine residue of a ubiquitin-conjugating enzyme (E2), and subsequently linked by an isopeptide bond to the ϵ -NH₂ group of a lysine residue in the substrate, through the action of a substrate-specific ubiquitin ligase (E3) (Pickart 2001). In a number of cases, ubiquitin is conjugated to the α -amino group of the target substrate (Hershko, Heller *et al.* 1984; Ciechanover and Ben-Saadon 2004), or may be conjugated to threonine, serine or cysteine residues (Cadwell and Coscoy 2005; Carvalho, Pinto *et al.* 2007; Wang, Herr *et al.* 2007; Shimizu, Okuda-Shimizu *et al.* 2010). Only two E1 enzymes have been discovered with individual E2s dictating the specific biological function of ubiquitin, with the specificity of the E2/E3 interaction limiting the final destination of the ubiquitin carried by a given E2 (McGrath, Jentsch *et al.* 1991). So far, three classes of E3 ligases have been identified: RING (Really Interesting New Gene), U-box and HECT (Homologous with E6-associated protein C-Terminus) domain ligases.

Ubiquitin can itself serve as an acceptor for additional ubiquitin molecules leading to the formation of polyubiquitin chains (Hoppe 2005). Chain formation can occur through seven lysine residues (at positions K6, K11, K27, K29, K33, K48, and K63), or the N-terminus of ubiquitin (M1), leading to the assembly of multiple chains with distinct topology (Komander and Rape 2012; Ye, Blaser *et al.* 2012). Quantitative analysis has determined K48 and K63 linkages to be the most abundant (Dammer, Na *et al.* 2011; Ziv, Matiuhin *et al.* 2011), with a polyubiquitin chain of four K48-linked ubiquitin molecules being sufficient for

targeting to the 26S proteasome (Chau, Tobias *et al.* 1989; Thrower, Hoffman *et al.* 2000), while K63-linked chains are involved in non-proteolytic events such as DNA damage responses and immune signalling (Komander and Rape 2012). Interestingly, a study in yeast showed enrichment of K11-linked chains upon ER stress (Xu, Duong *et al.* 2009). In addition, the roles of branched and mixed chain polyubiquitin are currently under investigation, with a recent study reporting that branched K11/K48 chains result in improved proteasomal substrate recognition (Meyer and Rape 2014).

Prior to degradation, ubiquitinated ERAD substrates must first be extracted from the ER membrane. While the polyubiquitin chain itself prevents backwards movement through the retrotranslocon, the AAA (ATPase associated with various cellular activities) ATPase VCP/p97 is believed to be the sole factor that actively ratchets proteins into the cytosol (Jarosch, Taxis *et al.* 2002; Rabinovich, Kerem *et al.* 2002). VCP/p97 is required for the dislocation of most luminal and membrane proteins in mammals (Ye, Meyer *et al.* 2001; Ballar, Pabuccuoglu *et al.* 2011). The enzyme contains two ATPase domains, with the first of these assembling VCP/p97 into a barrel-like hexamer with a central pore (DeLaBarre, Christianson *et al.* 2006), with a considerable fraction of this protein anchored to the ER membrane via the adapter protein VIMP (VCP interacting membrane protein) (Ye, Shibata *et al.* 2004). ATP-hydrolysis provides the energy to allow a change in VCP/p97 conformation (Zhang, Wang *et al.* 2000; Rouiller, DeLaBarre *et al.* 2002), resulting in the mechanical force required to extract substrates from the membrane. Recently, the importance of

deubiquitinating enzymes (DUBs), YOD1 and ataxin-3, for membrane extraction has been highlighted (Ernst, Mueller *et al.* 2009). With the Bag6 complex, previously introduced as a mediator of TA-protein insertion (Section 1.2) has been suggested to act in association with DUBs to facilitate proteasomal transport of dislocated ERAD substrates (Wang, Liu *et al.* 2011).

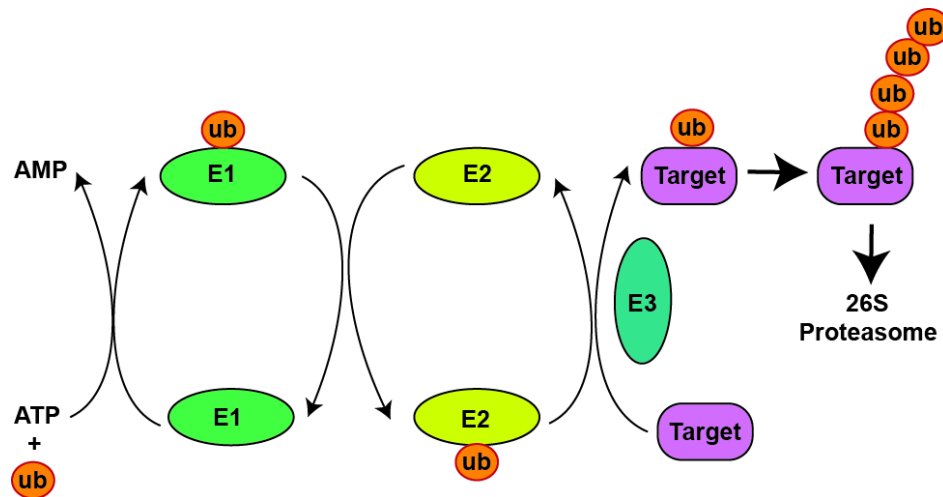


Figure 1.8 Conjugation of ubiquitin

Ubiquitin is first attached by a thioester bond to an ubiquitin-activating enzyme (E1) in an ATP-dependent manner. After transfer to an ubiquitin-conjugating enzyme (E2), the E2 binds to the substrate-specific ubiquitin ligase (E3) and transfers the activated ubiquitin moiety directly to the substrate that is also bound to the E3. Proteins carrying long chains of ubiquitin are delivered to the 26S proteasome for degradation.

1.10.1 The Hrd1-SEL1L ubiquitin ligase complex

To date, 26 ubiquitin ligases have been identified that localise to the ER membrane (Neutzner, Neutzner *et al.* 2011), and it is hypothesised that both distinct and overlapping pathways may be required to manage the large array of substrates trafficking through ERAD. However, the substrates and functions of

these ligases have yet to be clarified, with the two RING finger E3 ligases Hrd1 and gp78 (both orthologues of yeast Hrd1p) currently being the most well-defined of the family. Hrd1 (Synoviolin) is a multi-spanning ER protein with a RING domain at its C-terminus, which forms a complex with high similarity to its yeast counterpart (Figure 1.7) containing SEL1L (suppressor/enhancer of lin-12-like protein, the mammalian homologue of yeast Hrd3p) (Mueller, Klemm *et al.* 2008), HERP (homocysteine-inducible ER stress protein, Usa1 in yeast) (Lilley and Ploegh 2005; Schulze, Standera *et al.* 2005), Derlin 1-3 (Lilley and Ploegh 2004, Ye, Shibata *et al.* 2004) and the orthologues of Yos9p, OS-9 and XTP3-B (Christianson, Shaler *et al.* 2008; Hosokawa, Wada *et al.* 2008). The SEL1L-interacting partners AUP1 (ancient ubiquitous protein 1), UBXD8 and Ube2j1 were identified by the Ploegh lab (Mueller, Klemm *et al.* 2008) and confirmed by an integrated proteomics screen by the Kopito lab, which additionally identified FAM8A1 as a component of the Hrd1-SEL1 complex (Christianson, Olzmann *et al.* 2012).

SEL1L is a multiply glycosylated type I ER membrane protein, which functions as the adapter protein of membrane-embedded Hrd1. As mentioned previously, substrate ubiquitination is a key step in ERAD and subsequently both SEL1L and Hrd1 are required for the degradation of a variety of ERAD substrates (Kikkert, Doolman *et al.* 2004; Mueller, Lilley *et al.* 2006; Omura, Kaneko *et al.* 2006; Okuda-Shimizu and Hendershot 2007; Cattaneo, Otsu *et al.* 2008; Christianson, Shaler *et al.* 2008; Hosokawa, Wada *et al.* 2008; Bernasconi and Molinari 2010). Consequently, with the ability to interact with both misfolded

proteins and the Hrd1/SEL1L ubiquitin ligase complex, OS-9 potentially occupies a pivotal decision making role in protein fate; passing judgement on a protein's folding state and shuttling those condemned to downstream dislocation and the ubiquitination machinery. Interestingly, OS-9.2 but not OS-9.1 was shown to preferentially associate with overexpressed Hrd1-SEL1L (Iida, Fujimori *et al.* 2011), suggesting that individual OS-9 isoforms may function differentially.

1.11 Substrate degradation

Proteasomes are found within the cytoplasm and nucleus of all eukaryotic cells, and function to degrade intercellular proteins that have been modified with polyubiquitin chains. The eukaryotic 26S proteasome has a total molecular mass of approximately 1.2 MDa, and consists of a core of 20S particles and one or two 19S regulatory particles (Figure 1.9) (Voges, Zwickl *et al.* 1999; Goldberg 2003). The 19S cap is functionally divided into two parts, the base and the lid, which is involved in the recognition, binding and unfolding of ubiquitinated proteins, as well as regulating the opening of the gate of the 20S core particle.

The 20S core particle is a barrel-shaped structure composed of four stacked rings, which are highly conserved among all eukaryotes (Baumeister, Dahlmann *et al.* 1988; Lasker, Forster *et al.* 2012). The two outer rings each consist of seven unique α -subunits, and the two inner rings are each composed of seven unique β -subunits. The resultant structure is a hollow cylinder lined with the active sites of the catalytically active β 1 (peptidyl-glutamyl-peptide

cleaving activity), $\beta 2$ (trypsin-like cleaving activity) and $\beta 5$ (chymotrypsin-like cleaving activity) subunits (Groll, Ditzel *et al.* 1997). The combined activities of the three different active sites ensure high efficiency in protein degradation, digesting a broad variety of substrates into small oligopeptides, varying between 3 and 23 a. a. in length (Heinemeyer, Kleinschmidt *et al.* 1991; Nussbaum, Dick *et al.* 1998; Kisselev, Akopian *et al.* 1999; Borissenko and Groll 2007). Oligopeptides are subsequently released into the cytosol, where they are further cut into a. a. by abundant cytosolic endopeptidases and amino-peptidases, rendering them ready to be used in the synthesis of new proteins (Saric, Graef *et al.* 2004). Additionally, oligopeptides generated via the proteasomal degradation of “foreign” proteins may be presented on the cell surface by MHC-1 to T-cells, triggering an immune response (Wang and Maldonado 2006).

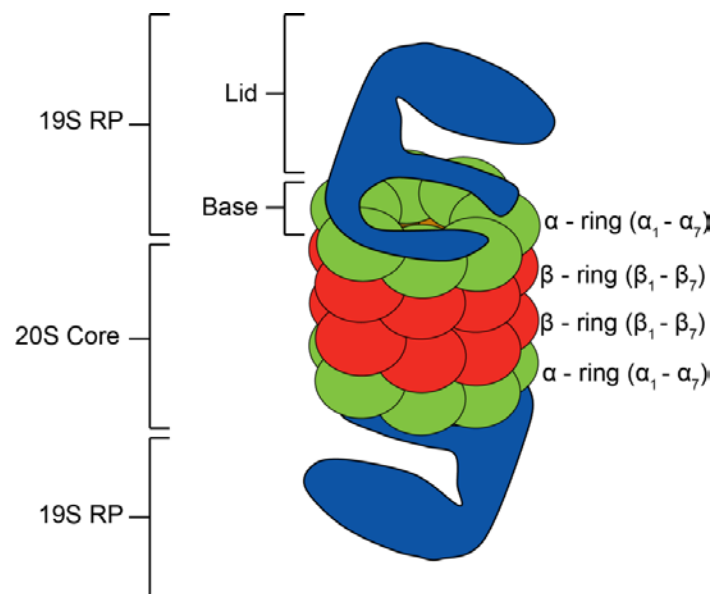


Figure 1.9: Structure of the 26S proteasome

The 26S proteasome consists of a 28-subunit catalytic core, composed of two outer and two inner heptameric rings stacked to form a hollow cylindrical structure in which proteolysis occurs. Each of the two inner rings of the 20S core is composed of seven different β -subunits, which host the three different catalytic sites on the inner surface. The outer rings comprise seven different α -subunits, none of which has catalytic activity, but which serve as an anchor for the multi-subunit ATPase-containing-19S regulator (700 kDa) lid.

1.12 ER Stress

Even an acute increase in the translation of secretory proteins may pose a major threat to the cell, due to the potential build-up of misfolded proteins. The situation has the potential to escalate if chaperone capacity is overwhelmed or perturbations in the ER environment occur, such as alterations in redox state, reduced calcium levels or a failure to post-translationally modify secretory proteins, compromising the cell's overall ability to produce properly folded proteins. Such perturbations can be the result of hypoxia, hypoglycaemia, acidosis, calcium imbalance and other factors, and can be pharmacologically induced by the drugs tunicamycin, thapsigargin or dithiothreitol, which inhibit protein glycosylation, disrupt ER Ca^{2+} levels, or perturbs ER redox state, respectively. In order to safeguard cellular homeostasis, a multi-faceted mechanism, the unfolded protein response (UPR) is employed to disperse the threat.

1.12.1 The unfolded protein response (UPR)

The disturbance of protein folding is readily sensed and interpreted by chaperones as ER stress, which activates signal transduction pathways jointly termed the unfolded protein response (UPR). The UPR attempts to reduce this burden through a multifaceted approach, inhibiting general translation but up-regulating the transcription and translation of specific ER chaperones, as well as facilitating the clearance of misfolded proteins from the lumen (Meusser, Hirsch

et al. 2005; Oyadomari, Yun *et al.* 2006). However, if the burden proves too great and misfolded proteins continue to accumulate, activation of the UPR may lead to programmed cell death (apoptosis). As illustrated in Figure 1.10, the three main branches of UPR are inositol-requiring protein-1(IRE- α), activating transcription factor-6 (ATF-6) and protein kinase-like ER kinase (PERK). The three active forms of these UPR proteins initiate a cascade of events leading to; attenuation of global mRNA translation so that the influx of proteins into the ER is halted temporary, an increase in the transcription of chaperone proteins to facilitate the increasing demand for protein folding, and an increase in the induction of the ERAD proteins. Finally, if all fails, pro-apoptotic pathways are activated.

1.12.2 The IRE-1 α pathway

Inositol-requiring enzyme 1 (IRE1), a type I transmembrane protein with serine/threonine kinase activity, was first identified as the ER stress sensor in yeast (Cox, Shamu *et al.* 1993; Mori, Ma *et al.* 1993). It monitors ER homeostasis via a luminal domain bound by BiP. Dissociation of BiP (when out-competed by accumulating misfolded proteins) triggers IRE-1 α oligomerisation and the activation of an endonuclease domain (Walter and Ron 2011). IRE1 subsequently initiates a diverse set of downstream signalling cascades through either modifications via Regulated IRE1 Dependent Decay (RIDD) of multiple substrates (Hollien and Weissman 2006; So, Hur *et al.* 2012), or unconventional splicing of the bZIP-family transcription factor, X-box-binding protein 1 (XBP1)

(Tirasophon, Welihinda *et al.* 1998; Marciniak and Ron 2006; Ron and Walter 2007; Yoshida 2007; Hummasti and Hotamisligil 2010; Hetz, Martinon *et al.* 2011). Spliced XBP1 translocates to the nucleus and binds the ER stress-response element (ERSE) promoter in target genes, up-regulating the expression of: i) ER chaperone proteins; ii) ERAD proteins; iii) lipid synthesis; proteins and iv) ER biogenesis proteins (Yoshida, Haze *et al.* 1998; Lee, Cho *et al.* 2003; Acosta-Alvear, Zhou *et al.* 2007; Yamamoto, Sato *et al.* 2007; Zhou, Lee *et al.* 2011). In addition, phosphorylated IRE1 activates JUN N-terminal kinase (JNK) through TRAF2 (tumour necrosis factor (TNF) receptor-associated factor 2) (Urano, Wang *et al.* 2000) and ASK1 (apoptosis signal-regulating kinase 1) (Nishitoh, Matsuzawa *et al.* 2002), promoting cell survival through the induction of autophagy (Ogata, Hino *et al.* 2006) or programmed cell death through the caspase-12-mediated apoptosis pathways (Yoneda, Imaizumi *et al.* 2001).

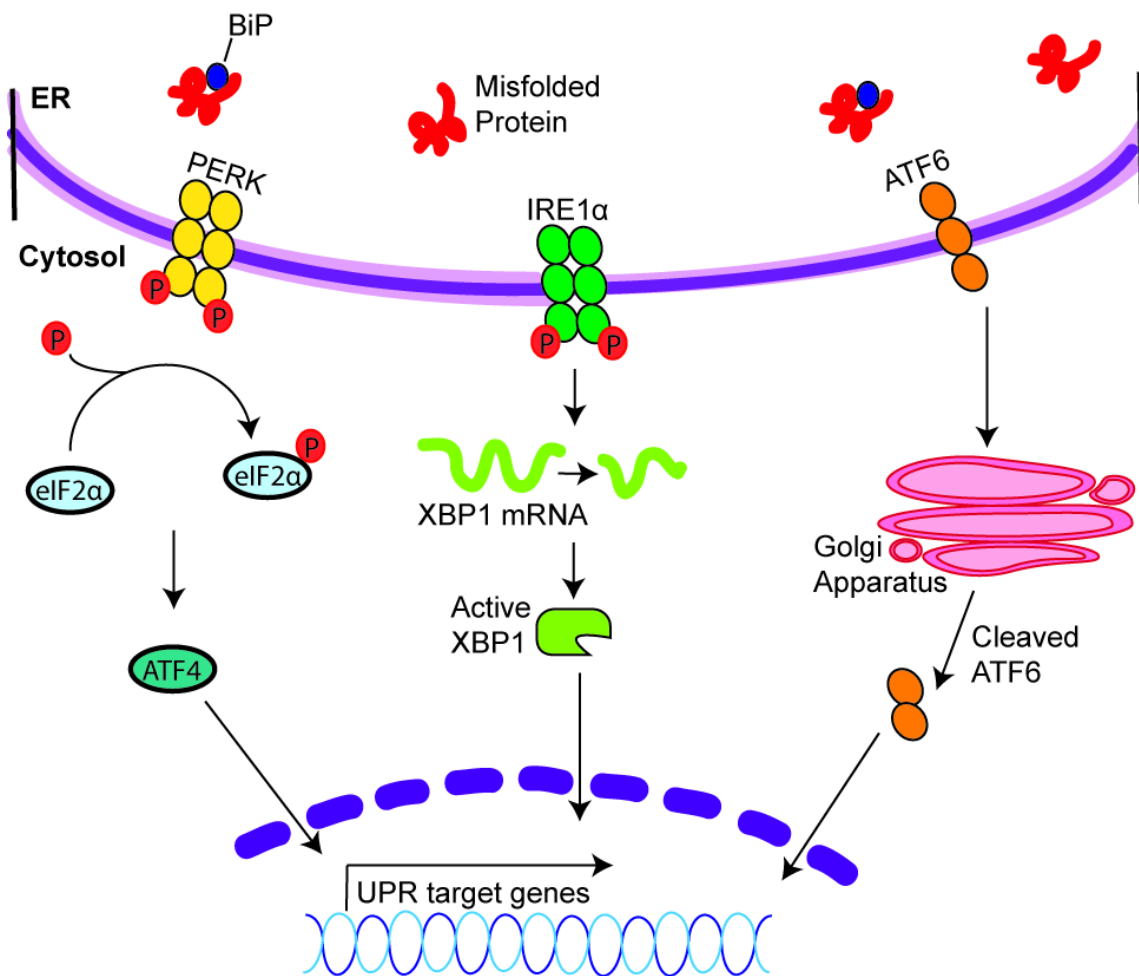


Figure 1.10: The unfolded protein response (UPR).

ER transmembrane proteins transduce signals to the cytosol and nucleus to restore protein-folding capacity through various pathways. 1) IRE1α RNase activity processes the mRNA encoding the transcription factor X-box binding protein 1 (XBP1). This leads to the expression of an active transcription factor (XBP1s) that up-regulates a subset of UPR target genes related to protein folding, ER-associated protein degradation (ERAD) and protein quality control. 2) Activation of PERK attenuates general protein synthesis through phosphorylation of the eukaryotic translation initiator factor 2α (eIF2α). EIF2α phosphorylation allows the selective translation of ATF-4 mRNA, which encodes a transcription factor that induces the expression of genes involved in antioxidant responses, a. a. metabolism, autophagy and apoptosis. 3) ATF-6 is localised at the ER in basal conditions and encodes a bZIP transcription factor in its cytosolic domain. Under the induction of ER stress, ATF6 translocates to the Golgi apparatus where it is processed by site 1 protease (S1P) and site 2 protease (S2P), releasing its cytosolic domain (ATF-6f). ATF-6f controls the up-regulation of select UPR target genes.

1.12.3 The PERK pathway

In mammalian cells, the initial response to ER stress is the reduction of ER protein load through transient global translation attenuation, mediated by the double-stranded RNA-activated protein kinase (PKR) – like ER kinase (PERK) signalling pathway. PERK's mechanism of activation is similar to that of IRE-1 α , as both possess a serine/threonine kinase domain extruding into the cytoplasm (Shi, Vatter *et al.* 1998; Harding, Zhang *et al.* 1999; Bertolotti, Zhang *et al.* 2000). Under normal conditions, PERK binds the chaperone protein BiP (GRP78). However, as unfolded proteins accumulate, BiP is sequestered and dissociates from PERK, resulting in its activation through oligomerisation and autophosphorylation (Bertolotti, Zhang *et al.* 2000; Liu, Schroder *et al.* 2000). Once activated, PERK phosphorylates the α -subunit of eukaryotic translational Initiation Factor 2 (eIF2 α) protein in the cytoplasm, inactivating eIF2 α and resulting in the attenuation of global protein translation, thus decreasing the protein folding load on ER chaperone proteins (Bastard, Prevost *et al.* 2006; Marciniak, Garcia-Bonilla *et al.* 2006; Ron and Walter 2007; Yoshida 2007, Kim, Kim *et al.* 2008; Hummasti and Hotamisligil 2010). Despite the blocking of translation; the transcription factor ATF-4 (activating transcription factor 4) is specifically unregulated (Harding, Zhang *et al.* 2000), the presence of upstream open reading frames (uORFs) in its 5'-untranslated region allowing escape from eIF2 α translation attenuation (Harding, Novoa *et al.* 2000; Vatter and Wek 2004) inducing expression of the pro-apoptotic factor CHOP (Fawcett, Martindale *et al.* 1999). PERK activity is negatively regulated by

dephosphorylation through an unknown mechanism (Bertolotti, Zhang *et al.* 2000), and binding of p58^{IPK}, a Hsp40 family member (Yan, Gale *et al.* 2002).

1.12.4 The ATF-6 pathway

Upon sensing ER stress, ATF-6 dissociates from BiP in a similar manner to the mechanism proposed for PERK and IRE1 (Chen, Shen *et al.* 2002; Shen, Chen *et al.* 2002). ATF-6 subsequently translocates to the Golgi apparatus for cleavage by resident proteins S1P (Site 1 Protease) and S2P (Site 2 Protease) (Shen, Chen *et al.* 2002; Nakanaka, Yoshida *et al.* 2004). Proteolytic processing results in the release of a 50 kDa cytosolic basic leucine zipper transcription factor, which translocates to the nucleus and regulates gene expression (Yoshida, Haze *et al.* 1998; Haze, Yoshida *et al.* 1999; Wang, Shen *et al.* 2000). ATF-6 pathway activation results in the up-regulation of several ER chaperone proteins including SEL1L (Kaneko, Yasui *et al.* 2007), BiP and Grp94 (Yamamoto, Yoshida *et al.* 2004; Yamamoto, Sato *et al.* 2007), XBP1 (Yoshida, Matsui *et al.* 2001), Herp (Kokame, Kato *et al.* 2001), CHOP (Ma, Brewer *et al.* 2002) and p58^{IPK} (van Huizen, Martindale *et al.* 2003). XBP-1 mRNA is also induced by ATF-6, and subsequently spliced by IRE during ER stress (Yoshida, Matsui *et al.* 2001).

1.13 ERAD in disease

Over 60 pathologies to date have been associated with ERAD (reviewed in (Guerriero and Brodsky 2012)). However, despite our ever expanding knowledge of the machinery and pathways involved, many questions still remain. The importance of ERAD is further underscored by the sheer range of diseases with which it is associated, including cancer, neurodegeneration, stroke, bipolar disorder, cardiac disease, diabetes and muscle degeneration.

In addition to loss-of-function disorders due to premature degradation of secretory or membrane proteins, preventing their deployment to distal compartments (as seen in CFTR), protein misfolding and aggregation are the basis of a number of pathological conditions including Alzheimer's (AD) and Parkinson's disease (PD) (reviewed in (Scheibel and Buchner 2006)), Huntington's disease (Qin and Gu 2004), cystic fibrosis (Kopito 1999; Sharma, Pampinella *et al.* 2004), α 1-antitrypsin deficiency (Lawless, Greene *et al.* 2004) and Creutzfeldt-Jakob disease (Aguzzi and Polymenidou 2004). All of these diseases are at least partly caused by a. a. changes that result in protein misfolding and/or degradation through the ERQC system. Failure or overwhelming of QC may result in unstable proteins being secreted, giving rise to amyloid-type formations in the extracellular environment. This abnormal accumulation, particularly in susceptible brain regions, can have fatal effects. The cell's capacity to handle aberrant proteins has been shown to diminish with age. Aged cells may also have a reduced degradative capacity (Shang, Gong *et al.* 1997; Ciechanover 2006). This could explain the late onset of

neurodegenerative diseases like PD or AD, where failures of protein quality control result in the formation of oligomers or amyloid inclusions of the associated proteins, α -synuclein in PD or A β and tau in AD.

As previously discussed in section 1.7.4, many viruses exploit the ERAD machinery for their maturation or immune evasion. In addition to HIV-1 and HCMV, polyomaviruses have been shown to utilise the unique ER environment to produce structural changes to their icosahedral capsids. Polyomaviruses are small, non-enveloped, DNA tumour viruses that infect a variety of animals (Norkin, Steinberg *et al.* 1985). Studies of JCV (JC virus), BKV (BK virus) and SV40 (simian virus 40) indicate that viral particles interact with PDI and ERp57 in the ER lumen to rearrange capsid proteins (Jiang, Abend *et al.* 2009; Rainey-Barger, Mkrtchian *et al.* 2009; Goodwin, Lipovsky *et al.* 2011; Inoue, Moore *et al.* 2011). The altered particles then appear to engage different retrotranslocons (dependent on either Derlin-1 or Derlin-2) to induce retrotranslocation to the cytosol, where the reduced calcium environment produces further capsid rearrangements (Schelhaas, Malmstrom *et al.* 2007; Tsai and Qian 2010). These particles then bind to the nuclear pore, where uncoating occurs to allow the passage of viral DNA into the nucleus. Coronaviruses (CoV), including SARS-CoV (Severe Acute Respiratory Syndrome CoV), are RNA viruses that have been recently shown to interact with components of ERAD. CoV replication occurs within membranous compartments that are enriched for EDEM1 and OS-9 (den Boon and Ahlquist 2010; Knoop, Swett-Tapia *et al.* 2010; Reggiori, Monastyrska *et al.* 2010). The intraluminal concentration of ERAD factors is

tightly regulated, and the importance of “ERAD tuning vesicles” or EDEMosomes in maintaining ER homeostasis has recently emerged (and will be discussed in more detail in Chapter 4 of this thesis) (Cali, Galli *et al.* 2008; Bernasconi and Molinari 2011). However, it has also recently transpired that the hijacking and/or induction of EDEMosome formation is key to the lifecycle of an ever increasing number of viruses, including Equine Arteritis Virus (Monastyrska, Ulasli *et al.* 2013) and Japanese Encephalitis Virus (JEV), a neurotropic flavivirus responsible for frequent epidemics of viral encephalitis in South East Asia, China and India (Sips, Wilschut *et al.* 2012; Sharma, Bhattacharyya *et al.* 2014).

A number of pathogenic bacteria co-opt components of the ERAD machinery for the mode of action of their toxins. Cholera toxin A1 from *Vibrio cholera* (Hazes and Read 1997; Tsai, Rodighiero *et al.* 2001; Rodighiero, Tsai *et al.* 2002; Bernardi, Williams *et al.* 2010; Williams, Inoue *et al.* 2013), Exotoxin A from *Pseudomonas aeruginosa* which results in respiratory/urinary tract infections (Koopmann, Albring *et al.* 2000; Ackerman, Giodini *et al.* 2006; Schauble, Cavalie *et al.* 2014), Pertussis toxin from *Bordetella pertussis* causing Whooping cough (Worthington and Carbonetti 2007), Ricin A chain from *Ricin communis* (Simpson, Roberts *et al.* 1999; Li, Spooner *et al.* 2010; Sokolowska, Walchli *et al.* 2011), Shiga toxin (SHT) from *Shigella dysenteriae* resulting in Shigellosis (Yu and Haslam 2005; Lee, Cherla *et al.* 2010) and Shiga-like toxins (SLT-I and -II) from *Escherichia coli* resulting in haemolytic uremic syndrome

(HUS) (LaPointe, Wei *et al.* 2005; Yahiro, Satoh *et al.* 2011; Li, Spooner *et al.* 2012), all manipulate components of ERAD.

In summary, ERAD is both directly and indirectly involved in a plethora of diseases, which together result in millions of deaths worldwide each year. Undoubtedly, the delineation and understanding of the molecular basis of ERAD, and the elucidation of its roles in disease, will aid our understanding of these diseases and assist in the development of successful treatments and therapies.

1.14 Aim of thesis

The aim of this thesis is to determine the molecular mechanisms underlying the targeting of misfolded proteins in the ER for degradation through ERAD. In particular this thesis will focus on the role of OS-9: its interaction with the Hsp90 homologue Grp94, its recognition of substrate and the delivery of said substrate to the Hrd1/SEL1 ubiquitin ligase complex. In addition, this thesis shall assess how OS-9 is itself degraded and shall investigate previously reported interactions of OS-9, in particular those reported to occur within the cytosol. We believe that delineation of the molecular arrangement of the SEL1L/OS-9/Grp94 complex will contribute substantially to understanding how protein triage decisions are made in the eukaryotic secretory pathway.

CHAPTER TWO

Materials and Methods

2.1 Materials

2.1.1 Reagents

All chemicals, unless otherwise stated, were obtained from Sigma or Fisher BioReagents[®]. All restriction enzymes and their buffers were purchased from New England Biolabs (UK). All tissue culture dishes, pipettes and Falcon tubes (15 ml and 50 ml) were obtained from Corning Incorporated. 1.5 ml and 2 ml reaction tubes were obtained from Eppendorf. PVDF membrane was purchased from GE Healthcare. N-ethylmaleimide (NEM) was purchased from Fluka analytical and phenylmethylsulfonyl fluoride (PMSF) from Boehringer Mannheim. U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) ethanolate (Sigma) was used at a final concentration of 20 μ M for the inhibition of Map kinase. The translation inhibitors emetine (75 μ M) and cycloheximide (100 μ M) (Sigma) were applied at various times as indicated in protein stability assays. Tunicamycin (TM) (Sigma) and thapsigargin (Tg) (Merck) were used to induce ER stress, and MG132 (Merck) was used at a final concentration of 25 μ M to inhibit the proteasome.

Alcian Blue solution

1 g Alcian Blue, 8GX (Sigma)

100 ml Acetic acid (3 % solution)

Mix well, adjust to pH 2.5 with acetic acid

5X Agarose sample buffer

50 % (w/v) Sucrose
100 mM EDTA, pH 8.0
0.1 % (w/v) Bromophenol blue
0.1 % (w/v) Xylene cyanol FF

Ammonium persulphate (APS)

10 % (w/v) stock solution was prepared in ddH₂O and stored at 4 °C.

Coomassie staining solution

10 % Acetic acid
20 % EtOH
0.02 % Coomassie Brilliant Blue
In ddH₂O, stored at room temperature (RT)

Complete Mini protease inhibitor cocktail

One cOmpleteTM protease inhibitor (Roche) tablet was dissolved in 1 ml of ice cold ddH₂O. 50 µl aliquots were stored at -20 °C.

DAPI staining solution

Powdered DAPI was dissolved at 1 mg/ml and stored at -20 °C in aliquots.

Destaining solution

10 % Acetic Acid

20 % EtOH

In ddH₂O, stored at RT

DNA Marker

1 kb DNA ladders were purchased from New England Biolabs (UK) and were used as a size standard for agarose gel electrophoresis.

Extraction buffer: GST recombinant protein purification

250 mM Tris HCl, pH 8.5

500 mM NaCl

5 mM EDTA

Stored at 4 °C

Added fresh: cOmplete EDTA-free protease inhibitor (1 tablet per 50 ml of extraction buffer), 1:500 PMSF, Triton X-100 to 1 % from a 10 % stock, and 1 mM DTT

Extraction Buffer: His recombinant protein purification

20 mM phosphate buffer, pH 7.2

500 mM NaCl

Added fresh: cOmplete EDTA-free protease inhibitor (1 tablet per 50 ml of extraction buffer), 1:500 PMSF, 1 % NP-40 (Sigma Chemicals), 1 mM DTT and 20 mM Imidazole.

Freezing medium

90 % (v/v) Foetal bovine serum (FBS)

10 % (v/v) DMSO

Stored at -20 °C

2X HEPES

8 g NaCl

0.105 g Na_2HPO_4

6.5 g HEPES

ddH₂O to 500 ml, pH 6.95 to 7.10

stored at RT

Mammalian cell lysis buffer

50 mM Tris, pH 7.4

5 mM EDTA

150 mM NaCl

Stored at 4 °C

Added fresh: 1X cOmplete Mini protease inhibitor cocktail (Roche), 2 mM NEM

Lipotransfection reagents

Lipotransfections were performed with Fugene 6 (Roche) or Lipofectamine[®]2000 (Invitrogen), following the manufacturer's recommendations or as otherwise stated.

Paraformaldehyde solution (4 %)

A 20 % paraformaldehyde stock solution was made by dissolving 20 g of paraformaldehyde powder in 700 ml of PBS. The solution was heated to dissolve the powder and the volume made up to 1 L with PBS. The solution was diluted to 4 % with PBS and stored in aliquots at -20 °C.

PBS-Tween (PBS-T)

0.02 % Tween (final) was diluted in 1X PBS and stored at RT.

Primers

Primers (Table 2.1.4) used for this study were synthesised by MWG, Invitrogen, and Integrated DNA technologies (IDT). The lyophilised primers were diluted in EB buffer (Qiagen) to obtain a concentration of 100 mM.

Sodium dodecyl sulphate (SDS) solution

A 10 % (w/v) solution of SDS was dissolved in ddH₂O and stored at RT.

2X Laemmli buffer

0.125 M Tris HCl, pH 6.8 Tris

4 % SDS

20 % glycerol

0.004 % Bromphenol blue

20 mM DTT

Stored in 2 ml aliquots at -20 °C

2X SDS-PAGE loading dye

100 mM Tris-HCl, pH 6.8

4 % (w/v) SDS

20 % (v/v) glycerol

0.2 % (w/v) Bromophenol Blue

Before use, 5 % (v/v) β -Mercaptoethanol was added fresh to the 2X SDS-PAGE loading dye.

10x SDS-PAGE transfer buffer

725 g glycine

145 g Tris

Final volume adjusted to 5 L with distilled water. The buffer was then used with 20 % ethanol as a 1X solution.

10X Transfer buffer

288.4 g glycine

60.6 g Tris Base

For 2 L, prepared in ddH₂O and stored at RT.

1X Transfer buffer

10 % 10x transfer buffer

20 % methanol

70 % ddh₂O (Stored at 4 °C)

Water

Nanopure water (Type I) generated from a MilliQ water system was used for all experiments.

2.1.2 Antibodies

Table 2.1: List of primary antibodies

Antigen	Host	Source	Application
αPCNA	rabbit pAb	Kind gift of Prof. Goding, LICR, Oxford	Wb (1:10,000)
α-tubulin	mouse mAb	Sigma-Aldrich	WB (1:10,000)
BiP	rabbit pAb	Kind gift of Prof. Braakman, Utrecht University	IF (1: 200)
BRN2	goat pAb	Kind gift of Prof. Goding, LICR, Oxford	WB (1:2,000)
Calnexin	mouse mAb	Enzo	IF (1:500)
Grp94 (9G10)	rat mAb	Cayman Chemical Company	WB (1:3,000)
Grp94	rabbit pAb	Kind gift of Prof Braakman, Utrecht University	WB (1:3,000) IF (1:200)
HA	mouse mAb	Sigma	WB (1:3,000)
Hrd1	rabbit pAb	Abgent	WB (1:2000)
KDEL	mouse mAb	Enzo Life Sciences	WB (1:3,000) IF (1:500)
LC3	rabbit mAb	Cell Signalling	IF (1:1,000)
LONP2	rabbit pAb	AbCam	WB (1:1,000) IF (1:500)
OS-9	Rabbit pAb	Lab Produced	WB (1:3,000)
OS-9	rabbit pAb	Novis Biologicals	IF (1:500)
myc	rabbit pAb	Abcam	WB (1:3,000)
PAX3	rabbit pAb	Kind gift of Prof. Goding, LICR, Oxford	WB (1:3,000)
SEL1L	goat mAb	Santa Cruz Biotechnology	WB (1:2,000)
S-tag	mouse mAb	Thermo Scientific	WB (1:10,000)

Abbreviations: monoclonal antibody (mAb), polyclonal antibody (pAb), western blot (WB), Immunofluorescence (IF).

Table 2.2: List of secondary antibodies

Ab name	Host	Source	Application
Anti-rabbit Immunoglobulins/HRP (H+L)	goat	Santa Cruz Biotechnology	WB (1:7,500)

Anti-mouse Immunoglobulins/HRP (H+L)	goat	Santa Cruz Biotechnology	WB (1:7,500)
Anti-goat Immunoglobulins/HRP (H+L)	donkey	Santa Cruz Biotechnology	WB (1:7,500)
Anti-sheep Immunoglobulins/HRP (H+L)	donkey	Santa Cruz Biotechnology	WB (1:7,500)
Anti-rat Immunoglobulins/HRP (H+L)	goat	GenScript Corp.	WB (1:7,500)
Alexa Fluor[®] 488 Anti-mouse IgG	rabbit	Invitrogen	IF (1:400)
Alexa Fluor[®] 647 Anti-rabbit IgG	mouse	Invitrogen	IF (1:400)
Alexa Fluor[®] 546 Anti-goat IgG	sheep	Invitrogen	IF (1:400)

Abbreviations: antibody (Ab), horseradish peroxidase (HRP), western blot (WB), Immunofluorescence (IF).

2.1.3 Plasmids

Table 2.3: List of plasmids

Name	Vector	Information	Tag	Source
Control	pcDNA3.1 (+)	Empty vector	-	Lab stock
S-SEL1L 1-794	pcDNA3.1 (+)	Human SEL1L	S-tag	Lab stock
S-OS-9.1	pcDNA3.1 (+)	Human OS-9 isoform 1	S-tag	Lab stock
S-OS-9.2	pcDNA3.1 (+)	Human OS-9 isoform 2	S-tag	Lab stock
S-OS-9.3	pcDNA3.1 (+)	Human OS-9 isoform 3	S-tag	Lab stock
S-OS-9.4	pcDNA3.1 (+)	Human OS-9 isoform 4	S-tag	Lab stock
S-OS-9.1_{ΔSS}	pcDNA3.1 (+)	Human OS-9 isoform 1 minus SS	S-tag	Lab stock
S-OS-9.2_{ΔSS}	pcDNA3.1 (+)	Human OS-9 isoform 2 minus SS	S-tag	Lab stock
S-OS-9.3_{ΔSS}	pcDNA3.1 (+)	Human OS-9 isoform 3 minus SS	S-tag	Lab stock
S-OS-9.4_{ΔSS}	pcDNA3.1 (+)	Human OS-9 isoform 4 minus SS	S-tag	Lab stock
S-OS-9.1 1-249	pcDNA3.1 (+)	Fragment (1-249) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 1-405	pcDNA3.1 (+)	Fragment (1-405) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 249-405	pcDNA3.1 (+)	Fragment (249-405) of human OS-9.1	S-tag	Lab stock

S-OS-9.1 249-485	pcDNA3.1 (+)	Fragment (249-485) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 249-497	pcDNA3.1 (+)	Fragment (249-497) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 249-507	pcDNA3.1 (+)	Fragment (249-507) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 249-517	pcDNA3.1 (+)	Fragment (249-517) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 249-527	pcDNA3.1 (+)	Fragment (249-527) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 249-611	pcDNA3.1 (+)	Fragment (249-611) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 249-667	pcDNA3.1 (+)	Fragment (249-667) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 330-405	pcDNA3.1 (+)	Fragment (330-405) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 330-527	pcDNA3.1 (+)	Fragment (330-527) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 330-611	pcDNA3.1 (+)	Fragment (330-611) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 443-507	pcDNA3.1 (+)	Fragment (443-507) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 443-527	pcDNA3.1 (+)	Fragment (443-527) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 443-611	pcDNA3.1 (+)	Fragment (443-611) of human OS-9.1	S-tag	Lab stock
S-OS-9.1 443-667	pcDNA3.1 (+)	Fragment (443-667) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 453-667	pcDNA3.1 (+)	Fragment (453-667) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 463-667	pcDNA3.1 (+)	Fragment (463-667) of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 473-667	pcDNA3.1 (+)	Fragment (473-667) of	S-tag	Cloned for this study

		human OS-9.1		
S-OS-9.1 486-667	pcDNA3.1 (+)	Fragment (486-667) of human OS-9.1	S-tag	Cloned for this study
S-OS9.1 P446L	pcDNA3.1 (+)	Mutant C1337T of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 S454L	pcDNA3.1 (+)	Mutant C1361T of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 Q486R	pcDNA3.1 (+)	Mutant G1664A of human OS-9.1	S-tag	Cloned for this study
S-OS-9.1 R188A	pcDNA3.1 (+)	Mutant of human OS-9.1 MRH	S-tag	Lab stock
Grp94 WT	pcDNA3.1(+) Zeo	Human Grp94	FLAG-tag	Kind gift of Dr. Argon, UPenn, USA
Grp94 NTD	pcDNA3.1 (+) Zeo	Fragment of human Grp94	FLAG-tag	Kind gift of Dr. Argon, UPenn, USA
Grp94 CTD	pcDNA3.1(+) Zeo	Fragment of human Grp94	FLAG-tag	Kind gift of Dr. Argon, UPenn, USA
Grp94 C2	pcDNA3.1 (+) Zeo	Fragment of murine Grp94	FLAG-tag	Kind gift of Dr. Argon, UPenn, USA
Grp94 C3	pcDNA3.1(+) Zeo	Fragment of murine Grp94	FLAG-tag	Kind gift of Dr. Argon, UPenn, USA
FLAG-HA-LONP2	pcDNA3.1 (+)	Human LONP2	HA-tag	Cloned for this study
S-LONP2	pcDNA3.1 (+)	Human LONP2	S-tag	Cloned for this study
Myc-Hif1α	pcDNA3.1 (+)	Human Hif1 α	Myc-tag	Kind gift of Prof. Xin Lu, LICR Oxford, UK
CPVL-S	pcDNA3.1 (+)	Human CPVL	S-tag	Cloned for this study
XTP3-B-myc	pcDNA3.1 (+)	Human XTP3-B	Myc-tag	Lab stock
XTP3-B-S	pcDNA3.1 (+)	Human XTP3-B	S-tag	Lab stock
XTP3-B-S N195Q	pcDNA3.1 (+)	Mutant of human XTP3-B MRH	S-tag	Lab stock
XTP3-B-S R207A	pcDNA3.1 (+)	Mutant of human XTP3-B MRH	S-tag	Lab stock
XTP3-B-S G379S	pcDNA3.1 (+)	Mutant of human XTP3-B MRH	S-tag	Lab stock
XTP3-B-S R428A	pcDNA3.1 (+)	Mutant of human XTP3-B MRH	S-tag	Lab stock
XTP3-B-S R207A/R428A	pcDNA3.1 (+)	Mutant of human XTP3-B MRH	S-tag	Lab stock

NHK-HA	pcDNA3.1 (+)	Null Hong-Kong protein	HA-tag	Lab stock
NHK-QQQ-HA	pcDNA3.1 (+)	Non-glycosylated NHK	HA-tag	Lab stock

Abbreviations: signal sequence (SS).

2.1.4 Primers

Table 2.4: List of primers

Name	Sequence
CMV Forward	5'-CGCAAATGGGCGGTAGGCGTG-3'
BGH Reverse	5'-TAGAAGGCACAGTCGAGG-3'
OS-9 330-667 (F2)	5'-GACATATGACCGGTAGCCCGACCAAGGATGACAGTAAG-3'
OS-9 442-667 (F3)	5'-GACATATGACCGGTGGGATCCTGCTTCCGTCAGACCGA-3'
OS-9 485-667 (F4)	5'-ATAACCGGTGAGCGGGATCGGGCAATGCTG-3'
OS-9 453-667 (F5)	5'-ATAACCGGTCGTTCCGAGGTGAAGGCTGGC-3'
OS-9 463-667 (F6)	5'-ATAACCGGTGAACTGGAAAACATCATCCAG-3'
OS-9 473-667 (F7)	5'-ATAACCGGTAAAGAGCTGGACCCAGATGGG-3'
OS-9 1-285 (R2)	5'-GGTCTAGATTAACCTCCACACTTGGGGGCCTAG-3'
OS-9 1-407 (R3)	5'-GGTCTAGATTATGGATCACCCCTTTCCTTCTC-3'
OS-9 1-527 (R4)	5'-GGTCTAGATTAGGGAGGCTTTTTGGGGACAAC-3'
OS-9 1-611 (R5)	5'-GGTCTAGATTAAGTCAGCCAACGTGCACCCTC-3'
OS-9 1-485 (R6)	5'-GGTCTAGATTATGACTCCTTCTTCAGCCCATC-3'
OS-9 1-497 (R7)	5'-GGTCTAGATTAGAGAGTGGATGTGAGAGCCAG-3'
OS-9 1-507 (R8)	5'-GGTCTAGATTATTTTCCCTCCAGTCTTTTGA-3'
OS-9 1-517 (R9)	5'-GGTCTAGATTACTTGTGCTTCTTCACCAGCTC-3'
OS-9term FW(248-667)	5'-GACATATGACCGGTCCCTCCCTACAGCCTGAGGAG-3'
OS-9term REV STOP(1-247)	5'-GGTCTAGATTAGTGACAGAGGATGGCCTGCGGTGC-3'
OS-9 P446L QC F	5'-GGGATCCTGCTTCTGTCAGACCGAGAC-3'
OS-9 P446L QC R	5'-GTCTCGGTCTGACAGAAGCAGGATCCC-3'
OS-9 S454L QC F	5'-GACCGGCTCCGTTGGAGGTGAAGGCTG-3'
OS-9 S454L QC R	5'-CAGCCTTCACCTCCAACGGAGCCGGTC-3'
OS-9RT-PCR-F	5'-CTTCCGTCAGACCGAGACC-3'
OS-9RT-PCR-R	5'-GGGCGGACAATTTTGATCT-3'
LG2-MEL-F	5'-ATGAGGGGTGATCCAGAACG-3'
LG20MEL-R	5'-AACGGAGCGGGTCTCGGTCTG-3'
OS-9 440 LIC up	5'-TACTTCCAATCCATGCTGGAAGGGATCCTGCTTCCGTCA-3'
OS-9 527 LIC down	5'-TATCCACCTTTACTGTCAGGGAGGCTTTTTGGGGACAAC-3'
OS-9 1 LIC UP	5'-TACTTCCAATCCATGGCGGCGGAAACGCTGCTGTCCAGT-3'
OS-9 667 LIC down	5'-TATCCACCTTTACTGTCAGAAGTCAAATTCGTCCAGGTCCCC-3'
pLIC-Forward	5'-TGTGAGCGGATAACAATTCC-3'
pLIC-Reverse	5'-AGCAGCCAACTCAGCTTCC-3'

Abbreviations: Quick-change (QC).

2.2 Methods

2.2.1 Mammalian cell methods

Mammalian cell lines

The cell lines used in this study were obtained from long term lab stocks. Human Embryonic Kidney (HEK) 293 cells and U2OS cells were maintained in Dulbecco's-modified Eagle medium (DMEM) (Lonza), supplemented with 10 % FBS (Biosera), 100 µg/ml penicillin-streptomycin (purchased from Gibco® by Life Technologies at a 200 nM concentration and stored at -20 °C) and 2 mM L-glutamine (Gibco® by Life Technologies) in a Heraeus incubator at 37 °C under 5 % CO₂. LG2-MEL-5-35 and LG2-MEL-207 (gift from Dr. Nathalie Vigneron) were maintained in IMDM supplemented with 10 % FBS, 100 µg/ml P/S and non-essential amino acids (Invitrogen). 501-MEL, SK-MEL 28, IGR 37 H21 and IGR 39 H21 were grown in RPMI supplemented with 10 % (501-MEL and SK-MEL 28) or 15 % (IGR 37 H21 and IGR 39 H21) FBS, 100 µg/ml P/S and L-glutamine.

Maintaining mammalian cell lines

Monolayer cell cultures were grown in 10 cm dishes (Nunc) in 10 ml medium until confluency (2 days on average). Cells were washed once with 1X PBS and incubated with 2 ml pre-warmed Trypsin-EDTA (Gibco® by Life Technologies) at 37 °C until the cells detached from the flasks or dishes. Trypsin was inhibited by the addition of an appropriate volume of fresh medium

and this culture was then seeded on to fresh flasks or dishes at the desired density.

Freezing/thawing of cells

Cells from a 70 % confluent culture were detached by trypsinisation then resuspended in 2 ml of freezing media at a concentration of approximately $1-5 \times 10^7$ cells per ml. 1 ml aliquots of the cell suspension were transferred into 2 ml freezing ampoules (Nunc). The vials were then labelled and cooled at the rate of 1 °C per minute in a Nalgene Cryo 1 °C freezing container in a -80 °C freezer (NEW Brunswick Scientific) for at least 24 hrs, before being transferred to a liquid nitrogen tank for long term storage.

To thaw cells from the liquid nitrogen stock, vials were transported on dry ice and flash thawed in a 37 °C water bath. The vials were spun down at 300 g for 3 minutes, the freezing media was aspirated and the cell pellet resuspended and transferred to a 10 cm dish with the appropriate pre-warmed fresh growth medium.

Calcium-phosphate transfection

Cells at 80-90 % confluence were transiently transfected with the expression plasmid of interest (4 µg total DNA; the DNA concentration was normalised with empty vector of the same backbone, each sample was co-transfected with 0.5 µg of sfGFP (super-folder green fluorescent protein) to

monitor transfection efficiency) using the calcium-phosphate co-precipitation technique. The DNA was diluted to 60 μ l with ddH₂O and 2.5 M CaCl₂. In another 1.5 μ l eppendorf, 60 μ l of 2X HEPES buffered saline (HBS) was gassed using a pasture pipette and, while gassing, the content of the first eppendorf was added drop-wise. The solution was incubated at RT for 15 minutes and then added drop-wise to the cells. After 5.5 hrs, the media on the cells was aspirated and fresh media was added. Cells were harvested 24 hrs post-transfection.

Lipofection

Cells were transiently transfected using the lipid-based FuGENE 6 transfection reagent (Promega). Cells were seeded in a 12-well plate and grown until 50-70 % confluency. On the day of transfection, 1.2 μ l of FuGENE transfection reagent was diluted in 20 μ l of OptiMEN and incubated for 5 minutes at RT. The mixture was added to 400 ng of DNA and incubated for 15 minutes at RT before addition to the cells.

Cycloheximide chase

Cells grown to confluency were treated with 100 μ g/ml of cycloheximide (or emetine) for the indicated period of time. Cells were harvested, lysed and 50 μ g of protein sample subjected to western blotting.

Immunofluorescent staining

Cells were grown in 6-well plates on coverslips (VWR), ideally to a 70-80 % confluency. U2OS cells were routinely used. After appropriate treatment, the cells were washed once with PBS and fixed in 4 % paraformaldehyde (diluted from paraformaldehyde 16 % solution pre-scored ampules – VWR) for 20 minutes at RT. Permeabilisation was performed using 0.1 % Triton (diluted in PBS) for 5 minutes on ice. Cells were subsequently washed twice in PBS prior to being blocked with 2 % BSA diluted in PBS for 60 min at RT. The appropriate primary antibody was diluted 1:50 - 1:200 in 2 % BSA and incubated with the cells for 60 minutes at RT. After two washes with PBS, the cells were incubated with the secondary fluorescent labelled antibody (Alexa Fluor[®]), diluted 1:400 in 5 % FCS (foetal calf serum) for 60 minutes in the dark. This was followed by an additional two washes with PBS. Where necessary, TO-PRO[®] (Molecular Probes) (1:5000) or DAPI (Sigma) (1:5000) was diluted in PBS and added for 10 minutes to stain the nuclei, followed by a wash with PBS and a final wash with H₂O. The coverslips were then mounted onto poly-lysine microscope slides using a mowiol-glycerol-based solution containing 2.5 % DABCO anti-fading agent with a drop of Fluoromount[™] (Sigma). After drying overnight, the slides were analysed by confocal microscopy (Zeiss), using LSM image viewer software. Images were processed using ImageJ software.

EDEMosome formation immunofluorescent staining

Prior to cell seeding, sterile (autoclaved) circular cover slips (VWR) were incubated with an Alcian Blue solution for 10 minutes at RT to assist in cell attachment. Alcian Blue was removed by rinsing with dH₂O until the water ran clear (typically 4 volume exchanges). 17 hrs post cell seeding, cells were treated with 100 μ M chloroquine (CQ) for 4 hrs. Cover slips were subsequently processed as above.

CFSE proliferation assay

A single-cell suspension was prepared for labelling. Cells were washed twice with PBS to remove all serum and re-suspended in 5×10^6 ml of PBS (pre-warmed to RT). CFSE was added to a final concentration of 1 μ M, mixed immediately and incubated for 10 minutes at RT in the dark. Labelling was stopped by the addition of 4-5 volumes of cold complete media and incubation on ice for 5 minutes. Cells were pelleted at 300 g for 4 minutes, washed three times in complete culture medium and seeded.

Preparation of samples for fluorescence activated cell sorting (FACS)

Flow cytometry is a fluidic- and laser-based method used for multiparameter analysis of single cells in suspension. Samples were washed once in PBS-EDTA (3 mM), taken up by pipetting into 1 ml of PBS-EDTA and transferred to FACS tubes (BD Falcon). The cells were washed by the addition

of 10 ml of PBS-EDTA and collected through centrifugation at 1000 rpm for 3 minutes at 4 ° C. The supernatant was removed and cells were re-suspended in 250 µl PBS-EDTA. FACS analysis was carried out immediately.

2.2.2 DNA and RNA techniques

Bacterial strains and culture

Chemically competent *E. coli* strain MC106 (araD139 Δ (araA-leu)7697 Δ lacX74 galK16 galE15(GalS) λ^- e14 $^-$ mcrAo relA1 rpsL150(strR) spoT1 mcrB1 hsdR2) was used as a host for the general cloning of recombinant plasmids (obtained from long term lab stocks). *E. coli* strain Rosetta (F $^-$ ompT hsdS_B(r_B $^-$ m_B $^-$) gal dcm pRARE (Cam^R)) cells were used for protein expression (kindly provided by Dr. Opher Gileadi, SGC Oxford, UK). The Rosetta strain is a BL21 strain derivative and supplies tRNAs for 7 codons rarely used in *E. coli* (AGA, AGG, AUA, CUA, GGA, CCC, and CGG) on a chloramphenicol-resistant plasmid.

Bacteria were cultured in Lennox L broth base (LB) media (1 % w/v SELECT peptone, 0.5 % w/v SELECT yeast extract, 0.5 % w/v NaCl) (Invitrogen) containing the required antibiotic (100 µg/ml ampicillin, 50 µg/ml kanamycin or 20 µg/ml chloramphenicol) at 37 °C in an orbital rotary shaker at 250 rpm. To grow single bacterial colonies on a solid substrate, LB-agar medium (LB broth containing 1.5 % bacto-agar) was melted and allowed to cool to 50 °C

prior to the addition of the selection antibiotic and pouring into petri dishes (Sterilin). Plates were stored at 4 °C and air-dried before use.

Bacterial stocks

A half volume of log phase bacteria liquid culture was mixed with a half volume of glycerol in a 1.8 ml polypropylene cryovial (Nunc) and stored at -80 °C.

Preparation of competent bacteria

1 ml of overnight liquid culture of the desired *E. coli* strain was inoculated into 200 ml of fresh LB medium. The culture was grown to an optical density of OD₅₅₀ = 0.5 at 37 °C. The conical flask was chilled by swirling in an ice-bath for 5 minutes before sedimentation by centrifugation (6,000 rpm, 4 °C, 5 minutes). The pellet was re-suspended in 80 ml of ice-cold filter sterilised transformation buffer (30 mM KOAc, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM MnCl₂ and 15 % glycerol) at pH 5.8. The solution was swirled on ice to chill for 5 minutes before sedimentation by centrifugation (6,000 rpm, 4 °C, 5 minutes). The pellet was then re-suspended in 8 ml filter sterilised ice-cold buffer (10 mM MOPS/KOH pH 6.5, 75 mM CaCl₂, 10 mM RbCl₂ and 10 % glycerol) and chilled by swirling on ice for 5 minutes. 100 µl aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

Transformation

Aliquots of competent cells were thawed on ice and the cell suspension was mixed gently by flicking the tube. 30 µl of this cell slurry was added to an eppendorf containing 100 ng of desired plasmid DNA pre-chilled for 1 minute on ice. The mixture was incubated on ice for 15 minutes and then subjected to heat shock at 42 °C for 50 seconds to enable uptake of the DNA, ice-cooled for 2 minutes and incubated, shaking, at 37 °C with 300 µl of pre-warmed SOC (super optimal broth with catabolite repression) for 1 hr. It was then spread or streaked on L-agar plates containing the appropriate antibiotic, inverted and incubated overnight at 37 °C to allow colony formation.

Colony screening

Colonies grown on agar plates were screened for positive clones with a 10 µl PCR reaction containing 5 µl 2X MyTaq™ Red Mix (Bioline), 0.08 µl each forward and reverse primer, 4 µl ddH₂O. Bacterial colonies were spiked directly into each reaction mix. Cycling parameters were as follows: initial denaturation at 95 °C for 2 minutes, 30 cycles of denaturation at 94 °C for 10 s, annealing at a temperature appropriate to the primers being used for 10 s, elongation at 72 °C for 30 s; final elongation step for 5 minutes at 72 °C.

Inserts cloned into the pNIC23-Bsa4 vector (pET expression vector with His6 tag in 22 a. a. N-terminal fusion peptide, with TEV protease cleavage site. Includes sites for LIC cloning, and a "stuffer" fragment that includes the SacB

gene, allowing negative selection on 5 % sucrose) were grown overnight in 5 ml LB with 5 % sucrose.

Annealing of complementary oligonucleotides

A solution of complementary oligonucleotides in an equal molar ratio was heated to 95 °C for 5 minutes to denature all secondary structures, then cooled down to RT to allow annealing.

DNA restriction digest

DNA (plasmid or PCR product) was digested with appropriate restriction enzymes (New England Biolabs), using the buffers and conditions recommended by the manufacturer. In the case of digestion of DNA with more than one restriction enzyme, a reaction buffer compatible with both enzymes was chosen. If no compatible buffer was found then a sequential digestion was performed. A typical reaction contained in a final volume of 20 µl was; 2-12 µl DNA, 2 µl 10x buffer, and 1 µl of restriction enzyme. The digestion was performed at 37 °C for 2-3 hrs.

Dephosphorylation

After restriction digest, if required, the terminal 5' phosphate groups were removed from the linearised plasmid DNA by treatment with Antarctic Phosphatase (NEB), (1 μ l per 20 μ l reaction with 2 μ l associated buffer) for 30 minutes at 37 °C.

Ligation of DNA fragments

Digested vector DNA was treated with Alkaline Phosphatase (Invitrogen) for 20 minutes at 37 °C, to catalyse the hydrolysis of the 5' phosphate in order to avoid re-ligation. Vector and insert DNA were used at a ratio of 1:3. The ligation reaction was performed in a total volume of 20 μ l containing T4 DNA ligase H. C. (Invitrogen) for 1 hr at RT.

Agarose gel electrophoresis

Agarose (at the appropriate concentration) was heated in 1X TAE buffer until completely dissolved. Ethidium bromide (Severn Biotech Ltd) was added to a final concentration of 20 μ g/ml. DNA samples containing 1X loading buffer were loaded into gels submerged in 1X TAE buffer and run next to a DNA ladder (1 kb ladder, Invitrogen) at 135 V. Bands were visualised via a UV-imaging system (UVP Bio Imaging Systems).

DNA extraction from agarose gels

The plasmid DNA that had been subjected to restriction endonuclease digestion (NEB) was loaded on an agarose gel and allowed to run until the fragments were well separated. The bands indicating digested fragments were visualised under UV, and the desired bands cut out using a clean scalpel. DNA was extracted from the gel slice using a QIAquick gel extraction kit, according to the manufacturer's instructions. This method is based on dissolving the agarose, with the mixture applied to a silica membrane. DNA binds to the membrane under the high-salt conditions provided by the buffer, impurities are washed away and pure DNA is eluted with 30 µl-50 µl water.

Polymerase chain reaction (PCR)

For cyclical amplification of a target DNA sequence, *Phusion*[®] *Hot Start II* DNA Polymerase (NEB), 50 ng of template plasmid DNA and 0.5 µM of each primer were used according to the manufacturer's instructions for reaction mix and cycling parameters.

Site-directed mutagenesis

To introduce a single a. a. change into a protein of interest, the nucleotide sequence of the corresponding codon was altered using the QuikChange site-directed mutagenesis system (Stratagene) according to the manufacturer's protocol. The method is based on a PCR using a complementary primer pair

containing the desired nucleotide change in the middle of the sequence (designed by PrimerX). The reaction is carried out with PfuUltra™ polymerase (Stratagene) to ensure amplification of the entire plasmid with high fidelity and without primer displacement. The 5' primer overhangs are ligated by endogenous ligases of the *E. coli* strain used. Following the PCR, the parental methylated plasmid DNA was digested with the restriction enzyme *DpnI*, the mutated plasmid DNA transformed into 50 µl XL10-Gold® Ultracompetent cells and plated on agar plates containing the appropriate antibiotic for selection. Colonies were picked, grown on a small scale (typically 5 ml LB) and plasmids purified using the QIAprep® Spin Mini Kit. The resulting plasmids were sequenced to check for appropriate mutation(s).

Small scale preparation of plasmid DNA (QIAprep® Spin Miniprep Kit)

5 ml of antibiotic-containing LB medium were inoculated with a single bacterial colony and incubated at 37 °C under agitation for 16 hrs. Bacteria were collected from 4 ml of culture by centrifugation at 14,000 rpm for 1 minute. DNA was extracted using the QIAGEN Miniprep kit following the manufacturer's instructions. The method is based on alkaline lysis followed by binding of plasmid DNA to an anion-exchange resin under low salt and pH-controlled conditions. Impurities are removed with medium salt washes. Plasmid DNA is finally eluted in 50 µl of elution buffer.

Large scale preparation of plasmid DNA (QIAfilter[®] Plasmid Midiprep Kit)

A single bacterial colony was used to inoculate 5ml of antibiotic-containing LB medium in a sterile test tube, and shaken at 37 °C for 8 hrs. The resulting bacterial suspension was used to inoculate a 250 ml flask of LB/antibiotic medium and shaken for a further 16 hrs at 37 °C. The cells were centrifuged at 5,000 g for 15 mins at 4 °C, and the plasmid DNA isolated from the bacterial pellet according to the manufacturer's protocol.

Determination of DNA concentration

The concentration and purity of a nucleic acid-containing solution was determined by measuring the absorbance at 260 nm and 280 nm with a Nanodrop spectrophotometer (Thermo Scientific).

RNA extraction and reverse transcription

RNA concentration was determined spectrophotometrically and quality was assessed (to exclude degradation and verify the absence of contaminating genomic DNA) by way of conventional agarose gel electrophoresis. cDNA was synthesised using 1 µg of total RNA, Reverse Transcriptase AMV (Roche) and Primer Random p[dN]₆ (Roche). To investigate the presence of OS-9 splice variants, whole cDNA pools were amplified with specific primers: 5'-AGCCCGACCAAGGATGATACAGTAAG-3' (forward) and 5'-AGTCAGCCAACGTGCACCCTC-3' (reverse). Semi-quantitative PCR was

performed using KAPA2G™ Fast PCR Kit (Kapa Biosystems). PCR products were separated by electrophoresis in a 1.5 % agarose gel.

Real-time PCR

Real-time quantitative PCR was performed on the 7500 real time PCR system (Applied Biosystem) using the QuantiTect SYBR Green PCR kit (Qiagen). Each reaction was performed in triplicate using 0.5 ml of cDNA in a final volume of 25 ml. The following thermal cycle was used for the samples: 10 minutes 95 °C; 40 cycles of 30 s at 95 °C, 40 s primer specific annealing temperatures, 40 s at 72 °C. For each experiment, the threshold was set to cross a point at which real-time PCR amplification was linear. The expression of GAPDH, a housekeeping gene, was used as an internal control to normalise differences in the efficiency of the reverse transcription reaction. The expression level of each target gene was measured as a fold relative to GAPDH, according to the following formula: $2^{[C(t)_{GAPDH} - C(t)_{target}]}$.

Confirmation of mutation in LG2-MEL cell lines

PCR-restriction fragment length polymorphism (PCR-RFLP) was used to confirm the presence of the C1337T point mutation in the cell lines LG2-MEL-5-35 and LG2-MEL-207, as described previously by Vigneron *et al.* 2002. Briefly, cDNA (which was generated from total RNA as previously outlined) was amplified with the primers: 5'-ATGAGGGGTGATCCAGAACG-3' (forward) and

5'- AACGGAGCGGGTCTCGGTCTGCC-3' (reverse). The reverse primer creates an *HpaII* restriction site CCGG in the 154 bp wild type PCR product, producing two fragments of 130 bp and 24 bp on digestion, whereas the mutated product yields a single band of 154 bp. Products were separated by electrophoresis on a 3 % agarose gel.

2.2.3 Protein manipulation

Preparation of cell extracts

Cells grown in monolayers were first washed and then taken up in cold 1X PBS. The cells were scraped with a sterile disposable cell scraper (Greiner), transferred to 1.5 ml eppendorf tubes and centrifuged for 1 minute at 2,000 rpm. The cells were resuspended in lysis buffer, vortexed and incubated on ice for 1 hr. The mixture was cleared by centrifugation at 14,000 rpm, at 4 °C for 20 minutes (eppendorf 5417R). The resulting lysate was removed to a fresh eppendorf tube and the cell debris discarded. 100 µl slurry of Sepharose[®] CL-4B beads (Pharmacia) were added to the supernatant and allowed to pre-clear at 4 °C under rotation for 2 hours or overnight. The lysate was centrifuged at 2,000 g for 2 minutes and the supernatant removed to a fresh tube. The pre-cleared lysate was then used for protein quantification.

Protein concentration determination

The protein concentrations of cell extracts were determined using the BioRad Bradford protein assay reagent system. 1 μ l of cell lysate was mixed with 200 μ l of 1x BioRad assay reagent, then measured at 595 nm in a spectrophotometer (Thermo Scientific). All samples were measured in triplicate, and the absorbance was compared against a standard curve made at the same time from known concentrations of bovine serum albumin (BSA; Sigma-Aldrich) in the same solutions, using the same method.

Affinity purification

Affinity purification was carried out using pre-cleared cell lysate. From each sample, 50 μ g of protein was collected as input sample and 1.5 mg for affinity purification. The following capture resins were used in accordance with the protein of interest's tag: S-protein agarose (Novagen) for S-tagged proteins or glutathione superflow resin (Fisher Scientific) for GST-tagged proteins. The appropriate amount of lysate was incubated with 60 μ l of bead slurry and the volume adjusted to 500 μ l with lysis buffer. Purification was performed for 4 hrs at 4 °C. Samples were washed three times in lysis buffer, resuspended in loading buffer (2X Laemmli buffer) containing 5mM dithiothreitol (DTT) and heated to 56 °C for 10 minutes. Samples were stored at -20 °C until required for further analysis.

Immunoprecipitation (with cell lysates)

2 µg of purified antibody or 2-5 µl of polyclonal antiserum, and 30 µl of protein G sepharose beads (50 % slurry in PBS) (Roche), was then added to 1 mg of pre-cleared lysate. The mixture was left on an eppendorf rotating wheel overnight at 4 °C. Immunocomplexes were collected by centrifugation at 2,000 rpm for 3 minutes and the supernatant discarded. The beads were washed with three successive changes of lysis buffer. After removing as much residual supernatant as possible (using gel loading tips), the IP beads were mixed with 20-60 µl of 5X sample buffer and heated at 56 °C for 10 minutes. The beads were centrifuged at 14,000 rpm for 15 seconds, and all or part of the sample loaded onto a SDS-polyacrylamide gel. The separated proteins were processed for immunoblotting.

De-glycosylation assay

Affinity-purified complexes coupled to beads and heated in 2x laemmli buffer were treated with 1 µl of EndoH (NEB) for 1 hour at 37 °C. Samples were subsequently analysed by western blotting.

Preparation of SDS-polyacrylamide gels

All plates were washed with water and detergent, dried and assembled in casting trays (BioRad). The acrylamide content of the gels varied between 6 % - 15 % depending on the size of the protein of interest. The acrylamide gels were

overlaid with 70 % isopropanol solution and left to polymerise. After polymerisation, the isopropanol was removed and a 4 % stacking gel was set with the appropriate number and size of wells.

Table 2.5: Composition of polyacrylamide gels

	Resolving Gels				Stacking
	7.5 %	10 %	12 %	15 %	4 %
1.5 M Tris HCl, pH 8.8	4.25 ml	4.25 ml	4.25 ml	4.25 ml	-
0.5 M Tris HCl, pH 6.8	-	-	-	-	2.52 ml
Acryl/Bis	3.19 ml	4.25 ml	5.10 ml	6.38 ml	1 ml
10 % SDS	0.17 ml	0.17 ml	0.17 ml	0.17 ml	0.1 ml
TEMED	0.01 ml	0.01 ml	0.01 ml	0.01 ml	0.01 ml
10 % APS	0.09 ml	0.09 ml	0.09 ml	0.09 ml	0.05 ml
ddH₂O	9.22 ml	8.16 ml	7.31 ml	6.04 ml	6.36 ml
Total volume	17 ml	17 ml	17 ml	17 ml	10 ml

Abbreviations: Ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), Tris (Tris(hydroxymethyl) aminomethane), sodium dodecylsulphate (SDS).

Preparation of SDS-polyacrylamide gels for the visualisation of hyper-glycosylated Grp94

4x Lower gel buffer (LGB)

181.6 g Tris base into 700 ml autoclaved milliQ water
pH to 8.8
Add 20 ml of 20 % SDS solution
Final volume adjusted to 1 L
(yields 1.5 M Tris pH 8.8, 0.4 % SDS)

4x Upper gel buffer (UGB)

30.3 g Tris base into 350 ml autoclaved milliQ water
pH to 6.8
Add 10 ml of 20 % SDS solution (or just 2 g powder)
Final volume adjusted to 500 ml
(yields 0.5 M Tris pH 6.8, 0.4 % SDS)

Table 2.6.1: Hyper-glycosylated Grp94 resolving gel

Gel:	Water (ml):	4x LGB (ml):	30 % acrylamide (ml):	10 % APS (μl):	TEMED (μl):
8 %	16.92	8.75	9.33	175	17.5
9 %	15.75	8.75	10.50	175	17.5
10 %	14.58	8.75	11.67	175	17.5

Table 2.6.2: Hyper-glycosylated Grp94 stacking gel

Gel:	Water (ml):	4x UGB (ml):	30 % acrylamide (ml):	10 % APS (μl):	TEMED (μl):
Stack	8.75	3.75	2.5	75	15

SDS-polyacrylamide gel electrophoresis (PAGE)

Proteins were separated on the basis of their molecular weight by two-phase denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Bio-Rad Mini-PROTEAN apparatus (Bio-Rad Laboratories, Ltd). Known concentrations of protein were mixed with appropriate volumes of 2x SDS-PAGE sample loading buffer and boiled for 10 minutes. The electrophoresis running buffer was purchased as a 10X stock from National Diagnostics. Electrophoresis was performed in a BioRad gel apparatus for 15 minutes at 100 V and subsequently at 40 mA (per gel) for 45 minutes. All blue pre-stained protein ladder (BioRad) marker was used as a size standard ladder.

Gels that were not subjected to western blotting were stained with Coomassie solution for 30 minutes and destaining solution for 3X 20 minutes. Gels were kept in ddH₂O and scanned the following day. Gels were dried onto Whatman paper using a GELMASTER[®] Gel Dryer Vacuum System (Welch[®]).

Western blotting

After separation by electrophoresis, proteins were transferred to a Hybond P PVDF membrane (activated for 2 minutes in methanol) using a semi-dry transfer method. The gel was first washed in purified deionised water, then assembled into a sandwich consisting of the membrane and 2 pieces of Whatman TM paper (cut to the size of the gel and soaked in the transfer buffer prior to use). The transfer was carried out by applying 110 V for 73 minutes.

After the transfer, the membrane was washed in 1x PBS-Tween for 5 minutes prior to blocking in either 5 % [w/v] non-fat milk powder (Marvel, UK) or 2 % BSA (Roche) in 1x PBS-T with gentle shaking for 1 hr at RT. The primary antibody was diluted in either 1.3 % milk in 1x PBS-T or 2 % BSA in 1x PBS-T according to the recommended, or that determined to be optimum, dilution. Membranes were incubated in primary antibody solution either over night at 4 °C or for 1 hr at RT, and subsequently washed 2 X for 10 minutes in 1X PBS-T. The secondary antibody tagged to horseradish peroxidase was diluted 1:7,500 in either 1.3 % milk in 1x PBS-T or 2 % BSA in 1x PBS-T, and incubated for 30 minutes with membrane, gently shaking at RT. Membranes were subsequently washed four times in 1x PBS for 15 minutes. Protein bands were visualised using Super Signal[®] West Pico Chemiluminescent substrate (Thermo scientific) according to the manufacturer's protocol. In this system, horseradish peroxidase and hydrogen peroxide are used to oxidise luminol, which decays to a ground state emitting light. This reaction was performed in the presence of phenols which extended the time of reaction and the intensity of light emission. The blots

were exposed using RX medical X-ray film (Fuji Photo Film Co.) for 1-30 minutes, or until a clear signal was detected.

Membrane stripping

The membrane was incubated in 2 ml stripping buffer (Thermo Scientific) for 20 minutes at RT, then washed in 1x PBS for 1 hour (the buffer was exchanged every 5 minutes for the first 20 minutes and then every 10 minutes). Prior to reprobing, the membrane was reblocked in 5 % milk or 2 % BSA, before incubation with the new primary antibody.

2.2.4 Protein expression systems

2.2.4.1 Bacterial expression systems

pGEX4T2 vector

pGEX vectors were designed to express proteins in *E. coli* as fusions to the C-terminus of Glutathione-S-Transferase (GST). GST is a 27 kDa protein, and the fusion protein usually remains soluble within the bacteria and can be purified from the lysed cells based on the affinity of the GST protein for glutathione, which is immobilised on agarose or sepharose beads. The fusion ORF is under the control of the IPTG-inducible *lac* promoter. The vector also expresses the *lac* repressor, the product of the *lac* I gene, to repress the expression of the GST-fusion protein. The fusion protein is produced upon induction with IPTG and can be purified from bacterial cell lysates. The

immobilised fusion protein can be eluted from the capture resin with free reduced glutathione at reduced pH.

Recombinant protein induction

E. coli cultures were inoculated into 1 L LB media in 2 L glass conical flasks, or TB media UltraYield baffled flasks (Thomson Instrument Company, CA, USA), with the appropriate antibiotics. 10 % inoculum from an overnight starter culture was used and cells grown to mid-log phase (OD₆₀₀ of 0.6 for LB or 2-3 for TB) at 37 °C with vigorous shaking. IPTG was added to 0.1-1 mM, thereby inducing recombinant protein expression. Induction was normally performed for up to 4 hrs, and cells harvested and stored at -20 °C.

Protein extraction

Harvested small-scale *E. coli* samples (1-2 ml culture) were lysed and the soluble proteins extracted with SDS-PAGE loading buffer. For large-scale extraction, lysis buffer consisted of 10 mM HEPES (pH 7.4), 5 % (v/v) glycerol, 100 mM NaCl, 1 mM Tris (2-carboxyethyl) phosphine (TCEP) and protease inhibitors (1 mM PMSF (phenylmethylsulphonyl fluoride), 1 mM Benzamidinium-HCl, 1 µM pepstatin and 1 mM EDTA). Cells were resuspended to 10 % (v/v) in lysis buffer and sonicated on ice with a MSE Soniprep 150 (Sanyo-Gallenkamp, Leicester, UK) for 10 cycles (20 seconds on, 20 seconds off), depending on the quantity of biomass. Lysate was clarified by centrifugation (20,000x g, 30 min, 4

°C) and supernatant decanted. Polyethyleneimine pH 7.5 (PEI) was optionally added at varying concentrations to precipitate DNA, which was removed by centrifugation (48,000x g, 20 min, 4 °C).

2.2.4.1 Protein chromatography

General FPLC high-pressure chromatography techniques

ÄKTA™ (GE Healthcare) was used for column chromatography. Buffers were prepared with ddH₂O, degassed by 0.22 µM filtration and FPLC procedures performed at 8 °C. Pumps were flushed with ddH₂O and air flushed from the system. The Superloop and sample lines were washed with ddH₂O. Columns were washed with 10 volumes ddH₂O, prior to column equilibration with 10 volumes of column buffer. Samples were 0.22 µM filtered prior to column loading. Flow rate and pressure were according to column type, with 1-2 ml elution fractions collected. Following elution, pumps and columns were flushed with ddH₂O, and columns cleaned by washing with salt, acid or alkali followed by ddH₂O, according to the manufacturers' instructions, and stored in 20 % (v/v) EtOH.

Cation exchange chromatography

Cation exchange was performed with 5 ml HiTrap HP columns. Buffer type, pH and ionic strength were chosen to allow adsorption of the target protein, generally identical to lysis buffer. Clarified lysates were applied to

columns and unbound proteins removed with 4 column volumes of lysis buffer. Bound protein was eluted with a linear gradient of 100-1000 mM NaCl (in column buffer) over 20 column volumes at 2 ml/min.

Size exclusion chromatography

Size exclusion was performed with Superdex75 HR media on HiLoad 16/60 columns (GE Healthcare). Columns were washed with 2 bed volumes of ddH₂O and equilibrated with 2 volumes of column buffer (10 mM HEPES pH 7.4, 100- 500 mM NaCl (where applicable), 5 % (v/v) glycerol, 1 mM TCEP and protease inhibitors. Pooled samples from upstream steps were concentrated, with buffer exchanged as necessary prior to column injection, with elution proceeding at 1.2 ml/min. Highest resolution was achieved with samples that were 0.1-1.0 % of the total bed volume.

Protein storage

Fractions of purified protein were stored at 4 °C for up to 48 hrs and at – 80 °C for longer periods. Samples were rapidly thawed in a 25 °C water bath. Storage buffer (10 mM HEPES pH 7.4, 200 mM KCl, 0.1 % (v/v) Triton X-100, 0.1 mM EDTA, 1 mM DTT, or 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 1 mM TCEP) was used for long-term storage of purified proteins at – 80°C, with optional glycerol to 50 % (v/v).

2.2.4.2 Mammalian expression vectors

pcDNA3.1 +/- vectors

The pcDNA3.1 vector was designed for high level and constitutive expression in many mammalian cell lines. The multiple cloning site is either in forward (+) or reverse (-) orientation and expression is driven from the promoter region of the human cytomegalovirus (CMV). The pcDNA3.1 vector used in this study was modified with an S- or myc-tag as either an N-terminal (in pcDNA3.1⁺) or C-terminal fusion (in pcDNA3.1⁻). The N-terminal fusion protein used in this study will be referred to as S-/myc-construct name, and C-terminal fusions as construct name-S-/myc. The vector was obtained from long term lab stocks.

pSUPER vector

The pSUPER vector was used to mediate shRNA-mediated knockdown of a specific gene in mammalian cell lines. Expression is driven by the polymerase III H1-RNA gene promoter and positive clones are selected with puromycin. The vector was obtained from long-term lab stocks.

Lentiviral transduction and stable cell line establishment

Vesicular stomatitis virus g pseudotyped lentiviral vectors were produced by transient transfection of 293FT cells (Invitrogen) with an HIV-1 based packaging vector, vesicular stomatitis virus g envelope vector, and transfer

vector containing either the short hairpin human OS-9 or luciferase. Forty-eight hours post-transfection, viral supernatants were filtered through a 0.45 µm filter and added to HEK293 cells. Transduced cells were gradually selected with puromycin, from 1 to 30 µg/ml over 2 weeks. The OS-9 short hairpin RNA-targeting sequence, targeting the 3' untranslated region, is as follows: 5'-GCTGCCTACCTGGAGATTCAG-3'.

Plasmids and constructs

PCR products of OS-9 were cloned into the pcDNA3.1(+) vector containing the signal sequence from bovine preprolactin (HindIII-EcoRI) and S-tag (EcoRI-AgeI). PCR products were introduced in frame between AgeI-XbaI. Truncations were introduced and mutants were cloned in a similar manner. For generation of the three point mutations S-OS-9.1_{P446L}, S-OS-9.1_{S454L} and S-OS-9.1_{Q486R} site-directed mutagenesis was employed, using Platinum[®] Pfx DNA Polymerase (Invitrogen) and generated via PrimerX software (Table 2.4).

CHAPTER THREE

Characterisation of the OS-9/Grp94 interaction and its role in ERAD

3.1 Introduction

ER-resident lectins play an essential role in ERAD, distinguishing terminally misfolded proteins from their correctly folded counter-parts or folding intermediates (Hosokawa, Kamiya *et al.* 2010). In *S. cerevisiae* Yos9p, a MRH (Mannose-6 phosphate receptor homology) domain-containing lectin, has been demonstrated to deliver bound ERAD substrates to the membrane-embedded ubiquitin ligase complex Hrd1p-Hrd3p (Carvalho, Goder *et al.* 2006; Denic, Quan *et al.* 2006; Gauss, Sommer *et al.* 2006). Subsequently, a mammalian orthologue of Yos9p, Osteosarcoma 9 (OS-9), has been established as occupying a nexus between the recognition of misfolded proteins and their delivery to the Hrd1 ubiquitin ligase complex and subsequent degradation (Bernasconi, Pertel *et al.* 2008; Christianson, Shaler *et al.* 2008; Hosokawa, Kamiya *et al.* 2009).

The OS-9 gene is located on chromosome 12q13 – q15, a region often amplified in sarcomas and gliomas and contains 15 exons spanning 30.4 kB (Su, Hutter *et al.* 1996; Kimura, Nakazawa *et al.* 1997). At least four OS-9 isoforms are predicted to result from alternative gene splicing (OS-9.1-4), in which either entire (ex. 13, 55 a. a.) or partial (ex. 11b, 15 a. a.) exons are removed to yield putative isoforms of unique lengths (667, 612, 597 and 652 a. a., respectively). All four alternatively spliced isoforms of OS-9 are predicted to contain a canonical N-terminal signal sequence, a glucosidase type II MRH domain and an N-linked glycan at N178 (Figure 3.1). However, previous studies

have not consistently detected all four isoforms, thus it remains uncertain when and under what conditions they may be produced.

In principal, OS-9 plays a key triage role, interrogating and passing judgement on the integrity of ERAD substrates. OS-9 has been proposed to bind directly to misfolded substrates as well as SEL1L (the adapter protein of the Hrd1 ubiquitin ligase) through modified oligosaccharides and/or its polypeptide chain (Bernasconi, Pertel *et al.* 2008; Christianson, Shaler *et al.* 2008; Bernasconi, Galli *et al.* 2012). In addition, OS-9 has been reported to interact with the ER-resident Hsp90 family member Grp94, with its binding to SEL1L and Grp94 displaying mutual exclusivity, while binding to the former requires OS-9's capacity to bind glycans (Bernasconi, Pertel *et al.* 2008; Christianson, Shaler *et al.* 2008). Grp94 is the sole Hsp90 family member present in the ER and is structurally similar to its cytoplasmic counterpart Hsp90 (Dollins, Immormino *et al.* 2005). However unlike cytoplasmic Hsp90, which has many identified client proteins and cofactors, and performs essential functions in the cytoplasm of cells from all kingdoms of life, Grp94 is only essential for metazoan viability, being absent in unicellular organisms and surprisingly non-essential in mammalian cell cultures (Randow and Seed 2001). Unlike BiP, CNX/CRT or PDI, Grp94 appears to be selective with a limited set of client proteins whose maturation depends upon it and who bear no obvious structural similarities with each other. Such a small client set is surprising considering the high expression levels of Grp94, constituting 5-10 % of the ER luminal contents (Koch, Smith *et al.* 1986; Ostrovsky, Ahmed *et al.* 2009; Marzec, Eletto *et al.* 2012). Indeed,

despite being one of the most abundant ER components, the physiological role and molecular mechanisms of Grp94 are not well understood (Eletto, Dersh *et al.* 2010; Mao, Wang *et al.* 2010).

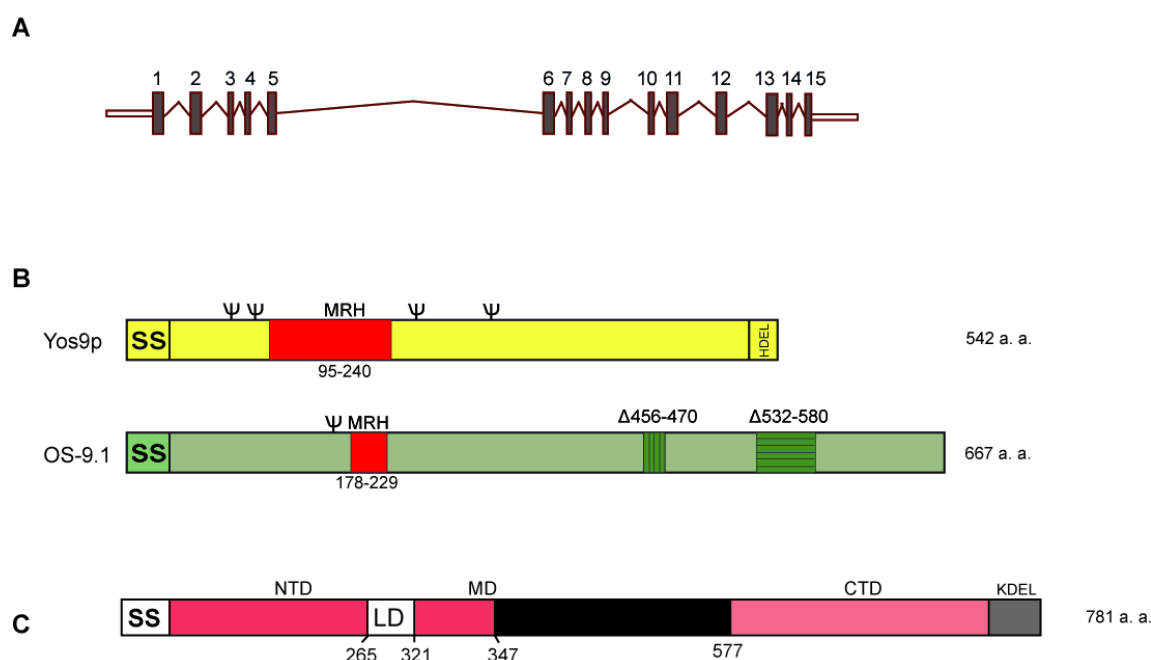


Figure 3.1: Schematics of OS-9, Yos9p and Grp94

A) Organisation of the OS-9 gene. B) Domain architecture of OS-9 and its yeast orthologue Yos9p. Conserved MRH domains (red) and potential N-linked glycosylation sites (Ψ) are indicated. C) Schematic representation of the domain organisation of human Grp94. N-terminal domain (NTD), charged linker domain (LD), middle domain (MD), and C-terminal domain (CTD). The signal sequence of Grp94 (SS) and KDEL, the C-terminal tetrapeptide of Grp94 that serves as the ER retention/retrieval ligand for the KDEL receptor, are also shown.

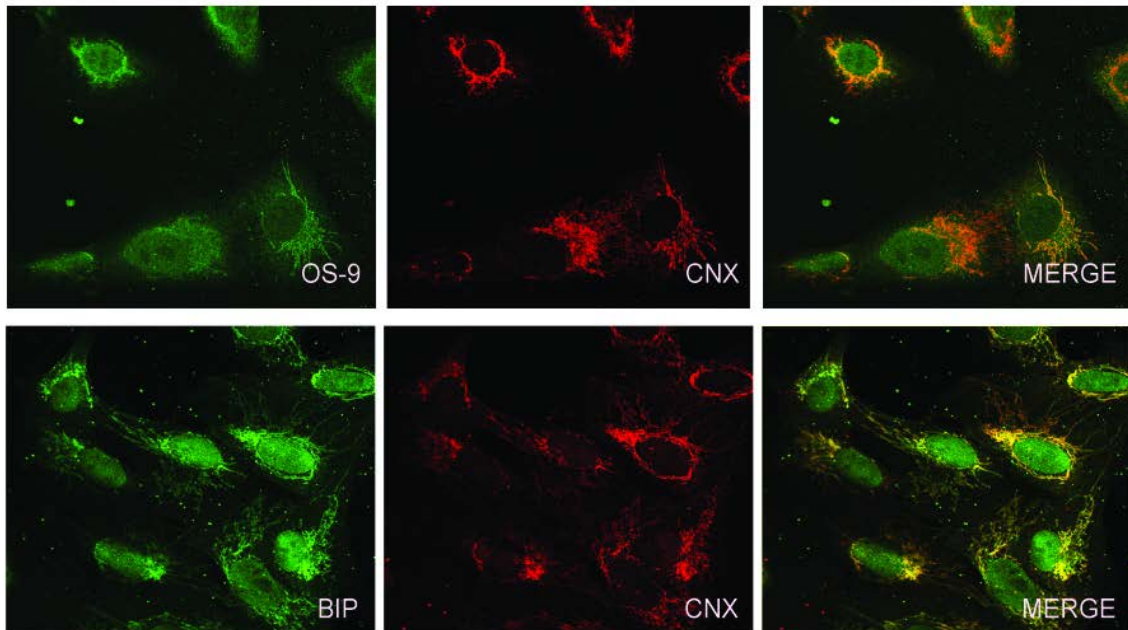
The functional role of the interaction that Grp94 has with OS-9 has also remained enigmatic. Lacking a canonical ER-retention/retrieval signal, Grp94 may confer ER localisation to OS-9. Similarly, Grp94 may sequester a pool of OS-9 held at the ready for ERAD or, conversely, Grp94 may in fact be degraded via OS-9 or may itself be involved in ERQC. In this chapter, the interaction between Grp94 and OS-9 shall be assessed, with a view to establishing where, how and when the interaction takes place, and ultimately delineating the purpose and consequences of this interaction. It is anticipated that extended understanding of the OS-9:Grp94 interaction will contribute substantially to understanding how protein triage decisions are made in the eukaryotic secretory pathway.

3.2 Results

3.2.1 OS-9 and Grp94 are resident in the ER

Multiple studies had supposed cytoplasmic localisation of OS-9 due to its interaction with proteins such as HIF-1 α , TrpV4 and MeprinA- β (Litovchick, Friedmann *et al.* 2002; Baek, Mahon *et al.* 2005; Wang, Fu *et al.* 2007). Unlike Yos9p which contains a carboxy-terminal tetrapeptide His-Asp-Glu-Leu (HDEL) ER retrieval signal (Munro and Pelham 1987), OS-9 lacks a canonical ER-retention/retrieval signal. However, it does contain a validated signal sequence sufficient for ER luminal targeting at its N-terminus (Christianson, Shaler *et al.* 2008). To resolve this discrepancy, immunofluorescence was employed and the glycosylation status of OS-9 investigated. A predominant ER residency for OS-9 was indicated by co-localisation of endogenous OS-9 with the ER-resident protein Calnexin, with a similar staining pattern seen for KDEL-containing, ER resident chaperone BiP (Figure 3.2 A). Moreover, OS-9 displayed sensitivity to endoglycosidase H (EndoH) (Figure 3.2 B). Endogenous OS-9 in HEK293 cells electrophoretically migrates as two bands that correspond to the two more predominant isoforms, OS-9.1 and OS-9.2. Upon addition of EndoH, a recombinant glycosidase which cleaves high mannose oligosaccharides but not complex oligosaccharides, the mobility of both isoforms was increased, indicating their modification via an *N*-linked glycan (as predicted at N178) indicative of ER-resident glycoproteins.

A



B

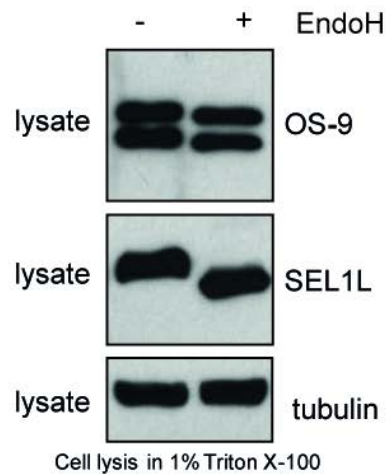


Figure 3.2: The lectin OS-9 is an ER-resident protein

A) Co-localisation of endogenous OS-9 and BiP (green) with the ER-resident protein Calnexin (red) by immunofluorescence in U2OS cells. B) 1 % Triton X-100 lysates from HEK293 cells untreated (lane 1) and treated with EndoH (lane 2), probed with anti-OS-9 and anti-SEL1L to demonstrate the glycosylation state of endogenous proteins.

Grp94 is targeted to the ER by its 21 a. a. N-terminal signal sequence and maintains its ER localisation via its C-terminal KDEL peptide, its localisation to the ER was confirmed by IF, co-localising with the ER marker CNX (Figure 3.3). However, there have been a number of reports of a small percentage of Grp94 escaping the ER, for example in association with bile-salt dependent lipase (BSDL) secreted by pancreatic acinar cells (Bruneau, Lombardo *et al.* 1998), and on the cell surface in tumour cell lines (Altmeyer, Maki *et al.* 1996; Frasson, Vitadello *et al.* 2009). The possible consequences of such potential mislocalisation of Grp94 and OS-9 will be discussed in further detail in chapters 4 and 5, respectively.

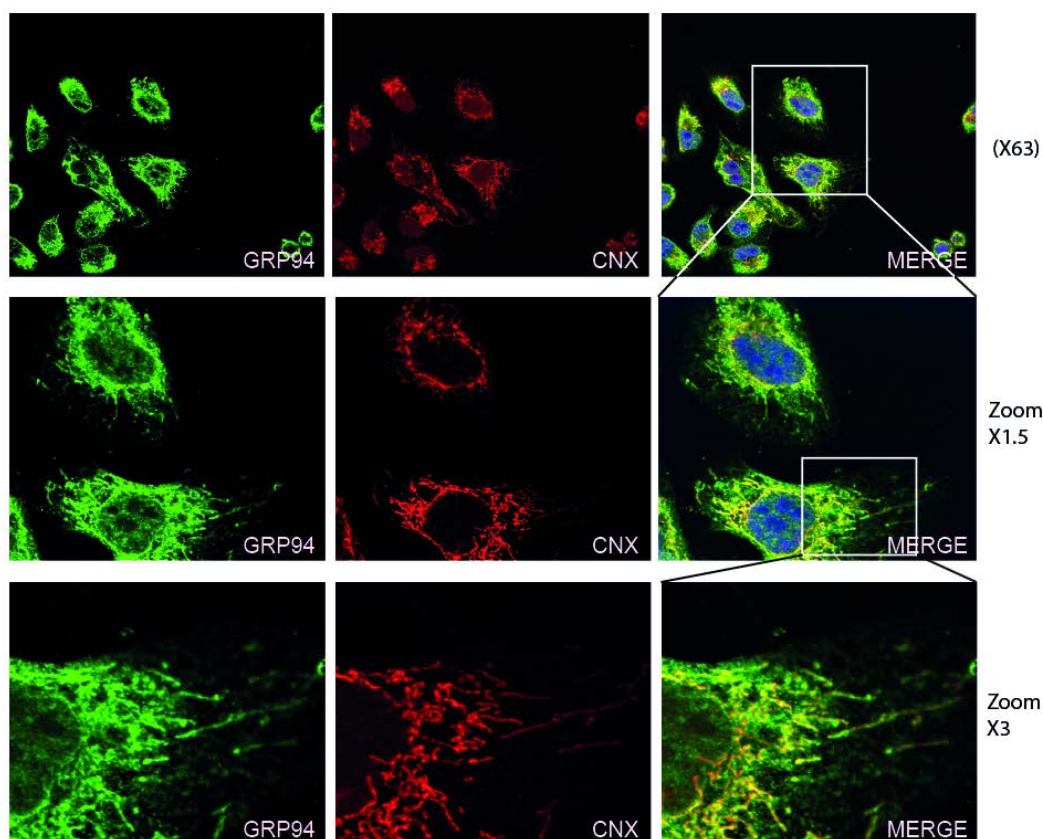


Figure 3.3: Grp94 is an ER-resident.

Co-localisation of endogenous Grp94 (green) with the ER-resident protein Calnexin (red) by immunofluorescence in U2OS cells.

3.2.2 Grp94 preferentially binds isoforms 1 & 2 of OS-9

Protein isoforms, produced by alternative splicing, can differ in various physiological aspects including ligand binding affinity, signalling activity, protein domain composition, subcellular localisation, and protein half-life (Kamatkar, Radha *et al.* 1996; Rudenko, Nguyen *et al.* 1999; Garcia, Gerber *et al.* 2004; Stamm, Ben-Ari *et al.* 2005). Indeed, bioinformatics analysis have shown that alternative splicing has a tendency to remove protein domains involved in protein-protein interactions or DNA binding (Resch, Xing *et al.* 2004). To investigate if differential splicing alters OS-9's ability to interact with components of the luminal ERAD surveillance machinery, S-tagged OS-9 isoforms were expressed in HEK293 cells, complexes were co-precipitated by S-protein agarose, and immunoblots of affinity captured OS-9 complexes were probed with antibodies against endogenous Grp94 and SEL1L (Figure 3.4 B). While SEL1L showed no clear differentiation between isoforms of OS-9, co-precipitated equally with all four splice variants, Grp94 preferentially interacted with the predominate isoforms 1 and 2. These data suggest that the 15 a. a. lost due to differential splicing of exon 11 in both OS-9.3 and OS-9.4 are essential for the interaction with Grp94, and present a context under which the interaction between OS-9 and Grp94 is lost.

To confirm that these interactions occur within the context of an intact ER and are not artefacts of post-lysis interactions, OS-9 isoforms were intentionally mis-localised to the cytoplasm through removal of their signal sequence (Δ ss). Removal of the signal sequence in all cases abolished both interaction with

SEL1L and Grp94 (Figure 3.4 B). These data demonstrate that interactions between OS-9 and Grp94 occur within the ER, that the interaction is isoform specific, and implicates the requirement of a region distal to the MRH domain for binding.

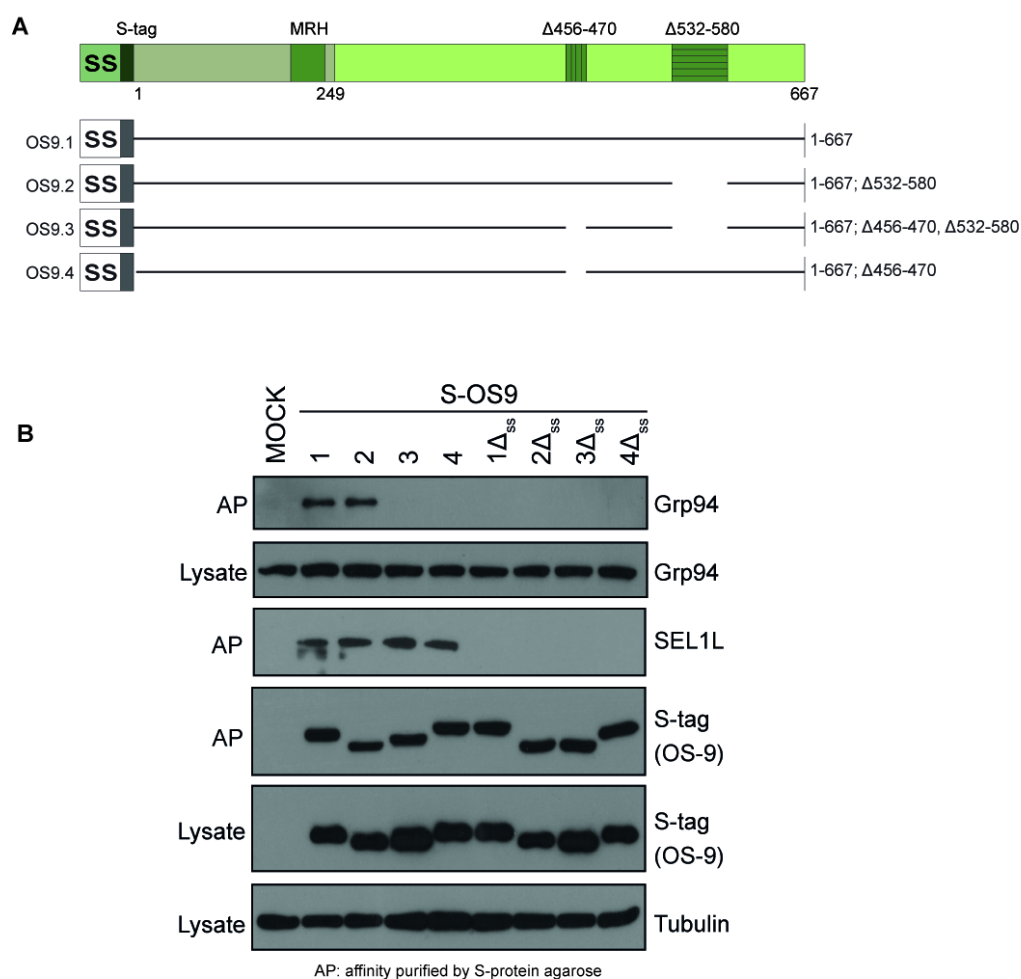


Figure 3.4: Alternative splicing of OS-9 and interaction with Grp94

A) Alternative splicing events that conform to the GT-AG rule could potentially generate 4 variants of OS-9. Signal sequence (SS) and Mannose-6-phosphate receptor homology domain (MRH) are common to all isoforms. B) S-tagged OS-9 constructs were affinity purified and blotted for interactions with endogenous Grp94. Δ ss constructs lack an ER-targeting signal sequence.

3.2.3 Identification of a Grp94 binding region within OS-9

To facilitate future studies aimed at understanding the functional significance of the OS-9/Grp94 interaction we identified the specific OS-9 sequence/region mediating interaction with Grp94. To map the interaction domain, a series of S-tagged truncations and nested deletions were generated for expression in HEK293 cells, and co-precipitation of both Grp94 and SEL1L was monitored (Figure 3.5/3.6).

As Grp94 is itself a glycoprotein, containing at least five confirmed *N*-linked glycosylation sites (of which only a fraction appear to be utilised) we questioned if the MRH domain of OS-9 contributes to the interaction. However, the N-terminal domain of OS-9 (1-248, containing the MRH) failed to co-precipitate Grp94 (Figure 3.6 C). Additionally, an MRH domain mutant OS-9_{R188A} (a mutant that abolishes glycan binding ability) (Hosokawa, Kamiya *et al.* 2009; Satoh, Chen *et al.* 2010), had no appreciable effect on OS-9's ability to co-precipitate Grp94 (Figure 3.7). These data indicate that the glycan binding ability of OS-9 may be independent of its interaction with Grp94, highlighting that the OS-9/Grp94 interaction is not simply an example of an OS-9/substrate interaction. These observations are consistent with reports that Grp94 displays a mannose₈ glycan (Lewis, Mazzaella *et al.* 1985), an oligosaccharide structure that is poorly recognised by OS-9's MRH domain (Hosokawa, Kamiya *et al.* 2009).

Neither OS-9.3 nor OS-9.4 were capable of co-precipitating Grp94 (Figure 3.4 B). As both isoforms are alternatively spliced at exon 11, particular attention was paid to this region (a. a. 456-470) as it appeared to be essential in the context of the OS-9/Grp94 interaction. Further truncations of the C-terminal domain identified a minimal fragment (443-507) that was both necessary and sufficient to co-precipitate Grp94 at levels comparable to full-length OS-9 (Figure 3.7). This region was designated the 94BR (Grp94 binding region) and shall subsequently be referred to as such. A sliding window approach, increasing or decreasing constructs by increments of 10 a. a., was used in the isolation of this Grp94 interaction domain (Figure 3.6 C). Incremental shortening from the N-terminus (S-OS-9.1₄₅₃₋₆₆₇, S.OS-9.1₄₆₃₋₆₆₇, S.OS-9.1₄₇₃₋₆₆₇) abolished the Grp94 interaction indicating that the 10 a. a. 443-453 (or one or more a. a. within this 10 a. a. run) are necessary for OS-9 binding of Grp94 (Figure 3.6 C). Collectively, these truncations indicate the 94BR is sufficient for interaction with Grp94, but more precisely that elements of at least three sections of this region 443-453, 497-507 and 456-470 (absent in OS-9.3 and OS-9.4) are necessary for the interaction (Figure 3.5/3.6).

With neither OS-9.3 nor OS-9.4 compatible for binding of Grp94 (Figure 3.4) it is unsurprising that the 15 a. a. removed through splicing lie within the 94BR (Δ 456-470). It is also noteworthy that fragments that bisect the 94BR (OS-9.1₂₄₉₋₄₈₆ and OS-9.1₄₈₇₋₆₆₇) result in loss of the Grp94 interaction (Figure 3.6 B), suggesting the disruption of an essential secondary structure or the possibility of a bipartite interaction domain within the region. Moreover, these observations

raise the possibility that conditional splicing to generate alternative isoforms could alter the functional capacities of OS-9.

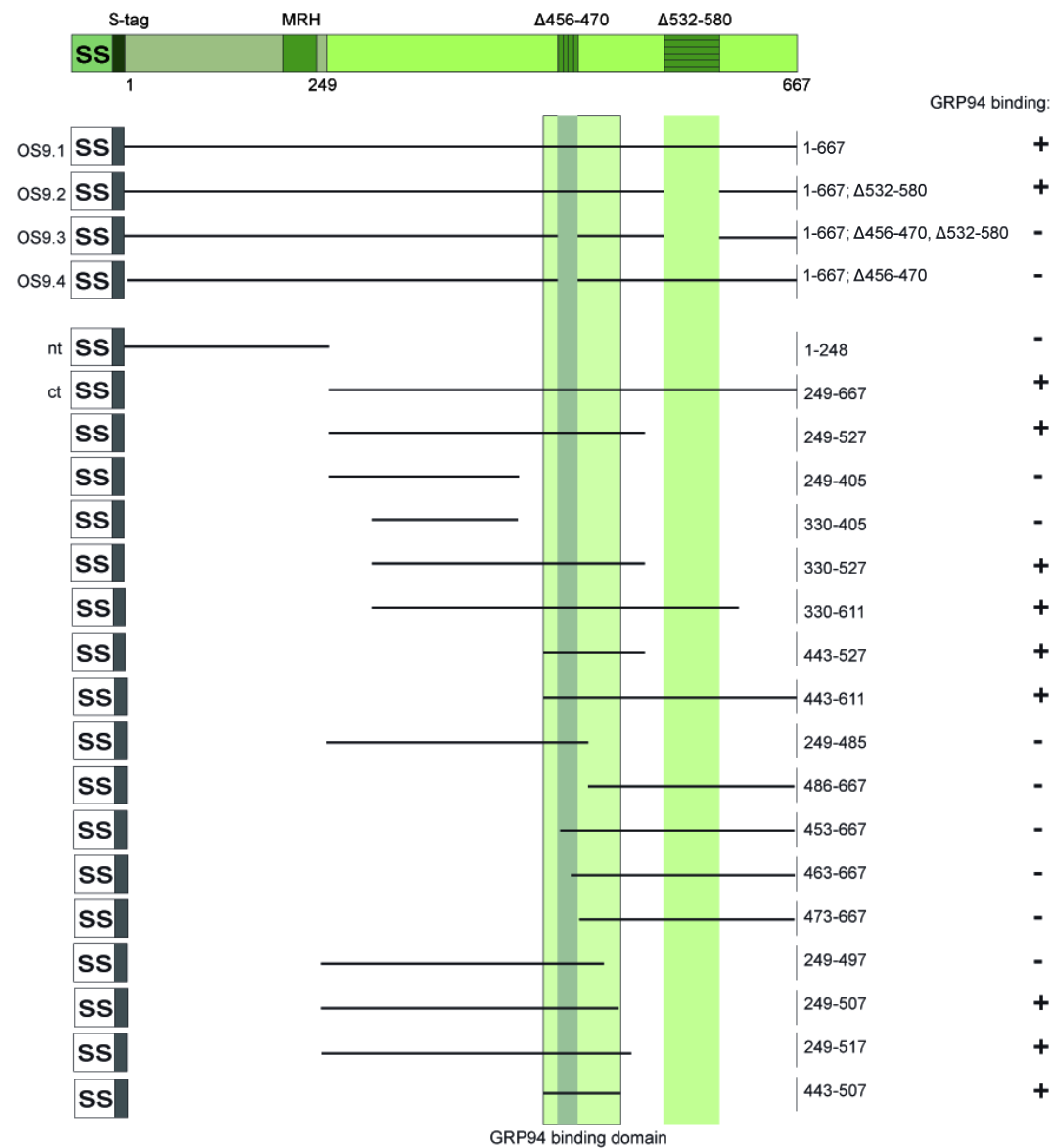


Figure 3.5: Truncation constructs of OS-9
Schematic representation of truncation constructs of OS-9 employed in defining the Grp94 interaction domain of OS-9.

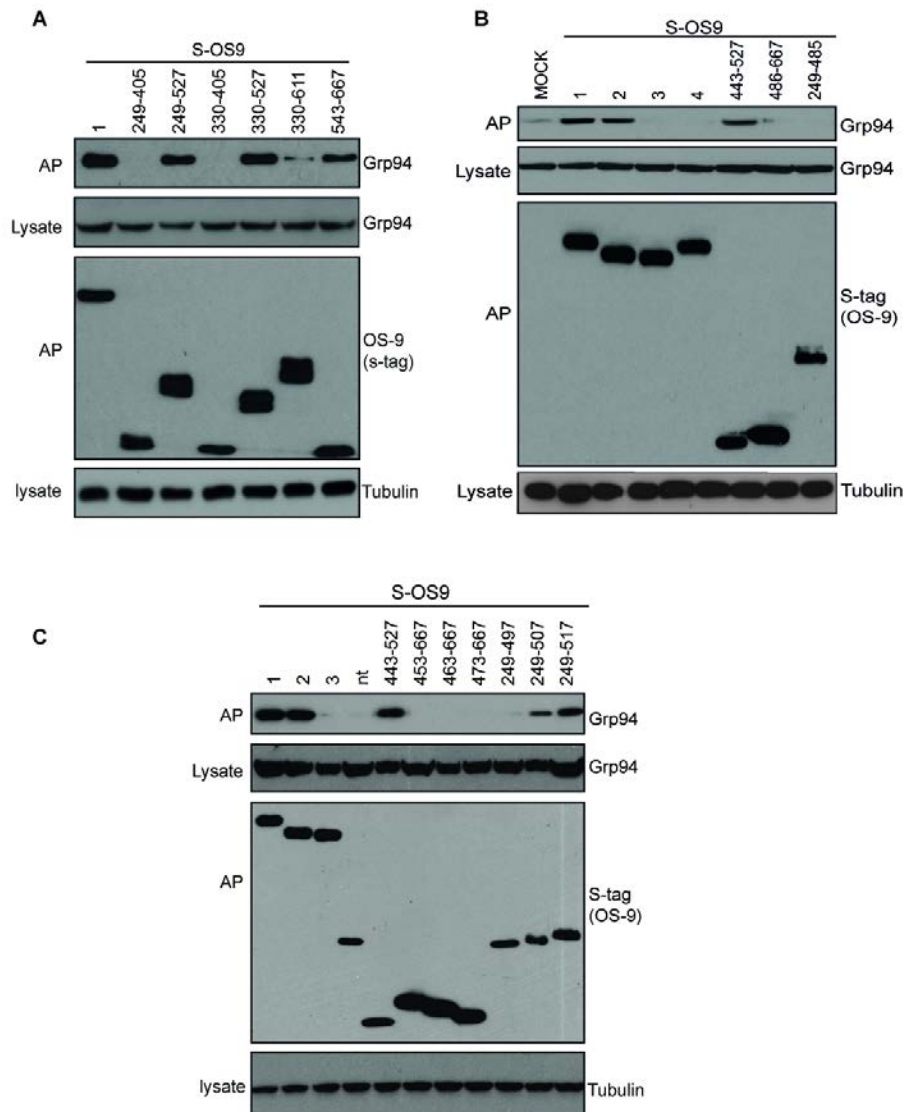


Figure 3.6: Interactions of OS-9 truncations with Grp94

A) Co-precipitation of Grp94 with S-tagged OS-9 truncation constructs, expressed in HEK293 cells, OS-9 complexes were affinity purified by S-protein agarose with western blots probed for Grp94 and S-tag. B) S-OS-9 fragments that bisected the region 443-527 both show an inability to interact with Grp94. C) Sliding window approach identifies a. a. 443-507 as the minimal region of OS-9 necessary for Grp94 interaction. Expression of S-tagged OS-9 isoforms 1-4, and multiple fragments decreasing in length by trimming upwards from a. a. (453, 463, 473-667) or downwards from a. a. 527 (248-517, 507, 497).

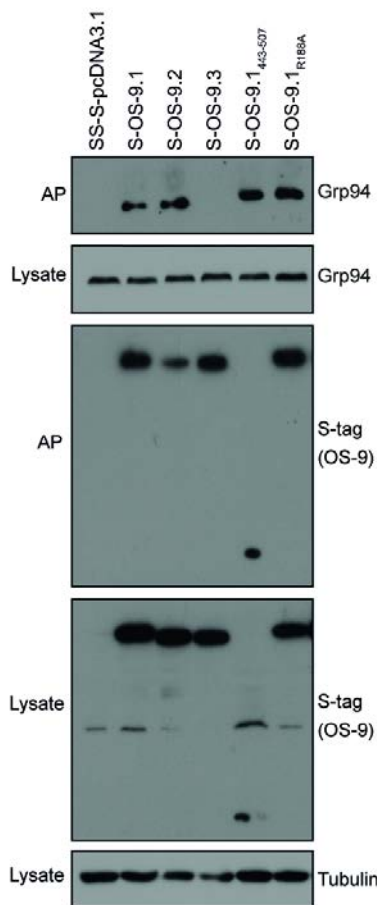


Figure 3.7: Interactions of 94BR and OS-9_{R188A} with Grp94

Co-precipitation of Grp94 with S-tagged OS-9 isoforms 1-3, 94BR and OS-9_{R188A}, expressed in HEK293 cells. OS-9 complexes were affinity purified by S-protein agarose with western blots probed for Gp94 and S-tag.

3.3.4 Architecture of OS-9

Secondary structural predictions for OS-9 (ProteinPredict) indicate an abrupt structural junction at a. a. 249. While the N-terminal region is predominated by the well-defined lectin-like MRH domain, the C-terminal region (249-667 for OS-9.1) is predicted to have lower overall complexity and multiple

regions with a high degree of intrinsic disorder (Disopred/PONDER) (Figure 3.8 A). Subsequently, the MRH domain of OS-9 has recently been crystallised in association with ligand, however, attempts to crystallise the C-terminal domain of OS-9 in its entirety have thus far failed (Sato, Chen *et al.* 2010). The presence of such disorder within the C-terminus raises the possibility that OS-9 may be a natively unfolded protein, and/or a member of the class of proteins known as “intrinsically disordered proteins” or IDPs. Indeed, the lack of a rigid three-dimensional structure may be fundamental to OS-9’s ability to identify and bind to a large, diverse client set. This structural plasticity for the purpose of functional plasticity is seen for example in p21, the cyclin-dependent kinase (CDK) inhibitor whose conformational disorder facilitates binding to and thus the inhibition of a diverse family of CDKs (Kriwacki, Hengst *et al.* 1996; Wang, Fisher *et al.* 2011). OS-9 also contains a disproportionately high quantity of negative charge in its non-MRH domain, including an acidic region, prevalent in Asp and Gln residues (a. a. 414-429, EEEEEDEDEDEDEDER). The overall protein (OS-9 isoform 1) is acidic (pI = 4.8) with more than 25 % of the C-terminal region (a. a. 249-667) comprised of E/D residues.

The 94BR resides within the C-terminal half of OS-9. Secondary structure algorithms (PsiPred (Jones 1999), Jpred (Cuff and Barton 2000), Phyre) predict two extended alpha-helical domains (448-471, 485-502), separated by a short unstructured linker region within this 65 a. a. stretch (Figure 3.8 B). The inability to bisect or reduce the domain without compromising Grp94 interaction suggests

that the interaction is not linear but conformational, with mutual contribution of both these helices to form the structure required for binding.

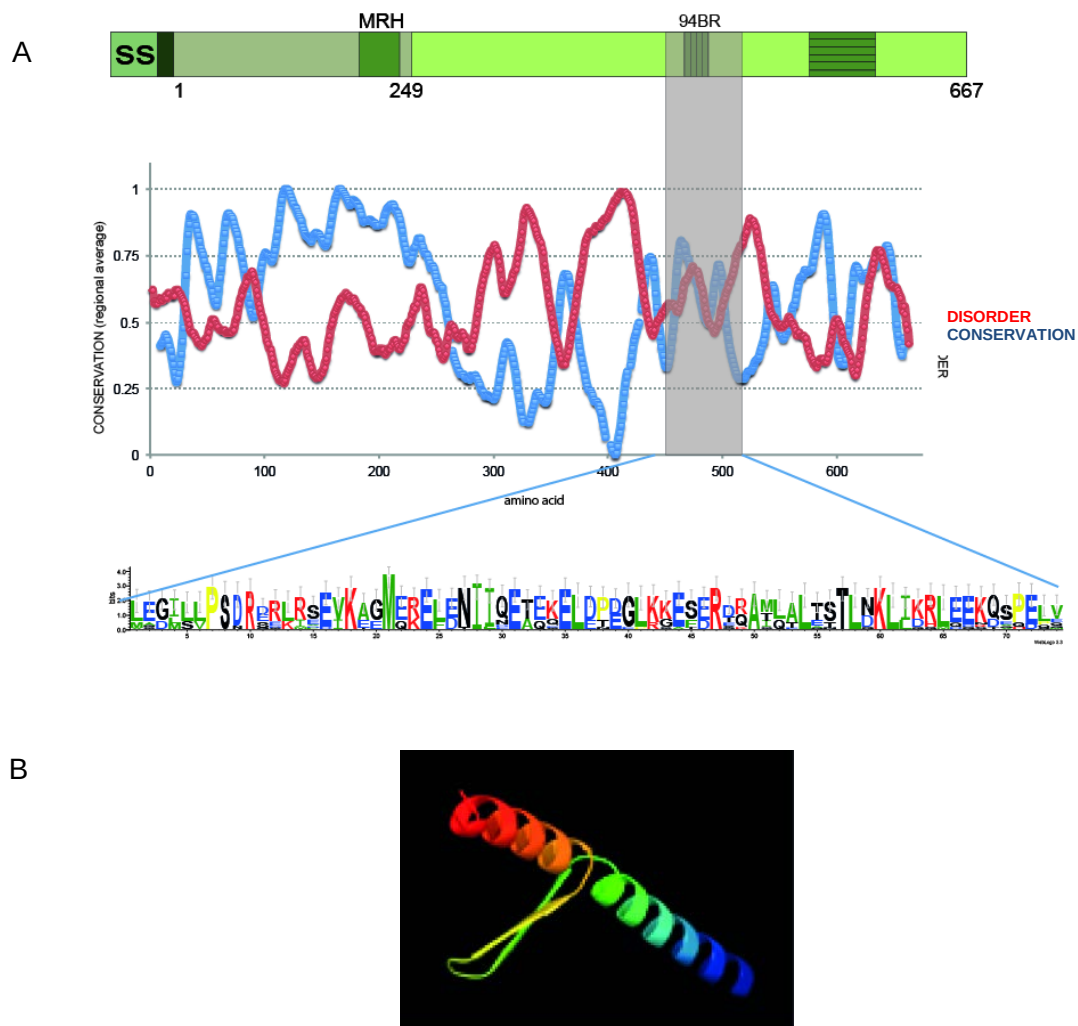


Figure 3.8: Prediction of conservation and disorder of OS-9_{94BR}

A) Prediction of disorder (red) and conservation (blue) of the 94BR. B) Two extended alpha-helical domains (448-471, 485-502) with a short unstructured linker region are predicted within the 94BR. Phyre prediction of OS-9 a. a. 435-510.

3.2.5 Conservation of OS-9 94BR

OS-9 is highly conserved at the a. a. level across metazoans and functionally in eukaryotes, with as much as 43 % identity between disparate chordates (Figure 3.9 A). OS-9 shares functional similarities with orthologues in

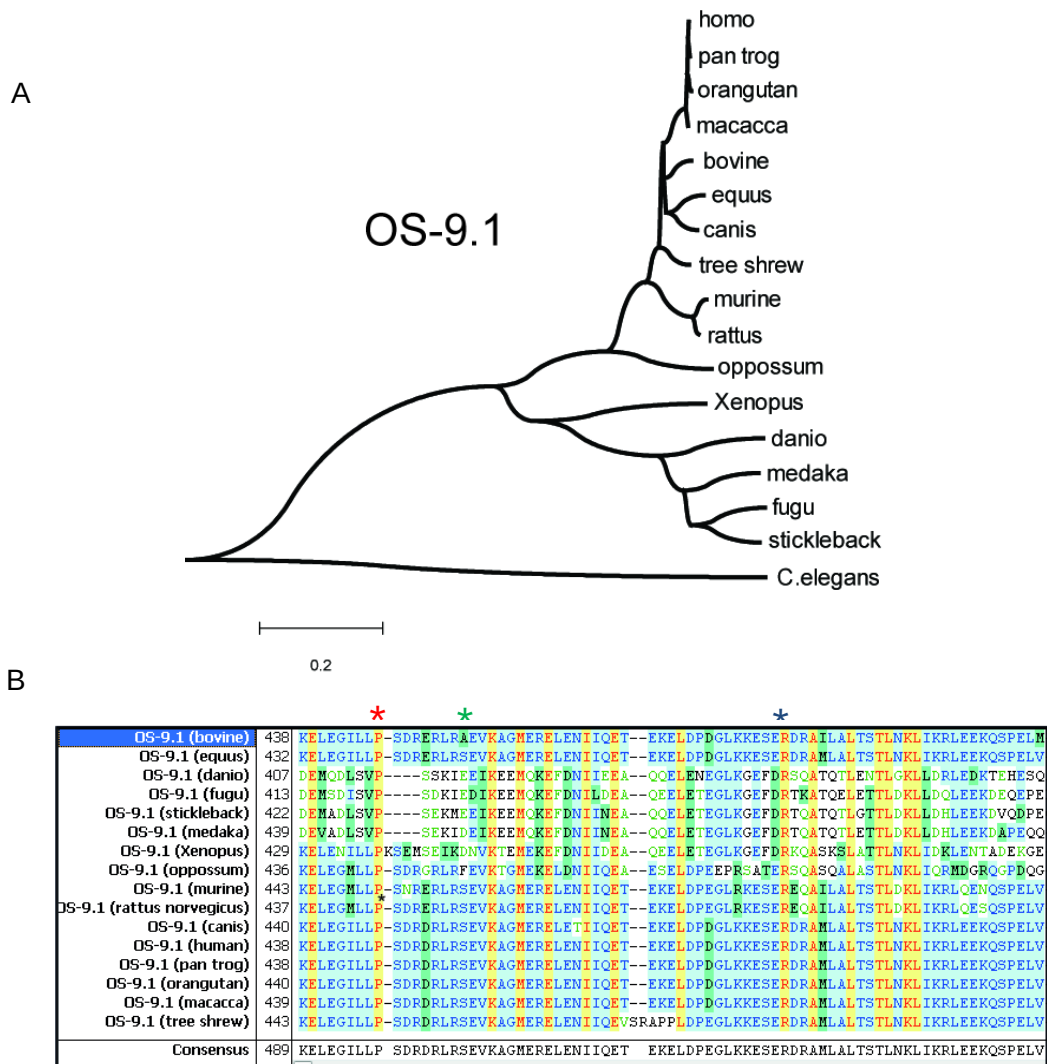


Figure 3.8: Evolutional conservation of OS-9_{94BR}

A) Evolutional conservation of the OS-9.1. B) Conservation of 94BR with point mutations highlighted, P446 (*), S454 (*) and R486 (*).

unicellular eukaryotes (Yos9p), however, their sequence similarity is approximately only 15 %. This is predominately reflective of their conserved MRH domain, with subsequently diminished sequence similarity within their C-termini. The 94BR sequence shows no appreciable homology to any known binding sites, however, it is highly conserved between higher eukaryotes (Figure 3.9 B). In particular, the proline residue at position 446 is highly conserved (Figure 3.9 B, indicated by red asterisk). The consequence of mutating this and other residues within the 94BR will be investigated in the following section (3.2.6).

3.2.6 Point mutations within the 94BR abolish Grp94 binding

Naturally occurring OS-9 variants of the 94BR detected in the human population in the forms of somatic mutations (P446L) (Vigneron, Ooms *et al.* 2002) and single nucleotide polymorphisms (SNPs) (S454L, Q486R) were employed to further investigate the specificity of OS-9's interaction with Grp94. The somatic mutation, P446L, was identified in the melanoma cell line LG2-MEL after an OS-9 peptide fragment was presented as a CTL-antigen (cytotoxic T-lymphocyte). In a screen of 185 tumour samples, Vigneron *et al.*, identified an antigen, LG2-C, which was recognised by autologous cytotoxic T-lymphocytes (CTLs) and is produced by a point mutation in the OS-9 gene (Vigneron, Ooms *et al.* 2002). Antigen processing occurs in the cytosol prior to TAP complex-mediated transport and loading of MHC molecules in the ER (Hansen and Bouvier 2009), raising the question of how an ER lumen protein gained access

to the cytosol in the first place (this will be address in further detail in Chapter 5). The OS-9 mutation in the melanoma cell line LG2-Mel is a C→T transition at base 1337 of the ORF, converting residue 446 from a proline to a leucine (P446L). As this mutation occurs within the 94BR, we sought to ascertain the P446L mutant's

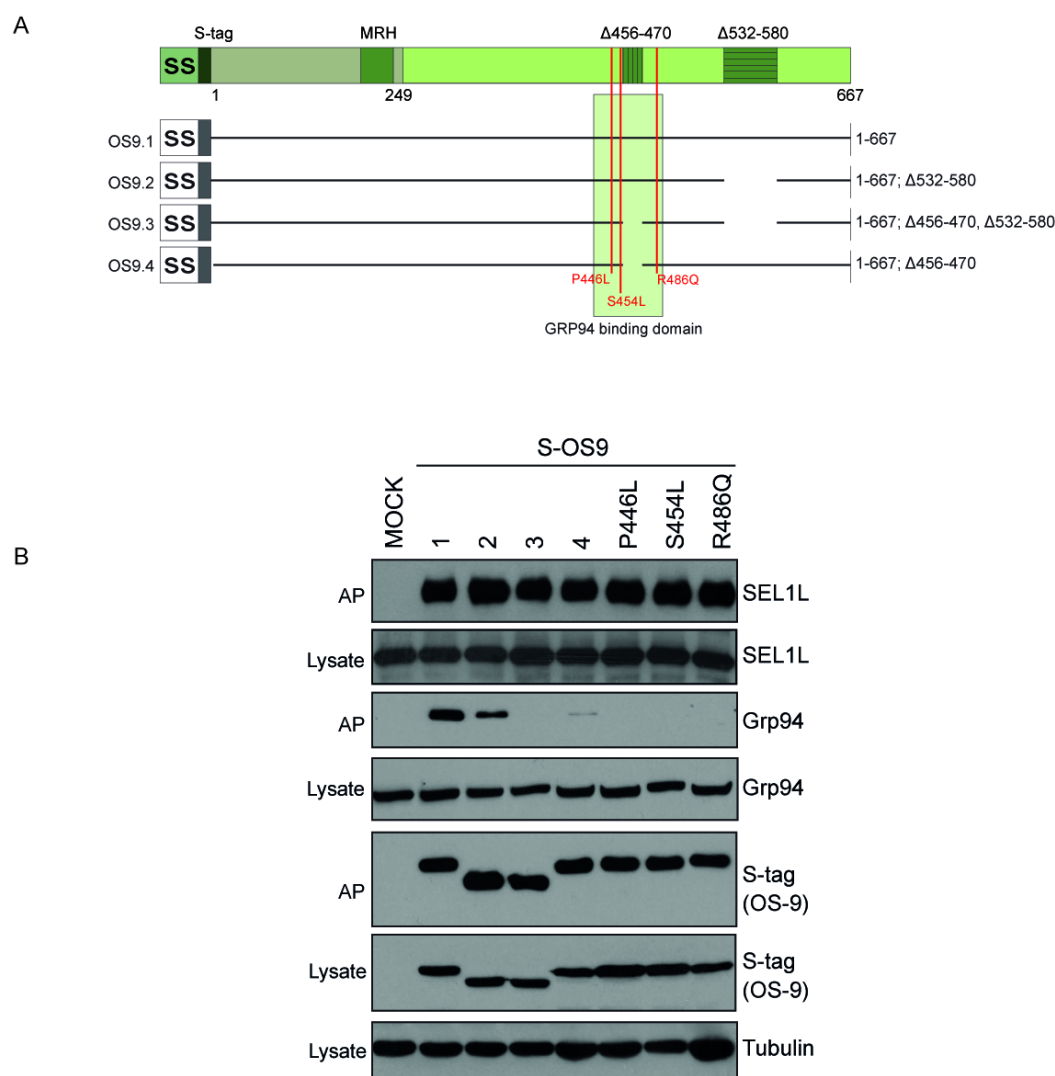


Figure 3.10: Point mutants within the 94BR abolish Grp94 binding

A) Schematic representing the locations of point mutants within the 94BR. B) Co-precipitation of Grp94 with S-tagged OS-9 truncation constructs and point mutant constructs P446L, S454L and R486Q, expressed in HEK293 cells. OS-9 complexes were affinity purified by S-protein agarose with western blots probed for Grp94 and S-tag.

ability to bind Grp94. Indeed, S-OS-9.1_{P446L} displayed a significantly reduced capacity to co-precipitate endogenous Grp94 when compared to the wild type (Figure 3.10 B). As mentioned, P446 is absolutely conserved in metazoans, down to fish (*Danio*, fugu, stickleback) (Figure 3.9 B).

A second naturally occurring variant of the 94BR was detected in the human population in the form of somatic mutation S454L. A point mutation reported in the International HapMap Project (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=76966193) occurs as a C→T mutation at base 1361 in the ORF, resulting in a serine to leucine conversion (S454L). S454 is not as highly conserved as P446, however, it does show a high degree of conservation in higher eukaryotes (Figure 3.11 B). This SNP conversion from a C to a T allele accounts for only 1 % of analysed cohorts, however, it was solely represented in cohorts of African descent. The African population data is composed of three cohorts; AWS (Americans of African ancestry in SW USA), LWK (Luhya in Webuya, Kenya) and YRI (Yoruba in Ibadan, Nigera) each with varying frequency of the T allele; 6 %, 2 % and 12 %, respectively (Figure 3.11 C). However, no correlation with disease has been reported for this mutation. The S-tagged OS-9_{S454L} mutant also showed drastically reduced binding to Grp94, comparable to OS-9.3 and OS-9_{P446L} (Figure 3.10 B).

Finally a third mutant, R486Q, was chosen as it too falls within the 94BR. This SNP, the result of a G to A conversion (G1664A), was reported in two cohorts from the NHLBI Exome Sequencing Project (National Heart, Lung, and

Blood Institute). Present in less than 0.001 % of analysed genomes of European-Americans, however, was absent from cohorts of individuals of

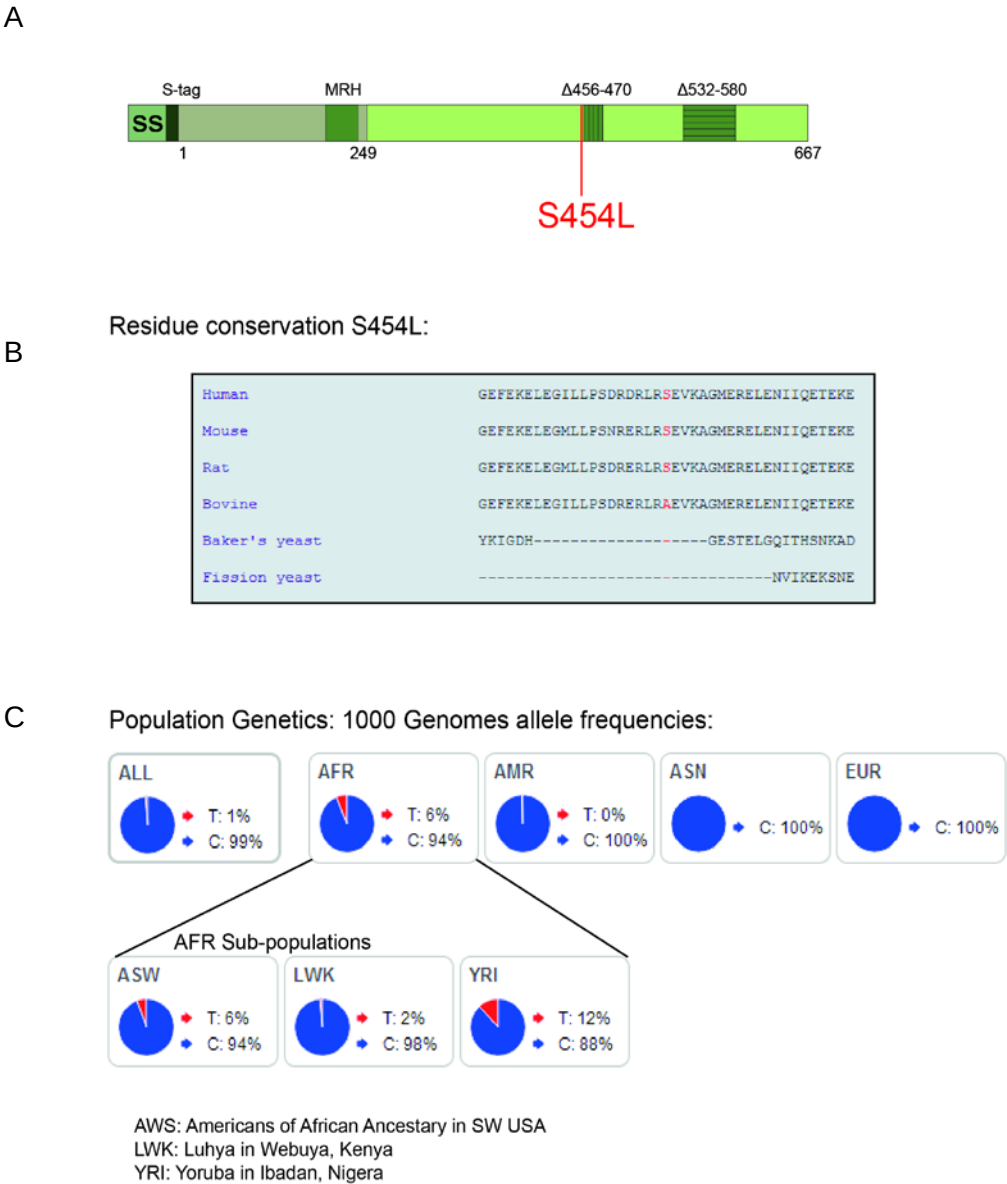


Figure 3.11: Conservation and incidence of the S454L mutant
 A) Schematic representing the location of the S454L mutant. B) Conservation of S454L. C) HapMap cohorts: AWS (Americans of African ancestry in SW USA), LWK (Luhya in Webuya, Kenya) and YRI (Yoruba in Ibadan, Nigeria). Expression of the S454L OS-9 SNP.

African-American origin. Similar to S454L, no correlation with disease has been reported for this mutation. Loss of interaction in all cases suggests that either these residues directly participate in binding to Grp94, or that their mutation indirectly disturbs a structural motif required for binding. Indeed, the replacement of a proline by a leucine residue, as seen in P446L, would be predicted to modify protein structure since proline residues are known to induce protein kinks due to their rigid cyclic lateral chain (Woolfson and Williams 1990). However, including the point mutations, together or separately, in the input sequence in the structural prediction algorithm PSIPRED had no effect on the predicted secondary structure, which remained as two extended alpha-helical domains with a short unstructured linker region within the 94BR (Figure 3.12 B). It should be considered though that structural prediction algorithms are far from absolute. To truly gain an understanding of the protein structures mitigating the OS-9/Grp94 interaction in vivo studies, e.g. NMR (Nuclear Magnetic Resonance), would be required however there were beyond the scope of this thesis.

3.2.7 OS-9 expression in LG2-MEL cells

To establish how a disruption of the OS-9/Grp94 interaction may affect function, mutant OS-9 in the melanoma lines LG2-MEL-5-35 and LG2-MEL-207 was compared to wild type OS-9 in HEK293 and 501MEL cells. LG2-MEL-5-35 and LG2-MEL-207 are both sublines of the melanoma line LG2-MEL (Vigneron, Ooms *et al.* 2002). Initially, to confirm the presence of the P446L mutation, a PCR of total cDNA was performed utilising a reverse primer that generated an

HpaII site (CCGG) on amplification of wild type OS-9, however the restriction site was abolished in the context of the P446L point mutation (Figure 3.13 A). LG2-MEL-207 is heterozygous, containing both wild type and mutant OS-9 transcripts, while homozygous LG2-MEL-5-35 expresses only OS-9_{P446L}. As the P446L mutation has previously been established as a disruptor of OS-9's interaction with Grp94, these two cell lines were used as a means to probe the consequences of a reduction or loss of interaction between OS-9 and Grp94 *in vivo*.

To assay the phenotypic consequences of the loss of the OS-9/Grp94 interaction, proliferation rates of the two cell lines were compared by direct counting of cells seeded at equal density over the course of five days (Figure 3.13 B). LG2-MEL-5-35 (P446L only) was found to have a faster doubling time when compared to LG2-MEL-207 (P446L and wild type) (Figure 3.13 B). The increased proliferation rate may be a consequence of disrupting the Grp94/OS-9 interaction in these cells, and could contribute to aspects influencing tumour progression. To confirm the initial finding, a second proliferation assay was used employing the intracellular fluorescent dye carboxyfluorescein succinimidyl ester (CFSE), with detection via flow cytometry. CFSE penetrates the plasma membrane and binds to cytosolic components when cell division takes place; CFSE is equally distributed to both daughter cells, leaving the daughter cells with half the original amount of CFSE. Thus, when analysed by flow cytometry, dividing cells will emit lower CFSE fluorescence than non-dividing cells, with a decreased CFSE reading proportional to the number of cell divisions. In

agreement with the prior experiment, the homozygous LG2-Mel-5-35 cell line demonstrated a decreased doubling time (and conversely a more rapid loss of CFSE) in comparison to that of heterozygous LG2-Mel-207 cells (Figure 3.14).

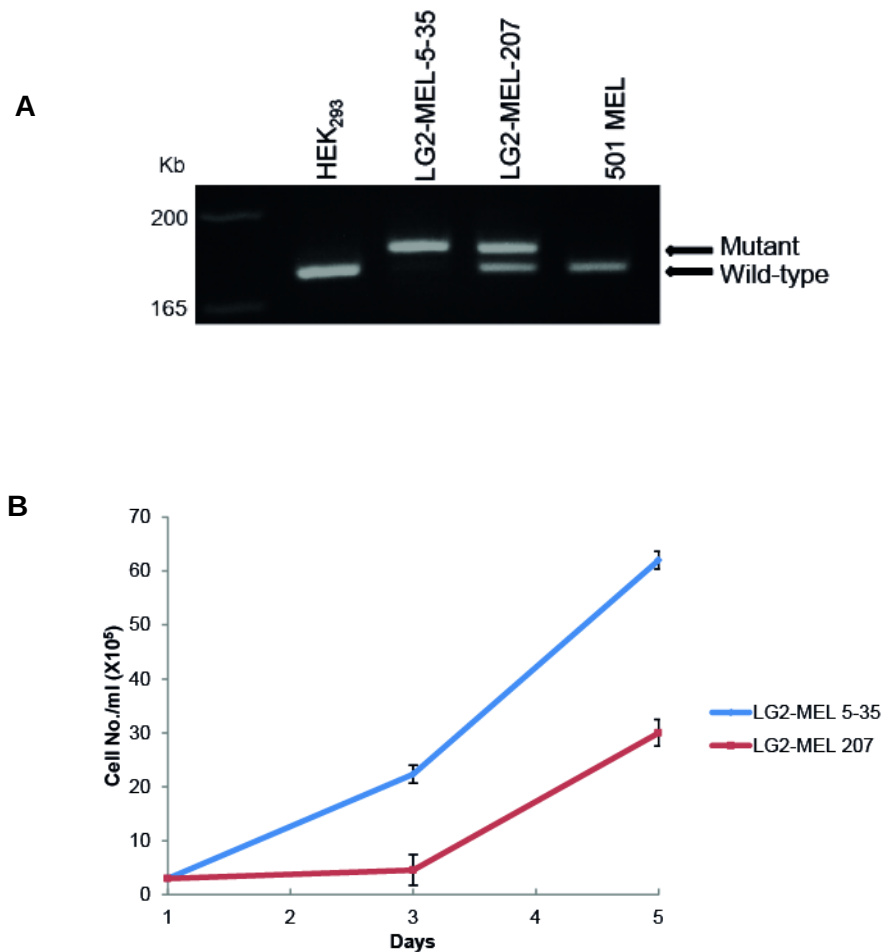


Figure 3.13: LG2-MEL cell lines

A) PCR of total cDNA from LG2-MEL-5-35 and LG2-MEL-207 cells. The reverse primer creates an *HpaII* restriction site CCGG in the 154 bp wild type PCR product, producing two fragments of 130 bp and 24 bp on digestion, whereas the mutated product yields a single band of 154 bp. Products were separated by electrophoresis on a 3 % agarose gel. B) Comparative growth of LG2-MEL-5-35 (blue) and LG2-MEL-207 (red) cells derived by directed cell counting over a five day period. Equal numbers of cells were seeded on day one.

CFSE 1: LG2-Mel-5-35 vs LG2 MEL 207

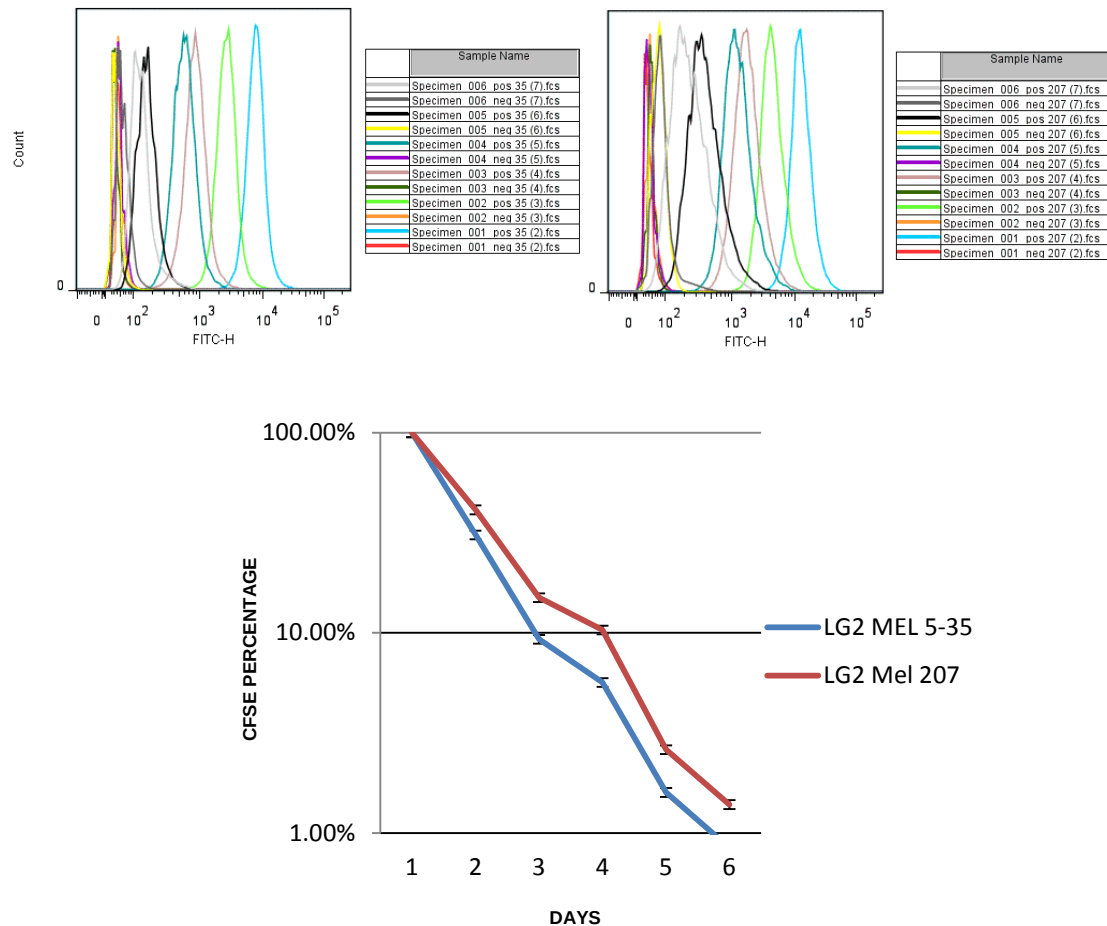


Figure 3.14: LG2-MEL cell lines comparative growth - CFSE

Comparative growth of LG2-MEL-5-35 (blue) and LG2-MEL-207 (red) using CFSE, analysed by FACS.

However, it is unclear whether this increase in growth rate could be directly attributed to loss of interaction between OS-9 and Grp94. To evaluate the expression of endogenous OS-9 in the LG2-MEL lines, western blots of cell lysates were performed with OS-9 antibody (Figure 3.15). OS-9 typically runs as two bands corresponding to OS-9.1 and OS-9.2 (as seen in the 501-MEL lane), however, both LG2-MEL lines displayed reduced levels of OS-9.1 (Figure 3.15 A). In addition, the membrane was probed with a panel of antibodies to known

transcription factors commonly elevated in melanoma including PAX3, BRN2 and TBX3 (Figure 3.15 A/B). Initial experiments displayed a reduction or lack of all three markers in LG2-MEL-207 when compared to LG2-MEL-5-35 and the positive control 501-MEL; indicating that LG2-MEL-207 lacks many of the hallmarks of melanoma. This finding is surprising, since both are presumably sublines derived from the same tumour. Conversely, LG2-Mel-5-35 expressed all three markers and had increased proliferation (Figure 3.15 A/B). However, the outstanding question remains whether this increased expression of melanoma markers and the increased proliferation phenotype observed are the cause or consequence of mutant OS-9 expression, and whether or not this is correlated with decreased Grp94 interaction.

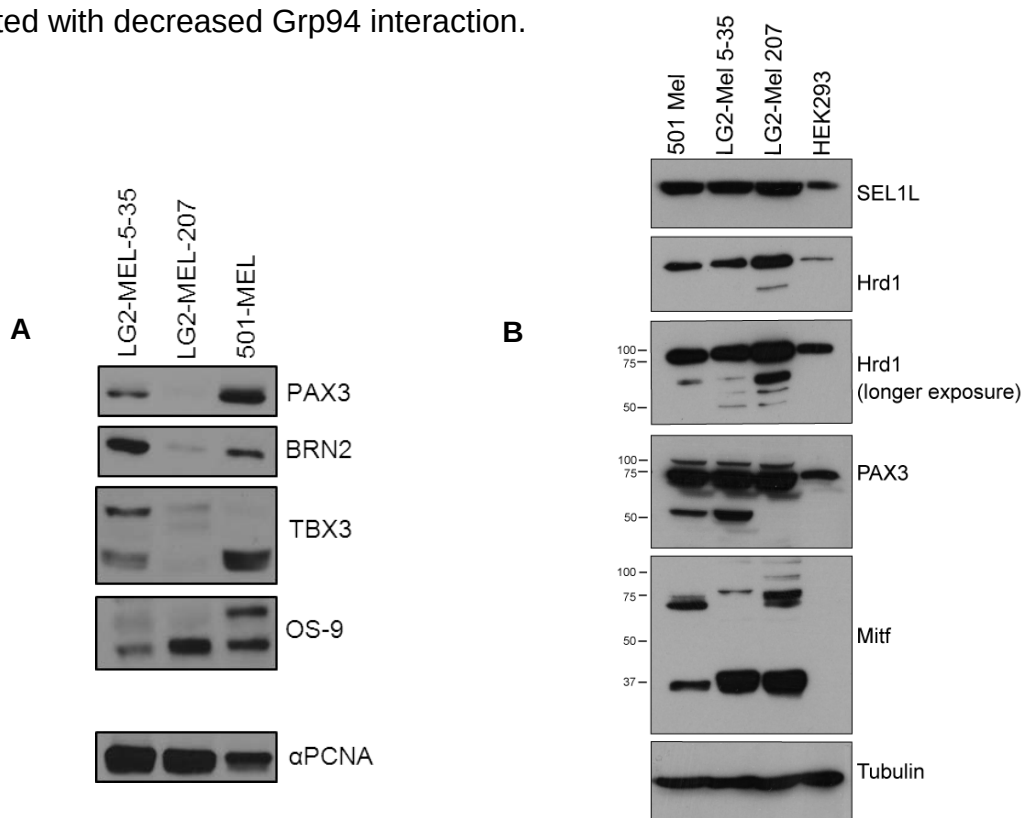


Figure 3.15: LG2-MEL cell lines expression analysis
Comparison of expression levels of ERAD components and melanoma markers from LG2-MEL-5-35, LG2-MEL-207, 501-MEL and HEK293 lysates.

3.2.8 Differential splicing of OS-9

Alternative splicing events that conform to the GT-AG rule could potentially generate four variants of OS-9 (Kimura, Nakazawa *et al.* 1997), where splicing of exon 13 ($\Delta 535-589$) yields OS-9.2, partial splicing within exon 11 ($\Delta 456-470$) produces OS-9.4 and a combination of the two splicing events ($\Delta 535-589$, $\Delta 456-470$) generates the OS-9.3 isoform. To date, however, the existence of OS-9.3 has been disputed and the existence of OS-9.4 has yet to be confirmed (Kimura, Nakazawa *et al.* 1997; Bernasconi, Pertel *et al.* 2008; Jansen, Eleveld-Trancikova *et al.* 2009). Early studies observed transcripts corresponding to three isoforms (OS-9.1, 2 and 3) (Kimura, Nakazawa *et al.* 1998), while more recent work detected only the products of exon 15 splicing, OS-9.1 and OS-9.2 mRNA and proteins (Bernasconi, Pertel *et al.* 2008), calling into question the existence of a second splicing event at exon 11b. To resolve this discrepancy and determine whether OS-9 splice variants are expressed in a cell-specific manner, total cellular cDNA pools from a panel of cell types were amplified with primers specific for sequences flanking the region of alternative splicing. Annealing sites more distant from putative splicing interfaces than the above mentioned studies were employed.

The initial results from total HEK293 cDNA produced four bands that migrated at sizes comparable to what would be expected for each of the four OS-9 isoforms (Figure 3.16 A). Bands were excised and cDNA sequenced with results identical to the sequences predicted for each isoform, confirming that four distinct isoform sequences of OS-9 had indeed been amplified. Although

not quantitative, the band corresponding to OS-9.2 was the most prominent in HEK293 and MCF7 cells, consistent with previous observations of OS-9.2 as the most prevalent isoform at the protein (Kimura, Nakazawa *et al.* 1997) and transcript level (Bernasconi, Pertel *et al.* 2008).

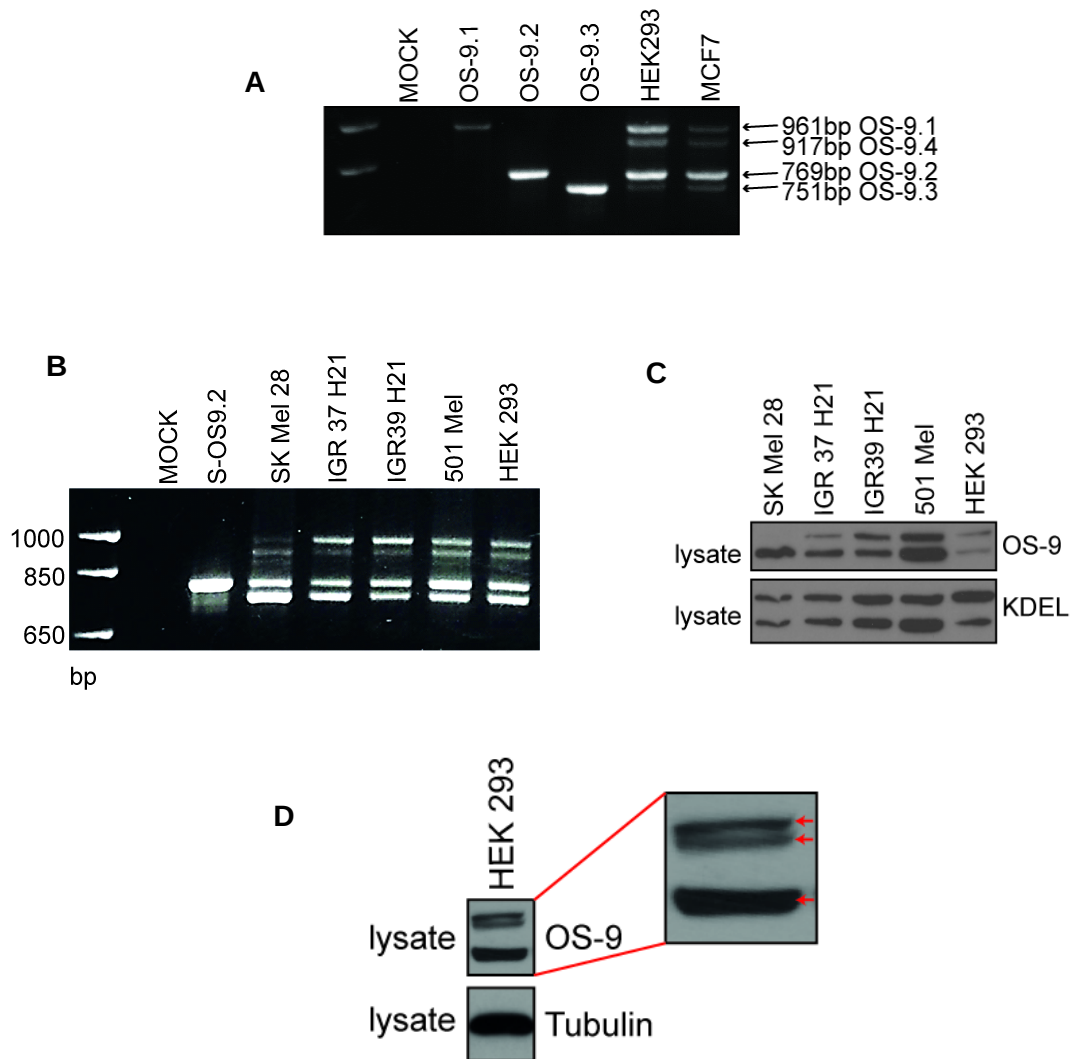


Figure 3.16: Differential splicing of OS-9 isoforms

A) PCR of total cDNA with primers specific for the differentially spliced region of OS-9, resulting in four different product sizes dependent on the isoform. Amplification of total cDNA in HEK293 and MCF7 cells revealed four bands corresponding to the expected sizes of each of the isoforms. B) Amplification of cDNA from a number of cell lines, most notably SK-Mel-28 cells, displays an increase in the band corresponding to OS-9.3. C) Panel of cell lysates probed for OS-9 and tubulin D) Separation of OS-9 into at least three bands.

Resolving all of the endogenous OS-9 isoforms from cell lysates can be problematic, as adequate separation by SDS-PAGE is difficult to attain. The problem is compounded by the aberrant migration of OS-9 by SDS-PAGE, producing an apparent molecular mass of approximately 97 kDa, despite having a predicted molecular mass of 73 kDa. A predicted/degenerate TEV site (consensus: ENLYLG/Y) at position 149 (ENLYFQ/G) was investigated as a possible strategy to reduce the molecular weight of OS-9, so as to enhance the differentiation of different isoform bands on SDS-PAGE. However, treatment of cell lysates with TEV protease induced no discernible effect on the migration pattern of the OS-9 isoforms. Nonetheless, utilisation of larger gel formats allowed the visualisation of at least three OS-9 antibody reactive bands corresponding to OS-9.1, OS-9.2 and OS-9.4 (Figure 3.16 D).

3.2.9 Modulation of OS-9 splicing

Both ectopically expressed OS-9.3 and OS-9.4 have already been demonstrated to be unable to co-precipitate Grp94 in this study, and alternative splicing of exon 11 of OS-9 has the potential to endogenously produce these non-Grp94-interacting isoforms. The presence of four OS-9 isoforms, only two of which interact with Grp94, raises the intriguing question of how alternative splicing of OS-9 may regulate OS-9 function by disrupting its interaction with Grp94 and, if OS-9 with varying functionality is differentially produced, whether this is dependent on cell type or due to specific triggers. Indeed, alternative splicing can be specific to a tissue or cell type, a developmental stage or be

induced by a particular external stimulus (Stamm 2002). To establish whether the expression pattern of OS-9 isoforms was generated in a cell type-specific manner, cDNA from a panel of cell lines was generated and analysed by RT-PCR (Figure 3.17). All cell lines examined produced four amplified bands, comparable to what would be expected for each of the four OS-9 isoforms. Interestingly, the human melanoma SK-MEL-28 line displayed an apparent altered ratio of cDNA expression in comparison to other cell lines tested, with an increase in the band corresponding to the OS-9.3 isoform, suggesting an increased level of exon 11 splicing in these cells (Figure 3.15). However, it is not clear what the cause and consequence of this apparent difference is.

Genomic editing is often a phenomenon that is dependent upon cellular conditions, so the possibility that splicing of exon 11b was a conditional event, occurring in response to cellular stress inducers, was explored. To determine whether a particular stress-inducing condition triggers differential splicing of OS-9, HEK293 cells were treated with a potent Map kinase inhibitor (U0126 ethanolate) and UV irradiation. MAP kinases (mitogen-activated protein kinases) are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens, osmotic stress, heat shock and proinflammatory cytokines) and are implicated in the regulation of various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis (Pearson, Robinson *et al.* 2001). The MAPK pathway has previously been demonstrated to regulate alternative splicing, for example it regulates splicing of CD44 through phosphorylation of the splicing factor SAM68 (Matter, Herrlich *et*

al. 2002). Treatment with U0126 ethanolate, a specific inhibitor of MEK1 and MEK2 (MAP kinase kinase; MAPKK) for 2 hrs, 24 hrs and 48 hrs did not alter the OS-9 splicing (Figure 3.17 A). Similarly, UV treatment of MCF7, 1205Lu and Colo-800 cells for 24 hrs showed no marked change in splicing (Figure 3.17 B). Although, a range of OS-9 isoform profiles were clearly discernible when different human cell lines were compared, a shift in splicing profiles was not detected under MAP kinase inhibition or UV irradiation. It should be noted that as only one dosage of UV was used, it may be that conditions employed in this analysis were not sufficient to induce a detectable change.

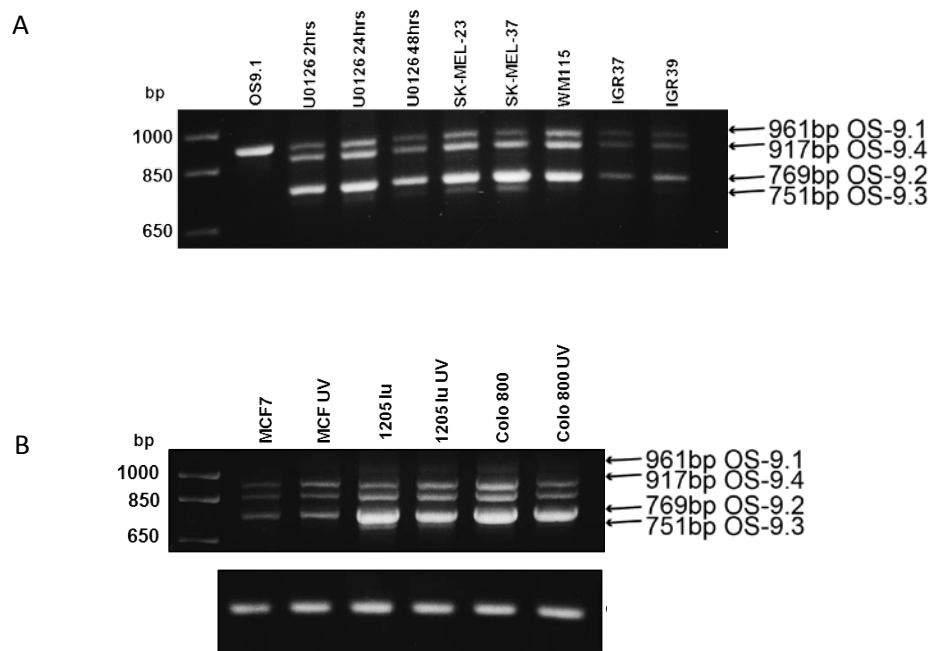


Figure 3.17: Modulation of differential splicing of OS-9 isoforms

A) Lanes 3, 4 and 5 show cDNA from HEK293 cells treated with the MAP kinase inhibitor U0126 ethanolate for 2, 24 and 48 hrs, respectively. Lanes 6-10 show amplification from a number of cell lines. B) MCF7, 1205Lu and Colo-800 cells both untreated and treated with UV for 24 hrs.

Similarly, OS-9 splicing may be induced under a specific stress: γ -irradiation, glucose deprivation, nutrient starvation and heat-shock are all possible candidates and their effect on OS-9 splicing will need to be investigated further.

2.2.10 Does OS-9 depend on Grp94 for localisation or stability?

The highly specific nature of the OS-9/Grp94 interaction has been highlighted in this chapter; however, the functional role of this interaction remains unknown. OS-9 lacks a canonical ER-retention/retrieval signal; Grp94, which contains a KDEL sequence, may confer ER localisation to OS-9, ensuring its retention within the ER. To determine the localisation and stability of OS-9 in the absence of Grp94, Grp94 knockdown cell lines were generated by shRNA

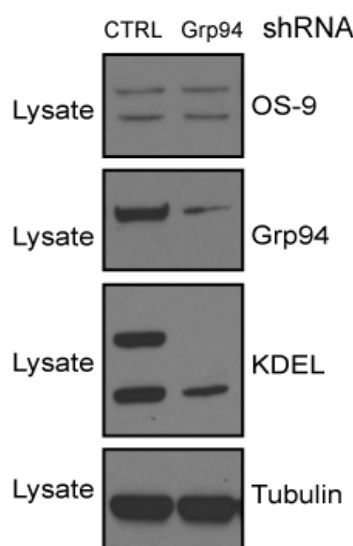


Figure 3.18: Knockdown of Grp94 in HEK293 cells

HEK293 cells stably down-regulated for endogenous Grp94. KDEL antibody is reactive to Grp94 (upper band) and BiP/HSP70 (lower band).

expression and selected under puromycin. Steady-state OS-9 levels were compared with those present in HEK293 cells, however, no difference could be observed via western blotting (Figure 3.18).

Localisation of transfected S-OS-9.1_{94BR} and S-OS-9.3 was assessed fluorescently. S-tagged constructs were transiently transfected into U2OS cells, probed with antibodies to the S-tag and the ER marker calnexin (CNX). S-OS-9_{94BR} was indeed seen to display a pattern indicative of ER localisation (Figure 3.19), while S-OS-9.3 showed a slightly more diffuse pattern. It is tempting to attribute OS-9.3 mislocalisation to loss of interaction with Grp94; however, it should be considered that transfection of OS-9.3 constructs may induce non-physiological effects.

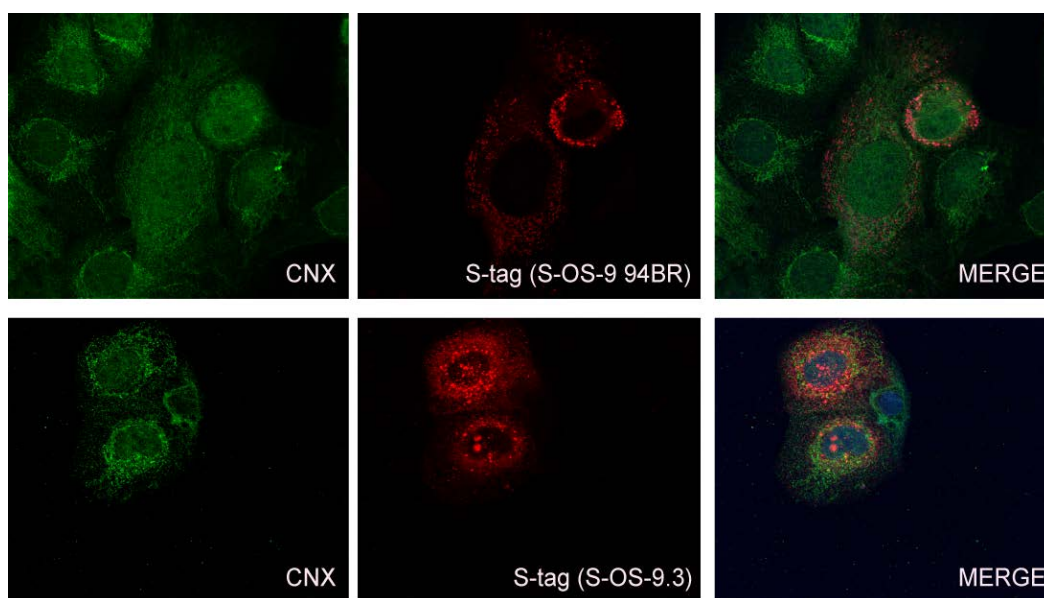


Figure 3.19: Localisation of OS-9.3 and 94BR.

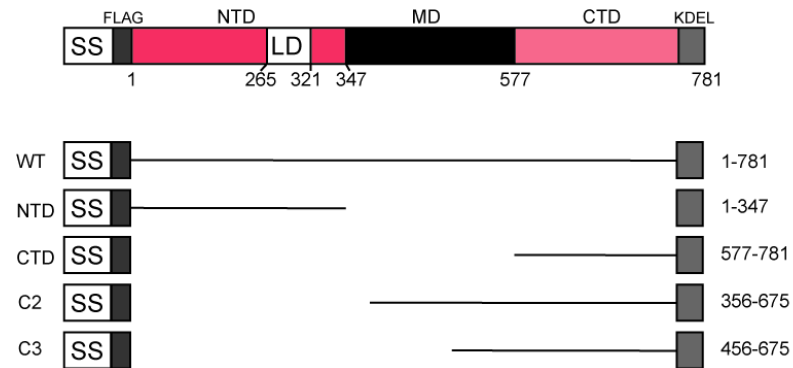
Co-localisation of transfected S-tagged S-OS-9 94BR with the endogenous ER-resident protein calnexin (red), visualised by immunofluorescence in U2OS cells.

3.2.11 Is Grp94 a substrate of OS-9?

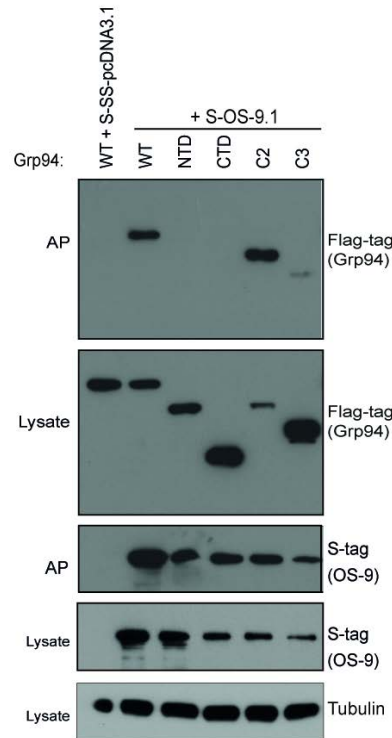
In conjunction to the work described to identify the region of OS-9 that mediates its interaction with Grp94, the lab of our collaborator Dr. Yair Argon sought to identify the region of Grp94 responsible for interacting with OS-9. This complementary work has helped to further expand our understanding of the OS-9/Grp94 interaction. Using Flag-tagged Grp94 truncation mutants (generously supplied by Dr. Yair Argon) co-transfected with S-tagged OS-9, it was confirmed that a region within the middle domain (MD) is required for co-precipitation of OS-9 (Dersh, Jones *et al.* 2014) (Figure 3.20). This region has not been implicated in binding to previously reported Grp94 interactors or clients, and maps to a small β -sheet and a long, exposed α -helix (Dersh, Jones *et al.* 2014).

Very little is known regarding the Middle domain of Grp94, apart from its requirement in association with the N-terminal domain (NTD) to form the ATPase active site of Grp94 (Figure 3.20) (Meyer, Prodromou *et al.* 2003; Chu, Maynard *et al.* 2006; Dollins, Warren *et al.* 2007). The corresponding MD of cytosolic Hsp90 contains multiple binding sites, including one for the cofactor Aha1, which accelerates the ATPase activity of Hsp90. However, as Aha1 shares no homology with OS-9 it is unlikely that the interaction functions in the same manner (Lotz, Lin *et al.* 2003, Meyer, Prodromou *et al.* 2003). Prior to this mapping, the only identified cofactor of Grp94 was CNPY3 (required for Toll-like receptor folding by Grp94), which binds within Grp94's NTD (Liu, Yang *et al.* 2010). OS-9, therefore, is the first protein identified to bind Grp94 via the MD (Dersh, Jones *et al.* 2014).

A



B



C

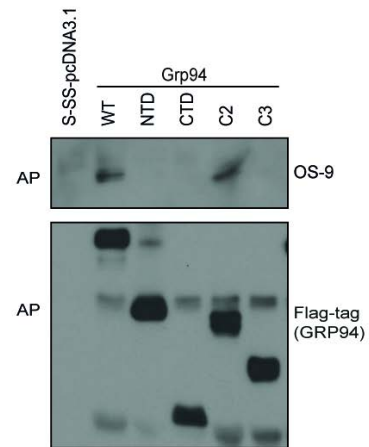


Figure 3.20: Mapping of OS-9 interaction domain on Grp94

A) Schematic representing truncation constructs of Grp94. B) Co-precipitation of Flag-tagged Grp94 truncation constructs with S-tagged OS-9.1 expressed in HEK293 cells. OS-9 complexes were affinity purified by S-protein agarose, with western blots probed for Grp94 and S-tag. C) Co-precipitation of endogenous OS-9 with Flag-tagged Grp94 constructs.

During the investigations of the Argon lab, the enrichment of higher-molecular weight forms of endogenous Grp94 were consistently observed (Dersh, Jones *et al.* 2014). While such forms of Grp94 were not initially observed in the experiments thus far described, detection was achieved under optimisation of immunoblotting sensitivity (Material & Methods: pages 91 and 92) with increased electrophoretic resolution (Figure 3.21). Mammalian Grp94 contains six canonical *N*-glycan acceptor sites (N41, N86, N196, N424, N460, N481; residue number starting from the first amino acid after the signal sequence) but is predominantly mono-glycosylated at N196 (mgGrp94; Qu *et al.* 1994). Therefore, owing to their ~3- to 12-kDa shift it was hypothesised that these species represent a minor cellular population of hyper-glycosylated Grp94 (hgGrp94), this was subsequently confirmed by the Argon lab through demonstration of their reactivity to endoglycosidase H (EndoH) and peptide-*N*-glycosidase F (PNGase F) (Dersh, Jones *et al.* 2014). Additionally, hgGrp94 was demonstrated to display decreased ATP binding and possess altered conformations (Dersh, Jones *et al.* 2014). Indeed, *N*-linked glycans are large, hydrophilic moieties which if placed in regions of a. a. packing or within active sites can disrupt protein conformation and activity. In the context of the predicted crystal structure of Grp94 (Dollins *et al.* 2007), only N434 and the constitutively modified N196 map to loop regions while other visible glycan acceptor sites map to α -helices, with N86 clearly predicted to disrupt the ATP-binding pocket. Taken together these data suggest that hgGrp94 forms might represent a pool of non-native and possibly non-functional Grp94. Critically, Dersh, Jones *et al.* 2014,

demonstrated that hgGrp94 is subject to enhanced turnover via OS-9, suggesting that hgGrp94 is indeed a substrate of OS-9 (Dersh, Jones *et al.* 2014). The turnover of which shall be discussed and investigated in further detail in Chapter 4 of this thesis.

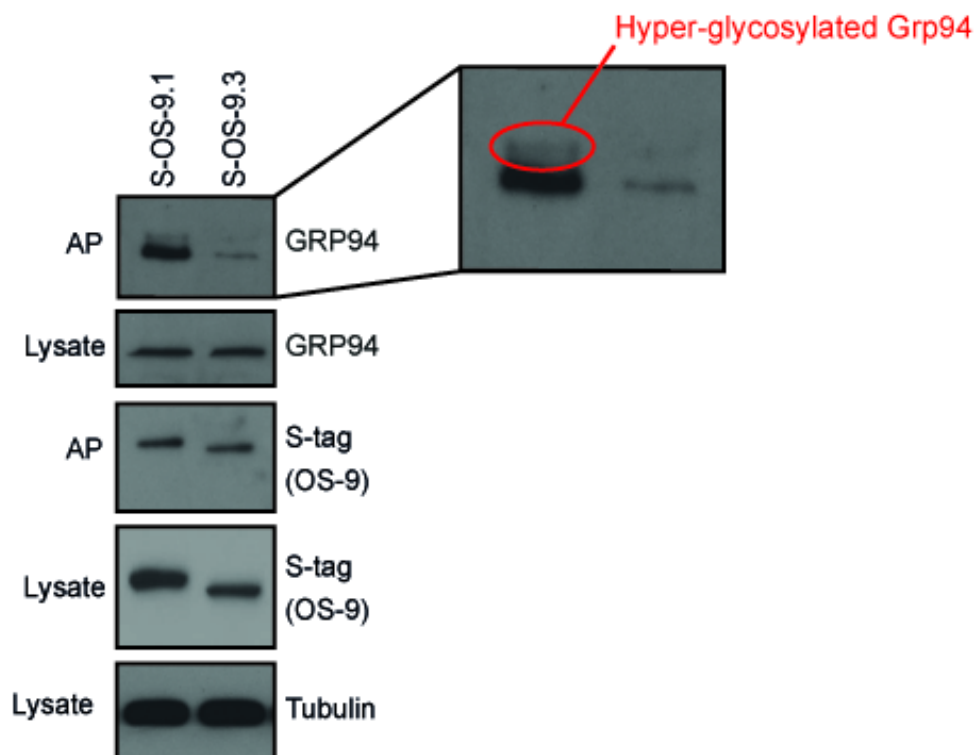


Figure 3.21: Detection of hyper-glycosylated Grp94

Co-precipitation of higher-molecular weight forms of endogenous Grp94 with S-tagged OS-9.1 in HEK293 cells

3.3 Discussion

In this study, the complex formed between Grp94 and OS-9 was examined in detail in an attempt to delineate its function, primarily through dissecting their interaction. It was found that the interaction occurs in the ER lumen and involves the C-terminal domain of OS-9. More precisely, a 65 a. a. region (443-507) is necessary for OS-9/Grp94 binding, which was designated the 94BR. The 94BR lies within the C-terminal half of OS-9 (248-617) where secondary structure algorithms (Jpred, PsiPred) predict two extended alpha helices within the OS-9 94BR (448-471, 485-502) linked by a short unstructured segment. Mutual contribution of these two helices in forming the structure needed for Grp94 binding is supported by the inability to bisect or reduce the domain without compromising the interaction. Indeed, the importance of maintaining the integrity of this region was demonstrated by the loss of interaction with Grp94 between OS-9.3/4 and the three point mutants P446L, S454L and Q486R. Together, these data demonstrate that a highly conserved domain of OS-9 (94BR) is responsible for binding Grp94. Moreover, these observations raise the possibility that conditional splicing to generate alternative isoforms could alter the functional capacities of OS-9.

Alternative splicing is not an uncommon event, particularly in multicellular eukaryotes, with current estimates suggesting that nearly 95 % of human genes are alternatively spliced (Pan, Shai *et al.* 2008; Barash, Calarco *et al.* 2010). This variability in splicing pattern facilitates the molecular diversity and organismal complexity generated from the limited number of genes present in

higher eukaryotes (McManus and Graveley 2011). Splice isoforms may possess differential and/or overlapping structure and function, and two reports have suggested differential functions of OS-9 splice variants (Molinari, Galli *et al.* 2002; Christianson, Shaler *et al.* 2008). Silencing of OS9.1 did not impair the degradation of NHK, while silencing of both OS-9.1 and OS-9.2 delayed ERAD (Christianson, Shaler *et al.* 2008). Additionally, BACE476 Δ (pancreatic isoform of human β -secretase degraded by ERAD) (Molinari, Galli *et al.* 2002) binds specifically to OS-9.1, but not to OS-9.2, when SEL1L is silenced by RNAi (Bernasconi, Galli *et al.* 2010). These observations raise the possibility that differential splicing to generate alternative isoforms could alter the interactions and thus function, of OS-9. In this study, neither OS-9.3 nor OS-9.4 was found to appreciably bind Grp94.

Prior to this study, OS-9.1 and in particular OS-9.2 have been reported as the most prominent isoforms, with doubt surrounding the existence of OS-9.3 and to a greater extent OS-9.4 as their detection by western blot has not been reported. The consistent detection of OS-9.2 mRNA and protein in multiple cell types indicates that excision of exon 13 (corresponding to 55 a. a., 535-589), occurs with regular frequency. In contrast, excision of exon 11b encoding 15 a. a. (456-471), appears to occur more rarely. Previous studies isolated and characterised three isoforms of OS-9 cDNA (OS-9.1, OS-9.2, OS-9.3) from a myeloid leukaemia HL-60 cDNA library, with similar results achieved in the human osteosarcoma cell line OsA-CL/SJSA-1 and the human rhabdomyosarcoma cell line RH30 (Kimura, Nakazawa *et al.* 1997). However,

similar experiments by Bernasconi *et al.* only revealed the expression of OS-9.1 and OS-9.2 transcripts in HEK293 cells (Bernasconi, Pertel *et al.* 2008). Neither bodies of work observed the OS-9.4 isoform. Using an improved amplification scheme, this study has succeeded in confirming the detection of four bands corresponding to all four OS-9 isoforms. Therefore conceivably, endogenously a situation which results in the loss of the OS-9/Grp94 interaction may occur. However, the purpose, trigger and frequency of alternative splicing of OS-9 have yet to be determined. Initial experiments employing UV irradiation and a MAP kinase inhibitor did not induce any marked change in the pattern of presented isoforms. Nonetheless, numerous other stress-inducing conditions remain to be tested, including: hypoxia, overgrowth/undergrowth, glucose starvation, tunicamycin treatment and γ -irradiation. Thus, a condition that stimulates the alternative splicing of exon 11 and hence the generation of isoforms OS-9.3 and OS-9.4, would result in an OS-9 pool with reduced or lost interaction with Grp94. Indeed, a comparable situation is present in the LG2-MEL cell lines, harbouring the OS-9_{P446L} mutant. The accelerated proliferation of the LG2-MEL-5-35 in comparison to LG2-MEL-207, which retains one wild type allele, may hint towards a consequential phenotype to the disruption of OS-9/Grp94, however, further studies will be required.

As to the function of the OS-9/Grp94 complex, initial results and particularly those conducted in collaboration with the lab of Yair Argon suggest that OS-9 is not reliant upon Grp94 for its own folding, nor is Grp94 required for OS-9 recognition of ERAD substrates. Intriguingly, despite the lack of

requirement of the MRH domain of OS-9 for Grp94 binding, OS-9 preferentially associates with a minor population of endogenous hyper-glycosylated Grp94. Analysis of the potential glycan acceptor sites on the crystal structure of Grp94 suggest extraneous glycans would indeed structurally disrupt Grp94, while hgGrp94 displayed increased sensitivity to trypsin proteolysis, had lower avidity to a conformation-specific (9G10) antibody and had impaired binding to ATP, all indications of the nonnative nature of hgGrp94 (Dersh, Jones *et al.* 2014). Additionally, hgGrp94 forms are degraded more rapidly than mgGrp94, suggesting a model whereby Grp94 is synthesised in abundance with the addition of extraneous glycans on cryptic acceptor sites signalling an increased rate of degradation (Figure 3.22).

Further investigation shall be required to establish if the soul function of the OS-9/Grp94 interaction is in the degradation, via OS-9, of misfolded and/or excess Grp94 and if additional factors are required in this degradation. Grp94 may indeed be a highly abundant ER chaperone, however, this does not exclude it from sensitive regulation which a loss of the OS-9/Grp94 interaction would disrupt. While this study has shed much light on the intricacies of the OS-9/Grp94, the question remains, what are the consequences of a loss of OS-9's interaction with Grp94?

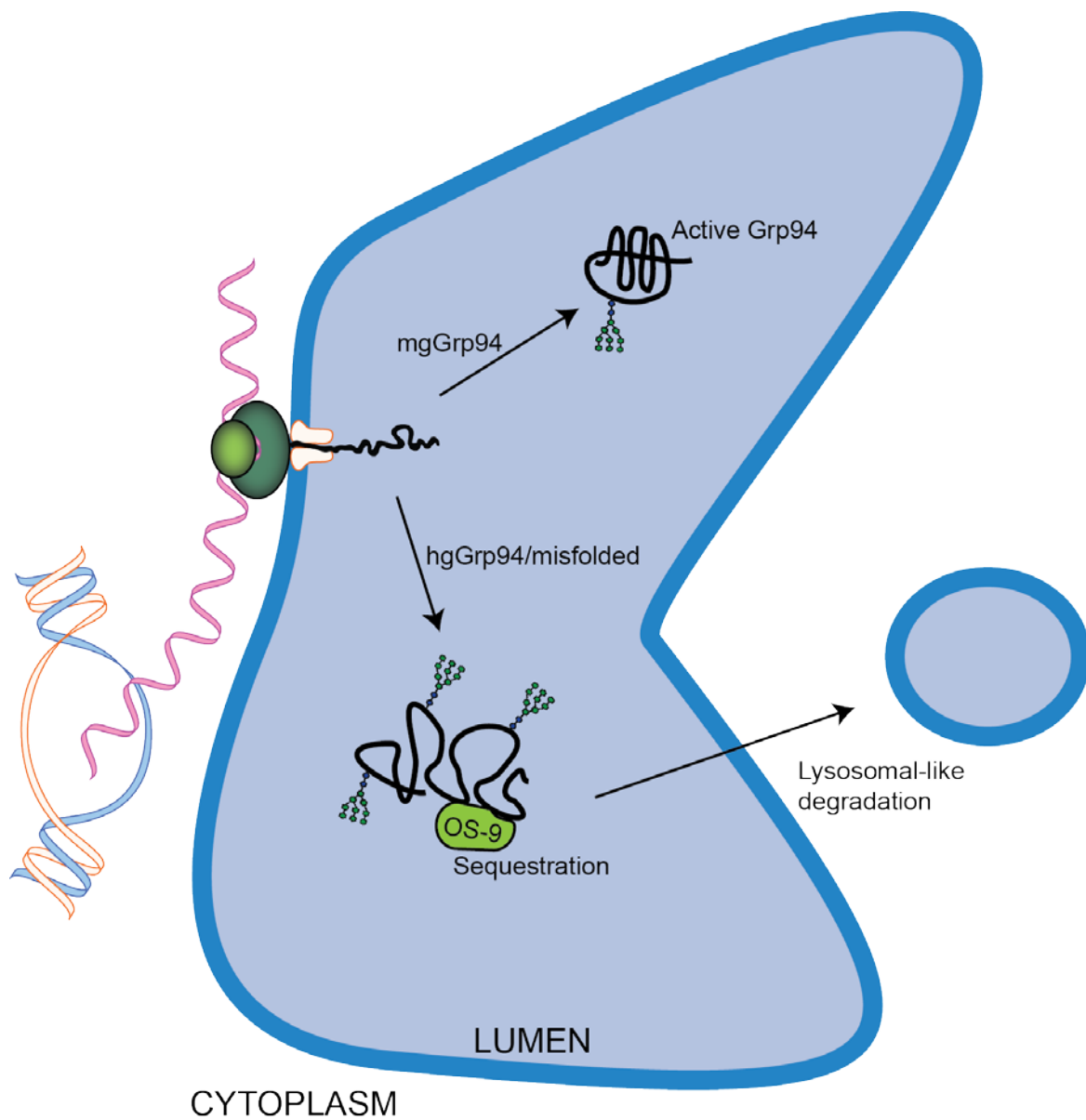


Figure 3.22: Model for the formation and disposal of hgGrp94

The hgGrp94 pool is preferentially sequestered by the ER-resident lectin OS-9 through a specialised polypeptide recognition domain (94BR) and not direct oligosaccharide binding.

CHAPTER FOUR

**The delivery of substrate by OS-9 to the
Hrd1/SEL1L ubiquitin ligase complex**

4.1 Introduction

The detection and destruction of aberrant proteins transiting the secretory pathway is essential in ensuring cellular homeostasis and disease evasion. However, detection by the intricate QC machinery is complex, with client proteins varying vastly in size, shape, number of subunits, folding time required, post-translation modifications and stability. The process is further complicated by each protein having the possibility to misfold in an immense number of ways. Despite the importance of protein degradation in nearly every aspect of cellular function, very little is known regarding the mechanisms by which ERQC and ERAD recognise their substrates, and the nature of substrate binding.

The yeast protein Yos9p, and its mammalian orthologue OS-9, have been established as ER-resident lectins that interrogate the folding competency of a subset of proteins during maturation in the ER, prior to their progression through the secretory pathway. Current models for ERAD of glycoproteins suggest the utilisation of a bipartite commitment signal, where substrates display a trimmed glycan with a terminal α 1,6-linked mannose (Quan, Kamiya *et al.* 2008), in addition to an unfolded peptide signal which is currently poorly defined (Bhamidipati, Denic *et al.* 2005; Denic, Quan *et al.* 2006; Bernasconi, Pertel *et al.* 2008; Christianson, Shaler *et al.* 2008; Hosokawa, Kamiya *et al.* 2009; Xie, Kanehara *et al.* 2009). The MRH domain of OS-9 (and Yos9p) is sufficient to selectively bind ER-derived, high mannose oligosaccharide forms with exposed α _{1,6} mannose moieties (Szathmary, Biemann *et al.* 2005). Both OS-9 (Hosokawa, Kamiya *et al.* 2009) and Yos9p (Quan, Kamiya *et al.* 2008) MRH

domains have been shown, via frontal affinity chromatography, to specifically recognise *N*-linked glycans trimmed of terminal branch C mannoses, while further trimming to the A and B branch of terminal mannoses, to Man7 and below (to Man5), is also bound by OS-9 (Hosokawa, Kamiya *et al.* 2009). In addition, the co-crystal structure of OS-9's MRH domain has been elucidated, in complex with α 3, α 6-mannopentose, with tandem tryptophan residues identified within the domain (WW) that are essential for coordinating-recognition of exposed $\alpha_{1,6}$ mannose moieties (Satoh, Chen *et al.* 2010). Indeed, the MRH domain of OS-9 has been shown to be critical for its role in ERAD (Hosokawa, Kamiya *et al.* 2009; Mikami, Yamaguchi *et al.* 2010). However, conversely, a functional MRH domain has been demonstrated to be dispensable for substrate association (Bernasconi, Pertel *et al.* 2008; Christianson, Shaler *et al.* 2008; Hosokawa, Kamiya *et al.* 2009), implying the involvement of a secondary component in substrate binding. In this chapter, the interaction of OS-9 with the model ER substrates NHK and the non-glycosylated NHK-QQQ is probed, with the aim of identifying the region(s) of OS-9 involved in their recognition.

The serine protease inhibitor α -1 antitrypsin (AAT) is prone to a naturally occurring point mutation at glutamine 342 (ATZ) that generates a mutant protein with a strong propensity to self-associate, which results in the formation of aggregates and consequently the α -1 antitrypsin deficiency disease state (DeMeo and Sliverman, 2004). However, in addition to the ATZ mutant, over 30 variants of α 1-AT have been identified that are allelic and co-dominantly expressed, yet only a few are associated with a deficiency phenotype (Gadek

and Crystal 1983). A second naturally occurring mutant, although with lower frequency, is the Null Hong Kong (NHK) variant, which was characterised from an individual (in Hong Kong) who exhibited a total absence of immunoreactive $\alpha 1$ -AT in serum (Sifers, Brashears-Macatee *et al.* 1988). NHK and a non-glycosylated counterpart QQQ (modified NHK where the asparagine residues of three possible *N*-glycosylation sites are substituted with glutamine) have commonly been employed as model ERAD substrates to probe the components and functionality of the ERAD pathway (Hosokawa, Wada *et al.* 2006; Hosokawa, You *et al.* 2007; Christianson, Shaler *et al.* 2008; Cormier, Tamura *et al.* 2009; Yamaguchi, Hu *et al.* 2010; Fujimori, Kamiya *et al.* 2013; Huang, Hsiao *et al.* 2013). The NHK mutant is generated by a TC dinucleotide deletion within the codon for Leu³¹⁸ in exon IV, which results in a frame shift mutation generating a premature termination codon at residue 334, upstream of the key residues for its function as a protease inhibitor, with the mutant gene being expressed as a truncated protein of 45 kDa that is retained within the ER (Sifers, Brashears-Macatee *et al.* 1988).

Additionally, this chapter shall assess OS-9s interaction with the adapter protein of the Hrd1 ubiquitin ligase complex SEL1L. For the set of proteins identified by OS-9, ubiquitination occurs through the Hrd1/SEL1L ubiquitin ligase complex with OS-9 delivering substrates through an interaction with SEL1L, but not directly with Hrd1 (Satoh, Chen *et al.* 2010). However, SEL1L has recently been suggested to be the ERAD tuning receptor, which selectively captures EDEM1 and OS-9 for constitutive clearance from the ER at steady state through

COPII-independent vesicle-mediated removal (Bernasconi and Molinari 2011; Bernasconi, Galli *et al.* 2012). Macroautophagy is a cellular process by which membrane-bound compartments engulf macromolecules and deliver them to lysosomes for destruction. It has been suggested that ERAD may be regulated or “tuned” through the rapid turnover of specific components through the proteasomes or autophagosomes/vesicular trafficking to lysosomes (Merulla, Fasana *et al.* 2013). Normal secretory vesicles released from the ER are 60-70 nm in diameter and have coatamer proteins, such as COPII, whereas the ER-derived tuning vesicles, known as EDEMosomes, lack coatamers and are 200-800 nm in diameter (Zuber, Cormier *et al.* 2007; Bernasconi, Galli *et al.* 2012).

Much of our understanding of EDEMosomes comes through the study of their hijacking by coronaviruses (Reggiori, Monastyrska *et al.* 2010; Reggiori, de Haan *et al.* 2011). During autophagy, a cytosolic form of LC3 (microtubule-associated protein 1A/1B-light chain 3), LC3-I, is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is inserted into both the inner and outer membranes of the autophagosome (Kabeya, Mizushima *et al.* 2000, Mizushima, Yamamoto *et al.* 2001). However EDEMosomes, unlike autophagosomes, do not contain LC3-II (Cali, Galli *et al.* 2008). Instead, membranes are non-covalently associated with LC3-I (Le Fourn, Gaplovska-Kysela *et al.* 2009), which has been suggested to be an unconventional, autophagy-independent use of LC3 for the vesicle-mediated removal of short-lived chaperones from the ER lumen (Figure 4.1) (Cali, Galli *et al.* 2008). Work by the Molinari lab has identified SEL1L as the

ERAD tuning receptor that “captures” OS-9 and EDEM1 while concurrently interacting with LC3-I through its cytosolic tail (Bernasconi, Galli *et al.* 2012). In the presence of misfolded proteins, the constitutive removal of OS-9 (and EDEM1) is blocked through competition with and the stabilisation of the SEL1L/Hrd1 ubiquitin ligase complex, thereby increasing their luminal levels and enhancing ERAD without requiring induction of the UPR (Bernasconi and Molinari 2011; Bernasconi, Galli *et al.* 2012). Within this chapter, the presence of OS-9 in EDEMosomes alongside whether or not Grp94 is targeted for degradation in a similar manner will be assessed.

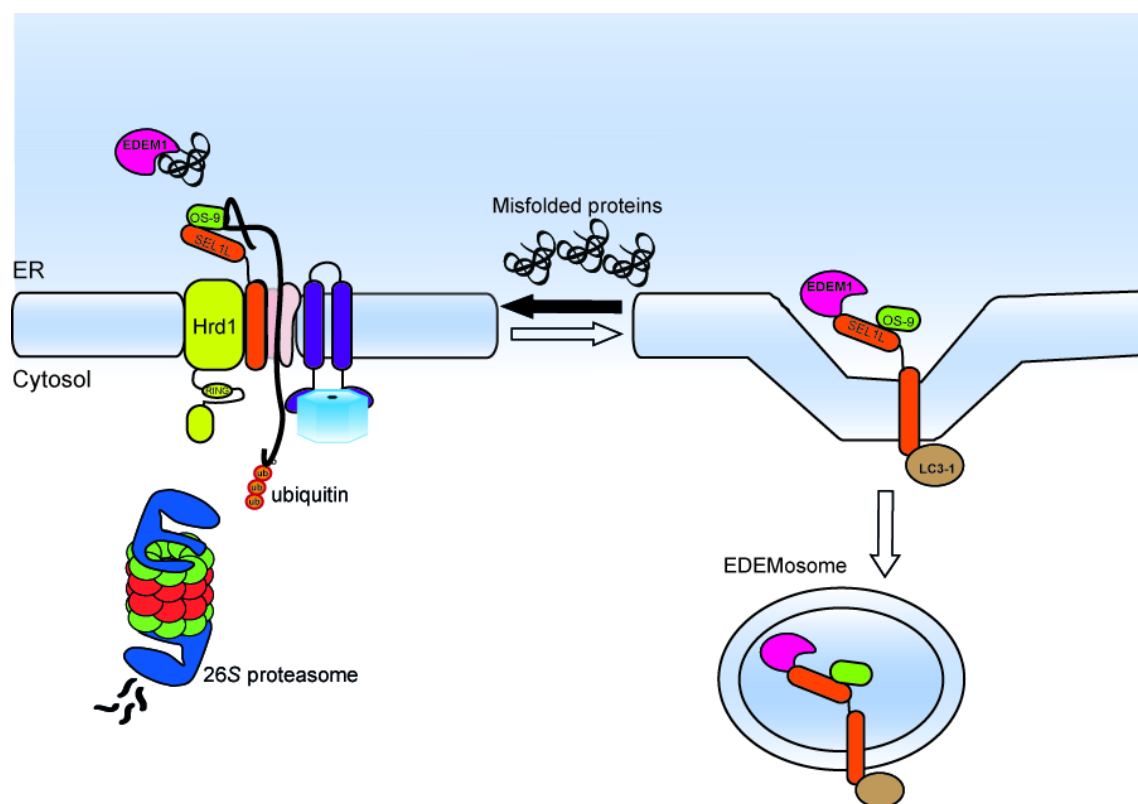


Figure 4.1 The ERAD tuning pathway

OS-9 and EDEM are involved in the delivery of misfolded proteins to the Hrd1/SEL1L ubiquitin ligase complex. The misfolded protein is subsequently dislocated, ubiquitinated and degraded by the 26S proteasome. Some ERAD chaperones are short-lived at steady-state. In the event of a lack of client proteins, EDEM1 and OS-9 segregate with SEL1L in small LC3-I coated vesicles, called EDEMosomes. EDEMosomes subsequently deliver their content to endo-lysosomal compartments for disposal.

4.2 Results

4.2.1 OS-9 co-precipitates NHK

A hallmark of a functional ERAD pathway is the ability to distinguish native from misfolded substrates, in addition to differentiating between slow-folding and terminally misfolded proteins. OS-9 has been demonstrated to detect misfolded proteins through the recognition of a trimmed glycan by its MRH domain (Hosokawa, Kamiya *et al.* 2009; Satoh, Chen *et al.* 2010). As previously demonstrated (Chapter 3), alternative splicing of exon 11 abolishes the interaction of OS-9 with Grp94. Similarly it was questioned whether OS-9 isoforms could differentially bind the model ERAD substrate NHK. S-tagged OS-9 isoforms were co-transfected with HA-tagged NHK and co-precipitated with S-protein agarose. Splicing of exon 11 and/or exon 15 of OS-9 had no appreciable effect on OS-9's interaction with NHK (Figure 4.2, OS-9.4 not shown). However, it should be noted that these data gave no appreciation for the degradation kinetics (i.e. if this interaction corresponds to increased/decreased degradation/retention of NHK). In addition, NHK is only one example of an ERAD substrate, therefore, the possibility that the deleted/partially deleted exons are required in the recognition of a different substrate cannot be ruled out.

Splicing of exon 11 had no effect on the interaction of OS-9 with NHK. To investigate if OS-9 recognises Grp94 and ERAD substrates via distinct regions, S-tagged 94BR was co-transfected with HA-tagged NHK and co-precipitated

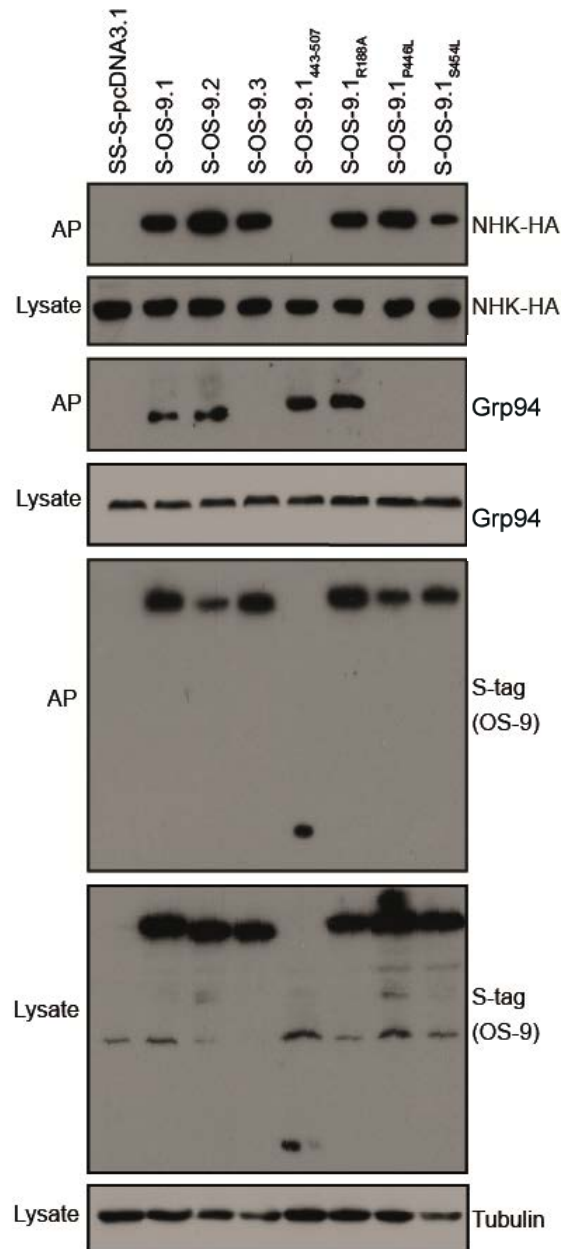


Figure 4.2 The 94BR is not sufficient for NHK binding

Co-precipitation of HA-tagged NHK with S-tagged OS-9 isoforms (1-3), truncations (94BR – Grp94 binding region) and the mutants R188A (disrupts MRH lectin activity), P446L and S454L (both mutants of 94BR that disrupt binding to Grp94). Constructs were expressed in HEK293 cells. OS-9 complexes were affinity purified by S-protein agarose with western blots probed for HA, Grp94, S-tag and tubulin.

with S-protein agarose. The 94BR retained its ability to co-precipitate endogenous Grp94, however, interaction with NHK was lost. Similarly, mutants of the 94BR (P446L and S454L) that failed to co-precipitate Grp94 do not disrupt co-precipitation of NHK (Figure 4.2). These data suggest that the region of OS-9 necessary and sufficient for interaction with Grp94 is not involved in, or itself sufficient for, binding to the model substrate NHK. Indeed, this in turn supports the conclusion that Grp94 is not recognised as a substrate of OS-9.

4.2.2 OS-9 co-precipitates non-glycosylated NHK-QQQ

This thesis has primarily focused on the identification and degradation of misfolded proteins through the recognition of a trimmed “glycan code”, however, not all secretory pathway proteins are glycosylated. A minority of proteins folded within the ER lack *N*-glycans, yet their recognition and degradation on misfolding is of equal importance. Indeed, a number of studies have demonstrated that the ER is endowed with the capability to both recognise and degrade non-glycosylated misfolded proteins (VanSlyke, Deschenes *et al.* 2000; Swanton, High *et al.* 2003; Sekijima, Wiseman *et al.* 2005; Kanehara, Xie *et al.* 2010). Additionally, non-glycosylated proteins have been shown to co-IP with OS-9 (Bernasconi, Pertel *et al.* 2008). All OS-9 isoforms (Figure 4.3, OS-9.4 not shown) are capable of co-precipitating with NHK-QQQ, a non-glycosylated form of NHK. This suggests that OS-9 substrate binding does not solely depend on

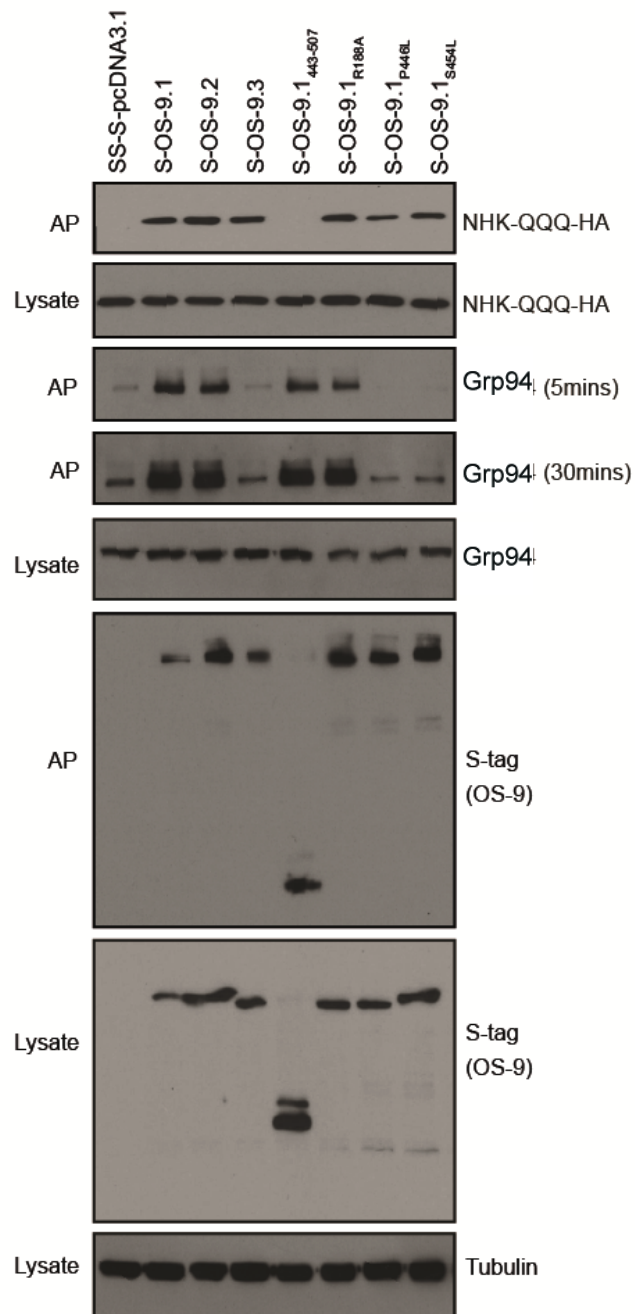


Figure 4.3 OS-9 co-precipitates NHK-QQQ

Co-precipitation of HA-tagged NHK-QQQ with S-tagged OS-9 isoforms (1-3), truncations (94BR – Grp94 binding region) and the mutants R188A (disrupts MRH lectin activity), P446L and S454L (both mutants of 94BR that disrupt binding to Grp94). Constructs were expressed in HEK293 cells. OS-9 complexes were affinity purified by S-protein agarose with western blots probed for HA, Grp94, S-tag and tubulin.

the recognition of *N*-linked glycans by its MRH domain. Similarly the R188A OS-9 mutant (a mutation predicted to disrupt the lectin activity of the MRH) (Christianson, Shaler *et al.* 2008) retained its ability to co-precipitate both NHK and NHK-QQQ (Figures 4.2/3). These data support a mechanism where, in addition to the lectin activity of the MRH domain, there is a secondary peptide recognition aspect to the interaction (investigated in Section 4.2.3). This is in agreement with results previously published by Christianson *et al.* (Christianson, Shaler *et al.* 2008). Additionally, these data suggest that this peptide recognition, of both glycosylated and non-glycosylated substrates, is not provided through the assistance of Grp94.

4.2.3 Region of OS-9 required for substrate binding

The interaction of OS-9 with terminally misfolded proteins is bipartite, with OS-9 binding to the trimmed glycans via its MRH domain and to the unfolded peptide through an as-yet unknown region (Bernasconi, Pertel *et al.* 2008, Quan, Kamiya *et al.* 2008; Hosokawa, Kamiya *et al.* 2009). In an attempt to identify a peptide-binding region of OS-9, truncations of OS-9 used previously (with Figures 3.5/3.6) were co-transfected with HA-tagged NHK and NHK-QQQ. Each of the constructs containing the MRH domain (S-OS9.1, S-OS-9.3 and S-OS-9.1_{NT}) was able to co-precipitate both NHK and NHK-QQQ (Figure 4.4/4.5). Constructs lacking the region 443-667 retained the ability to co-precipitate NHK, while expression of this region alone was insufficient to precipitate NHK. This suggests that this region does not participate in NHK binding. The most

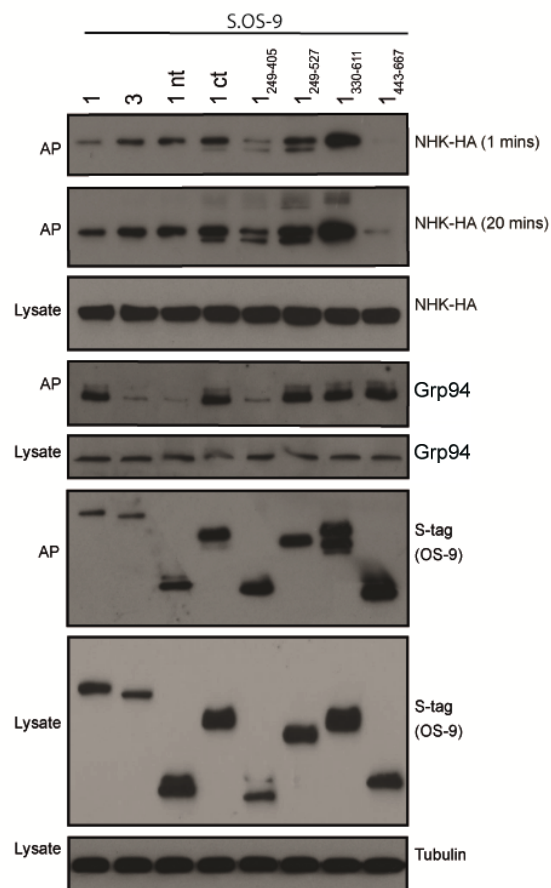
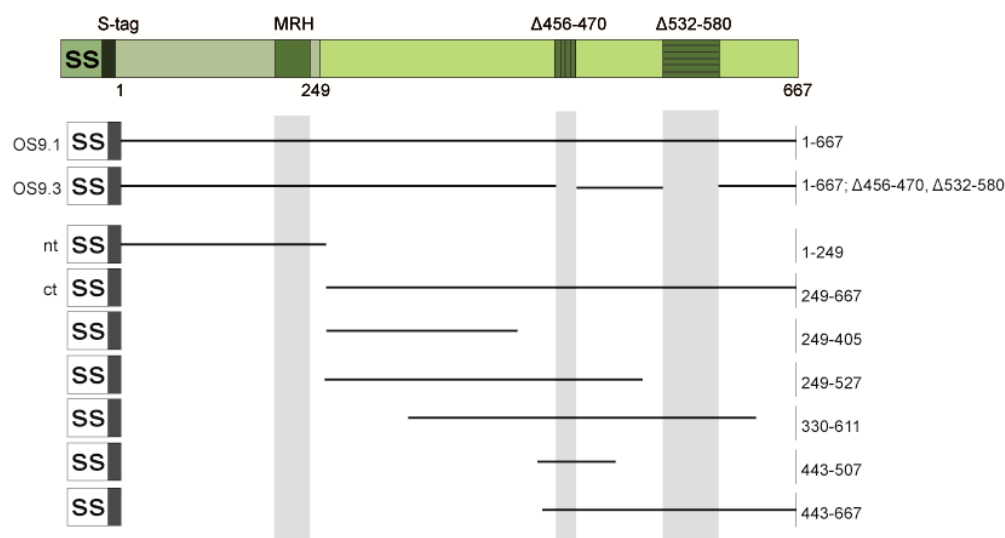


Figure 4.4 Peptide binding of OS-9 to NHK

A) Schematic representation of truncation constructs of OS-9 employed in defining the NHK/OS-9 interaction. B) Co-precipitation of HA-tagged NHK with S-tagged OS-9 isoforms, truncations. Constructs were expressed in HEK293 cells, OS-9 complexes were affinity purified by S-protein agarose and visualised by western blot.

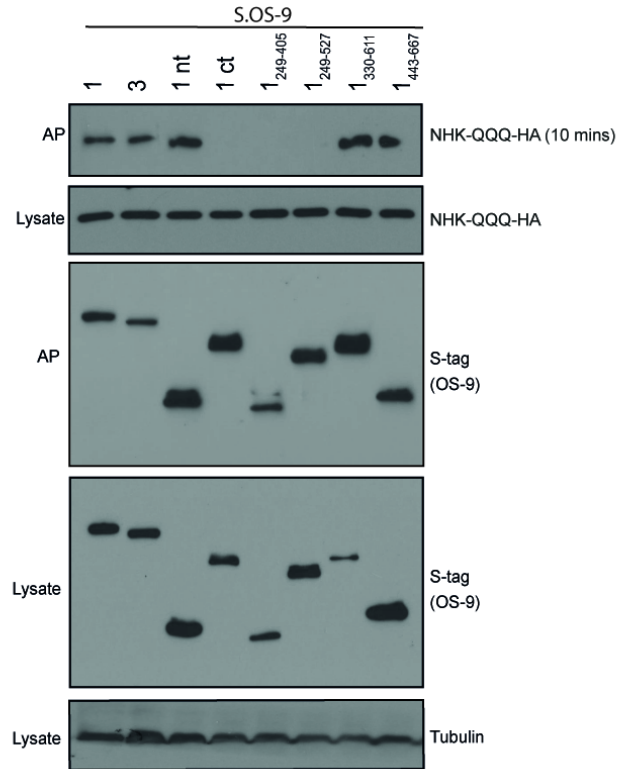


Figure 4.5 Peptide binding of OS-9 to NHK-PPP

Co-precipitation of HA-tagged NHK-PPP with S-tagged OS-9 isoforms, truncations. Constructs were expressed in HEK293 cells. OS-9 complexes were affinity purified by S-protein agarose and visualised by western blot.

intriguing results were seen with the constructs containing a. a. 249-405 (S-OS-9.1_{CT}, S- OS-9₂₄₉₋₄₀₅ and S-OS-9₂₄₉₋₅₂₇), which all lack the MRH domain. These forms failed to co-precipitate the non-glycosylated NHK-PPP, however, they did pull down NHK. This coincided with the appearance of an additional band of reduced molecular weight. It is tempting to postulate that the N-terminus is required for OS-9's interaction with non-glycosylated substrate. However, the construct OS-9.1₃₃₀₋₆₁₁ still co-precipitates both NHK and NHK-PPP, while lacking the entire N-terminus (Figures 4.4/5). Additional experiments are

required to probe and clearly define the interaction of OS-9 with both glycosylated and non-glycosylated substrates, but this initial data suggests that there may be a number of regions of OS-9 that are capable of interacting independently with both/either glycosylated or/and non-glycosylated substrates.

4.2.4 Is OS-9 a substrate of the Hrd1 ubiquitin ligase complex?

As previously introduced, in addition to engaging with misfolded proteins, OS-9 interacts with the Hrd1 ubiquitin ligase through its cofactor SEL1L. Several components of the ERAD machinery have been shown to be substrates of their own degradation pathway. Mammalian SEL1L has a $t_{1/2}$ of ~3 hrs (Mueller, Lilley *et al.* 2006), while Ube2j1 (cognate E2 of Hrd1) is also degraded rapidly (Lenk, Yu *et al.* 2002). *In vitro*, ERAD-implicated Ub-ligases Hrd1 and gp78 autoubiquitinate suggesting that degradation may regulate their intracellular levels (Fang, Ferrone *et al.* 2001; Kaneko, Ishiguro *et al.* 2002; Nadav, Shmueli *et al.* 2003; Kikkert, Doolman *et al.* 2004). Similarly, OS-9 may be an ERAD substrate, as well as a functional component, degraded constitutively in the absence of substrate. In this way, the ER maintains its degradation capacity without the need to transcriptionally regulate expression. To determine the intrinsic half-life of OS-9, cycloheximide chase experiments were performed on HEK293s, detected by western blot using a pan-OS-9 antibody and tubulin as a control. To ascertain whether OS-9 is a *bona fide* ERAD substrate, degradation was measured in the presence of the proteasome

inhibitor MG132, with Grp94 half-life being measured concurrently (Figure 4.6 A). OS-9.1 and OS-9.2 were demonstrated to have a half-life of approximately 7 hrs, while Grp94 remained stable over the duration of the time course, consistent with previous reports of a 3-4 day half-life (Staron, Wu *et al.* 2011). Indeed, OS-9 was stabilised by the addition of MG132 suggesting that OS-9, or at least a portion of OS-9, is degraded in a proteasome-dependent manner.

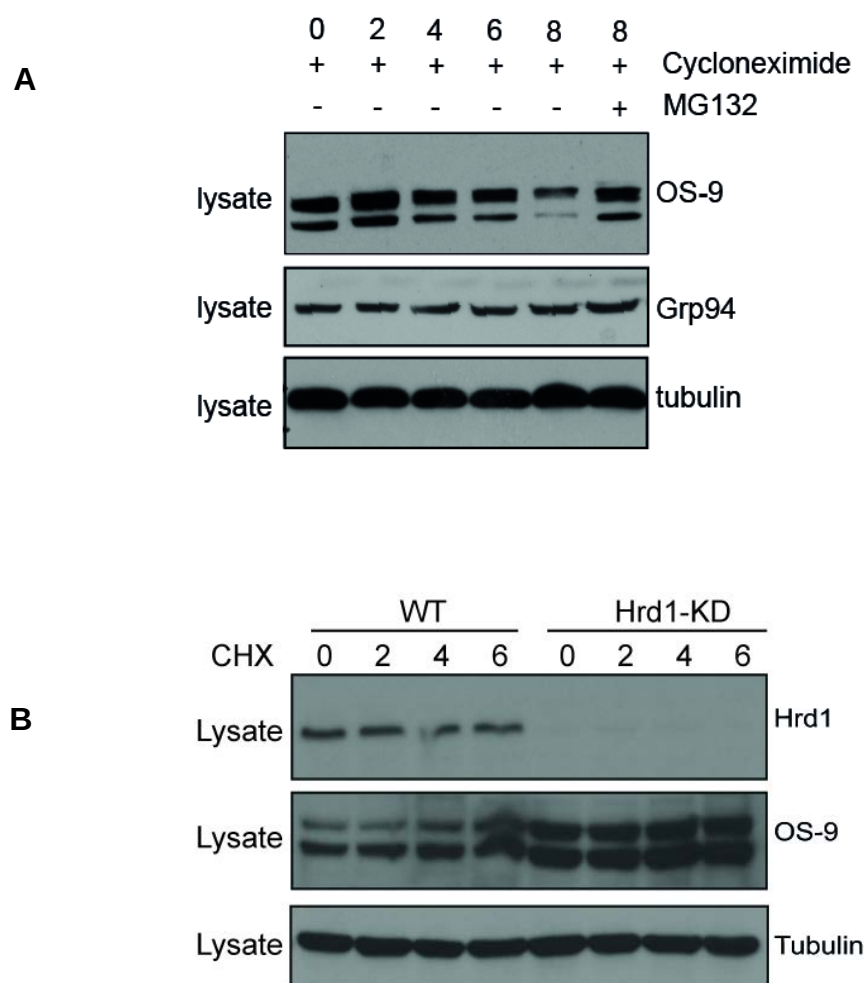


Figure 4.6 Protein stability assay of endogenous OS-9

A) HEK293 cells were treated with cycloheximide (100 μ M) and the proteasomal inhibitor MG132. At the indicated time points, whole cell lysates were analysed by immunoblotting and probed with antibodies to endogenous OS-9, Grp94 and tubulin. B) Comparison of wild type HEK293 cells treated with cycloheximide (100 μ M) and Hrd1-KD (knockdown) for the indicated time points. Whole cell lysates were analysed by immunoblotting and probed with antibodies to endogenous OS-9, Hrd1 and tubulin.

If OS-9 is itself degraded by ERAD, is its degradation mediated by the SEL1L/Hrd1 ubiquitin ligase complex? HEK293 cells stably knocked down for Hrd1 and subjected to a protein stability assay with cycloheximide showed a substantial increase of both prominent OS-9 isoforms (Figure 4.6, B). The time course was limited to 6 hrs as Hrd1 knockdown cells, which display a higher sensitivity to ER stress in comparison to the wild type, experience cell death when exposed to cycloheximide in excess of this duration. Consequently, degradation of OS-9 is not clearly visualised in this blot, however there is clear stabilisation of OS-9 upon knockdown of Hrd1. Similar elevation and stabilisation of OS-9 levels have been noted on knockdown of SEL1L both *in vivo* and *in vitro*, suggesting that OS-9 may itself be degraded in a SEL1L/Hrd1-dependent manner (Tyler, Pearce *et al.* 2012; Sun, Shi *et al.* 2014).

OS-9 has previously been shown to be transcriptionally upregulated under ER stress (Alcock and Swanton 2009; Sun, Shi *et al.* 2014), however somewhat paradoxically, when HEK293 cells were subjected to the ER stress inducer tunicamycin (TM) (which blocks *N*-linked glycosylation) for 4 hrs, OS-9 levels were seen to be reduced (Figure 4.7). Similar results were seen under treatment with thapsigargin (TG) (an inhibitor of Ca^{2+} ATPase) and dithiothreitol (DTT) (prevents disulphide bond formation) (Figure 4.7). In addition, the observed reduction of OS-9 was reversed on treatment with MG132 in all cases, reinforcing the proposal that OS-9 was degraded in a proteasome-dependent manner. These results shall be discussed in further detail in section 4.3.

However, to fully appreciate the dynamics, maintenance and degradation of OS-9, the recently proposed “ERAD tuning” pathway must first be considered.

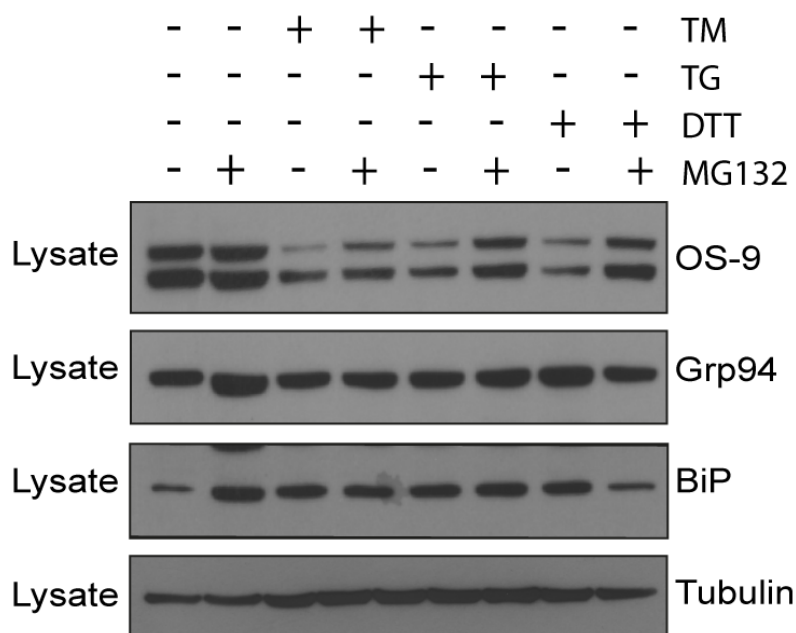


Figure 4.7 Stability of OS-9 under ER stress

HEK293 cells were treated with tunicamycin (TM), thapsigargin (TG) or dithiothreitol (DTT) for 4 hrs and the proteasomal inhibitor MG132, with whole cell lysates analysed by immunoblotting.

4.2.5 ERAD tuning

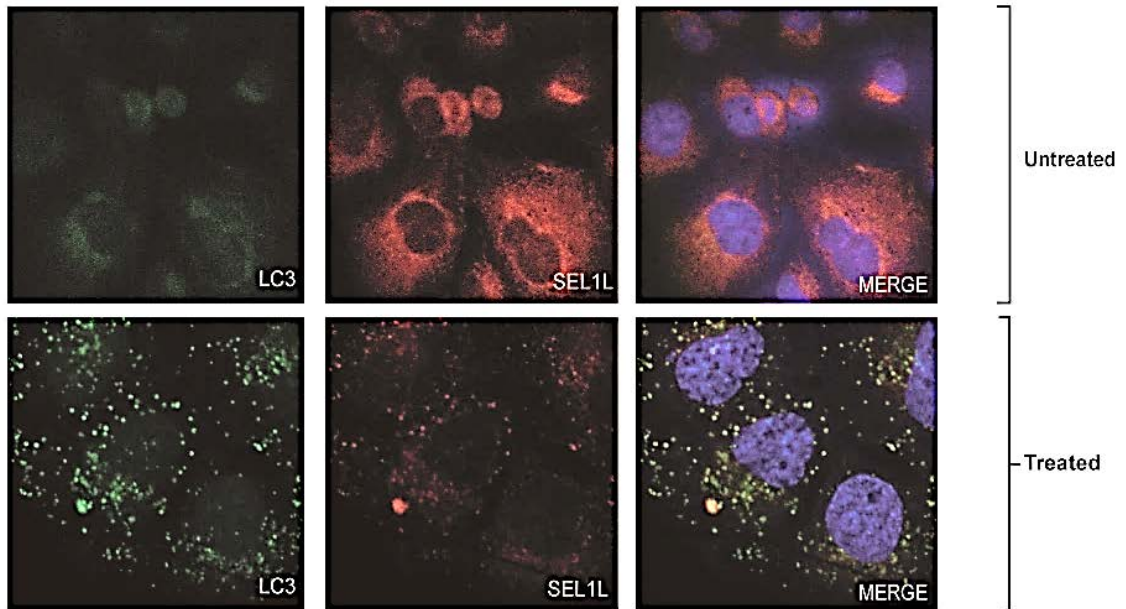
Luminal levels of OS-9 have been proposed to be regulated by so-called “EDEMosomes” or “ERAD tuning vesicles” (Reggiori, Monastyrska *et al.* 2010; Bernasconi and Molinari 2011; Bernasconi, Galli *et al.* 2012; Bernasconi, Noack *et al.* 2012). EDEMosomes thus far have been shown to contain a number of short-lived components of the Hrd1 complex; namely SEL1L, EDEM1, and OS-9, and are believed to reduce ERAD by disposal in acidic organelles

(Bernasconi, Galli *et al.* 2012) or allow for a rapid increase (bypassing transcription/translation) of ERAD factors when required (Reggiori, de Haan *et al.* 2011).

In order to confirm the localisation of endogenous OS-9 in EDEMosomes, vesicles were visualised by IF post stabilisation by the lysomotropic drug chloroquine (CQ) in cultured U2OS cells, which prevents the fusion of EDEMosomes with LAMP1-containing degradative organelles (Cali, Galli *et al.* 2008; Reggiori, Monastyrska *et al.* 2010), in a protocol adapted from that employed by Bernasconi *et al.* (Bernasconi, Galli *et al.* 2012). Co-localisation of SEL1L and LC3 (Figure 4.8 A), and the exclusion of calnexin from these vesicles (Figure 4.8 B) were utilised as positive and negative controls, respectively. As shown in Figure 4.9, OS-9 does indeed co-localise with SEL1L in vesicles when cells are exposed to 100 μ M CQ for 4 hrs.

Next, it was queried whether Grp94 is also present within EDEMosomes. Grp94 is a long-lived protein: it is bound by OS-9 but this interaction is mutually exclusive from the OS-9/SEL1L interaction (Figure 4.10) (Christianson, Shaler *et al.* 2008), and subsequently Grp94 is unaffected by the depletion of SEL1L (Bernasconi, Galli *et al.* 2012). The long-lived nature of Grp94 makes its presence in EDEMosomes unlikely, however, recent work published by Dersh *et al.* (as discussed in chapter 3 of this thesis) identified a hyper-glycosylated form of Grp94 that preferentially associated with OS-9 and was degraded with a reduced half-life compared to mono- glycosylated Grp94 (~20 hr, while mono-glycosylated Grp94's half-life is in excess of 30 hr) (Dersh, Jones *et al.* 2014).

A



B

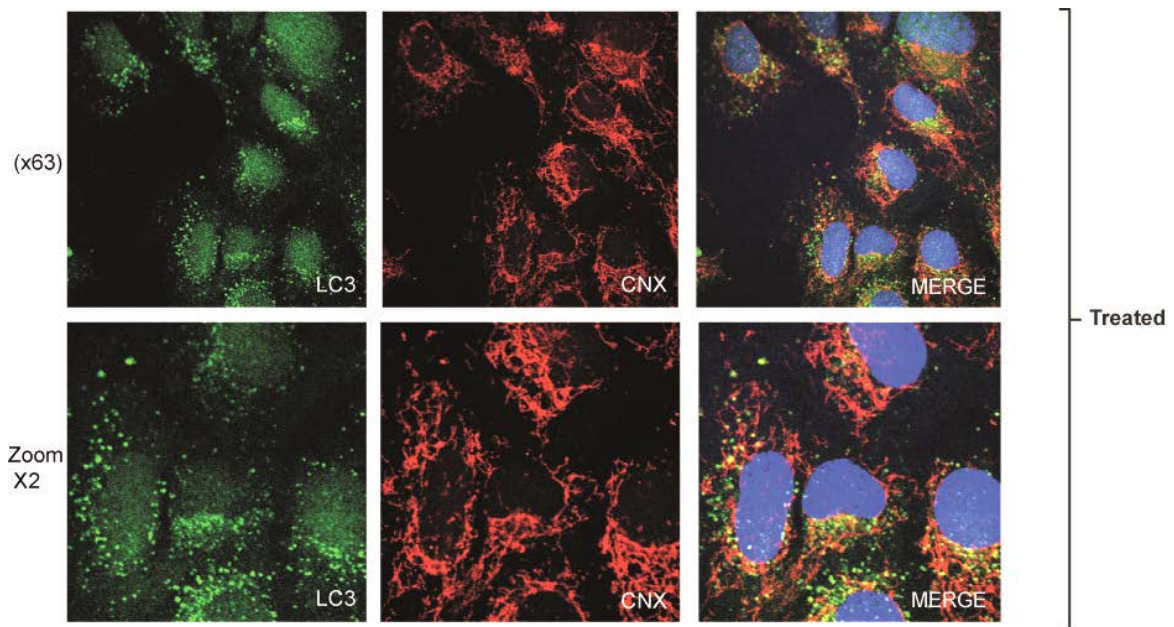


Figure 4.8 EDEMosome formation

A) HEK293 cells treated or untreated with CQ for 4 hrs visualised by immunofluorescence of LC3 (green) and SEL1L (red). Co-localisation of SEL1L with LC3 during induction of EDEMosome formation under CQ treatment is demonstrated. B) Negative control. HEK293 cells treated with CQ for 4 hrs display lack of co-localisation of calnexin (red) in LC3-positive EDEMosomes.

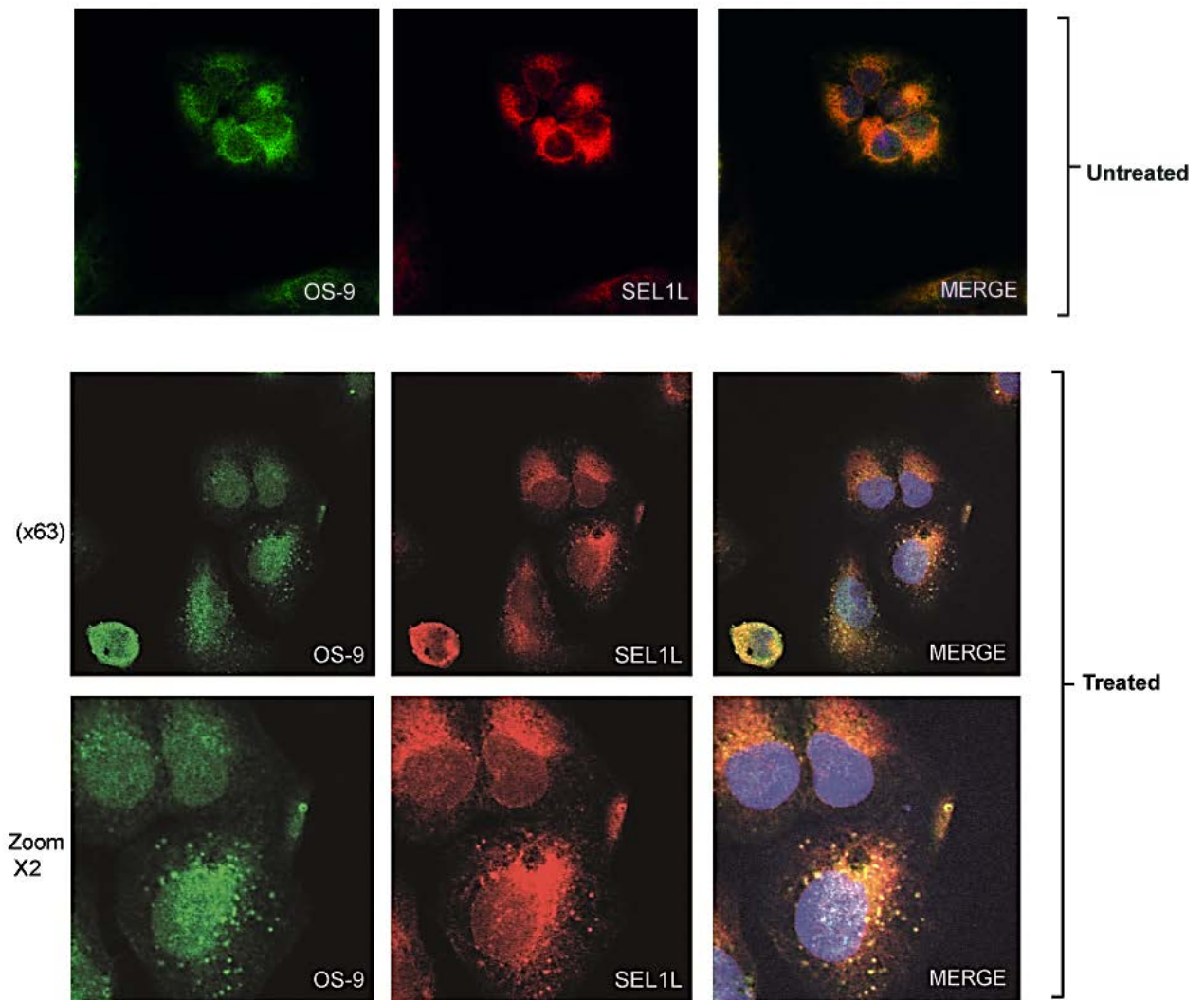


Figure 4.9 OS-9 is present in EDEMosomes

HEK293 cells treated or untreated with CQ for 4 hrs visualised by immunofluorescence of OS-9 (green) and SEL1L (red). Co-localisation of SEL1L with OS-9 in punkta under CQ treatment is demonstrated.

Additionally, hyper-glycosylated Grp94 (hgGrp94) was stabilised (and showed an increased percentage of hgGrp94 to total Grp94) in cells treated with bafilomycin A1, which inhibits the acidification of lysosomes, while hgGrp94 levels were not appreciably affected by treatment with the proteasome inhibitor

MG-132 (Derseh, Jones *et al.* 2014). U2OS cells treated with CQ to visualise EDEMosomes showed that, while the majority of Grp94 remained in the ER, there was indeed a small percentage of Grp94 that was seen to co-localise with SEL1L in these vesicles (Figure 4.11). While we cannot be certain of the glycosylation status of the Grp94 that was seen to co-localise, it is tempting to speculate that this population represents the shorter-lived hgGrp94. HgGrp94 has a more open confirmation and has impaired ATP binding in comparison to mono-glycosylated Grp94 (Derseh, Jones *et al.* 2014), suggesting impaired function and thus a requirement for degradation.

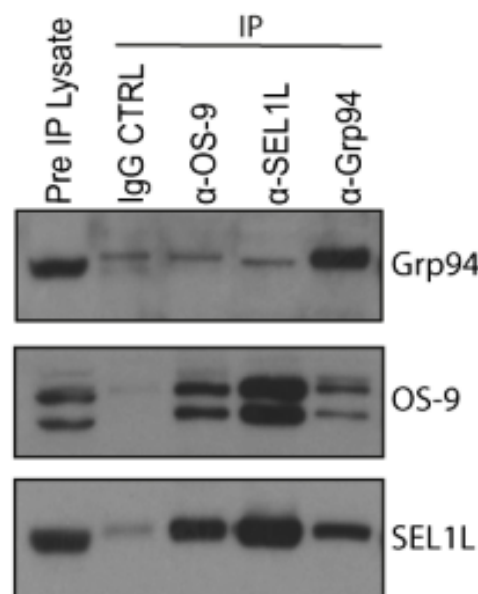


Figure 4.10 Mutual exclusivity of OS-9/SEL1L and OS-9/Grp94 interactions

Immunoprecipitations from 1 % Triton X-100 detergent lysates of HEK293 cells with anti-OS-9, anti-Grp94 and anti-SEL1L. Western blots were probed with antibodies against OS-9, Grp94 and SEL1L.

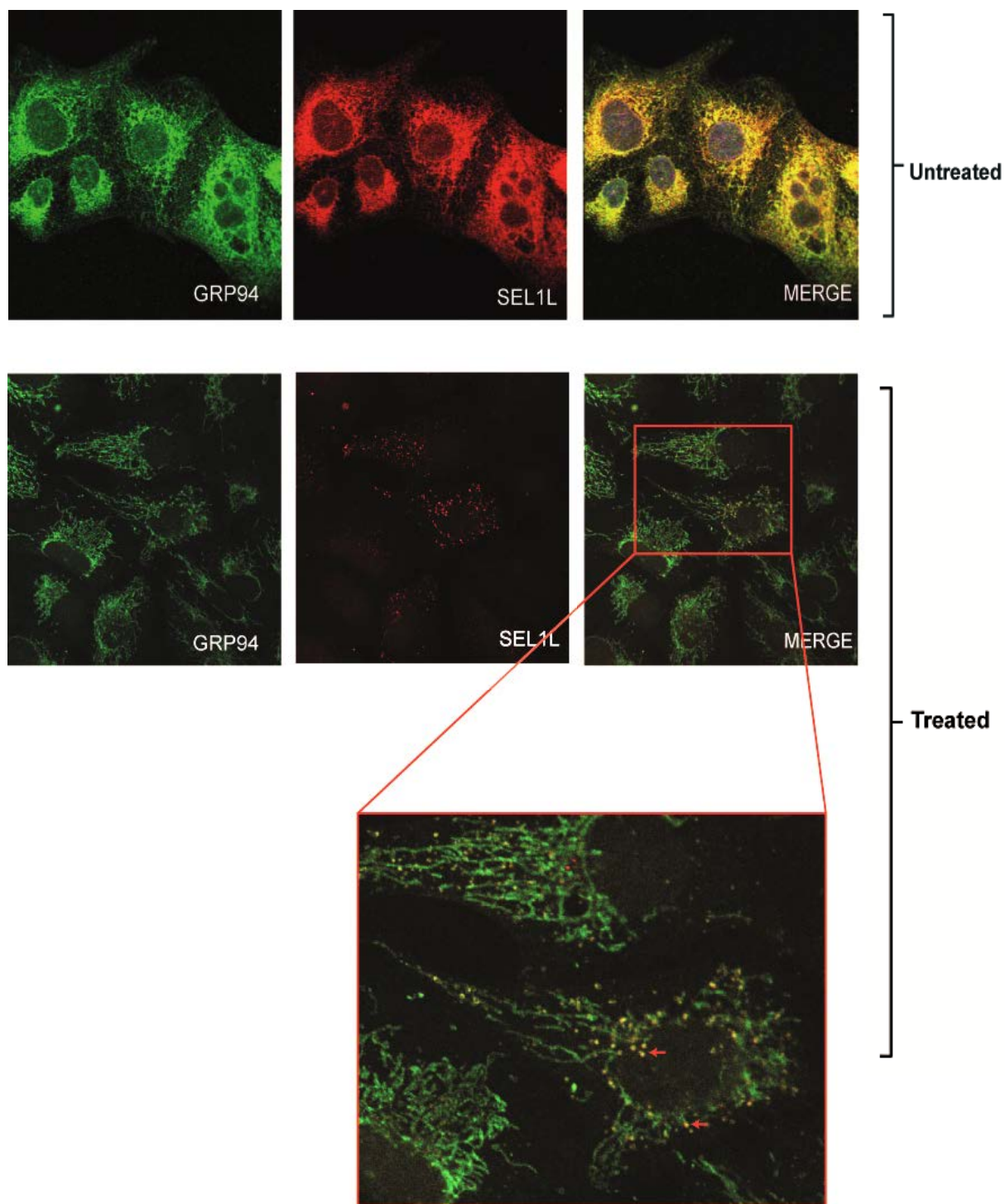


Figure 4.11: A sub-population of Grp94 co-localises to EDEMosomes

HEK293 cells treated or untreated with CQ for 4 hrs visualised by immunofluorescence of Grp94 (green) and SEL1L (red). Co-localisation of a fraction of Grp94 with SEL1L in EDEMosomes (red arrows) is demonstrated.

4.3 Discussion

OS-9 is a well-established component of the ERAD pathway, which delivers substrate to the Hrd1/SEL1L ubiquitin ligase complex. However, the means by which OS-9 differentiates misfolded proteins from their correctly folded counterparts is not yet fully understood. Much research has been focused on the recognition of trimmed *N*-linked oligosaccharides as “degradation signals” and indeed OS-9 has been demonstrated to recognise specific trimmed glycans through its MRH domain (Hosokawa, Kamiya *et al.* 2009). However, studies in both yeast and mammalian cells suggest a bipartite nature to the recognition of substrate. Work in *S. cerevisiae* has shown that both wild type and a lectin mutant (R200A) of Yos9 immunoprecipitate CPY* with or without its glycans (Bhamidipati, Denic *et al.* 2005; Denic, Quan *et al.* 2006; Jaenicke, Brendebach *et al.* 2011; Izawa, Nagai *et al.* 2012; Kostova and Wolf 2005; Spear and Ng 2005). Similar results have been demonstrated for OS-9 (Bernasconi, Pertel *et al.* 2008; Christianson, Shaler *et al.* 2008; Hosokawa, Kamiya *et al.* 2009) and indeed have been confirmed in this chapter. OS-9 bound NHK, however, this interaction was preserved after mutations to the MRH domain. Additionally, OS-9 was competent for the co-precipitation of the non-glycosylated NHK-QQQ, suggesting a secondary detection factor in addition to glycan recognition. This would not be the first example of bipartite recognition employed by members of the ERAD machinery, recently EDEM1 & 2 which are implicated in mannose trimming, were shown to bind ricin A chains in a hydrophobic dependent manner (Sokolowska, Walchli *et al.* 2011; Slominska-

Wojewodzka, Pawlik *et al.* 2014), suggesting the possibility of bipartite recognition of not just sugar structure but also protein structure. This study has demonstrated that a number of distinct regions of OS-9 are capable and sufficient for co-precipitation of both glycosylated and non-glycosylated NHK, nonetheless, further studies will be required to truly elute the nature of this bipartite interaction. Of particular note, is the insufficiency of the 94BR to co-precipitate neither NHK nor NHK-PPP, suggesting Grp94 is not merely a substrate of OS-9 but also Grp94 does not assist OS-9 in binding non-glycosylated substrate.

Previous studies have speculated that the degradation of OS-9 may depend on the SEL1L-Hrd1 complex (Tyler, Pearce *et al.* 2012). Here, evidence is presented that OS-9 is degraded in a proteasome-dependent manner, stabilised by treatment with MG132 and accumulated in a Hrd1-deficient environment, suggesting that the Hrd1 ubiquitin ligase plays a role in the degradation of OS-9. These results are complimented by previous studies which have demonstrated that cells with a low SEL1L content, displayed a delayed turnover of OS-9 (Bernasconi, Galli *et al.* 2012). This data is consistent with OS-9 being degraded in a Hrd1/SEL1L-dependent manner, however, this may be only half of the story. Depletion of SEL1L and the subsequent impeded degradation of OS-9 may also be attributed to the identification of SEL1L as the ERAD tuning receptor, regulating the selective removal of OS-9 (and EDEM-1) from the ER (Bernasconi and Molinari 2011). Several reports demonstrate that lysosome and autophagy inhibitors do not normally have an effect on ERAD

(Klausner and Sitia 1990; Meusser, Hirsch *et al.* 2005; Romisch 2005; Ruddock and Molinari 2006), while interference with the ubiquitylation or proteasomal machineries severely hinders the pathway. Where autophagy and lysosomes may not play a direct role in ERAD, recent studies suggest that they may play a role in the “tuning” of a number of components of the ERAD machinery (Reggiori, Monastyrska *et al.* 2010; Bernasconi and Molinari 2011; Reggiori, de Haan *et al.* 2011; Bernasconi, Galli *et al.* 2012). Constitutive degradation and selective removal of ERAD machinery, that if present in excessive concentrations, could prematurely interrupt productive polypeptide maturation, allows for “fine tuning” of ERAD, ensuring ER homeostasis. Additionally, it provides a rapid response mechanism whereby components of the ERAD machinery are post-translationally regulated, allowing for their prompt up regulation, bypassing transcription without activation of the UPR. Intriguingly, one would assume that knocking out Hrd1 would affect the formation of the Hrd1 ubiquitin ligase complex, increasing the levels of OS-9/SEL1L targeting to EDEMosomes and therefore, reducing, the level of OS-9. However, as already demonstrated in Figure 4.7, this is not the case. An explanation for this may be that a build-up of misfolded substrates bound to OS-9 excludes OS-9 from EDEMosome formation. As misfolded substrates accumulate as a consequence of their failure to progress through the ERAD pathway due to the absence of Hrd1, increased quantities of OS-9 are required to bind the ever-increasing load of misfolded proteins.

Dersh *et al.* recently reported the existence of hyper-glycosylated forms of Grp94 (hgGrp94), and that these are bound preferentially by OS-9 (Dersh, Jones *et al.* 2014). Initial experiments in this study failed to identify these higher molecular weight forms of endogenous Grp94, as their detection displayed dependency on the sensitivity of immunoblotting, as well as sufficient electrophoretic resolution. Alterations to gel composition and conditions allowed visualisation of hgGrp94 (Figure 3.21). Mammalian Grp94 contains six canonical *N*-glycan acceptor sites (N41, N86, N196, N424, N460, N481; residue number starting from the first a. a. after the signal sequence), but is the mono-glycosylated (at N196) form predominates (Qu, Mazzearella *et al.* 1994). As outlined in Chapter 3 of this thesis, hgGrp94 displays characteristics of non-native molecules which are subsequently degraded in a more rapid manner in comparison to mgGrp94. Dersh *et al.* noted that hgGrp94 was degraded in a lysosomal dependent manner (this hypothesis is reflected in Figure 3.22), complementarily this thesis reports that a sub-population of Grp94 co-localises with OS-9 in EDEMosomes. Further studies will be required to confirm if the observed Grp94 is indeed hyper-glycosylated and if its sole function in these vesicles is to facilitate its degradation. Indeed, the presence of “misfolded” Grp94 in EDEMosomes would seem to be counter-intuitive to the concept of ERAD “tuning” and therefore further characterisation of these vesicles is required. However, this initial finding adds an intriguing component to our knowledge of the turnover of components of the ERAD machinery.

CHAPTER FIVE

**Investigation of previously reported
cytoplasmic and endoplasmic OS-9
interaction partners**

5.1 Introduction

As established in the previous two chapters, OS-9 is a component of the ERAD pathway that is primed in a central triage position, interacting with substrates, chaperones and the SEL1L/Hrd1 ubiquitin ligase complex. In addition to these well reported interactions, numerous other associations of OS-9 have been reported. OS-9 is a *bona fide* ER-resident protein, targeted to the lumen by a validated signal sequence and glycosylated at N178 (Christianson, Shaler *et al.* 2008). Yet many proteins lacking canonical ER-targeting features have been purported to be OS-9 interactors including: HIF-1 α (Baek, Mahon *et al.* 2005), TRPV4 (Wang, Fu *et al.* 2007), Meprin β (Litovchick, Friedmann *et al.* 2002) and LONP2 (Christianson, Olzmann *et al.* 2012), although the validity of some interactions has been questioned (Brockmeier, Platzek *et al.* 2011). Given the specificity demonstrated for Grp94 by the OS-9 94BR, whether these other interactors shared a structural motif with Grp94 that might explain their co-precipitation, or indeed if OS-9 showed similar isoform-dependent differential binding, was questioned.

LONP2 (Lon protease homologue 2, peroxisomal) is a 852 a. a. protein containing a LON domain, nucleotide binding region and a microbody targeting signal (Figure 5.1). LON proteases are part of the AAA+ (ATPases Associated with various cellular Activities) family, containing a highly conserved ATPase domain and a proteolytic domain (Nagy, Akoev *et al.* 2006). LON proteases have been demonstrated to function in the cytosol of prokaryotes and in the mitochondria and peroxisomes of eukaryotes (Maurizi 1992; Goldberg,

Moerschell *et al.* 1994; Gottesman 2003; Takaya, Suzuki *et al.* 2003; Maupin-Furlow, Gil *et al.* 2005; Aksam, Koek *et al.* 2007). Of particular interest in the context of this thesis is the observation that LON proteases are essential for the removal of misfolded and oxidatively damaged proteins (in particular substrates harbouring stretches of hydrophobic a. a. residues), as well as certain short-lived regulatory proteins in the mitochondrial matrix (Goldberg and Waxman 1985; Suzuki, Suda *et al.* 1994; Bota and Davies 2002; Botos, Melnikov *et al.* 2004). The bacterial LON protease mediates approximately half of all protein degradation (Ondrovicova, Liu *et al.* 2005; von Janowsky, Knapp *et al.* 2005; Gur and Sauer 2008), while a decrease in the concentration of LON protease in human cells causes defects in the mitochondrial morphology and an increased rate of apoptosis (Bota and Davies 2002; Bota, Ngo *et al.* 2005). LONP2 is a peroxisome-specific isoform of LON protease, suggested to mediate the selective degradation of misfolded and unassembled polypeptides in the peroxisomal matrix (Kikuchi, Hatano *et al.* 2004), and may also indirectly regulate peroxisomal fatty acid-oxidation (Okumoto, Kametani *et al.* 2011).

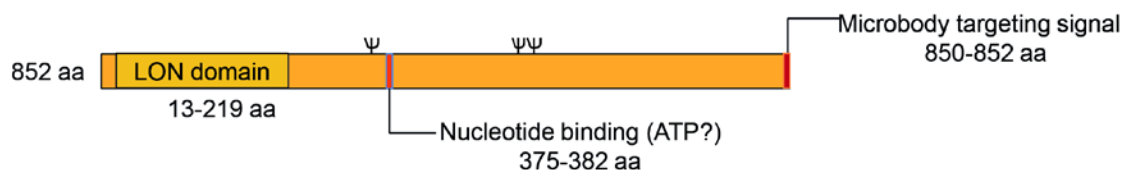


Figure 5.1: The LON protease.

Domain architecture of LONP2 (852 a. a.) containing a LON domain (13-219), nucleotide binding domain (375-382) and a microbody targeting domain (850-852). Three potential N-linked glycosylation sites (Ψ) are shown.

The hypoxia response (HR) is a cellular stress response that is initiated when an organism is subjected to a low oxygen environment. The transcription factor HIF (hypoxia-inducible factor) plays a key role in the maintenance of oxygen homeostasis in all metazoans (Semenza 2007). HIF target genes mediate systemic and local adaptation to hypoxia, inducing enhanced O₂ delivery by increasing erythropoietin, blood vessel dilation, and angiogenesis (Kelly, Hackett *et al.* 2003; Pugh and Ratcliffe 2003; Semenza 2003). HIF is a heterodimer that consists of an oxygen-regulated α subunit and a constitutively expressed HIF-1 β subunit, with molecular masses of 120-130 and 91-94 kDa, respectively (Figure 5.2). Both the α and β subunits belong to the family of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain-containing transcription factors (Wang, Jiang *et al.* 1995), with the BHLH and PAS domains mediating DNA binding and dimerisation. The α subunit is highly oxygen sensitive, whereas the β -subunit is oxygen insensitive and constitutively expressed in the nucleus, serving as the DNA-binding partner for the α subunit. To date, three HIF- α isoforms (HIF-1 α , HIF-2 α and HIF-3 α) have been described, with HIF-1 α being ubiquitously expressed (Wiesener and Maxwell 2003). Under normoxia, HIF-1 α is continuously synthesised and instantaneously degraded in an ubiquitin/proteasome dependent manner (Salceda and Caro 1997; Huang, Gu *et al.* 1998; Kallio, Wilson *et al.* 1999). In contrast, in hypoxic conditions, degradation of HIF-1 α is suppressed resulting in its stabilisation and translocation to the nucleus, where it binds to HIF- β and acts as a transcriptional

activator for target genes containing a hypoxia response element (HRE) (Semenza and Wang 1992; Semenza 2011).

The human HIF-1 α subunit is an 826 a. a. protein (Figure 5.2) which, in addition to the bHLH and PAS domains, contains a unique O₂-dependent degradation domain (ODDD) that is responsible for HIF-1 α degradation in the presence of oxygen (Huang, Gu *et al.* 1998), and two transactivation domains NTAD (N-terminal transactivation domain) and CTAD (C-terminal transactivation domain) which are essential for HIF activity (Jiang, Agani *et al.* 1997; Pugh, O'Rourke *et al.* 1997).

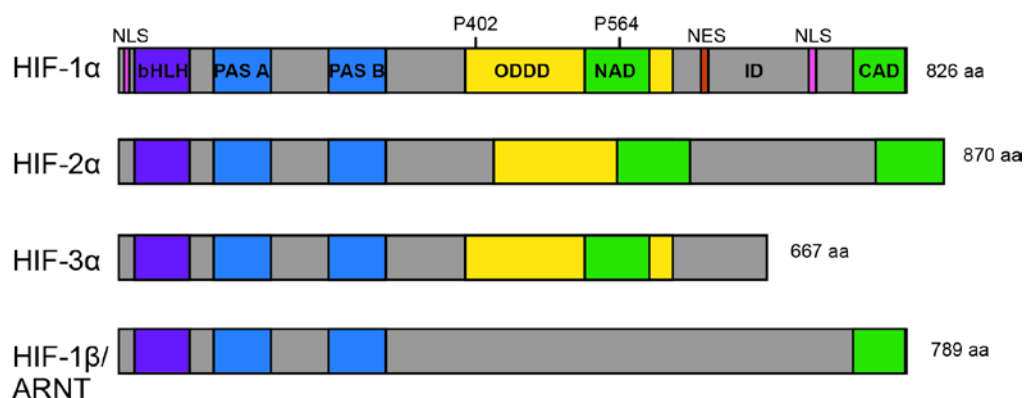


Figure 5.2: Schematic representation of human HIF proteins.

HIFs are members of the basic helix-loop-helix (bHLH)/PER-ARNT-SIM (PAS) domain family of transcription factors. They bind to DNA as heterodimers composed of an oxygen-sensitive HIF- α subunit (HIF-1 α , -2 α or 3 α) and a constitutive HIF-1 β (ARNT) subunit. The bHLH (purple) and PAS (blue) domains mediate DNA binding and dimerisation respectively. HIF- α subunits contain a unique oxygen-dependent degradation domain (ODDD – in yellow) that is responsible for stability in an oxygen-dependent manner. The N-terminal transactivation domain (NAD) and C-terminal transactivation domain (CAD) (both in green) mediate the transcriptional activation of HIF target genes. Additionally, two nuclear localisation signals (NLS), one nuclear export signal (NES) and the location of two hydroxylation sites (prolines 402 and 564) in HIF-1 α are shown.

Additionally in this chapter, the interactions of the ER lectin XTP3-B will be discussed. Both OS-9 and XTP3-B are mammalian homologues of Yos9p,

displaying 15 % sequence similarity to their yeast counterpart and approximately 23 % sequence similarity to each other (Christianson, Shaler *et al.* 2008). XTP3-B is composed of 483 a. a. and contains two MRH domains (Figure 5.3). Similar to OS-9, XTP3-B recognises high-mannose-type sugar chains, such as the Man₅GlcNAc₂-containing terminal Man α _{1,6}Man sequence (Figure 1.4), and is capable of co-precipitating the model ERAD substrate NHK (Yamaguchi, Hu *et al.* 2010). XTP3-B has additionally been shown to interact with the Hrd1/SEL1L ubiquitin ligase complex but does not interact with Grp94 (Christianson, Shaler *et al.* 2008). How substrates partition between OS-9 and XTP3-B remains unclear, however, previous studies have highlighted differences between the behaviour and predicted structure of the two lectins (Bernasconi, Galli *et al.* 2010; Satoh, Chen *et al.* 2010; Fujimori, Kamiya *et al.* 2013). Here, the association of XTP3-B with the carboxypeptidase CPVL (Carboxypeptidase Vitellogenic -Like Protein) reported previously will be examined.

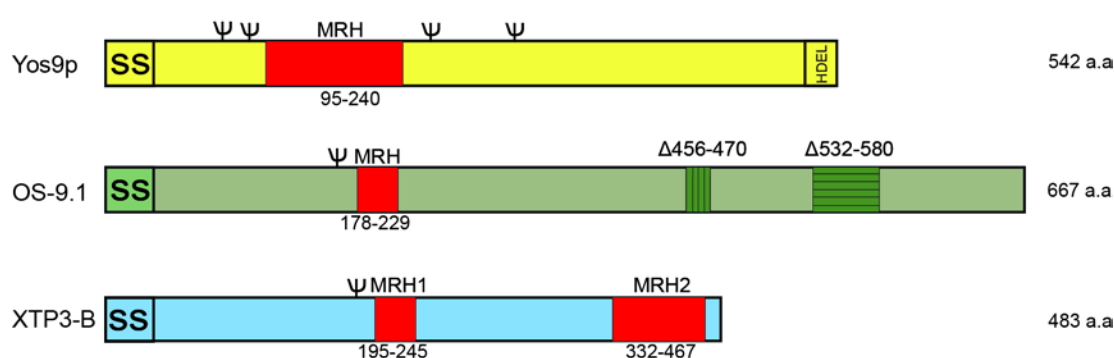


Figure 5.3: MRH domain containing ER proteins

Schematic comparison of yeast Yos9p and its two mammalian homologues XTP3-B and OS-9. Signal sequences (SS), conserved MRH domains (red) and potential *N*-linked glycosylation sites (Ψ) are shown.

5.2 Results

5.2.1 LONP2 interacts with cytoplasmically directed OS-9

LONP2 was identified as an interaction partner of OS-9 in a large proteomics study of the mammalian ERAD interaction network, in which protein complexes captured by S-protein affinity purification from detergent-solubilised lysates were analysed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) (Christianson, Olzmann *et al.* 2012). Indeed, the association of OS-9 with the protease LONP2 suggests the possibility that some substrates may be partially degraded prior to dislocation. However, LONP2 does not contain a predicted ER signal sequence, has previously been demonstrated to locate to peroxisomes and contains a typical peroxisomal targeting signal-1 (PTS-1) (Kikuchi, Hatano *et al.* 2004; Omi, Nakata *et al.* 2008). To establish whether OS-9 and LONP2 do interact and if they do so in the context of an intact ER, S-tagged isoforms of OS-9 were co-transfected with FLAG-HA-tagged LONP2 and affinity purified with S-agarose. Under these conditions, LONP2 was co-precipitated with all four isoforms of OS-9 (Figure 5.4 B). However, co-precipitation of endogenous LONP2 by transfected S-tagged isoforms of OS-9 gave conflicting results. LONP2 was only precipitated by OS-9 constructs (OS-9.1-4_{Δss}) that were intentionally mislocalised to the cytosol through deletion of their ER targeting sequence. This suggests that the interaction seen in Figure 5.4 may in fact be an artefact of LONP2 over-expression, and that the interaction does not occur within the ER. In an attempt to clarify LONP2's localisation, immunofluorescence was carried out, however, the data (Figure 5.4

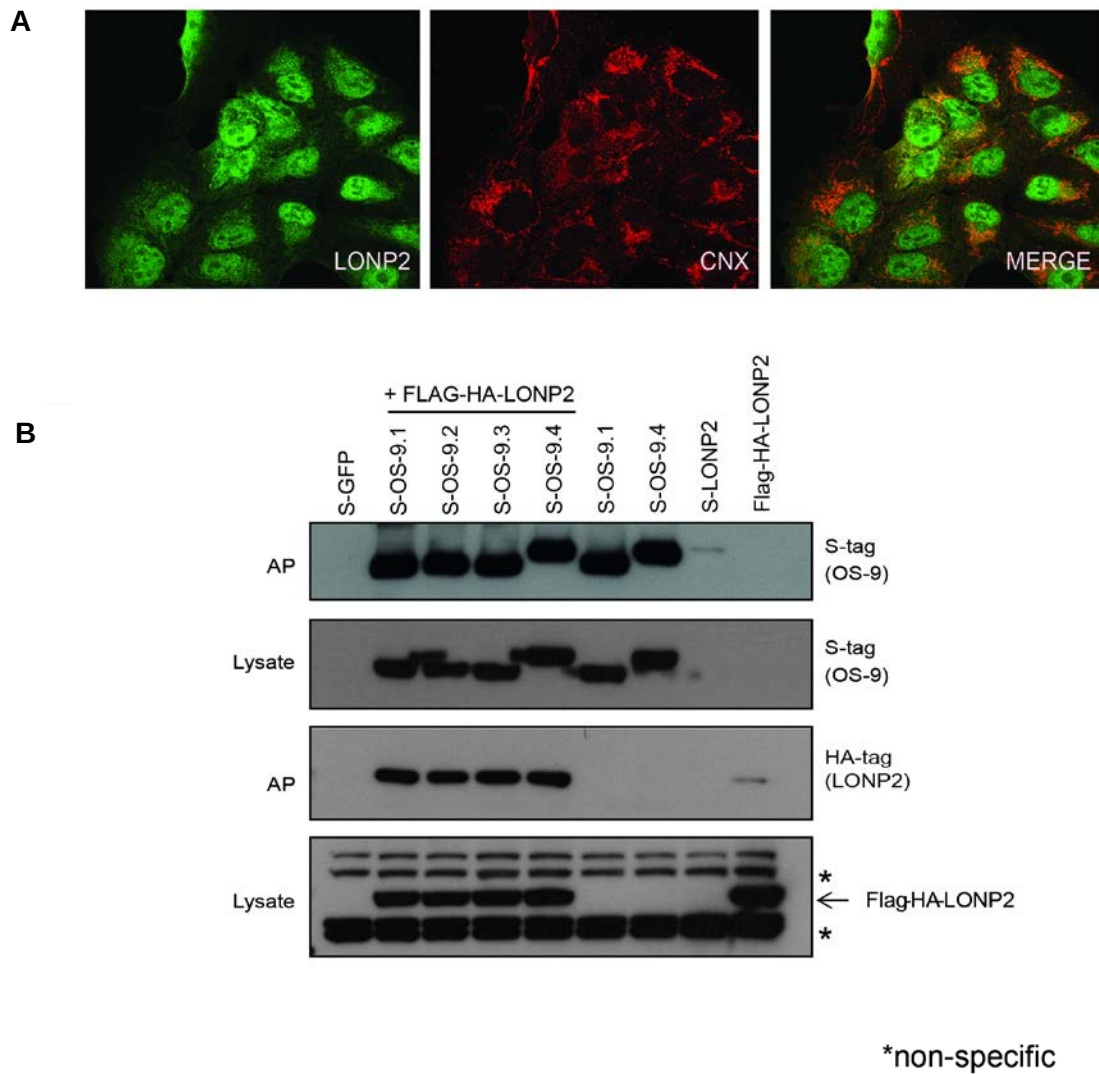


Figure 5.4: The LON protease LONP2 co-precipitates with S-OS-9

A) Co-localization of endogenous LONP2 (green) with the ER-resident protein Calnexin (red) by immunofluorescence in U2OS cells. B) Co-precipitation of FLAG-HA-LONP2 with S-tagged OS-9 isoforms, expressed in HEK293 cells, OS-9 complexes were affinity purified by S-protein agarose with western blots probed for HA-tag and S-tag.

B) were inconclusive due to an inability to discern whether the resulting pattern reflected a lack of specificity of the LONP2 antibody or *bona fide* LONP2 localisation (Figure 5.4 A). However, LONP2 co-precipitation with OS-9 was

unaffected by EndoH treatment despite the presence of three putative acceptor sites, suggesting that LONP2 was not glycosylated, supporting the finding that the OS-9/LONP2 interaction does not occur within the ER (Figure 5.5 B). These data suggest that OS-9 may have alternate interactions if cellular conditions cause it to mislocalise to outside the ER.

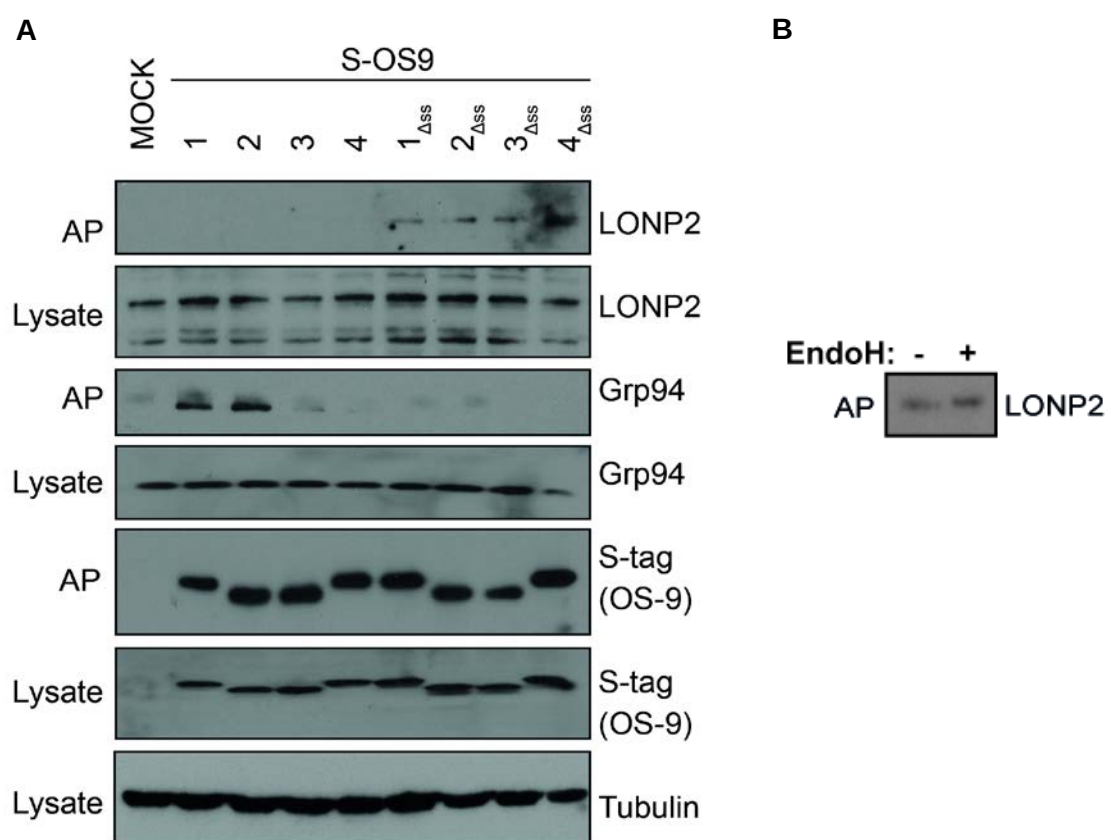


Figure 5.5: LONP2 co-precipitates with cytoplasmically localised S-OS-9.

A) Co-precipitation of endogenous LONP2 with S-tagged OS-9 isoforms and S-tagged OS-9 isoforms lacking a signal sequence (Δ ss), expressed in HEK293 cells. OS-9 complexes were affinity purified by S-protein agarose with western blots probed for HA-tag and S-tag. B) Treatment of endogenous LONP2 post-precipitation with S-OS-9, with S-agarose with EndoH.

5.2.2 HIF-1 α interacts with cytoplasmically directed OS-9.4

In yeast two-hybrid screens, OS-9 was identified as a protein that represses the transcription factor HIF through the activation of two enzymes that initiate oxygen-dependent degradation of HIF- α subunits (Baek, Mahon *et al.* 2005). The oxygen sensing prolyl-hydroxylases PHD2 and PHD3 initiate the degradation of HIF-1 α through its hydroxylation at proline 402 and/or 564 (Epstein, Gleadle *et al.* 2001; Hoffman, Ohh *et al.* 2001; Jaakkola, Mole *et al.* 2001; Yu, White *et al.* 2001) which results in the binding of von Hippel-Lindau protein (VHL), the recognition component of an E3 ubiquitin ligase, which targets HIF-1 α for subsequent degradation (Huang, Gu *et al.* 1998; Kallio, Wilson *et al.* 1999; Kamura, Sato *et al.* 2000). Baek *et al.* have suggested a role for OS-9 as a negative regulator of HIF-1, promoting the oxygen-dependent degradation of HIF-1 α by the proteasome by facilitating prolyl-hydroxylation through interaction with HIF-1 α and PHDs (Baek, Mahon *et al.* 2005).

To confirm the interaction of OS-9 with HIF-1 α , and to assess if this interaction is affected by alternative splicing of OS-9 in a similar manner to Grp94, HEK293 cells were co-transfected with S-tagged isoforms of OS-9 (1-4) either containing or lacking a signal sequence (Δ ss), the latter of which were mislocalised to the cytosol, with myc-tagged HIF1- α . Cells were lysed in TX-100 and precipitated with S-agarose. HIF-1 α failed to interact with any ER-targeted OS-9 isoforms (OS-9.1-4), however, when the signal sequence was removed, HIF-1 α could be co-precipitated but only with isoform 4 (OS-9.4 Δ ss, Figure 5.6). These data suggest reiterate the possibility that OS-9 may have alternative

interactions if cellular conditions cause it to mislocalise outside the ER. However, in contrast to LONP2, the OS-9/HIF-1 α interaction occurred only in the context of the OS-9.4 isoform, suggesting the splicing of exon 15 may result in the formation of a binding site or a modification to the structural confirmation that permits the interaction. Interestingly, OS-9.2 also lacks exon 15, yet fails to precipitate HIF-1 α , suggesting that the partial splicing of exon 11 prevents the interaction, perhaps again through influencing the structure of OS-9.

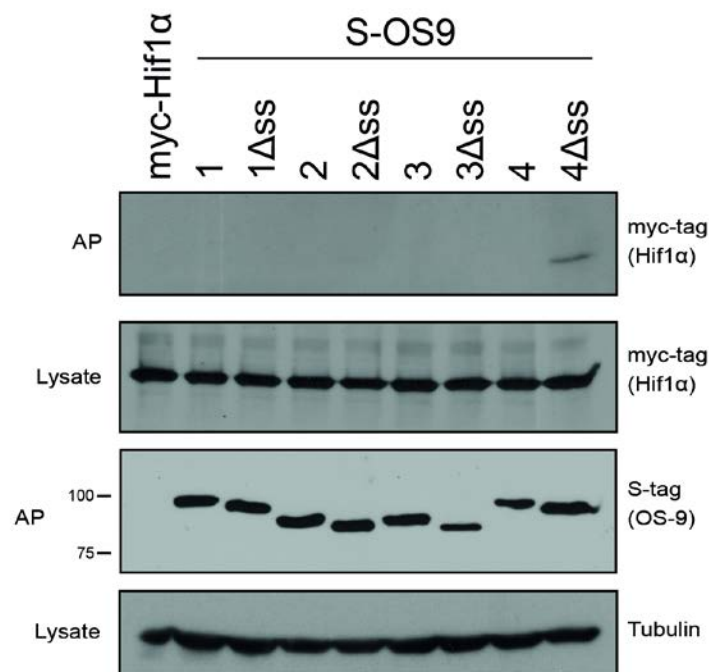


Figure 5.6: HIF-1 α interacts with cytoplasmically directed OS-9.4

Co-precipitation of myc-tagged Hif-1 α with S-tagged OS-9 isoforms, and S-tagged OS-9 isoforms lacking a signal sequence (Δ ss), expressed in HEK293 cells. OS-9 complexes were affinity purified by S-protein agarose, with western blots probed for myc-tag and S-tag.

5.2.3 Post-translational OS-9 targeting to the cytoplasm

If an isoform of OS-9 (or all isoforms) did reach the cytosolic environment after passing through the ER lumen, NGly1 would presumably cleave the *N*-linked glycan at N178 in a manner similar to that which occurs for ERAD substrates. An intriguing observation over the course of this study was that expression of S-OS-9 isoforms led to the detection of two bands of ~40 kDa that corresponded to N-terminal fragments (positive for S-tag). The source of these bands is not clear and could result from either; 1) cleavage within full-length OS-9; or 2) premature termination. If it was the former, then an intact C-terminal fragment may be datable by western blot. Indeed, the rabbit α -OS-9 from the Kopito lab recognises the C-terminus, however, due to time limitations these experiments could not be carried out.

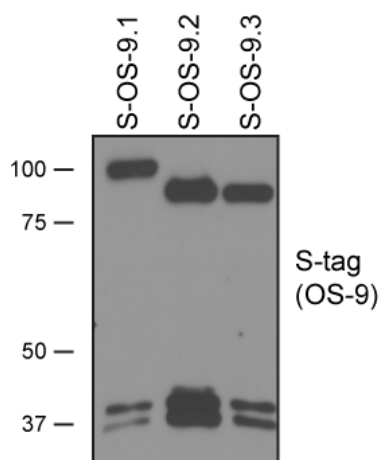


Figure 5.7: Potential N-terminal fragments of OS-9

Expression of S-OS-9 isoforms (1, 2, 3), precipitation via S-agarose and probing with S-tag. Bands of ~40 kDa that correspond to N-terminal fragments were detected in addition to bands corresponding to OS-9 isoforms.

5.2.4 CPVL is an ER carboxypeptidase that interacts with XTP3-B

Similar to OS-9, XTP3-B is an ER-resident lectin that interacts with the HRD1 ubiquitin ligase complex (Christianson, Shaler *et al.* 2008; Hosokawa, Kamiya *et al.* 2009; Bernasconi, Galli *et al.* 2010) and is upregulated by UPR through Ire1/Xbp-1 activation (Bernasconi, Pertel *et al.* 2008). XTP3-B contains two MRH domains (Figure 5.3), however, only the C-terminal MRH domain has been demonstrated to recognise high-mannose-type sugar chains such as the Man₅GlcNAc₂-containing a terminal Man α _{1,6}Man sequence (Yamaguchi, Hu *et al.* 2010; Fujimori, Kamiya *et al.* 2013). Additionally, XTP3-B can recognise not only glycosylated misfolded proteins, but also non-glycosylated misfolded species in the HRD1 complex (Christianson, Shaler *et al.* 2008) and has lectin activity that specifically recognises M9 glycans (Fujimori, Kamiya *et al.* 2013).

Previous work from the Christianson lab, which employed a strategy of integrating proteomic, functional genomic and gene expression data sets to construct a comprehensive map of the ERAD interaction network (Christianson, Olzmann *et al.* 2012), revealed a novel interaction partner of XTP3-B: CPVL (Carboxypeptidase, vitellogenic-like) named due to its 43 % a. a. sequence similarity to a serine carboxypeptidase expressed in mosquito ovaries. CPVL is a carboxypeptidase and bears strong sequence similarity to serine carboxypeptidases. Carboxypeptidases are a large class of proteases that act to cleave a single a. a. from the C-termini of proteins or peptides, and have been shown to activate or inactivate bioactive peptides such as angiotensin, bradykinin, and endothelin I (Mahoney, Ntolsi *et al.* 2001). The CPVL gene

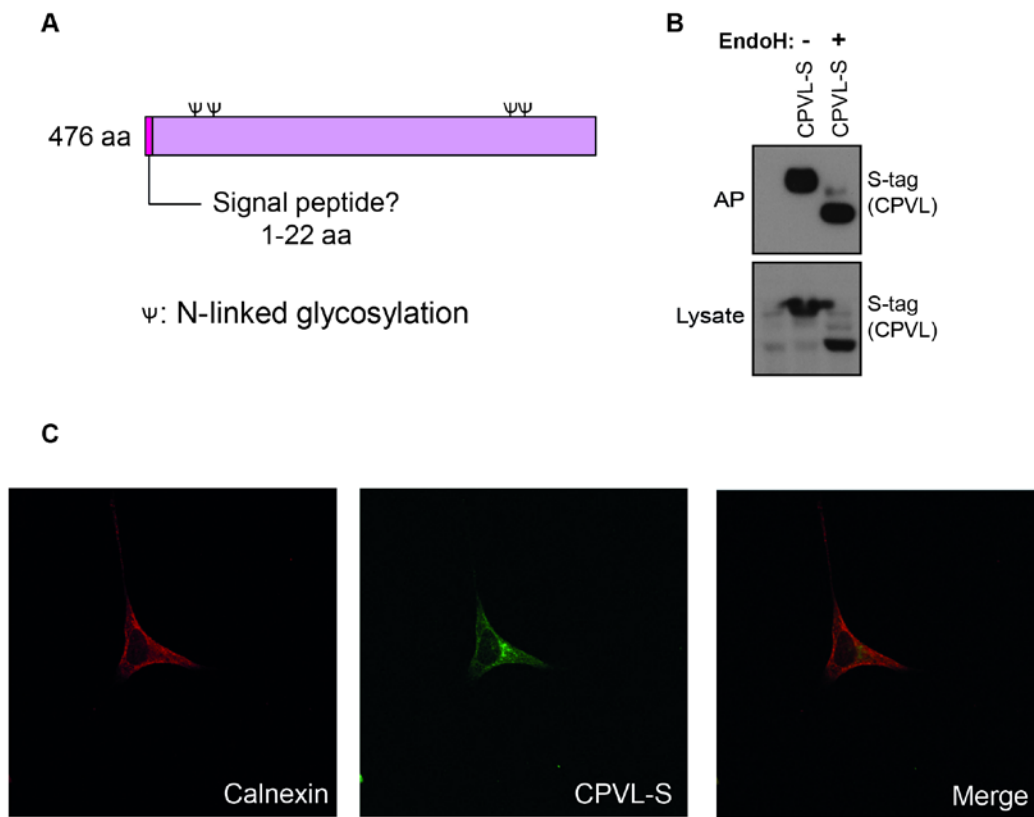


Figure 5.8: CPVL displays ER localisation. A) Domain architecture of CPVL (476 a. a.) containing a putative signal peptide (1-22) and four potential N-linked glycosylation sites (Ψ). C) Treatment of S-tagged CPVL post-precipitation with S-agarose and EndoH. B) Co-localization of S-tagged CPVL (green) with the ER-resident protein Calnexin (red) by immunofluorescence in U2OS cells.

contains at least 13 exons spread over more than 150 kb on human chromosome 7p14–p15 (Mahoney, Ntoli *et al.* 2001). The 476 amino acid gene product has a putative signal sequence but no transmembrane domain, and has four predicted glycosylation sites (Figure 5.8 A). To ascertain the localisation of CPVL, S-tagged constructs of CPVL (which had been precipitated from lysates via S-agarose) were treated with EndoH to assess their

glycosylation status (Figure 5.8 B). CPVL showed sensitivity to EndoH, displaying a clear mobility shift in its electrophoretic band, indicating that the protein was in the ER. Additionally, to confirm ER localisation, co-localisation of CPVL with the ER marker and resident protein calnexin was visualised by immunofluorescence, indicating that CPVL is a *bona fide* ER resident (Figure 5.8 C). However, it should be noted that IF was performed on U2OS cells transiently transfected with S-tagged CPVL (probed for the S-tag), and not on endogenous CPVL, as the available antibodies were unable to produce interpretable IF data. In the above experiments, CPVL was tagged at its C-terminus with an S-tag. Intriguingly, positioning the S-tag at the N-terminus of CPVL proved toxic to cells transfected with the construct, which displayed massive cell death. A possible explanation could be that the tagged N-terminus interfered with CPVL's signal sequence, resulting in mislocalisation of the construct and the acquisition of toxic functions.

5.2.4.1 XTP3-B interacts with CPVL in a lectin-independent manner

To confirm the reported XTP3-B/CPVL interaction (Christianson, Olzmann *et al.* 2012), HEK293 cells were transfected with S-tagged XTP3-B or co-transfected with S-tagged CPVL and myc-tagged XTP3-B. Cell extracts were precipitated S-protein agarose, followed by western blotting. CPVL-S co-precipitated XTP3-B, and XTP3-B was competent for co-precipitation of endogenous CPVL (Figure 5.9 B), confirming that XTP3-B and CPVL do indeed interact.

XTP3-B contains two MRH domains (Figure 5.3), with only the C-terminal MRH domain demonstrated to recognise high-mannose-type sugar chains, with specific co-precipitates with NHK (in a glycan-dependent manner) (Yamaguchi, Hu *et al.* 2010; Fujimori, Kamiya *et al.* 2013). As demonstrated above, CPVL is glycosylated (Figure 5.8 B), but it was questioned whether CPVL is a substrate of XTP3-B that is bound via its *N*-glycan in a MRH domain-dependent manner. HEK293 cells were transfected in parallel with five constructs harbouring mutations to one or both MRH domains of XTP3-B (R207A, R428A, R207A/R428A), which were reported to disrupt the lectin properties of that domain (Figure 5.9 A) (Christianson, Shaler *et al.* 2008). None of these mutants appreciably disrupted the interaction between CPVL and XTP3-B (Figure 5.9 C), while R207A did appear to display reduced binding this appears to be the result of a fault in the gel (bubble) which is enforced by the lack of disruption to binding of the R207A/R428A mutant (Figure 5.9 C). These data suggests that either the MRH domains are entirely dispensable for the interaction with CPVL or that the interaction is bipartite, involving a peptide recognition domain in addition to interaction through the MRH domain(s). Further experiments will be required to fully characterise the XTP3-B/CPVL interaction, however, the association of the lectin XTP3-B with the carboxypeptidase suggests the possibility that some ERAD substrates may be partially degraded prior to dislocation.

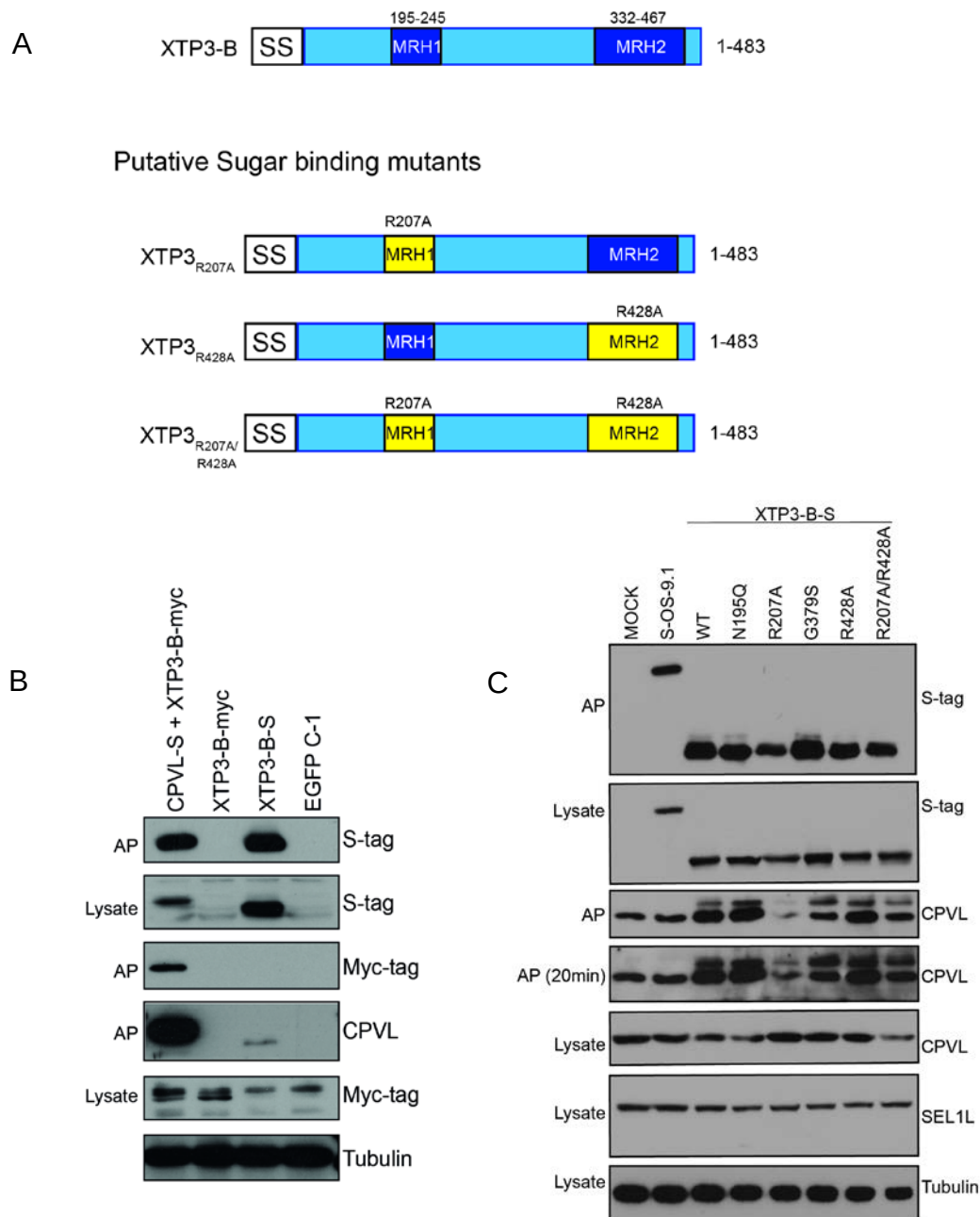


Figure 5.9: The carboxypeptidase CPVL co-precipitates with XTP3-B.

A) Domain architecture of XTP3-B (483 a. a.), containing a putative signal peptide (1-22) and two MRH domains, in addition to the locations of mutations that disrupt one or both MRH domains. B) Co-precipitation of XTP3-B-myc with S-tagged CPVL (lane 1) and endogenous CPVL with XTP3-B-S (lane 3), expressed in HEK293 cells. Complexes were affinity purified by S-protein agarose, and western blots probed for myc-tag, S-tag and CPVL. C) Co-precipitation of XTP3-B-S and a panel of XTP3-B MRH domain mutants with endogenous CPVL, expressed in HEK293 cells. Complexes were affinity purified by S-protein agarose, with western blots probed for S-tag, CPVL and SEL1L.

5.5 Discussion

OS-9 is a *bona fide* ER-resident protein, targeted to the lumen by a validated signal sequence and glycosylation at N178 (Christianson, Shaler *et al.* 2008) (confirmed in Figure 3.2). Yet early reports supposed that OS-9 localised cytoplasmically due to its interaction with proteins including HIF-1 α , TrpV4 and MeprinA- β . Despite this apparent incongruity in locale, interaction domains were identified. A yeast two-hybrid screen identified an OS-9 fragment, 357-667, sufficient for interaction with HIF-1 α (Baek, Mahon *et al.* 2005). While truncations and alanine scanning of MeprinA- β 's C-terminal, cytosolic tail identified a minimal stretch sufficient to interact with OS-9 (Litovchick, Friedmann *et al.* 2002). OS-9 also bound fragments of HIF-1 α (692-794/826) and TrpV4 (237-468), with no obvious sequence similarity existing between these fragments making it is unlikely that a common OS-9 binding motif exists. Do these reports represent physiological interactions or are they the product of experiment flaws? Indeed, yeast two-hybrid screens are notoriously error prone; however, what would the consequences of a non-ER localised population of OS-9 be?

In addition to the above reports, a proteomics screen by Christianson *et al.*, identified LONP2, a peroxisomal protease involved in the degradation of misfolded proteins, in complex with OS-9 (Christianson, Olzmann *et al.* 2012). In this study, OS-9 and LONP2 are shown to be capable of interaction. However, the interaction occurs only in the context of cytoplasmically localised OS-9. The results can be interpreted in a number of ways: 1) mislocalisation of OS-9,

through a yet undefined mechanism, results in a gain of function; 2) the interaction is an artefact of cell lysis and does not occur natively; or 3) over expression of LONP2 results in increased turnover of LONP2, driving association with S-OS-9. Levels of endogenous LONP2 interacting with ER-localised OS-9 may be so low that they failed to be identified by the detection method employed in this study, and the artificial increase of LONP2 expression may have led to increased turnover to maintain a basal level of LONP2, hence driving interaction with OS-9. Similarly, it has been suggested that HIF-1 α is under surveillance of the ER-associated protein quality control system, where OS-9 directly interacts with HIF-1 α in ER-derived vesicles and promotes its degradation by the proteasome. Here it is demonstrated that HIF1- α failed to interact with any ER-targeted OS-9 isoforms, but that HIF-1 α could be co-precipitated when the signal sequence was removed, but only with isoform 4 (OS-9.4 $_{\Delta SS}$). Both the interaction of OS-9 with LONP2 and HIF-1 α suggest possible gain of function of OS-9 upon mislocalisation.

Is it possible that under certain circumstances a subset (or potentially a specific isoform) of OS-9 may be intentionally or unintentionally mislocalised to the cytosol, or are these interactions merely a by-product of the experimental conditions applied? OS-9 exhibits a high degree of disorder (as predicted by PONDR, DisoPred – Figure 3.8) in the C-terminus, suggestion that it may be unstructured in a way that confers non-specific or “sticky” binding properties. This aspect may be functional in the proper cellular context (i.e. OS-9 binding of ERAD substrates or GRP94), but aberrant if incorrectly exposed to an

alternative environment, a situation that may occur under stress or a loss of translation fidelity. Moreover, the sticky nature of OS-9 may yield non-native interactions following cell lysis and compartmental mixing. However, it may be possible that a sub-population of OS-9, or a particular OS-9 isoform, may form a distinct cytosolic sub-population. Indeed, minor cytosolic populations of two ER luminal proteins, calreticulin and BiP, have been reported (Shaffer, Sharma *et al.* 2005; Ni, Zhou *et al.* 2009). Calreticulin, normally a calcium-dependent, ER-resident chaperone that participates in the folding of secreted proteins, has been demonstrated to mislocalise to the cytosol where it exhibits a number of gain of functions. Trace amounts of calreticulin are present in the cytosol, implicating a cytosolic integrin-binding protein (Leung-Hagesteijn, Milankov *et al.* 1994; Coppolino, Migliorini *et al.* 1995; Coppolino, Woodside *et al.* 1997), nuclear export factor (Holaska, Black *et al.* 2001), regulator of steroid hormone receptor activity (Burns, Duggan *et al.* 1994; Dedhar 1994), and an mRNA binding protein that regulates p21 translation (Iakova, Wang *et al.* 2004). Speculation regarding the mode of mislocalisation of calreticulin is attributed to an inherently inefficient signal peptide (Shaffer, Sharma *et al.* 2005). However, the OS-9 signal sequence is not uncommonly short and is able to target an unrelated polypeptide (e.g. TCR α) to the ER lumen (Christianson, Shaler *et al.* 2008), arguing against this explanation in the context of OS-9.

Of particular note is the absence of a KDEL sequence in OS-9. KDEL, the sequence Lys-Asp-Glu-Leu, or a closely related sequence is present at the C-terminus of soluble ER resident proteins and some membrane proteins: Grp78,

Grp94, PDI, have C-terminal KDEL sequences. The sequence is necessary for ER-retention, and sufficient to reduce the secretion of proteins from the ER through its recognition by a membrane bound receptor that continually retrieves proteins from later compartments of the secretory pathway. The means, by which OS-9 is retained within the ER, in the absence of a canonical ER retention motif, is unknown. However, it may be provided by an atypical retention motif or conferred through an interaction with a KDEL-containing protein (e.g. Grp94 or BiP). In the latter case, loss of interaction with or reduced expression of the interaction partner may result in the mislocalisation of OS-9, indeed as may be the case with OS-9.3 and OS-9.4 in the context of Grp94 interaction.

This thesis presents the ER localisation of OS-9 (Figure 3.2), however, the intensity of IF may have failed to identify small sub-populations of OS-9 present outside the ER. Additionally, mislocalisation of OS-9 (or one of its isoforms) may only occur under certain stimuli which had not been incorporated into our analysis. Further studies are certainly required to verify if these interactions occur endogenously. However, if proven, the degradation of HIF-1 α via OS-9 linking the hypoxia response and ERAD would be an intriguing field of research.

CHAPTER SIX

Discussion/Conclusions

This study aimed to characterise the OS-9/Grp94 interaction and its role in the ERAD protein degradation pathway. The results outlined in this thesis provide evidence that: 1) OS-9 is indeed a resident of the ER lumen and that the OS-9/Grp94 interaction occurs in the context of an intact ER; 2) a 65 a. a. region of OS-9 (OS-9₄₄₃₋₅₀₇), designated the 94BR (Grp94 binding region), is necessary and sufficient for OS-9 to interact with the chaperone Grp94 (Chapter 3); 3) the LG2-MEL-5-35 cell line, which is homozygous for a OS-9 mutant (P446L) that is not competent for interaction with Grp94, displayed increased proliferation when compared to the LG2-MEL-207 cell line heterozygous for the mutation; and that 4) the 94BR is not sufficient for interaction with the model substrate NHK (Chapter 4). In addition, this study demonstrates that the previously reported interactions of OS-9 with LONP2 and HIF-1 α occur, but only in the context of localisation of OS-9 to the cytosol. The following chapter will discuss these results and their implications on our understanding of ERAD.

6.1 ERAD is essential for cellular homeostasis

The protein folding, quality control and disposal machineries that operate in the mammalian ER lumen determine the fate of thousands of proteins, each one characterised by unique structural, biophysical and biochemical properties. Protein biosynthesis is a complex and error-prone process, which is further complicated by the propensity for transcriptional and translational errors and mutations. ERQC and ERAD are essential to cellular homeostasis; ensuring that misfolded proteins are blocked from progressing

through the secretory pathway and are degraded, mitigating their congestion of the ER.

Recognition of misfolded substrates through characteristics that differentiate them from correctly or partially folded proteins, their dislocation (membrane proteins) or retrotranslocation (luminal proteins) across the lipid bilayer, ubiquitination via membrane-embedded ubiquitin ligases and their eventual degradation via the 26S proteasome are the steps that combine to constitute ERAD. Despite the many advances in defining the interactions and mechanisms involved in coupling misfolded protein recognition and degradation, many aspects of ERAD remain unresolved. Of particular interest has been OS-9, an ER-resident lectin potentially functioning as a link between the recognition and delivery of aberrant proteins to the SEL1L/Hrd1 ubiquitin-ligase complex. Current models place OS-9 in a position to shuttle a subset of misfolded proteins to the E3 ubiquitin ligase Hrd1, through interaction with its membrane adapter protein SEL1L. Additionally, OS-9 interacts with Grp94, the ER-resident Hsp90 family member, in a manner that is mutually exclusive of its interaction with SEL1L (Christianson, Shaler *et al.* 2008). It is anticipated that a deeper understanding of the molecular arrangement of the SEL1L:OS-9:Grp94 complex, how they interact with each other and aid in the degradation of substrate, will contribute to our understanding of how protein triage decisions are made in the eukaryotic secretory pathway.

6.2 The OS-9/Grp94 interaction is mediated through the 94BR

We report that the interaction between OS-9 and Grp94 takes place in the ER lumen and involves the C-terminal domain of OS-9. More precisely, a 65 a. a. region (443-507), is necessary and sufficient for Grp94 binding by OS-9. The importance of maintaining the integrity of this region was demonstrated by the loss of interaction with Grp94 between OS-9.3 and OS-9.4, both of which experience partial splicing of exon 11 resulting in the loss of residues 456-470. Additionally, three point mutations have been reported within the OS-9_{94BR}: a somatic mutation identified in a melanoma cell line LG2-MEL (P446L), discovered after an OS-9 peptide fragment was presented as a CTL-antigen; a SNP identified as prevalent in populations of African descent (S454L); and a second SNP (R486Q), with minor occurrence in populations of European-American decent. Indeed, the absolute conservation of both P446 and R486 in chordates, hints to their importance, while S454 is also conserved in mammals and other chordates. None of the three mutants within the 94BR are capable of co-precipitation of Grp94, however, as our analysis did not include structural data, it is not clear whether these mutants are direct contact-points for Grp94 binding or if their mutation interferes with the secondary structural conformation of the binding domain. Indeed, the replacement of a proline by a leucine residue, as seen in P446L, would be predicted to modify the protein's structure, as proline residues are known to induce protein kinks due to their rigid cyclic lateral chain (Woolfson and Williams 1990). Additionally, fragments bisecting the 94BR (OS-9₂₄₉₋₄₈₆, OS-9.1₄₈₇₋₆₆₇) also fail to support Grp94 binding, suggesting that an

essential secondary structure may be disrupted. Secondary structure algorithms (Jpred, PsiPred) predict two extended alpha helices joined by a short linker segment within this region. However, analysis using each of the mutant sequences did not alter the predicted structure. A more in-depth *in vivo* analysis (e.g. NMR – Nuclear magnetic resonance), beyond the scope of this thesis, would be required to truly appreciate their structural effects. Together, these data demonstrate that a highly conserved region within OS-9 (94BR) is responsible for binding Grp94. Moreover, these observations raise the possibility that conditional splicing to generate alternative isoforms could alter the functional capacities of OS-9. The results of our mapping of the 94BR were recently published in the paper “OS-9 facilitates turnover of non-native Grp94 marked by hyperglycosylation” (Dersh, Jones *et al.* 2014) and mapping of the Grp94 to the C-terminus of OS-9 has recently been confirmed by Seidler *et al.* (Seidler, Shinsky *et al.* 2014).

6.3. Expression of OS-9 isoforms that do not interact with Grp94

Four potential splice variants of OS-9 were observed in this study using isoform-specific PCR. Prior to our analysis, OS-9.1 and in particular OS-9.2 had been reported as the most prominent isoforms, with doubt surrounding the existence of OS-9.3 and to a greater extent OS-9.4 (Kimura, Nakazawa *et al.* 1998; Bernasconi, Pertel *et al.* 2008). Using an improved amplification scheme, with primer annealing sites more distal to the region of splicing than previous studies, four bands were detected that migrated at molecular weights

corresponding to those predicted for each isoform. Each amplification product was isolated and sequenced, and confirmed to correspond to the four predicted OS-9 isoforms. While the presence of four isoforms of OS-9 was verified at the mRNA level, confirmation of the translation of the four isoforms to proteins proved more problematic. This may be due to intrinsic instability of the mRNA of both OS-9.3 and OS-9.4. However, as detection of three bands that were attributed to OS-9.1, OS-9.2 and OS-9.4 was achieved (Figure 3.16 D), thus confirming the existence at the protein level of an OS-9 isoform that lacks the region 456-470, the difficulties in detection were conceivably due to poor resolution under the SDS-PAGE conditions used in this analyses.

The trigger, frequency and physiological role of alternative splicing of OS-9 have yet to be determined. Indeed, the regulation and mechanisms of splicing are extremely intricate, carried out by the spliceosome, a macromolecular machinery composed of five small nuclear ribonucleoprotein particles (U1, U2, U4, U5, and U6 snRNPs) and more than 200 auxiliary proteins (Jurica and Moore 2003; Will and Luhrmann 2011). Regulation occurs through splicing enhancers or silencers that are bound by serine-arginine rich (SR) proteins, the SR proteins are themselves regulated via their phosphorylation states, which can in turn change their activities, localisation or interactions with other proteins or RNA (Stamm 2008; Chen and Manley 2009). The matter is further compounded by each cell type having a unique repertoire of SR proteins, and several SR proteins being themselves alternatively spliced (Black 2003; Kashima and Manley 2003). Initial experiments employing UV irradiation and a

MAP kinase inhibitor did not induce a marked change in the isoform profile. However, other stress-inducing conditions yet to be tested, may prove more efficacious, including; hypoxia, overgrowth/undergrowth, glucose starvation, ER stress and γ -irradiation. Several of these conditions are particularly relevant for tumour cells. For instance, the vigorous growth of tumours may expose cells to regions with insufficient blood supply and therefore low oxygen and glucose availability which, in turn, may result in alternative splicing.

A number of reports have already suggested that OS-9 splice variants may function differentially. Silencing of OS-9.1 did not impair degradation of NHK, while silencing of both OS-9.1 and OS-9.2 delayed ERAD (Christianson *et al.*, 2008). Additionally BACE476 Δ , the pancreatic isoform of human β -secretase degraded by ERAD (Molinari, Galli *et al.* 2002), binds specifically to OS-9.1 but not to OS-9.2 when SEL1L is silenced by RNAi (Bernasconi, Galli *et al.* 2010). Therefore, a decrease in OS-9.1 levels, or conversely an increase OS-9.3/OS-9.4, could have a significant impact on overall fidelity. Hence, potential deregulation of splicing may serve to fuel cancer progression, altering the proportions of isoforms expressed, resulting in the increased/decreased degradation ability of components involved in growth regulation.

6.4 Consequences of OS-9 splicing

This study has shown that neither OS-9.3 nor OS-9.4 can appreciably bind Grp94. Thus, a condition that stimulates the alternative splicing of exon 11,

and hence the generation of isoforms OS-9.3 and OS-9.4, would result in an OS-9 pool with reduced or lost interaction with Grp94. This poses interesting questions regarding the consequences of loss of interaction with Grp94 in relation to ERAD, and also its effect on cellular function, viability and proliferation. In particular, considering its relative abundance in the ER and its potential role in ERAD, loss of interaction with Grp94 could conceivably play a major role in regulating OS-9-mediated substrate delivery to the SEL1L/Hrd1 ubiquitin ligase complex. A potential hint to the metabolic consequences of disruption to the OS-9/Grp94 interaction is provided by comparative proliferation assays between the melanoma cell lines LG2-MEL-207 and LG2-MEL-5-35. LG2-MEL is a melanoma cell line derived from a lymph node metastasis that was noted to express several antigens recognised by autologous CTLs (Vigneron, Ooms *et al.* 2002). This study observed an increased proliferation rate of the homozygous LG2-MEL subline LG2-MEL-5-35, harbouring two mutant alleles of OS-9 in comparison to heterozygous LG2-MEL-207, which retained one wild type OS-9 allele (Figure 3.13 B), suggesting that this loss of heterozygosity may favour tumour progression. However, it should be noted that common melanoma markers (PAX3, BRN2, and TBX3) were expressed abundantly in LG2-5-35 lysates but were lacking (or expressed at significantly lower levels) in LG2-MEL-207 lysates (Figure 3.15), which raises the question of whether the cellular phenotypes observed are a cause or consequence of mutant OS-9 expression, and whether or not this is correlated with decreased Grp94 interaction. Encouragingly, the C-to-T transition at position 1422 of OS-9

is not a one off event, with reports in uterine and thyroid tumours (GenBank Database, Accession Nos. [AW797189](#) and [AW607948](#)). In addition, a C-to-T transition at a dipyrimidine site, as in the case of P466L, is a mutation typically induced by UV exposure and frequently is observed in skin cancers (Brash, Rudolph *et al.* 1991; Ikehata, Kudo *et al.* 2003). Unsurprisingly due to the widespread nature of alternative splicing, its aberrant regulation leads to human disease and indeed the subject has been covered in numerous reviews (Cooper, Wan *et al.* 2009; Barta and Schumperli 2010; Dhir and Buratti 2010; Raponi and Baralle 2010; Irimia and Blencowe 2012; Gamazon and Stranger 2014; Scuderi, La Cognata *et al.* 2014). Indeed, the link between aberrant alternative splicing and cancer has been established (Xu and Lee 2003; Kalnina, Zayakin *et al.* 2005; Grosso, Martins *et al.* 2008; David and Manley 2010). Genome-wide studies have revealed more than 15,000 tumour-associated splice variants in a wide variety of cancers (He, Zhou *et al.* 2009; Shapiro, Cheng *et al.* 2011), with the potential employment of splice variants as cancer biomarkers being investigated (Venables, Klinck *et al.* 2008; Lenos, Grawenda *et al.* 2012). As the chromosomal region containing OS-9 (ch12.q15) is frequently targeted for genetic rearrangement and often amplified in human sarcomas (soft-tissue, osteo-) and gliomas, further investigation of the alternative expression of isoforms of OS-9, particularly in the context of cancer, may be warranted.

Of particular interest in the context of OS-9 is the recent suggestion that polypeptide segments affected by alternative splicing are most often intrinsically disordered, enriched in target sites for post-translational modification and

binding motifs that mediate protein-protein interactions, which increases the functional versatility of the corresponding proteins (Barash, Calarco *et al.* 2010; Ellis, Barrios-Rodiles *et al.* 2012). Indeed, analysis of OS-9 protein order by the PONDR algorithm indicates a high degree of disorder in a. a. 248-617, which contains the regions of alternative splicing, suggesting that the region may be natively unfolded. Currently, no structural information exists for the C-terminal half of OS-9 (248-617), and it shares no homology with Yos9p or any other known protein motifs. Indeed, attempts to isolate a recombinant C-terminal and 94BR protein over the course of this study proved unsuccessful, potentially due to the instability of this region. Intrinsically disordered regions (IDR) are commonly responsible for important protein functions, particularly signalling and regulation, which typically involve multiple partners and require high specificity and low affinity (Dunker, Brown *et al.* 2002; Iakoucheva, Brown *et al.* 2002; Uversky 2002; Dyson and Wright 2005). Computational predictions of protein disorder found that more than 30 % of eukaryotic proteomes are natively disordered (Ward, Sodhi *et al.* 2004). This intrinsic disorder may provide OS-9 with the plasticity and flexibility required to identify and bind a wide array of substrates. Intriguingly, a recent study characterising the entropy of IDRs, suggested their functions may be controlled by environmental factors such as changes in temperature, redox-potential and pH (Flock, Weatheritt *et al.* 2014). For example, decreased pH in the *cis*-Golgi promotes unfolding of the C-terminal of ER-resident protein 44 (Erp44), permitting its recognition and trafficking of substrate back to the ER (Vavassori, Cortini *et al.* 2013). Therefore,

it should be kept in mind that the complexity of OS-9's interactions, both with components of the ERAD pathway and substrates, may be further compounded through a similar form of regulation.

6.5 Function of the OS-9/Grp94 interaction

This work suggests that the interaction between OS-9 and Grp94 is a particularly specific one. However, when this project was begun, a functional role for the interaction was yet to be ascertained. Suggested possible functions were: 1) OS-9 may be an obligate client of Grp94; 2) the interaction may fulfill a role in ERAD; or 3) Grp94 may be degraded via OS-9. A notable difference between Yos9 and its mammalian counterpart is the absence of a KDEL or other potential ER retention sequences from mammalian proteins. Without a canonical retrieval sequence, it has been hypothesised that OS-9 retention may be conferred by virtue of its interaction with Grp94 (or BiP). Hence a loss in binding, as seen in OS-9.3/4 and the point mutants, may result in constitutive secretion of OS-9 and potentially a compromise to Hrd1-mediated ERAD. When localisation of S-OS-3 and S-OS-9_{94BR} was compared (Figure 3.20), S-OS-9_{94BR} appeared to reside within the ER and while the majority of S-OS-9.3 was also predominantly ER localised, it additionally displayed a higher degree of mislocalisation outside of the ER. This may conceivably be an artifact of the artificial up-regulation of OS-9.3 through transfection, or may offer a hint to Grp94's influence on OS-9's localisation. To eliminate the caveats and artifacts of overexpression, antibodies directed against specific isoforms of endogenous

OS-9 would prove a powerful tool, however, attempts to generate such antibodies over the course of this thesis proved unsuccessful. Further investigation shall be required to establish if Grp94 has a role in the ER's retention of OS-9, however, it was demonstrated in this thesis that Grp94 showed no effect on OS-9 stability, with steady-state levels of OS-9 not affected by a loss of Grp94.

Although both Grp94 and SEL1L bind to OS-9, neither protein precipitates the other, indicating that they must form distinct complexes with OS-9 (Christianson, Shaler *et al.* 2008). Therefore, perhaps by retaining a sequestered population of OS-9 with Grp94, it's available on demand if a need for degradation arises. A role for Grp94 in ERAD is logical as it has thus far been characterised primarily as a pro-folding component of the ER, it is required to assist in the folding of a select subset of client proteins that includes immunoglobulins (Melnick, Dul *et al.* 1994), insulin-like growth factors (Wanderling, Simen *et al.* 2007), some toll-like receptors and integrins (Randow and Seed 2001; Yang, Liu *et al.* 2007) and select members of the Wnt pathway (Liu, Staron *et al.* 2013). Indeed, Christianson *et al.* observed an impairment in NHK degradation upon Grp94 silencing (Christianson, Shaler *et al.* 2008). However, a recent study found that Grp94 bound poorly to misfolded α -1 antitrypsin mutants (NHK, NHK-QQQ and the aggregation-prone PI Z mutant), and that Grp94-deficient cells were able to degrade glycosylated NHK and non-glycosylated NS1 (misfolded immunoglobulin light chain) with the same kinetics as Grp94-sufficient cells (Dersh, Jones *et al.* 2014). This recent data suggests

that Grp94's role is not in ERAD, or at least not in the context of the investigated substrates.

As this study has demonstrated, the OS-9/Grp94 interaction is highly specific, rendering the possibility of Grp94 being a substrate of OS-9 an unlikely explanation. Despite Grp94 being itself a glycoprotein, the MRH domain, through which OS-9 selectively recognises trimmed *N*-linked glycans during ERAD, is dispensable for binding of Grp94. Indeed, the MRH domain is absent from the 94BR, and it was demonstrated that OS-9_{R188A} retained the ability to co-precipitate Grp94. Conversely, this study has shown that OS-9 co-precipitates non-glycosylated substrates (NHK-QQQ), and that OS-9_{R188A} retains the ability to co-precipitate NHK. Therefore, OS-9 demonstrates a polypeptide-binding capacity in the context of substrate recognition. However, this region is distal from that which binds Grp94, with the 94BR demonstrated to be insufficient to co-precipitate either NHK or NHK-QQQ. Nonetheless, a recent study compiled in association with this work, suggests a role for OS-9 in the degradation of a sub-population of Grp94 (Dersh, Jones *et al.* 2014). Dersh *et al.* reported the existence of hyper-glycosylated forms of Grp94 (hgGrp94) that were preferentially bound by OS-9. The results of this study suggest that hgGrp94 represents a pool of non-native Grp94, with altered conformations and decreased ATP binding affinity, which is subsequently degraded more rapidly (Dersh, Jones *et al.* 2014). In addition, Dersh *et al.* proposed that hgGrp94 is degraded via OS-9 in a lysosomal-like pathway and not by the proteasome via ERAD. Indeed, this thesis has subsequently demonstrated the co-localisation of

a sub-population of Grp94 in SEL1L-containing vesicles, in a similar manner and under the same conditions as OS-9 and SEL1L co-localising in LC3-1 positive vesicles (Figure 4.11). This data suggests that hgGrp94 is degraded along with OS-9 in ERAD “tuning” vesicles, so called EDEMosomes (which shall be discussed in further detail in the subsequent section) (Reggiori, Monastyrska *et al.* 2010; Bernasconi and Molinari 2011; Bernasconi, Noack *et al.* 2012). Additionally, mapping of the region of Grp94 which interacts with OS-9 suggests a region normally buried within the structure of Grp94 is responsible for OS-9 binding (Dersh, Jones *et al.* 2014).

Combined, these data suggest that the Grp94/OS-9 interaction plays a role in the degradation of “misfolded”, hyper-glycosylated forms of Grp94, which are degraded via OS-9 in an EDEMosome-dependent manner. However, this does not account for the entirety of the OS-9/Grp94 interaction, with hgGrp94 representing only a small sub-population of Grp94 that co-precipitates with OS-9. Therefore, additional roles for Grp94 in the context of its interaction with OS-9 cannot be excluded and require further investigation.

6.6 Interaction of OS-9 with substrate

It is now well established that OS-9 is a lectin that interacts with, and is required for, the degradation of some ERAD substrates (Bernasconi, Pertel *et al.* 2008; Christianson, Shaler *et al.* 2008; Hosokawa, Kamiya *et al.* 2009; Satoh, Chen *et al.* 2010). In particular OS-9 is recognised as performing a role in

luminal substrate recognition. A study assaying the requirements for efficient disposal of two canonical, *N*-glycosylated, membrane-anchored ERAD substrates, BACE476 (Molinari, Galli *et al.* 2002) and CD3- δ (Yang, Omura *et al.* 1998), demonstrated that OS-9 only played a role in the degradation of soluble variants that were lacking the transmembrane anchor (Bernasconi, Galli *et al.* 2010). However, OS-9 has also been shown to capture CD147 (EMMPRIN/Basigin), a ubiquitously expressed TM protein that is a member of the Ig superfamily. In particular, OS-9 was shown only to interact with only core-glycosylated (CG) CD147 (which is endoH sensitive) and not the endoH-resistant “fully glycosylated” (FG) form (Tyler, Pearce *et al.* 2012). Additionally, while knock down of OS-9 delays degradation of NHK (Bernasconi, Pertel *et al.* 2008, Christianson, Shaler *et al.* 2008), it does not affect the degradation of several other ERAD substrates such as RI₃₃₂ (Christianson, Shaler *et al.* 2008).

Despite the implications of its involvement in the recognition and degradation of numerous substrates, the exact method by which OS-9 differentiates misfolded proteins from their correctly folded counterparts remains unclear. Recognition by OS-9 was previously attributed to the recognition of a trimmed *N*-glycan by its MRH domain but, conversely, a number of proteins lacking *N*-glycans are still degraded in a manner dependent on OS-9 (Denic, Quan *et al.* 2006; Christianson, Shaler *et al.* 2008). Additionally, it has been shown that mutation to the MRH domain that disrupts conserved contacts with the glycan block the interaction of OS-9 with SEL1L, which is itself glycosylated (Christianson, Shaler *et al.* 2008). This does not rule out the requirement for *N*-

glycan recognition, indeed, the MRH domain has been shown to be critical for ERAD of some substrates (Hosokawa, Kamiya *et al.* 2009; Mikami, Yamaguchi *et al.* 2010) but it does suggest that the interaction of OS-9 with substrate is likely bipartite, with the involvement of a yet undefined interaction between unfolded peptide segments and a region(s) of OS-9. A similar situation exists in regard to yeast Yos9p, which has been shown to associate specifically with CPY* bearing Man₈- and Man₅-oligosaccharides (Szathmary, Biemann *et al.* 2005). With mutations of critical residues within the MRH domain abrogate degradation of CPY* but do not disrupt its interaction with Yosp9, additionally unglycosylated CPY* can be crosslinked to Yos9p (Bhamidipati, Denic *et al.* 2005). A recent study which probed a peptide array of linear epitopes of CPY* for Yos9p interaction, reported four regions of CPY* which were bound preferentially by Yos9p (Smith, Rodriguez *et al.* 2014). However, the study did not explore the corresponding region of Yos9p orchestrating the interactions or indeed if more than one region was responsible.

This study reports that a number of regions of OS-9 are capable and/or required for binding of the model substrates NHK and NHK-QQQ. A number of distinct regions across OS-9 retained the capacity to co-precipitate NHK, including the N-terminal (that included the MRH) and C-terminal (lacking the MRH domain). These data reinforce the redundancy of the MRH domain, at least in the context of substrate binding. Similarly, OS-9 retained its ability to co-precipitate non-glycosylated NHK-QQQ. While fewer regions of OS-9 bound NHK-QQQ in comparison to NHK, both segments containing and lacking the

MRH interacted, suggesting that the MRH is unsurprisingly redundant in OS-9's interaction with non-glycosylated substrate but also that different regions of OS-9 are involved in binding of glycosylated and non-glycosylated substrate. In addition, the failure of both NHK and NHK-QQQ to bind the 94BR also suggests that substrate binding, particularly binding to non-glycosylated forms, is not facilitated secondarily through an interaction with Grp94. This does not rule out the possible utilisation of BiP by OS-9 as a means of substrate recognition, however, this possibility was not explored due to time limitations. Further research will be required to definitively map the region(s) of OS-9 required for substrate binding, possibly through the employment of a peptide arrays of epitopes of OS-9 probed with a variety of substrates, however, this study has provided insight into the complexity of the OS-9 substrate interaction.

6.7 Degradation and regulation of OS-9 luminal levels

It is not unknown for components of the ERAD machinery to be substrates of their own degradation pathway. Is OS-9 itself a substrate of the pathway of which it is a key functional component? It is intuitive that those providing the "policing" must themselves be "policed" and maintained at levels that ensure ER homeostasis, where excessive quantities of ERAD pathway components may result in overly stringent regulation, but likewise must be sufficient to eliminate potentially harmful, misfolded polypeptides. Mammalian SEL1L has a $t^{1/2}$ of ~3 hours (Mueller, Lilley *et al.* 2006) while Ube2j1 (cognate E2 of Hrd1) also is degraded rapidly (Lenk, Yu *et al.* 2002). In vitro, Hrd1 has

been shown to auto-ubiquitinate (Carroll and Hampton 2010), while the E3 gp78 is thought to be degraded in a Hrd1-dependent manner (Shmueli, Tsai *et al.* 2009; Ballar, Ors *et al.* 2010), suggesting that their intracellular levels are regulated through their degradation (Weissman, Shabek *et al.* 2011).

Is OS-9 similarly synthesised in excess and degraded/removed constitutively when not needed (i.e. in the absence of substrates)? Indeed, it has previously been speculated that the degradation of OS-9 may depend on the SEL1L-Hrd1 complex (Tyler, Pearce *et al.* 2012). In this thesis, it has been demonstrated that OS-9 is degraded in a proteasome-dependent manner and that it accumulates upon proteasome inhibition with MG132. Additionally, OS-9 levels were dramatically increased in cells diminished for Hrd1. Complimentary results have been published by Sun *et al.* who, through the utilisation of tamoxifen-inducible SEL1L knockout mice (IKO), noted elevated protein levels of EDEM1, OS-9.1 and OS-9.2 by over 50-, 5-, and 2- fold, respectively when SEL1L was knocked out (Sun, Shi *et al.* 2014). This expansion of the OS-9 pool was uncoupled from transcriptional regulation as mRNA levels were not increased (in the pancreas of Sel1L^{IKO} mice) (Sun, Shi *et al.* 2014). Thus, SEL1L and Hrd1 deficiency result in the accumulation of OS-9, suggesting that OS-9 may indeed be a substrate of the Hrd1 ubiquitin ligase complex, co-degraded perhaps in association with the misfolded protein to which it has bound.

Bernasconi *et al.* have suggested that surplus SEL1L removal to vesicles from the ER via an LC3-I-dependent pathway also removes OS-9 (and EDEM1) along with it, as a mechanism that “tunes” ERAD’s capacity to meet demand. In

this way, components of the ERAD machinery are degraded constitutively because if present in excessive concentration, they may prematurely interrupt productive polypeptide maturation. While it has been demonstrated that OS-9 is transcriptionally induced upon activation of the Ire1/Xbp1 ER-stress pathway (Bernasconi, Pertel *et al.* 2008), the localisation of OS-9 in EDEMosomes has been suggested to provide a means of “rapid response”, where the ER maintains its degradation capacity without the need for transcriptional upregulation of expression. In fact it has been reported that, at steady state, as much as 20 % of OS-9.1, and to a lesser extent OS-9.2, was found in LC3-I-positive EDEMosomes (Cali, Galli *et al.* 2008; Reggiori, Monastyrska *et al.* 2010). In this study, the localisation of OS-9 in LC3-I-positive vesicles, in association with SEL1L has been confirmed. Therefore, the data presented here suggests a model by which OS-9 is degraded via two methods. In the presence of unfolded substrate, OS-9 is degraded in association with its substrate by the 26S proteasome in a Hrd1/SEL1L-dependent manner. Alternatively, in the absence of substrate, OS-9 is constitutively degraded through its incorporation with SEL1L in EDEMosomes. However, the molecular mechanism of EDEMosome formation has yet to be defined, and it is also unclear whether the OS-9-associated in EDEMosomes represents a correctly folded and functional population or is in fact misfolded. In the latter case, EDEMosomes may not provide a means of “rapid response” to ER stress but may simply degrade misfolded forms of the ERAD machinery. Indeed, further research shall be required regarding the formation and purpose of EDEMosomes.

6.8 Interaction of OS-9 with cytoplasmically localised proteins

Many studies reporting OS-9's interaction with proteins lacking canonical ER-targeting features have been published (Litovchick, Friedmann *et al.* 2002; Baek, Mahon *et al.* 2005; Wang, Fu *et al.* 2007). In this thesis, the validity and consequences of these interactions is questioned, with particular focus on the interactions of OS-9 with the cytoplasmically localised HIF-1 α and LONP2 (Baek, Mahon *et al.* 2005; Christianson, Olzmann *et al.* 2012).

OS-9 has been identified as a novel HIF-1 α interacting protein, which promotes the degradation of HIF-1 α through interactions with HIF-1 α prolyl hydroxylases (PHDs) (Baek, Mahon *et al.* 2005). The association is surprising, as HIF-1 α and the PHDs predominantly localise to the cytoplasm and the nucleus. HIF-1 α contains both a nuclear localisation signal (NLS) and a nuclear export signal (NES) that are critical for its function, where under hypoxic conditions it translocates to the nucleus to form a heterodimer with ARNT (HIF-1 β) (Kallio, Okamoto *et al.* 1998; Romero, Irisarri *et al.* 2008; Chachami, Paraskeva *et al.* 2009). The localisation of OS-9 had yet to be firmly established at the time of this study, with OS-9 previously described as an ER membrane protein exposed to the cytoplasm that interacted with meprin β (Litovchick, Friedmann *et al.* 2002). However, numerous studies have since established OS-9 as a component of ERAD that is localised to the ER lumen (Christianson, Shaler *et al.* 2008; Alcock and Swanton 2009; Hosokawa, Kamiya *et al.* 2009; Bernasconi, Galli *et al.* 2010). The physical separation of OS-9 and HIF-1 α (and PHDs) in different sub-cellular compartments would suggest that the interaction

does not occur endogenously, and was an artifact of the original yeast-two-hybrid screen. Indeed, the interaction between PHDs and OS-9 has been challenged by the Metzen group, who observed no significant physical interaction between the proteins by fluorescence resonance energy transfer (FRET) (Brockmeier, Platzek *et al.* 2011). The OS-9/HIF-1 α interaction is additionally challenged by the overexpression of OS-9 in osteosarcomas. The majority of human cancers are characterised by overexpression of HIF-1 α (Zhong, De Marzo *et al.* 1999), with inhibition of HIF-1 impairing tumour growth (Semenza 2003). If OS-9 regulates HIF-1 α degradation, its over-expression in cancer would seem counter-intuitive. To compound matters further, it has also been reported that Grp94 is up-regulated via HIF-1 transactivation in response to hypoxia, with three hypoxia responsive elements (HRE) present in the Grp94 promoter (Paris, Denis *et al.* 2005). Additionally, Grp94 has demonstrated a protective effect against hypoxia-induced apoptosis, suggesting that its overexpression in different cancers may provide enhanced tumour cell survival (Bando, Katayama *et al.* 2003). Similarly, a number of studies suggest that calcium depletion and ER stress can also affect hypoxia signalling, either via interference with the regulation of HIF (Berchner-Pfannschmidt, Petrat *et al.* 2004; Liu, Moller *et al.* 2004; Werno, Zhou *et al.* 2008) or via HIF-independent pathways (Bi, Naczki *et al.* 2005). As discussed previously, the inducer of OS-9 splicing has yet to be identified, however, a small number of studies have analysed the changes in pre-mRNA splicing on a genome-wide scale, in the context of hypoxia (Hang, Li *et al.* 2009; Moller-Levet, Betts *et al.* 2009;

Weigand, Boeckel *et al.* 2012). OS-9 was not identified in these screens, but a more directed analysis may shed light on the interplay of OS-9/HIF1- α and hypoxia.

Previous work from the Christianson lab employing a strategy of integrating proteomic, functional genomic and gene expression data sets to establish a more comprehensive overview of the ERAD network reported another interaction between OS-9 and a non-ER localised protein, the peroxisomal Lon peptidase LONP2 (Christianson, Olzmann *et al.* 2012). LONP2 is an ATP-dependent serine protease that mediates the selective degradation of misfolded and unassembled polypeptides in the peroxisomal matrix (Aksam, Koek *et al.* 2007, Omi, Nakata *et al.* 2008). To date, the LONP2/OS-9 interaction and its consequences have not been explored. In this study, it is reported that all four isoforms of OS-9 interact with LONP2, but only in the event of their loss from the ER. Similarly, HIF-1 α bound OS-9 in the context of its mislocalisation but in an isoform-dependent manner, only binding appreciably to OS-9.4. Does such mislocalisation or delivery to the cytoplasm, with possible gain of function consequences, occur physiologically? A precedent for normally ER-resident proteins to mislocalise to the cytosol where they exhibit gain of function activities already exists. The best example is that of calreticulin (CRT), normally a calcium-dependent, ER-resident chaperone that participates in the folding of secreted proteins. Trace amounts are present in the cytosol and have been implicated in the regulation of steroid growth hormone receptors (Burns, Duggan *et al.* 1994; Dedhar, Rennie *et al.* 1994), integrin function (Dedhar 1994;

Coppolino, Leung-Hagesteijn *et al.* 1995) and enhancement of thyroid transcription factor-1 (TTF-1) transcriptional activity (Perrone, Tell *et al.* 1999). In addition, CRT has been demonstrated to bind core histones and its supercoiling activity assists the incorporation of core histones into nucleosomes (Kobayashi, Uchiyama *et al.* 2006). CRT mislocalisation appears to arise from a failure to fully translocate into the ER due to an inefficient signal sequence (Kang 2005). However, the OS-9 signal sequence is not uncommonly short and is sufficient to target an unrelated polypeptide (e.g. TCR α) to the ER lumen (Christianson *et al.* 2008). Nonetheless, we have already proposed that Grp94 may play a role in the retention of KDEL-lacking OS-9 in the ER, and that OS-9.4 lacks the 94BR required for Grp94 binding. This also raises the question of why only OS-9.4 $_{\Delta SS}$ and not OS-9.3 $_{\Delta SS}$ is competent for HIF-1 α binding. Indeed, the study has already demonstrated that the OS-9 isoforms exhibit differential binding properties; perhaps the additional splicing of exon 13 in OS-9.3 confers a structural change that does not permit HIF-1 α binding.

An alternative mechanism by which OS-9 may reach the cytosol would be as a substrate via ERAD. For this to occur, OS-9 would either become a substrate or act as a “chaperone” for a *bona fide* substrate during retrotranslocation. One prediction of this would be that a cytosolic form of OS-9 may be present. If the N-terminus were exposed to the cytosol, it may become a target for the cytosolically localised NGly1/PNGase1, which is in fact an amidase. Cleavage of OS-9’s N-linked glycan by NGly1 would effectively convert the asparagine (N) at position 177 to an aspartate (D), and would thus

distinguish OS-9 that had been exposed to the ER and escaped, (retrotranslocated) from molecules that had never entered the ER lumen (e.g. were mislocalised). However, this difference would only be observed by LC-MS/MS as a mass change, and so was beyond the scope of this study. Indeed, the LG2-MEL cell lines, that were employed in probing a phenotype for the loss of interaction between OS-9 and Grp94, were noted to express several antigens recognised by autologous CTLs (Vigneron, Ooms *et al.* 2002). Of particular interest was the antigen resulting from a point mutation at position 446 of OS-9, which was presented as a peptide by HLA-B*4403 (Vigneron, Ooms *et al.* 2002). How a portion of OS-9 came to be present as a tumour antigen is itself intriguing. Antigen processing occurs in the cytosol prior to TAP complex-mediated transport and loading of MHC molecules in the ER. Are these antigens sampled from a population of OS-9 that exists in the cytosol or has P446L mutation resulted in a loss of OS-9 from the ER due to its loss of interaction with Grp94? Further studies are required to differentiate between these two possibilities.

6.9 Conclusions

The work presented in this thesis provides insight into the organisation of the ERAD network. In particular, the OS-9/Grp94 interaction is demonstrated to be dependent upon a 65 a. a. region within the C-terminal domain of OS-9, which has been designated the 94BR. The interaction is extremely specific, with naturally occurring point mutations, P449L, S454L and Q486R disrupting binding. Moreover, this work highlights the diversity in protein function provided by alternative splicing, confirming the presence of a splicing event that generates OS-9 isoforms incompetent for interaction with Grp94. The causes and consequences of loss of the OS-9/Grp94 interaction were explored over the course of this thesis. While further studies are still required to establish the inducer of OS-9 splicing, it was observed that a cell line harbouring a mutant form of OS-9 that lacked the ability to bind Grp94 (LG2-MEL-5-35) displayed an increased rate of proliferation when compared to a sub-line that retained a wild type allele for OS-9 (LG2-MEL-207), suggesting a possible consequence of loss of the OS-9/Grp94 interaction. This may be particularly relevant in the context of tumour progression, with OS-9 overexpression detected in a number of cancers and Grp94 knockdown demonstrated to dramatically decrease proliferation rates in two aggressive breast cancer and multiple myeloma cell lines (Dejeans, Glorieux *et al.* 2012; Hua, White-Gilbertson *et al.* 2013). Further studies are required for role(s) of the OS-9/Grp94 interaction to be fully appreciated, however, we report here that Grp94's interaction with the 94BR is distinct from OS-9's interaction with substrate. Additionally, the results presented hint to a

role for Grp94 in OS-9 ER retention. Nonetheless, this study has ultimately confirmed the presence of a sub-population of Grp94 in EDEMosomes, adding weight to the recent study by Dersh *et al.* that reported a role for OS-9 in the degradation of hyper-glycosylated forms of Grp94 in a lysosomal-dependent pathway (Dersh, Jones *et al.* 2014). In addition, this study investigated the interactions of OS-9 with glycosylated and non-glycosylated substrate, confirming previous reports of the bipartite nature of OS-9/substrate binding. Indeed, this thesis identifies a number of distinct regions of OS-9 with the capacity to co-precipitate substrate. To date, only a small number of endogenous ER quality control substrates have been reported in the literature. This limited number of substrates restricts the investigation of the ERAD pathway, and work to expand this knowledge will aid in our research and understanding of substrate recognition.

In summary, this thesis has contributed a small amount of understanding to the complex story that is the ERAD degradation pathway, and the roles that OS-9 plays within it. However, many questions still remain: are the interactions of OS-9 with SEL1L and/Grp94 stable or dynamic? What induces splicing of OS-9? Similar to splicing of exon 11b, does the splicing of exon 13 have interaction consequences? Through what means can OS-9 become cytoplasmically localised? Ultimately, a deeper understanding of ERAD substrate recognition will bring with it the means to manipulate this pathway, offering new approaches for the development of new treatments for protein misfolding diseases and disorders.

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