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Review

Endoplasmic reticulum stress responses

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Abstract. In homeostasis, cellular processes are in a dynamic equilibrium. Perturbation of homeostasis causes stress. In this review I summarize how perturbation of three major functions of the endoplasmic reticulum (ER) in eukaryotic cells – protein folding, lipid and sterol biosynthesis, and storing intracellular Ca^{2+} – causes ER stress and activates signaling pathways collectively termed the unfolded protein response (UPR). I discuss how the UPR reestablishes homeostasis, and summarize our current understanding of how the transition from protective to apoptotic UPR signaling is controlled, and how the UPR induces inflammatory signaling.

Keywords. Apoptosis, Ca^{2+} store, cholesterol, endoplasmic reticulum, membrane fluidity, molecular chaperone, protein folding, unfolded protein response.

Introduction

The endoplasmic reticulum (ER) of eukaryotic cells is a separate metabolic compartment [1] and the first compartment in the secretory pathway of eukaryotic cells. The ER has four major physiological functions. First, the ER is the site of membrane and secretory protein synthesis. Second, it is the place where the majority of secretory and transmembrane proteins fold into their native conformation [2, 3]. At the same time, these nascent proteins undergo a series of post-translational modifications, most notably the addition of asparagine-linked oligosaccharides [4] and the formation of disulfide bonds [5]. Quality control mechanisms ensure that only correctly folded proteins exit the ER. Slowly folding or permanently unfolded proteins are retained in the ER and targeted for proteasomal degradation *via* ER-associated protein degradation (ERAD) [6]. Third, the ER is also the place where intracellular Ca^{2+} is stored in higher eukaryotes [7]. Forth, the ER membrane is involved in the biosynthesis of lipids and sterols [8]. I discuss here how perturbation of homeostasis of these physiological functions of the ER causes ER stress, how this stress is sensed, and how this stress signal is transduced across the ER membrane to initiate cellular and organismal responses to ER stress.

Homeostasis of protein folding in the ER

For cytosolic proteins, the thermodynamic driving force in protein folding is the hydrophobic effect, i.e., the minimization of exposure of hydrophobic amino acid residues at the surface of the protein. For many proteins the native conformation is also the conformation with the lowest free Gibbs enthalpy. Unfolded conformations are characterized by a higher number of hydrophobic surface patches than the native conformation [9], making them prone for interaction and aggregation with other unfolded proteins in the crowded environment of a cell. Molecular chaperones promote productive protein folding by preferentially interacting with hydrophobic surface patches on

unfolded proteins. In this way this protein class shields unfolded proteins from interaction and aggregation with other unfolded proteins by creating a private folding environment or Anfinsen cage. Kinetic control of protein folding through catalysis of otherwise rate-limiting protein folding reactions, for example the *cis-trans* isomerization of peptidyl-prolyl bonds by *cis-trans* peptidyl-prolyl isomerases (PPIs; abbreviations of gene and protein names are summarized in Table 1), is superimposed over governance of protein folding by the hydrophobic effect.

The hydrophobic effect is also a major driving force for conformational protein folding in the ER. All newly synthesized proteins destined for the secretory pathway enter the ER in a completely unfolded conformation through the Sec61p translocation channel. As in the cytosol, catalysis of *cis-trans* isomerization of peptidyl-prolyl bonds is essential to conformational protein folding in the ER. Post-translational modification of nascent polypeptide chains in the ER, i.e., *N*-linked glycosylation and formation of disulfide bonds, makes protein folding in the ER more complex than in the cytosol. Attachment of hydrophilic *N*-linked oligosaccharides directs their attachment sites to the surface of the protein, shields the vicinity of the attachment sites from hydrophobic interactions with other proteins, and thereby may impose irreversibility onto conformational protein-folding reactions. Formation of the native set of disulfide bonds in proteins that have more than one option for disulfide bond formation is superimposed over the search for the thermodynamically most stable conformation, and precedes final conformational folding steps [10]. Disulfide bond formation and isomerization is slow, and requires catalysis by protein disulfide isomerases (PDIs).

An essential component of a functional protein-folding machinery is the ability to not only extract properly folded proteins from the machinery, but also the ability to extract slowly folding or folding-incompetent polypeptide chains and to target these polypeptide chains for proteolytic degradation. This ability prevents stalling or poisoning of the folding machinery by accumulation of folding-incompetent polypeptide chains interacting with components of the folding machinery. Poisoning of degradation routes disrupts the protein-folding machinery of the ER and causes ER stress [11]. Homeostasis of protein folding in the ER is the balance between the influx of newly synthesized, completely unfolded polypeptide chains into the ER and the sum of the effluxes of correctly folded proteins to the Golgi complex and unfolded proteins targeted for proteasomal degradation.

Chaperone-assisted conformational protein folding in the ER

The ER harbors at least three general hierarchical protein-folding machineries, (i) the HSP70 molecular chaperones BiP/GRP78/Kar2p [12] and Lhs1p/GRP170/ORP150 [13, 14], (ii) the HSP90 chaperone GRP94 [15], and (iii) the lectin chaperones calnexin, calreticulin, and calmegin [4, 16]. These three chaperone systems work in a hierarchical order (Fig. 1). BiP prefers completely unfolded or unstructured polypeptide chains as substrates, whereas GRP94 and the lectin chaperones preferentially work on partially folded proteins.

HSP70 class ER-resident molecular chaperones

A conserved ~44-kDa N-terminal adenosine triphosphatase (ATPase) domain and a conserved ~15-kDa C-terminal substrate-binding domain characterizes HSP70 class chaperones [12]. Two sheets each of four antiparallel β-strands and a α-helical lid form the substrate-binding pocket. These chaperones exist in two states, an adenosine diphosphate (ADP)-, and an ATP-bound state. In protein folding they cycle through rounds of ATP hydrolysis and ADP-ATP exchange [12] (Fig. 1). The ADP-ATP exchange reaction induces conformational changes that open the lid of the substrate-binding pocket, whereas ATP hydrolysis closes the lid [12]. Thus, HSP70 chaperones have low affinity for unfolded substrates in the ATP-bound form [12], and high affinity in the ADP-bound form. Substrate binding to HSP70 chaperones stimulates the ATPase activity of HSP70 chaperones [12]. Substrates bound to HSP70 chaperones are conformationally locked and cannot progress on their folding pathway [12]. Upon release from the chaperone the substrate continues to fold. HSP70 class chaperones actively promote protein folding, because of their ability to cycle between substrate-bound and substrate-free states. Chaperones that can actively promote protein folding are called chaperone foldases [17]. Both ATP hydrolysis and the ADP-ATP exchange reactions of BiP are stimulated by co-chaperones (Fig. 1). DnaJ co-chaperones, such as ERdj1/MTJ1, ERdj3/HEDJ/Scj1p, ERdj4/MDG1, ERdj5, Jem1p, and Sec63p [18], stimulate the ATPase activity of BiP. The GrpE co-chaperone Sil1p/BAP is a nucleotide exchange factor and catalyzes the ADP-ATP exchange reaction in BiP [18]. BiP stimulates the ATPase activity of Lhs1p, and Lhs1p is a nucleotide exchange factor for BiP [19]. Interaction of DnaJ co-chaperones also increases the stability of the chaperone-substrate complex [20] and affects the substrate specificity of HSP70 class chaperones [20]. This regulation of HSP70 chaperone activity is the molecular basis for ATP consumption in protein folding.

Table 1. Abbreviations and synonyms of gene and protein names used in the text. Approved gene and protein names are from the HUGO gene nomenclature committee web site (HUGO GNC, http://www.genenames.org/index.html), the *Saccharomyces* Genome Database (SGD, http://www.yeastgenome.org/), or the *Escherichia coli genom*e website (Colibri, http://genolist.pasteur.fr/Colibri/).

Abbreviation used in text	Explanation	HUGO GNC abbreviation
A1		BCL2A1
ACAT1	Acyl-CoA:cholesterol acyltransferase 1	ACAT1
AKT	AKR/J mice transforming retroviral oncogene	AKT1
AP-1	Activation protein 1	
APAF1	Apoptotic peptidase-activating factor 1	APAF1
aPKC	Atypical protein kinase C	
ASK1	Apoptosis signal-regulating kinase 1	MAP3K5
ATF3	Activating transcription factor 3	ATF3
ATF4	Activating transcription factor 4	ATF4
ATF6	Activating transcription factor 6	ATF6
ATG1	Autophagy-related 1	ULK1
ATG5	Autophagy-related 5	ATG5
ATG7	Autophagy-related 7	ATG7
ATG8	Autophagy-related 8	GABARAPL1
BAD	BCL-x_L/BCL-2 associated death promoter	BAD
BAG-1	BCL-2-associated athanogene	BAG1
BAK	BCL-2 homologous antagonist/killer	BAK1
BAP	BiP-associated protein	SIL1
BAX	BCL-2-associated X protein	BAX
BBC3	BCL-2-binding component 3	BBC3
BBF2H7	Box B-binding factor 2 H7	CREB3L2
BCL-2	B cell leukemia/lymphoma 2	BCL2
BCL-w	BCL-2 widely expressed	BCL2L2
BCL-x_L	BCL-x long	
BCL-x_S	BCL-x short	
BFL-1		BCL2A1
BHRF1		
BID	BH3 interacting domain death agonist	BID
BIM	BCL-2 interacting mediator of cell death	BCL2L11
BiP	Heavy chain binding protein	HSPA5
BIK	BCL-2-interacting killer	BIK
BLK	BIK-like killer	
BMF	BCL-2-modifying factor	BMF
BNIP	BCL-2 and E1B 19-kDa interacting protein	BNIP
BOD	BCL-2 related ovarian death gene	BCL2L11
BOK	BCL-2-related ovarian killer	BCL2L9
BOO	BCL-2 homolog of ovary	BCL2L10
C/EBP	CCAAT-enhancer binding protein	CEBP
C-reactive protein		CRP
Calmegin		CLGN
Calsequestrin		CASQ
CDK4	Cyclin-dependent kinase 4	CDK4
CDK6	Cyclin-dependent kinase 6	CDK6
CHIP	C terminus of heat shock cognate protein of 70-kDa (HSC70) interacting protein	

Table 1 *(Continued)*

Abbreviation used in text	Explanation	HUGO GNC abbreviation
CHOP/CHOP-10	CCAAT/enhancer-binding protein (C/EBP) homologous protein	DDIT3
c-JUN	Cellular ju-nana	JUN
CNX	Calnexin	CANX
CREB3	CRE-binding protein 3	CREB3
CREB4	CRE-binding protein 4	CREB4
CREB-H	CREB homolog	CREB3L3
CRT	Calreticulin	CALR
Cue1p	Coupling of ubiquitin conjugation to ER degradation	
Cul3	Cullin 3	CUL3
Der1p	Degradation in the ER 1 protein	
DERLIN-1	Degradation in the ER 1 protein (Der1p)-like protein 1	DERL1
DERLIN-2	Degradation in the ER 1 protein (Der1p)-like protein 2	DERL2
DERLIN-3	Degradation in the ER 1 protein (Der1p)-like protein 3	DERL3
DIABLO	Direct IAP binding protein with low p*I*	DIABLO
DIVA	Death inducer binding to *v*BCL-2 and APAF1	BCL2L10
DnaJ	2'-Deoxyribonucleic acid chain elongation J	
Dsk2p	Dominant suppressor of *kar1* 2 protein	
ECH	Erythroid cell-derived protein with CNC homology	
EDEM1	ER degradation enhancer, mannosidase α-like 1	EDEM1
EDEM2	ER degradation enhancer, mannosidase α-like 2	EDEM2
EDEM3	ER degradation enhancer, mannosidase α-like 3	EDEM3
eIF2α	Eukaryotic translation initiation factor 2α	EIF2A
eIF-4E	Eukaryotic translation initiation factor 4E	EIF4E
ELOVL1	Elongation of very long chain fatty acids 1	ELOVL1
ELOVL2	Elongation of very long chain fatty acids 2	ELOVL2
ELOVL3	Elongation of very long chain fatty acids 3	ELOVL3
ELOVL4	Elongation of very long chain fatty acids 4	ELOVL4
ELOVL5	Elongation of very long chain fatty acids 5	ELOVL5
ELOVL6	Elongation of very long chain fatty acids 6	ELOVL6
Eps1p	ER-retained Pma1p suppressing 1 protein	
ER-UDPase	UDPase in the ER	
ERdj1	ER DnaJ protein 1	DNAJC1
ERdj3	ER DnaJ protein 3	DNAJB11
ERdj4	ER DnaJ protein 4	DNAJB9
ERdj5	ER DnaJ protein 5	DNAJC10
ERGIC-53	ER Golgi intermediate compartment protein of 53 kDa	LMAN1
ERK1	Extracellular signal regulated kinase 1	MAPK3
ERK2	Extracellular signal regulated kinase 2	MAPK1
Erlectin	ER lectin	
ERManI	α(1,2)-ER mannosidase I	
Ero1p	ER oxidation 1 protein	
ERO1-Lα	Ero1p-like α	ERO1L
ERO1-Lβ	Ero1p-like β	ERO1LB
ERp57	ER protein of 57 kDa	PDIA3
ERp72	ER protein of 72 kDa	PDIA4
Erv2p	Essential for respiration and viability 2 protein	

Table 1 *(Continued)*

Abbreviation used in text	Explanation	HUGO GNC abbreviation
Eug1p	ER protein unnecessary for growth under standard laboratory conditions 1 protein	
Fkb2p	FK506-binding protein 2 protein	
GAB1	GRB2-associated binding protein 1	GAB1
GADD34	Growth arrest and DNA damage gene 34	PPP1R15A
GAM1	γ-Ray mutator 1	
GCN2	General control non-derepressible 2	EIF2AK4
Gcn4p	General control non-derepressible 4 protein	
α-Glc I	α-Glucosidase I	
α-Glc II	α-Glucosidase II	
GLUT4	Insulin-responsive glucose transporter	SLC2A4
gp78	Glycoprotein of 78 kDa	
GRB2	Growth factor receptor-bound protein 2	GRB2
GRP78	Glucose-regulated protein of 78 kDa	HSPA5
GRP94	Glucose-regulated protein of 94 kDa	HSP90B1
GRP170	Glucose-regulated protein of 170 kDa	HYOU1
GrpE	Growth after phage induction E	HSPA
GSK3	Glycogen synthase kinase 3	GSK3
Hac1p	Homologous to ATF/CREB1 1 protein	
$Hac1^{i}p$	Homologous to ATF/CREB1 1 protein, induced form	
HACA	Homologous to ATF/CREB1 A	
HEDJ	Human ER-associated DnaJ	DNAJB11
HERP	Hyperhomocysteinemia-induced ER stress-responsive protein	HERPUD1
HRC	Histidine-rich calcium-binding protein	HRC
Hrd1p	HMG-CoA reductase degradation 1 protein	
Hrd3p	HMG-CoA reductase degradation 3 protein	
HRK	Harakiri	HRK
HSC70	Heat shock cognate gene 70	HSPA8
11βHSD1	11β-Hydroxysteroid dehydrogenase type 1	HSD11B1
HSF1	Heat shock transcription factor 1	HSF1
HSP70	Heat shock protein of 70 kDa	
HSP90	Heat shock protein of 90 kDa	HSP90
HTRA2	High temperature requirement A2	HTRA2
Htm1p	Homologous to mannosidase I	EDEM1
IAP	Inhibitor of apoptosis	
IκB	Inhibitor of NF-κB	NFKBI
IKK	IκB kinase	
IL-2	Interleukin-2	IL-2
IL-6	Interleukin-6	IL-6
IL-8	Interleukin-8	IL-8
INO1	Inositol requiring 1 protein	
INSIG1	Insulin-induced gene 1	INSIG1
INSIG2	Insulin-induced gene 2	INSIG2
IP_3R	Inositol 1,4,5-trisphosphate receptor	ITPR
IR	Insulin receptor	INSR
IRE1α	Inositol requiring 1α	ERN1α
IRE1β	Inositol requiring 1β	ERN1β

Table 1 *(Continued)*

Abbreviation used in text	Explanation	HUGO GNC abbreviation
IRS1	Insulin receptor substrate 1	IRS1
IRS2	Insulin receptor substrate 2	IRS2
IRS3	Insulin receptor substrate 3	IRS3
IRS4	Insulin receptor substrate 4	IRS4
Kar2p	Karyogamy 2 protein	HSPA5
KEAP1	Kelch-like ECH-associated protein 1	KEAP1
JAB1	Jun activation domain binding protein 1	COPS5
Jem1p	DnaJ-like protein of the ER membrane	
JNK1	Jun N-terminal kinase 1	MAPK8
JNK2	Jun N-terminal kinase 2	MAPK9
JNK3	Jun N-terminal kinase 3	MAPK10
JUN-B	Ju-nana (Japanese for 17) B	JUNB
JUN-D	Ju-nana D	JUND
Junctin	Integral component of the junctional sarcoplasmic reticulum membrane	ASPH1
LDL	Low-density lipoprotein	
LDLR	Low-density lipoprotein receptor	LDLR
Lhs1p	Luminal HSP70 1 protein	HYOU1
MCL1	Myeloid cell leukemia sequence 1	MCL1
MCP-1	Monocyte chemoattractant protein 1	CCL2
MDG1	Microvascular differentiation gene 1	DNAJB9
MEK	MAP or ERK kinase	
Mga2p	Multicopy suppressor of *gam1* 2 protein	
Mnl1p	Mannosidase-like 1 protein	EDEM1
MTD	Matador	BCL2L9
MTJ1	Murine tumor cell DnaJ-like protein 1	DNAJC1
mTOR	Mammalian target of rapamycin	FRAP1
MyD116	Myeloid differentiation primary response gene 116	PPP1R15A
NBK	New BCL-2 interacting killer	BIK
NF-κB	Nuclear factor-κB	NFKB
NF-Y	Nuclear factor-Y	NFY
NIPK	Neuronal cell death-inducible putative kinase	
NOXA	NADPH oxidase activator	NOXA1
NR-13	Neuroretina 13	
NRF2	Nuclear factor erythroid 2-related factor 2	NFE2L2
OASIS	Old astrocyte specifically induced substance	CREB3L1
Ole1p	Oleic acid requiring 1 protein	
OMI		HTRA2
Opi1p	Overproduction of inositol 1 protein	
ORP150	150-kDa oxygen-regulated protein	HYOU1
p38	Protein of 38 kDa	MAPK14
P5	50 kDa protein	PDIA6
p53	53 000 M_r component	TP53
$p58^{IPK}$	58-kDa inhibitor of PKR	DNAJC3
$p70^{S6K}$	70 000 M_r 40 S ribosomal protein S6 kinase	RPS6KB2
PDI	Protein disulfide isomerase	
PDK1	Phosphoinositide-dependent kinase 1	PDPK1

Table 1 *(Continued)*

Abbreviation used in text	Explanation	HUGO GNC abbreviation
PDK2	Phosphoinositide-dependent kinase 2	
PEK	Pancreatic eIF2α kinase	
PEPCK	Phosphoenolpyruvate carboxykinase	PCK
PERK	PKR-like ER kinase	EIFAK3
PI3K	Phosphatidylinositol 3-kinase	PIK3
PKB	Protein kinase B	AKT1
PKR	Double-stranded RNA-activated protein kinase	EIF2AK2
Pma1p	Plasma membrane ATPase	
PNGase	*N*-Glycanase	NGLY1
PP1	Protein phosphatase 1	
PPI	Peptidyl prolyl *cis-trans* isomerase	
PUMA	p53 upregulated modulator of apoptosis	BBC3
Rad23p	Radiation sensitive 23 protein	
RAF		RAF1
RAS	Rat sarcoma	RAS
RB	Retinoblastoma protein	RB1
RelA	Reticuloendotheliosis viral oncogene homolog A	RELA
Rpd3p	Reduced potassium dependency 3 protein	HDAC
RyR	Ryanodine receptor	RYR
S1P	Site 1 protease	MBTPS1
S2P	Site 2 protease	MBTPS2
Sarcalumenin		SRL
SCAP	SREBP cleavage-activating protein	SCAP
SCD1	Δ^9-Stearoyl-CoA desaturase 1	
Scj1p	*Saccharomyces cerevisiae* DnaJ 1 protein	DNAJB11
Scs2p	Suppressor of choline sensitivity	
Sec61p	Secretory 61 protein	
Sec63p	Secretory 63 protein	
SERCA1	Sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase 1	ATP2A1
SERCA2	Sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase 2	ATP2A2
SERCA3	Sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase 3	ATP2A3
Serum amyloid A	Serum amyloid A	SAA
SHP2	SH-2 domain-containing tyrosine phosphatase	PTPN11
Sil1p	Suppressor of *IRE1*/*LHS1* synthetic lethality 1 protein	
Sin3p	Switch independent 3 protein	SIN3
SMAC	Second mitochondria-derived activator of caspase	DIABLO
SOS	Son of sevenless	SOS
SPIKE	Small protein with inherent killing effect	
Spt23p	Suppressor of Ty 23 protein	
SRC	Sarcoma formation	SRC
SREBP1a, c	Sterol response element binding protein 1a, c	SREBF1
SREBP2	Sterol response element binding protein 2	SREBF2
tBID	Truncated BID	
TNF-α	Tumor necrosis factor-α	TNF
TNF-β	Tumor necrosis factor-β	LTA
TNFR	Tumor necrosis factor receptor	TNFRSF

Table 1 *(Continued)*

Abbreviation used in text	Explanation	HUGO GNC abbreviation
TRAF2	TNFR-associated factor 2	TANK
TRB3	Tribbles homolog 3	TRIB3
Triadin		TRDN
Ubc7p	Ubiquitin-conjugating enzyme 7 protein	UBE2G
UGGT	UDP-glucose:glycoprotein glucosyltransferase	
Ume6p	Unscheduled meiotic gene expression 6 protein	
*v*BCL-2	Viral BCL-2	
VCP	Valosin-containing protein	VCP
VIP36	36-kDa vesicular integral membrane protein	LMAN2
v-JUN	Viral ju-nana	JUN
XBP1	X-box-binding protein 1	XBP1
XIAP	X-linked IAP	BIRC4
Yos9p	Yeast osteosarcoma 9 homolog protein	

Indeed, depletion of cellular ATP levels inhibits protein folding *in vivo* [21, 22]. The Michaelis-Menten constant (K_M) of HSP70 chaperones for ATP is ~1–2 μM and well below the cytosolic ATP concentration of 1–2 mM [23]. ATP is transported into the ER, most likely by an ADP/adenoside monophosphate (AMP) antiport mechanism. In *in vitro* experiments ATP is concentrated ~30-fold in ER-derived vesicles [24–26]. It is possible that the protein folding capacity of the ER is limited by ATP import, especially under conditions of high secretory activity or ER stress. In an inactive state, HSP70 chaperones, including BiP, form stable dimeric and oligomeric complexes [23, 27, 28]. HSP70 chaperones are bound to ADP, phosphorylated in their substrate binding domain [27–29], and ADP-ribosylated [28, 30, 31] in the oligomeric state. The oligomeric forms do not interact with unfolded substrates and function as a reserve pool. In *in vitro* experiments both peptides [23] and ATP [27] dissociate the oligomeric form of BiP. The monomeric and oligomeric BiP pools are readily interconverted. *In vivo*, conversion of BiP to the monomeric, unmodified form precedes induction of expression of its gene [28, 32].

The HSP90 class ER-resident molecular chaperone GRP94

GRP94 consists of an N-terminal nucleotide binding domain [15], a C-terminal domain essential for substrate binding and dimerization [15], and a central imperfect basic leucine zipper (bZIP). GRP94 prefers partially folded substrates and acts after BiP in protein folding [15] (Fig. 1). The K_M of GRP94 for ATP is >100 μM [33, 34], and its intrinsic ATPase activity is barely above background ATP hydrolysis. Co-chaperones that stimulate the ATPase activity of GRP94 are not known. GRP94 appears to be a chaperone holdase, a chaperone that binds to unfolded proteins, but does not cycle through substrate binding-release cycles and therefore, does not actively promote protein folding [17]. Holdases present unfolded substrates to foldases, PDIs, and PPIs, which promote the folding of the substrate [17]. Structural maturation of the bound substrate terminates the interaction of the substrate with the holdase [17]. Under conditions unfavorable to protein folding holdases provide a buffer capacity that sequesters unfolded proteins and only releases these unfolded proteins to associated foldase systems when conditions favorable to protein folding have been reestablished [17]. Ligand binding to the N-terminal regulatory domain of GRP94 inhibits a conformational change associated with increased chaperone activity [35, 36]. *In vitro*, both ADP and ATP are equally efficient in inhibiting this conformational change [36]. Increased ATP-consuming folding attempts of substrate proteins interacting with the ER luminal HSP70 foldase machinery may deplete ER luminal adenosine nucleotide pools and result in activation of GRP94. In this view GRP94 provides additional chaperone buffer capacity through its holdase activity during periods of stress (Fig. 1).

The chaperone foldase cycle of the lectin chaperones

Many proteins entering the ER are co-translationally modified by transfer of the oligosaccharide $Glc_3Man_9GlcNAc_2$ (Glc, D-glucose; GlcNAc, 2-*N*-acetylamide-D-glucose; Man, D-mannose) from dolicholpyrophosphate-$GlcNAc_2Man_9Glc_3$ located in the ER membrane to consensus Asn-X-Ser/Thr (X represents

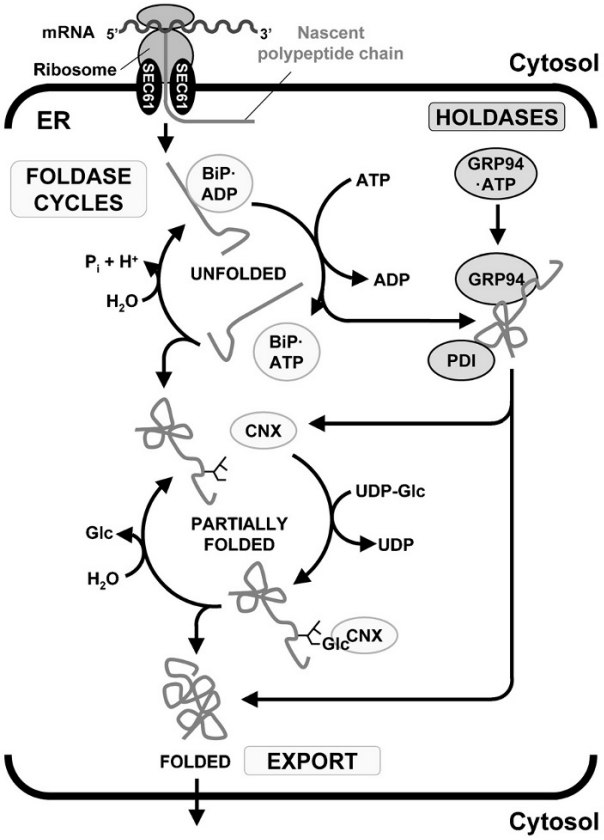

Figure 1. Hierarchy of general chaperone systems in the mammalian endoplasmic reticulum (ER) and chaperone foldase cycles for heavy chain binding protein (BiP) and calnexin/calreticulin/calmegin. A nascent, unfolded polypeptide chain enters the ER cotranslationally through the Sec61p translocation channel and engages in ATP-consuming BiP ATP-ADP hydrolysis cycles, which facilitate the translocation reaction [268] and assist the initial folding of the polypeptide chain [12]. Partially folded proteins may be transferred to the HSP90 chaperone GRP94 in analogy to transfer of partially folded substrates from HSP70 to HSP90 chaperones in the cytosol [269, 270], or the calnexin cycle. While associated with GRP94 or while cycling in the calnexin cycle, the substrate continues to fold. Folding in the calnexin cycle consumes ATP, because of UDP-D-glucose consumption by this cycle. The chaperone activity of protein disulfide isomerase (PDI) also assists in folding of the native protein. When the protein is no longer recognized as unfolded by any one of the ER-resident chaperone systems, it is exported from the ER if it does not carry specific retention or retrieval signals, such as a C-terminal (K/H)DEL sequence [271]. Glc, D-glucose; P_i, inorganic phosphate (HPO_4^{2-}).

any amino acid except L-proline) residues by oligosaccharyltransferase. The two terminal D-glucose residues are immediately (half-life, $t_{1/2}$, <3 min [37]) removed by sequential action of α-glucosidases I and II [4], triggering interaction of the monoglucosylated form with the lectin chaperones calnexin, calreticulin, or the testis-specific calnexin homolog calmegin (Fig. 1) [4]. These chaperones consist of a long, extended P domain, which mediates interactions with other components of the ER-resident protein-folding machinery [38] and may contribute to recognition of substrate proteins [39, 40]. Further deglucosylation by α-glucosidase II releases the unfolded substrate from calnexin/calreticulin/calmegin (Fig. 1) [4]. If still unfolded, uridine diphosphate (UDP)-glucose:glycoprotein glucosyltransferase (UGGT) reglucosylates the $Man_9GlcNAc_2$ oligosaccharide, triggering a second round of interaction with the lectin chaperones [4]. UGGT preferentially recognizes partially unfolded protein structures, which can be close to or far away from the acceptor oligosaccharide [41, 42]. The D-glucose donor in the reglucosylation reaction catalyzed by UGGT is UDP-D-glucose. UDP-D-glucose is synthesized in the cytosol from α-D-glucose-1-phosphate and uridine triphosphate (UTP) by UDP-glucose pyrophosphorylase and imported into the ER by antiport with uridine monophosphate (UMP) [43]. UMP is formed by hydrolysis of UDP by the Mg^{2+}/Ca^{2+}-dependent apyrase UDPase in the ER (ER-UDPase) [44]. Apyrases usually require millimolar Ca^{2+} concentrations to exhibit activity [44]. Thus, Ca^{2+} depletion of the mammalian ER may stall the calnexin cycle (Fig. 1). ATP is only a poor substrate for ER-UDPase [44]. Regeneration of UTP from UMP in the cytosol consumes ATP. Thus, the calnexin cycle can be considered a chaperone foldase cycle that promotes the folding of its substrates (Fig. 1).

Oxidative protein folding in the ER

Formation and isomerization of disulfide bonds is superimposed over conformational folding in the ER. Formation of the native conformer can precede or follow formation of a largely native set of disulfide bonds. *In vitro* folding experiments favor a 'quasi-stochastic' mechanism that couples conformational and oxidative protein folding [10]. In this mechanism conformational folding follows formation of a conformationally stable set of disulfide bonds. These disulfide bonds become 'locked', i.e., protected from further rearrangements by subsequent local or global conformational folding [10]. Formation of the native set of disulfide bonds largely occurs by random encounters of reactive groups determined by polypeptide chain loop entropies and conformational constraints imposed on the movement of the reactive groups by the conformation of the folding intermediate. The quasi-stochastic mechanism implies cooperativity of chaperones and PDIs *in vivo*. Many PDIs have chaperone activity [5], or form complexes with chaperones as in the case of ERp57, which interacts with calnexin [45]. ERdj5, which contains a DnaJ domain, is likely to interact with BiP and Lhs1p through its DnaJ domain. Structurally, PDIs are characterized by several thioredoxin-like domains

[5]. The catalytic domains are characterized by the amino acid motif CXXC (C, Cys; X, any amino acid) containing two redox-active cysteines [5]. The chaperone activity of the founding member of the PDI family, PDI, can be separated from its redox activity by mutating one of the two cysteines in both CGHC (C, Cys; G, Gly; H, His) motifs [46], suggesting that PDI functions as a chaperone holdase. In unfolding of cholera toxin, PDI is a redox-regulated chaperone foldase [47]. Most redox-active PDIs have two activities, an oxidase activity required for the *de novo* formation of disulfide bonds, and a disulfide isomerase activity. The isomerase activity is critical for oxidative protein folding in light of the random search for the native set of disulfide bonds in the quasi-stochastic mechanism. This conclusion is also supported by the observation that the majority of PDI is found in the reduced dithiol form in the ER [48]. Isomerase activity is redox neutral, but continued oxidase activity requires reformation of the oxidized, disulfide form of PDI by the flavin adenine dinucleotide (FAD)-dependent oxidases Ero1p/ERO1-Lα, ERO1-Lβ, and Erv2p [49]. These enzymes utilize molecular oxygen as final electron acceptor. Oxidative protein folding in the ER is responsible for ~25% of all reactive oxygen species (ROS) in eukaryotic cells [50, 51].

ERAD and autophagy

To prevent poisoning of the ER-resident protein-folding machinery, mechanisms exist that extract slowly folding and folding-incompetent substrates from chaperone folding machineries and target them for proteolytic degradation. Two degradation routes are known: (i) retrotranslocation of the unfolded polypeptide chain into the cytosol followed by ubiquitination and proteasomal degradation in a process termed ERAD (Fig. 2a, reviewed in [6]) and (ii) targeting of parts of the ER to lysosomes or vacuoles in autophagy (Fig. 2b, reviewed in [52]). The ERAD pathway is constitutively active, because of synthetic lethalities of loss-of-function mutations in ERAD and the unfolded protein response (UPR) in yeast [53, 54]. ER stress signaling induces several ERAD components to increase the degradative capacity of ERAD in ER-stressed cells [53, 54]. ER stress also induces autophagy, but it is not yet established to which extent autophagy contributes to clearance of misfolded secretory cargo proteins in unstressed or stressed cells. Slowly folding proteins are recognized by BiP, PDI [47, 55], the PDI homolog Eps1p [56], and lectins probing the extent of demannosylation of the *N*-linked oligosaccharide such as EDEM1–3/Htm1p/Mnl1p, Yos9p, and Erlectin [4]. BiP and PDIs may select ERAD substrates on the basis of the time spent in their foldase cycles. Association of the nucleotide exchange factor BAG-1 [57] and the DnaJ co-chaperone $p58^{IPK}$ [58] with cytosolic HSP70s and of the ubiquitin ligase CHIP with cytosolic HSP90s [59] selects substrates for proteasomal degradation. In the simplest model an excess of folding-promoting co-chaperones over degradation-promoting co-chaperones functions as a timer to measure the time a substrate protein spends in an HSP70 foldase cycle (Fig. 1). Regeneration of oxidized PDI by Ero1p and/or Erv2p from reduced PDI interacting with a substrate may signal conformational inaccessibility of disulfides in a folding intermediate and target the PDI-bound substrate to the ERAD pathway. In the calnexin cycle, slow demannosylation reactions [60] superimposed over the de- and reglucosylation reactions catalyzed by α-glucosidase II and UGGT function as a timer that triggers targeting of slowly folding proteins for retrotranslocation and proteasomal degradation [61–64] (Fig. 3). Mannose trimming creates poorer substrates for α-glucosidase II [65], UGGT [66], calnexin, and calreticulin and should result in a net slow down of the calnexin cycle [67, 68]. In autophagy, a phagophore or isolation membrane is formed from an unknown source, engulfs cytosol and organelles such as mitochondria, peroxisomes, parts of the ER or foreign particles, and then fuses with lysosomes or vacuoles [52], where these organelles are then degraded (Fig. 2b). Selective substrate degradation by autophagy requires compartmentalization of the ER, recognition of these compartments by the phagophore, and separation of these subcompartments from the remainder of the ER (Fig. 2b). Induction of ER subcompartments termed Russell bodies [69], BiP bodies [70, 71], ER-associated compartments (ERACs) [72], proliferation of stacked ER cisternae [73] and proliferation of the ER-Golgi intermediate compartment (ERGIC) [74] is triggered by accumulation of misfolded protein aggregates in the secretory pathway. These compartments are enriched in aggregates of misfolded proteins and molecular chaperones such as BiP [70, 72], calnexin and calreticulin [75]. Selective enrichment of lectin chaperones [75] or soluble ER-resident proteins [71] or inaccessibility for ER-resident proteins [69] of some of these structures suggests that sorting mechanisms and subcompartmentalization of the ER exist.

Ca^{2+} homeostasis

In most eukaryotes the ER or its specialized subcompartment, the sarcoplasmic reticulum (SR) in muscle cells, is an important intracellular Ca^{2+} store involved in Ca^{2+} signaling. Three types of transmem-

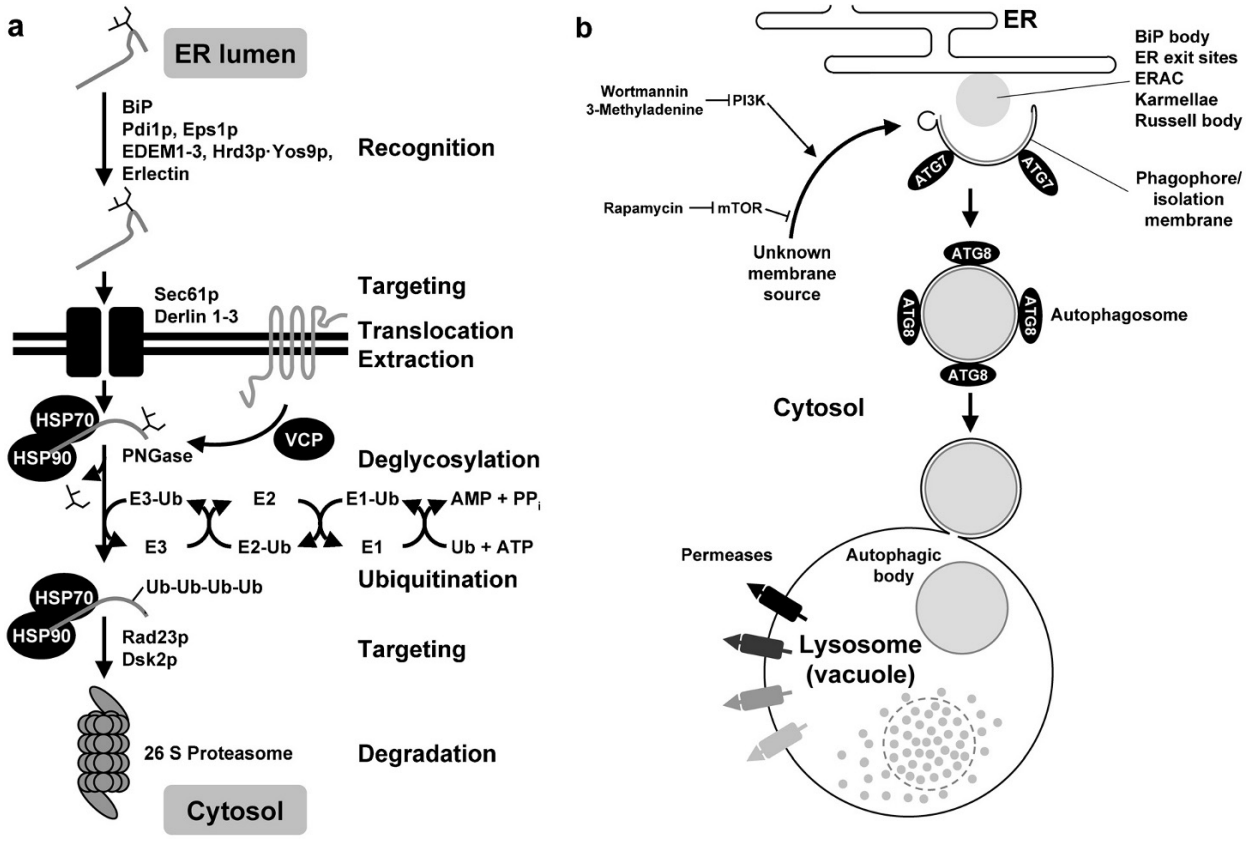

Figure 2. (*a*) ER-associated protein degradation (ERAD). Retrotranslocation followed by ubiquitination and proteasomal degradation. Permanently unfolded and slowly folding proteins are recognized by chaperones such as BiP, PDI [47, 55, 56], and lectins such as EDEM, the Hrd3p·Yos9p complex, and Erlectin [4] and targeted to the ER membrane for retrotranslocation into the cytosol. ER-resident molecular chaperones also keep degradation substrates in a soluble form in the ER during targeting to the ER membrane [272–274]. The nature of the retrotranslocation channel is not resolved. Retrotranslocation of *N*-glycosylated proteins is difficult to reconcile with retrotranslocation through a channel, because of the large size and hydrophilic nature of *N*-glycans [275, 276]. As an alternative to a retrotranslocation channel, release of proteins from the ER *via* budding of lipid droplets from the ER membrane, either as components of these lipids or *via* release through pores formed transiently in the ER membrane by the lipid droplet budding process was recently proposed [277]. After export from the ER or extraction from the ER membrane by the ATPases associated with diverse cellular activities (AAA) ATPase VCP [278, 279] proteins are deglycosylated by *N*-glycanase (PNGase) [280, 281], ubiquitinated, and targeted to the proteasome by Rad23p and Dsk2p [282–284]. Cytosolic HSP70 and HSP90 chaperones prevent aggregation of some degradation substrates in the cytosol [285–289]. E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; PP_i, pyrophosphate ($HP_2O_7^{3-}$); Ub, ubiquitin. (*b*) Autophagy. The phagophore or isolation membrane is formed from an unidentified membrane source. The phagophore sequesters cytosol and other organelles. Phagophore formation is inhibited by mTOR signaling and stimulated by a class III, wortmannin- and 3-methyladenine-sensitive PI3K. The ubiquitin-activating enzyme-like protein ATG7 initiates covalent attachment of ATG8 to phosphatidylethanolamine, which is critical for formation and maturation of autophagosomes. After fusion with the vacuole or lysosomes an autophagic body, surrounded by a phospholipid monolayer, is released into the vacuolar lumen and degraded in the vacuole. Permeases in the vacuolar membrane export degradation products such as amino acids and sugars.

brane proteins control Ca^{2+} in- and efflux into the ER/SR, the ryanodine receptor (RyR) [76], the inositol 1,4,5-trisphosphate receptor (IP_3R) [77], and the SR/ER Ca^{2+} ATPases (SERCA) 1–3 [78]. A free Ca^{2+} concentration of ~1–2 mM is maintained in the ER, in contrast to a resting cytosolic Ca^{2+} concentration of 0.1 μM. The SERCA pumps are responsible for most Ca^{2+} influx into the ER. As they pump Ca^{2+} against the Ca^{2+} concentration gradient, ATP hydrolysis is coupled to Ca^{2+} transport by these pumps [78]. The pump can operate in reverse mode, synthesizing ATP and contributing to Ca^{2+} leakage from the ER. Humans possess three *SERCA* genes, *ATP2A1* encoding SERCA1, *ATP2A2* encoding SERCA2, and *ATP2A3* encoding SERCA3. All have several splice variants at the mRNA level with tissue-specific expression patterns. SERCA2b and SERCA3 are widely expressed. Association of calnexin [79] or calreticulin [80] with a 12-amino acid luminal extension tail specific to SERCA2b inhibits SERCA2b when ER luminal Ca^{2+} concentrations are high [81]. When the Ca^{2+} store is full a disulfide bond between cysteines 875 and 887 is formed, which is catalyzed by the ERp57·calnexin complex, resulting in inhibition of SERCA2 [82]. Disruption of this interaction upon depletion of the Ca^{2+} store activates SERCA2b [81].

Phosphorylation of a protein kinase C consensus site in the cytosolic tail of calnexin is also required for its interaction with SERCA2b [79].

Ca^{2+} efflux is mediated by the RyR [76] and the IP_3R [77]. RyRs and IP_3Rs can coexist in the same cell type. Three isoforms each with one or two alternative splice variants of the IP_3R are expressed at varying levels in many cell types. The IP_3R forms heterotetramers, which are opened by binding to the second messenger inositol 1,4,5-trisphosphate generated at the plasma membrane. Three isoforms (RyR1, RyR2, and RyR3) and several splice variants, functioning as homotetramers, are expressed in many tissues, but at the highest level in striated muscles [76]. In mammals, RyR1 is the predominant isoform in skeletal muscle. RyR2 is predominant in cardiac muscle. In the SR, the RyR is activated by excitation-contraction coupling through interaction with voltage-dependent Ca^{2+} channels in the t-tubule membrane, i.e., the dihydropyridine receptors. A conformational change is induced in the dihydropyridine receptor by depolarization of the t-tubule membrane activating the RyR in the SR membrane, resulting in Ca^{2+} release from the SR [76].

Many SR luminal high-affinity and high-capacity Ca^{2+}-binding proteins allow storage of up to 20 mM Ca^{2+} in the ER/SR [7], even when a free Ca^{2+} concentration of 1 mM is maintained. Calsequestrin is the most abundant Ca^{2+}-binding protein in the SR, besides the 165-kDa histidine-rich Ca^{2+}-binding protein (HRC) and sarcalumenin. Calsequestrin has ~50 Ca^{2+}-binding sites. Its dissociation constant (K_d) for Ca^{2+} is ~1 mM. Calsequestrin is anchored *via* the transmembrane proteins triadin and junctin to the RyR. At low (<10 mM) Ca^{2+} concentrations calsequestrin inhibits the RyR. In the ER molecular chaperones serve as Ca^{2+}-binding proteins in place of calsequestrin [7].

Homeostasis of composition of the ER membrane

The ER is the site where biological membranes are assembled and involved in (i) elongation of fatty acids (FAs) by FA elongases [83], (ii) desaturation of FAs [84], (iii) cholesterol metabolism [8], and (iv) phospholipid biosynthesis [85] (Fig. 4). An increase in membrane fluidity, either by depletion of cholesterol or saturated FAs results in proteolytic activation of type II transmembrane transcription factors localized in the ER membrane, the basic helix-loop-helix (bHLH) transcription factors SREBP-1a, SREBP-1c, and SREBP2 in mammals [8]. The RelA family transcription factors Spt23p and Mga2p are activated by a decrease in fluidity of ER membrane in yeast [86]. If cholesterol levels are above physiological levels, cholesterol is esterified in the ER membrane with unsaturated FAs [84] by acyl-coenzyme A (CoA):cholesterol acyltransferase 1 (ACAT1) [8]. Cholesteryl esters are stored in lipid droplets in the cell [8]. Excess accumulation of these cholesteryl ester lipid droplets, for example in macrophages in atherosclerotic lesions, results in a foamy macrophage morphology called a foam cell [8]. Cholesterol is an allosteric activator of ACAT1 [8]. In addition, sterols stimulate ubiquitination and subsequent proteasomal degradation of 3-hydroxy methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the rate-limiting step in cholesterol synthesis in the mevalonate pathway, by a complex of the ubiquitin ligase gp78 and INSIG1 [87, 88].

The SREBPs are key regulators of lipid metabolism in mammalian cells [89, 90]. These proteins consist of an N-terminal, cytosolic bHLH domain, two transmembrane domains, and a regulatory cytosolic tail [89, 90]. When cholesterol levels are high, SREBPs are localized in the ER in a complex with SCAP, a protein with eight transmembrane domains, and INSIG1 or its minor isoform INSIG2 [90]. Upon cholesterol depletion a conformational change in SCAP dissociates a SREBP·SCAP complex from INSIG1 [90]. The SREBP·SCAP complex then translocates to the Golgi complex [91, 92]. Free INSIG1 is ubiquitinated by gp78 and degraded by the proteasome [90]. The Golgi-resident proteases S1P and S2P release the cytosolic bHLH domain of the SREBPs in two ordered proteolytic cleavages [89, 90]. After translocation to the nucleus the N-terminal cytosolic domains activate expression of genes involved in the biosynthesis of cholesterol, triacylglycerols, phospholipids, saturated and unsaturated FAs, i.e., the gene encoding Δ^9-stearoyl-CoA desaturase 1 (*SCD1*) [84, 89]. Spt23p and Mga2p are two homologous type II transmembrane transcription factors distantly related to the RelA transcription factor family, which includes the NF-κB transcription factors. Both are activated by limited proteolysis of their C-terminal transmembrane domains by the proteasome when unsaturated FAs are depleted and fluidity of the ER membrane decreases [93]. Both transcription factors stimulate transcription of *OLE1* encoding the yeast functional homolog of *SCD1* [94]. Opi1p is a transcriptional repressor of several phospholipid biosynthetic genes required for the synthesis of phosphatidylinositol [95, 96], phosphatidyl-L-serine [95, 97], phosphatidylethanolamine, and phosphatidylcholine [95] from their common precursor phosphatidic acid [85]. Opi1p is bound to ER membranes enriched in phosphatidic acid in an inactive complex with Scs2p [98]. Conversion of phosphatidic acid into phospho-

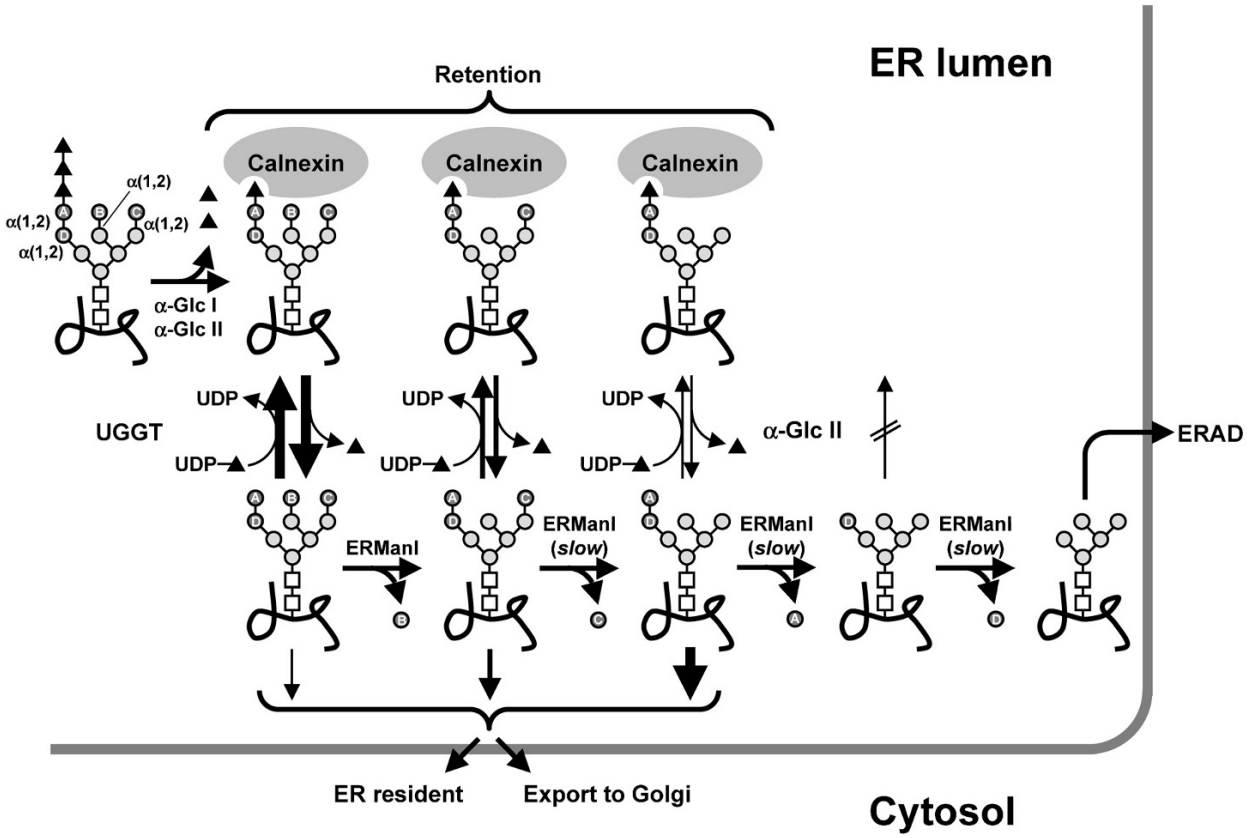

Figure 3. Recognition of *N*-linked oligosaccharides as a marker for protein-folding status [61, 290]. Oligosaccharyltransferase transfers the oligosaccharide $Glc_3Man_9GlcNAc_2$ (left: ▲, D-glucose; ○, D-mannose; □, 2-*N*-acetyl-D-glucosamine) onto a newly synthesized polypeptide chain. α-Glucosidases I and II (α-Glc I, α-Glc II) remove the two terminal D-glucose moieties. The monoglucosylated form is recognized by calnexin, calreticulin, or calmegin and retained in the ER. Upon removal of the terminal D-glucose moiety by α-glucosidase II, the protein is released from the lectin chaperone, but if still unfolded is reglucosylated by UDP-glucose:glycoprotein glucosyl transferase (UGGT). Removal of D-mannose B and C converts the oligosaccharide into a poorer substrate for UGGT and α-glucosidase II and decreases the affinity of the oligosaccharide for calnexin and calreticulin and indirectly promotes export to the Golgi complex *via* recognition of oligosaccharides containing α(1,2)-linked D-mannose residues by ERGIC-53 and VIP36. Trimming of D-mannose A removes the D-glucose acceptor from the oligosaccharide, efficiently blocks the reglucosylation reaction, inhibits the interaction with ERGIC-53 and VIP36 and triggers retrotranslocation and proteasomal degradation of slowly folding proteins [61–64]. The demannosylation reactions are slower than the deglucosylation reactions [291, 292]. α(1,2)-ER mannosidase I (ERManI) preferentially removes D-mannose B [37, 293, 294], and more slowly the other D-mannose moieties [295]. Golgi-resident mannosidase exhibit specificity for D-mannoses A, C, and D and may be involved in trimming of the α(1,2)-linked D-mannose residues.

lipids, i.e., when cellular inositol and choline concentrations are elevated decreases the phosphatidic acid content of the ER membrane and releases Opi1p from the ER membrane. Opi1p then translocates to the nucleus and represses transcription of phospholipid biosynthetic genes [99]. Thus, Opi1p regulates *de novo* membrane biosynthesis.

ER stress

ER stress is caused by perturbation of any of the three homeostatic functions of the ER, protein folding, functioning as an intracellular Ca^{2+} store, and as a site for synthesis of unsaturated FAs, sterols, and phospholipids. Accumulation of unfolded proteins in the ER lumen is a shared hallmark of perturbation of any of these three physiological functions of the ER and results in activation of a common response to this stress situation, the UPR.

In ER storage diseases, mutant folding-incompetent proteins are retained in the ER, and, if resistant to proteasomal degradation, accumulate in the ER [2, 3]. Strong overexpression of wild-type (WT) proteins in eukaryotic expression systems for heterologous secretory proteins [2, 3], in differentiation into a secretory cell type, i.e., in terminal B cell differentiation [2, 3], or in viral infections associated with synthesis of large amounts of highly glycosylated capsid proteins in a short amount of time [2, 3], perturbs ER homeostasis. In plants, the response to microbial infections is associated with increased

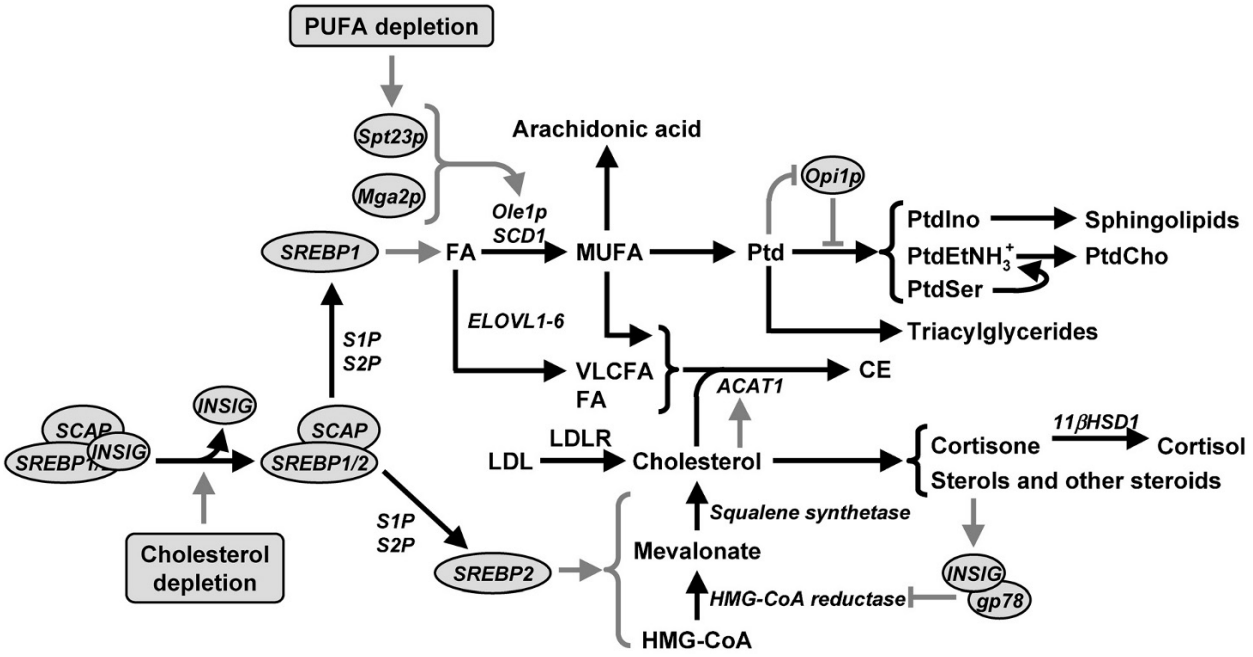

Figure 4. Changes in the lipid composition of the ER membrane regulate several aspects of eukaryotic lipid metabolism. Cholesterol depletion activates the basic helix-loop-helix (bHLH) transcription factors SREBP1 and SREBP2. SREBP1 stimulates fatty acid (FA) and triacylglyceride synthesis, and SREBP2 cholesterol synthesis. Polyunsaturated FA (PUFA) depletion activates the RelA transcription factors Spt23p and Mga2p in yeast, which induce *OLE1* expression to stimulate FA desaturation. High cholesterol levels allosterically stimulate ACAT1 and cholesterol esterification with FAs or very long chain FAs (VLCFAs) and proteasomal degradation of HMG-CoA reductase after ubiquitination by the ubiquitin ligase gp78. Elevated levels of phosphatidic acid (Ptd) sequester the transcriptional repressor of several phospholipid biosynthetic genes, Opi1p, to the ER membrane to stimulate phospholipid biosynthesis when levels of the phospholipid precursor Ptd are high. Enzymes discussed in the text are shown. Proteins localized at the ER are shown in italics. Regulatory proteins are shown in gray ellipses. CE, cholesteryl ester; 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; MUFA, monounsaturated FA; PtdCho, phosphatidylcholine; $PtdEtNH_3^+$, phosphatidylethanolamine; PtdIno, phosphatidylinositol; and PtdSer, phosphatidyl-L-serine.

secretory activity [2, 3]. Reducing agents, such as β-mercaptoethanol or 1,4-dithio-DL-threitol (DTT) reduce disulfide bonds and interfere with oxidative protein folding in the ER. The drug tunicamycin irreversibly [100] inhibits *N*-linked glycosylation of newly synthesized proteins, resulting in accumulation of unfolded proteins in the ER. Likewise, genetic depletion of ER resident molecular chaperones [101] perturbs the balance between folding demand and capacity and causes ER stress. Finally, poisoning of the ERAD machinery, i.e., through expression of polyglutamine repeat proteins [11], results in accumulation of unfolded proteins in the ER.

Ca^{2+} depletion inhibits protein folding [102, 103], interferes with retention of ER-resident proteins [104], ER-Golgi trafficking [105], and chaperone function, *e.g.*, the function of BiP [106] and calnexin [107]. Calreticulin reversibly binds to and inhibits PDI below 50 μM Ca^{2+} [108, 109]. Calreticulin also interacts with ERp57 in a Ca^{2+}-dependent manner [109–111]. ER-α(1,2)-mannosidase [112], UGGT [113], and ER-UDPase [44], but not α-glucosidase II [114], require millimolar amounts of Ca^{2+} to display significant enzymatic activity. Depletion of ER-luminal Ca^{2+} stores inhibits reglucosylation by UGGT by inactivating UGGT and depletes the ER of UDP-glucose by inhibiting the antiport import of UDP-glucose for UMP. Ca^{2+} depletion abrogates the interaction of calnexin and calreticulin with unfolded substrates [68], and inhibits targeting of unfolded proteins for ERAD, because of decreased ER-α(1,2)-mannosidase activity.

The interaction of the hydrophobic rings of cholesterol with saturated fatty acyl groups is important for the formation of rigid lipid-ordered rafts [115]. The rigidity of these rafts may be too high at higher than physiological cholesterol or saturated fatty acid levels, resulting in inhibition of proper function of integral membrane proteins that require conformational freedom for proper function [116]. Elevated ER-luminal Ca^{2+} concentrations may further decrease membrane fluidity by neutralizing the polar head groups of phospholipids, facilitating their tighter packaging. Alternatively, direct sterol/phospholipid interactions regulate protein function [116]. Examples for ER-resident integral membrane proteins whose conformational freedom is restricted by a decrease in

membrane fluidity are UDP-glucuronyltransferase and SERCA2b [117]. Increased membrane rigidity may inhibit conformational changes in SERCA2b associated with its Ca^{2+}-pumping activity, and in turn cause ER stress by depleting the ER luminal Ca^{2+} store. Examples for regulation of integral ER-membrane proteins by direct lipid-protein interactions are ACAT1, for which cholesterol is an allosteric stimulator [8], and ER-α(1,2)-mannosidase. ER-α(1,2)-mannosidase is a type II transmembrane protein, whose activity is dependent on an ordered lipid bilayer and zwitterionic phospholipids, but inhibited by negatively charged phospholipids [112].

How ER stress is sensed

Perturbation of any of the physiological functions of the ER results in accumulation of unfolded proteins. Unfolded proteins are harmful to cells because they display non-native hydrophobic patches at their surface. Through these patches they interact and inhibit the function of other proteins, especially those proteins for which a major aspect of their function is to interact with other proteins, i.e., transcription factors [118], molecular chaperones [118], and the proteasome [11, 118]. For the same reason, unfolded proteins interact with cellular membranes and interfere with maintenance of gradients across the affected membrane [118]. Accumulation of unfolded proteins may disrupt the Ca^{2+} gradient over the membrane, resulting in Ca^{2+} leaking out of the ER, inhibition of chaperone and degradation systems, and accelerated accumulation of unfolded proteins. To maintain homeostasis of the ER, monitoring of the protein-folding status and of membrane integrity of the ER is critical. This function is fulfilled by several ER-resident transmembrane proteins [2, 3], bZIP transcription factors synthesized as type II transmembrane precursors, the type I transmembrane protein kinase PERK/PEK and the type I transmembrane protein kinase endoribonucleases ERN1α/IRE1α and ERN1β/IRE1β. Several type II transmembrane bZIP transcription factors have been identified in the ER membrane, ATF6α, ATF6β, OASIS, CREB3/Luman, CREB-H, CREB4, and BBF2H7. The ER luminal domains of these proteins serve as ER stress-sensing domains. The ER luminal domains of IRE1 and PERK regulate the protein kinase activity of these proteins by functioning as ER stress-regulated di- and oligomerization domains. Two substrates for the protein kinase activity of PERK are known, the cap'n'collar bZIP transcription factor NRF2 [119, 120] and eIF2α. The protein kinase activity of IRE1 controls its endoribonuclease activity, which cleaves exon-intron junctions in mRNAs encoding the bZIP transcription factors Hac1p in yeast, HACA in filamentous fungi, and XBP1 in metazoans. The mechanism of this unusual splicing reaction has been reviewed in detail recently [2, 3]. The function of the luminal domains of IRE1 and PERK is conserved [121]. For example, chimeric Ire1p, in which the luminal domain of Ire1p has been replaced with the luminal domain of PERK is functional in yeast [121]. This functional conservation is most likely a consequence of small, but significant sequence homology in the luminal domains of IRE1α/β and PERK in regions of the luminal domains required for ER stress signaling [121]. The ER luminal domain of the transmembrane bZIP transcription factors functions as an ER stress-regulated ER retention motif. In unstressed cells, the transcription factors are retained in the ER, but upon ER stress translocate to the Golgi complex [91, 122, 123]. There ordered proteolysis by S1P and S2P releases the cytosolic bZIP transcription factor domain from the Golgi membrane to allow its translocation to the nucleus [91].

Three models can explain how the ER stress sensors monitor protein-folding status in the ER, (i) the competition model, in which molecular chaperones probe protein-folding status and report on protein-folding status to the stress sensors (Fig. 5a), (ii) the ligand-binding model, in which unfolded proteins directly interact with the ER stress-sensing domains, and (iii) the probing model, in which newly synthesized stress-sensing proteins probe the efficiency of the ER-resident protein-folding machinery by presenting themselves as substrates to the folding machinery (Fig. 5e). In the competition model, the ER luminal domains of the stress sensors compete with unfolded proteins for binding to the general chaperone BiP [124, 125]. In unstressed cells, BiP is associated with the ER luminal domains of the stress sensors [125, 126], because of the low concentration of unfolded proteins under these conditions. Accumulation of unfolded proteins triggers their interaction with BiP, depletes the free BiP pool, and sequesters BiP from the ER luminal domains of the stress sensors, resulting in their activation [125, 126]. Consistent with this model are the observations that increased expression of secretory proteins induces BiP [124, 127]; that more slowly folding proteins displaying a prolonged association with BiP induce BiP more efficiently than more rapidly folding proteins [124, 128]; that overexpression of BiP attenuates its induction [129]; and that depletion of ER luminal BiP pools in cells constitutively secreting BiP mutants lacking their C-terminal HDEL (D, Asp; E, Glu; H, His; L, Leu) retrieval signal [130] induced BiP expression. Overexpression of PDI [131] and calreticulin [132] did not

mitigate the ER-stress response, suggesting a specific role for BiP in initiation of the UPR. A prediction of the competition model is that discrete BiP binding sites exist in the ER luminal domains of the stress sensors (Fig. 5a). Binding of BiP to ATF6α masks two Golgi localization sequences, GLS1 and GLS2 [92]. GLS1 overlaps with a BiP binding site. Deletion of GLS1 and the BiP binding site in ATF6α triggers constitutive export of ATF6α-ΔGLS1 to the Golgi complex. GLS2, but not GLS1, is conserved in ATF6β. BiP binding sites in ATF6β or the other bZIP transcription factors have not yet been mapped. Binding of BiP to the luminal domains of IRE1 and PERK prevents their di- and/or oligomerization [126, 133–135]. BiP binds to Ire1p *via* its ATPase domain, most likely in its ATP-bound state [136]. Substrate binding to the substrate-binding domain stimulates ATP hydrolysis and a conformational change in the ATPase domain, releasing BiP from Ire1p (Fig. 5b) [136]. More recent experimental evidence suggests an active role for the ER luminal domains of ATF6 and IRE1α in their activation (Fig. 5c) [92, 134, 135, 137]. Three independent regions, luminal domains 1–3, exist in ATF6 that bind to BiP and can induce BiP release [137]. Likewise, in yeast Ire1p conformational changes in the ER luminal domain appear to coincide with or precede BiP release (Fig. 5d). An Ire1p mutant that did not bind to BiP was inactive in unstressed cells and activated upon induction of ER stress [134, 135], suggesting that the conformation of the luminal domain of Ire1p directly inhibits its dimerization and that ER stress, at least partially independent of BiP release from the ER luminal domain of Ire1p, induces conformational changes in the ER luminal domain that release this conformational repression.

Functional evidence for the competition model, i.e., activation of the UPR by increased secretory activity or genetic depletion of ER luminal BiP levels is also consistent with increased display of unfolded regions at the surface of proteins. These unfolded regions may directly activate the ER stress sensors. The crystal structure of the core ER luminal domain sufficient for signal transduction by yeast Ire1p has revealed the presence of a major histocompatibility complex (MHC)-like peptide-binding groove in an Ire1p dimer [138]. Ire1p signaling was abolished in mutants in which amino acid residues lining the bottom of the groove or located at the interface of the dimer were mutated [138], providing causal evidence for the importance of the groove and dimerization of Ire1p. The depth of the peptide binding groove precludes interaction with compactly folded protein structures, but should allow association with less compact or unfolded regions. Binding of Ire1p to unfolded regions may trigger dimerization of Ire1p, because two Ire1p monomers are required to form the groove. Six pairs of core luminal domain monomers formed one turn of a continuous helix in the crystal structure providing evidence for fibrillation of Ire1p during ER stress. Mutation of amino acid residues lining the second interface required for formation of these higher order structures also interfered with Ire1p signaling. In contrast to these findings, the crystal structure of human IRE1α [139] and the phylogenetic conservation of IRE1 function from yeast to humans [121] suggest that the MHC-like groove and fibrillation of IRE1α in the ER membrane do not contribute to IRE1 function. The MHC-like groove in human IRE1α is too narrow to allow for peptide binding and is oriented towards the ER membrane [139], making it difficult for unfolded proteins to bind to this groove. Amino acid residues lining the MHC-like groove in yeast Ire1p are either buried in human IRE1α or not conserved. IRE1α monomers do not display a higher order arrangement in the crystal structure [139].

The probing model may contribute to regulation of ATF6α (Fig. 5e). In unstressed cells interaction of fully glycosylated ATF6α with calreticulin retains ATF6α in the ER [140]. In ER-stressed cells, newly synthesized ATF6α is underglycosylated, preventing the interaction with calreticulin. ER stress induces the degradation of fully glycosylated ATF6α to prevent retention of heterodimers of underglycosylated and fully glycosylated ATF6α *via* their bZIP domains [141]. In this way, ATF6α may probe the efficiency of the glycosylation, folding, and degradation machinery of the ER. ATF6 also probes the functionality of the oxidative protein folding machinery of the ER [142]. In unstressed cells, ATF6 exists as disulfide-bonded dimers and oligomers, or as a monomer containing an intramolecular disulfide bond, indicative of an advanced folding status of the protein. Upon ER stress, the oxidized species disappear, either because of an inability of the oxidative protein-folding machinery to efficiently introduce disulfide bonds into newly synthesized ATF6 or because of reduction of already formed disulfide bonds. Disulfide bond formation does not block translocation of ATF6 to the Golgi complex, but interferes with cleavage by S1P, probably because of the more compact conformation of the disulfide bonded species compared to the reduced species. Because of the tight coupling of conformational and oxidative folding, disulfide bond formation in ATF6 may also indirectly probe for the efficiency of conformational folding in the ER.

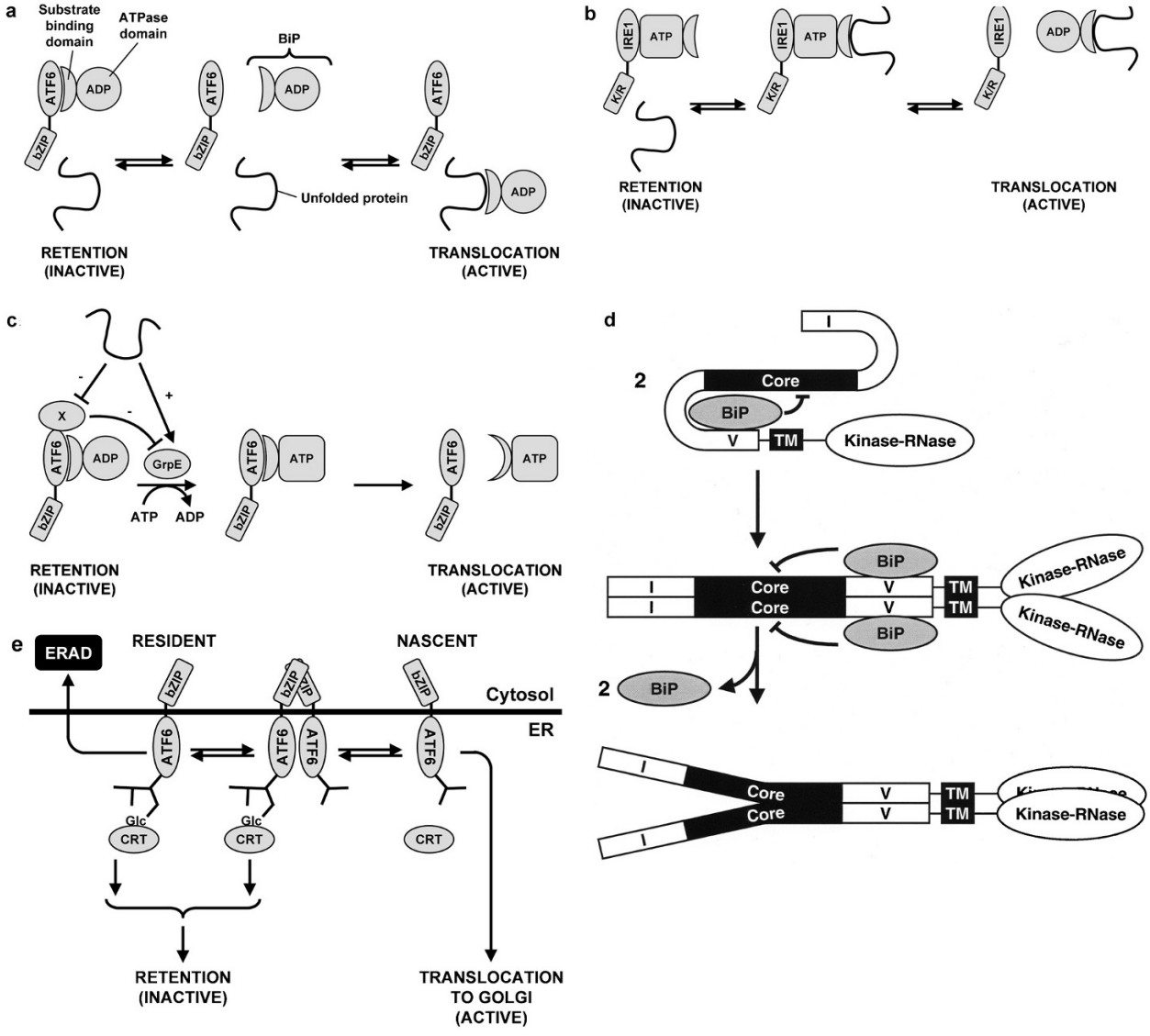

Figure 5. ER stress-sensing models. (*a*) The competition model is based on the law of mass action. The competition model assumes that a dynamic equilibrium of binding of the luminal domains of the ER stress sensors, i.e., ATF6 and unfolded proteins to the substrate-binding domain of BiP exists in the ER. The abundance of unfolded proteins in the ER then determines if the ER stress sensors are active or inactive. If the concentration of unfolded proteins is low, BiP effectively binds to and inhibits activation of all ER stress sensors. Through an increase in their concentration unfolded proteins sequester BiP from the stress-sensing domains, resulting in activation of the ER stress sensors. The *in vitro* stability of BiP·IRE1, BiP·PERK, and BiP·ATF6 complexes suggests that the *in vivo* dissociation rates of these complexes may be too small to allow for dynamic regulation of the ER stress sensors [125]. (*b*) In the ATPase domain-binding model, the stress-sensing domain interacts with the ATPase domain of ATP-bound BiP. Unfolded protein binding to the substrate binding domain of BiP stimulates ATP hydrolysis and induces a conformational change in BiP, resulting in release of BiP from the stress-sensing domain [136, 137]. (*c*) The BiP ATPase cycle restarting model [137] is based on the competition model, but because of the stability of BiP binding to the stress-sensing domains, proposes that unfolded proteins reactivate a stalled BiP ATPase cycle. Unfolded proteins may stimulate the activity of GrpE co-chaperones, or inhibit unknown factors, possibly a phosphatase or ADP-ribosylase, associated with the BiP·stress-sensing domain complex that inhibit the GrpE co-chaperones. Alternatively, the stress-sensing domain may directly inhibit the GrpE co-chaperones. (*d*) Activation of yeast Ire1p by ER stress [134, 135]. In the first step, an unknown ER-stress signal induces conformational changes in subdomains I and V that relieve inhibition of the core domain by these subdomains. For subdomain V this conformational change may be triggered by release of BiP, or may trigger BiP release from subdomain V. In the second step, an unknown ER stress signal induces a conformation change in the core domain. This conformational change in the core domain is repressed by BiP associated with subdomain V. The conformational change in the core domain is transduced across the ER membrane and contributes to activation of the kinase domain. (*e*) In the folding machinery probing model [140, 141], the stress sensor presents itself as a substrate to the ER-resident protein-folding machinery. ATF6 newly synthesized in unstressed cells is retained in the ER by interaction with the chaperones calnexin and calreticulin, because it is fully glycosylated. In ER-stressed cells the folding and glycosylation machinery works less efficiently. ATF6 synthesized in ER-stressed cells escapes retention because it is only partially glycosylated. Degradation of fully glycosylated ATF6 in ER-stressed cells prevents retention of underglycosylated ATF6 in the ER through interaction of the bZIP domains. K/R, protein kinase endoribonuclease domain; I, subdomain I; TM, transmembrane domain; and V, subdomain, V.

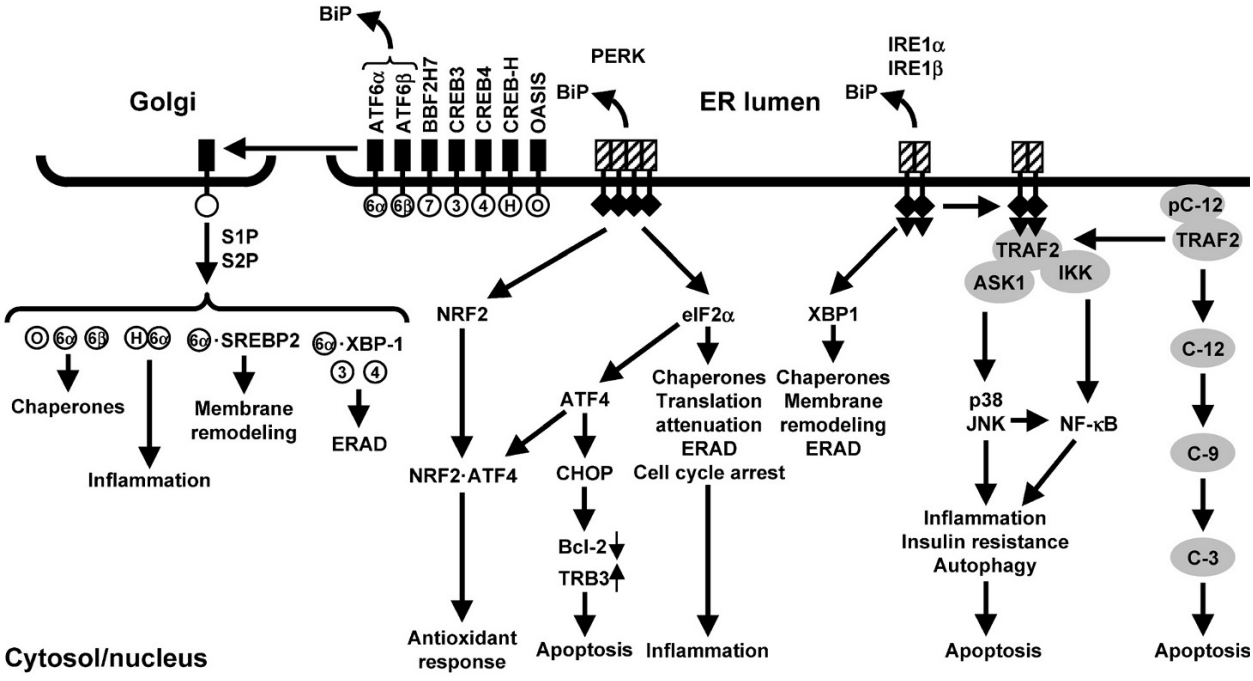

Figure 6. Principal unfolded protein response (UPR) signal transduction pathways. In humans, caspase-4 substitutes for caspase-12 [257, 258]. In yeast, filamentous fungi, and plants the IRE1-XBP1 (Hac1p/HACA) pathway is the only known UPR signal transduction pathway. Our knowledge of downstream signaling events, i.e., the functions of the bZIP transcription factors activated by ER stress is still incomplete. Genes regulated by BBF2H7 in response to ER stress have not yet been characterized. Reprinted in modified form with permission from Bentham Science Publishers from [18].

Remedial action – return to homeostasis

To return cells to homeostasis, the type II bZIP transcription factors, PERK, and IRE1α/β initiate and coordinate a series of protective responses to ER stress (Fig. 6). On a cellular level, these responses include the transcriptional induction of chaperone gene expression to increase the protein-folding capacity of the ER [143, 144], the transcriptional induction of genes involved in ERAD [53, 54, 145, 146], stimulation of autophagy [147–149], transient attenuation of general translation [150–152] and of transcription encoding secretory proteins [153, 154] to decrease the unfolded protein load of the ER, the stimulation of phospholipid biosynthesis to increase the size of the ER [73, 155, 156] and to dilute its unfolded protein load, and induction of an antioxidant response [119, 120, 157] to counteract ROS formed in increased repetitive oxidative protein folding attempts in ER-stressed cells [50, 51].

Increase of the folding capacity of the ER

The increase of the folding capacity of the ER involves up-regulation of ER-resident chaperone foldases, their co-chaperones, and co-factors, such as Ca^{2+} and ATP, of oxidoreductases and holdases. Several UPR pathways have been implicated in transcriptional induction of these genes, especially the type II transmembrane transcription factors ATF6 and OASIS, and the IRE1-Hac1p/XBP1 pathway. ATF6α induces expression of several chaperones [158], including BiP, GRP94, calreticulin, and the DnaJ co-chaperone $p58^{IPK}$ [159], which was recently localized to the ER [160], several PDIs, including PDI, ERp72, and P5 [161, 162] and the cargo receptor ERGIC-53 [163]. ATF6α acts on several promoter elements, i.e., the ATF/cyclic AMP (cAMP)-response element [CRE, TGACGT(C/A)(G/A)] [164], ER stress-response elements I (ERSE-I, CCAAT-N_9-CCACG) [165] and II (ERSE-II, ATTGG-N-CCACG) [166]. ATF6α binds to ERSE-I and –II in a complex with NF-Y [166]. Consistent with the Ca^{2+} requirement of most chaperones is induction of SERCA2 by ATF6α [161]. ATF6β also activates transcription of BiP on ERSE-I in a complex with NF-Y [167, 168], but can also act as a repressor of *BiP* [165, 169]. Pathways parallel to ATF6α/β exist that contribute to chaperone induction, because knockdown of ATF6α/β by RNAi did not reveal specific ATF6α/β targets [162]. OASIS contributes to BiP induction by binding the CRE and ERSE-I sites, with CRE being the major OASIS-binding site [122].

A second UPR pathway contributing to induction of ER-resident chaperones is the IRE1-Hac1p/XBP1

pathway. The IRE1-Hac1p/XBP1 pathway is the only known UPR pathway present in yeast and plants, which makes this pathway the only known UPR pathway conserved in all eukaryotes. In yeast, spliced Hac1p (Hac1^{i}p) induces expression of many ER-resident chaperones, through UPR elements (UPRE) 1 (CAGCGTG) and 2 (TACGTG) [170] as a heterodimer with the bZIP transcription factor Gcn4p [170]. Hac1^{i}p induces expression of BiP [129, 171], Lhs1p [171], Pdi1p [171], Eug1p [171], and Fkb2p [171, 172], and of co-chaperones such as Scj1p [171], Jem1p [54], and Sil1p [54]. Likewise, XBP1 is required for full induction of many chaperone genes, including BiP and GRP94, the DnaJ co-chaperones p58^{IPK}, and ERdj4, and the oxidoreductases ERO1-Lα [162] and -β [156]. XBP1 binds to ERSE-I in a complex with NF-Y [173]. In summary, induction of chaperone foldases, their co-chaperones, chaperone holdases, oxidoreductases, and increased supply of cofactors such as Ca^{2+} suggests that the protein-folding capacity of the ER is augmented by the UPR in ER-stressed cells.

ERAD and autophagy

Both ATF6α and XBP1 also increase expression of genes encoding proteins involved in ERAD. ATF6 induces HERP through an ERSE-II site [166, 174, 175]. Increased UPR signaling and ER stress sensitivity of *herp*$^{-/-}$ embryonic carcinoma cells suggests a role for HERP in ERAD [176]. XBP1 contributes to HERP induction through NF-Y-independent binding to mammalian UPREs (TGACGTGG/A) [175]. XBP1 also stimulates expression of *EDEM1* [146], and *DERLIN-2* and *-3* [145]. CREB4 activates expression of *EDEM1* [177]. In yeast, several ubiquitin-conjugating enzymes and ligases, including Ubc7p, Cue1p, and Hrd1p are induced in ER-stressed cells in an *IRE1*-dependent manner [53]. Proteolytic activation of ATF6 kinetically precedes endonucleolytic activation of XBP1, suggesting that a folding only recovery phase precedes a folding and degradation recovery phase in the UPR [146]. A second aspect of ERAD is selective degradation of mRNAs encoding secretory and transmembrane proteins [178].

The UPR also induces autophagy [147–149]. *atg5*$^{-/-}$ mouse embryonic fibroblasts, which are defective in autophagy [147], and several yeast strains in which the genes required for autophagy, i.e., *ATG1*, were deleted [149] displayed increased sensitivity to ER stress-inducing drugs, showing that autophagy is important for survival of ER stress. Whether autophagy contributes to clearance of misfolded secretory cargo proteins from ER-stressed cells is unknown. Autophagy may play other roles in ER-stressed cells, e.g., counterbalancing ER expansion in these cells [149]. In mammalian cells, induction of autophagy by the UPR required the protein kinase, but not the endoribonuclease activity of IRE1 [147]. These data show that a second signaling activity of IRE1, which is independent of its endoribonuclease activity, is important to regulate autophagy (Fig. 7). Phosphorylated IRE1 assembles a signaling complex consisting of TRAF2 [179] and the protein kinase ASK1 [11], and the IκB kinase IKK [180]. The IRE1·TRAF2·ASK1 complex activates two mitogen-activated protein (MAP) kinase modules, resulting in activation of p38 and JNK1–3 (Fig. 7) [179]. JNK inhibitors and a dominant-negative TRAF2, which lacks its N-terminal effector domain, blocked activation of autophagy by IRE1α [147]. In unstressed cells IRE1α is associated with JAB1 [181]. JAB1 is a selective transcriptional coactivator that stabilizes binding of *c*-JUN and JUN-D, but not of JUN-B or *v*-JUN, to AP-1 sites [182]. Release of JAB1 from IRE1 during ER stress may be a positive feed forward loop to potentiate JNK signaling and may modulate JNK signaling towards the need to respond to ER stress.

Attenuation of cap-dependent translation

Phosphorylation of eIF2α by PERK at Ser51 inhibits cap-dependent translation, and therefore translation of the majority of cellular mRNAs. The mechanism of this translational attenuation mechanism has been reviewed in some detail before [3, 18, 183]. Attenuation of general translation clears short-lived proteins from the cell. Important examples in the UPR are clearance of D-type cyclins, resulting in G_1 arrest of the cell cycle [184, 185], and of IκBs, resulting in activation of the proinflammatory transcription factor NF-κB. D-type cyclins activate cyclin-dependent kinases 4 and 6 (CDK4, CDK6), which then phosphorylate and inactivate the transcriptional co-repressor retinoblastoma protein (RB) [186, 187]. RB represses transcription of cyclin E required for passage through the restriction point and entry into S phase [186]. Overexpression of D-type cyclins [184], cells expressing a dominant negative PERK lacking its protein kinase domain [185] or *rb*$^{-/-}$ cells [185] did not display an ER stress induced G_1 arrest. Ser51 phosphorylation of eIF2α by a second eIF2α kinase, GCN2, contributes to depletion of D-type cyclins during ER stress [188]. NF-κB is a member of the RelA transcription factor family [189]. These transcription factors share a RelA homology domain involved in homo- and heterodimerization, DNA binding, and interaction with IκBs. Interaction with IκBs restricts NF-κB dimers to the cytosol. IκBs are short-lived proteins that are cleared from the cell during translational attenuation in the UPR, resulting in release of NF-κB from NF-κB·IκB complexes, translocation of NF-κB to the nucleus, and activation of genes involved in immune and inflam-

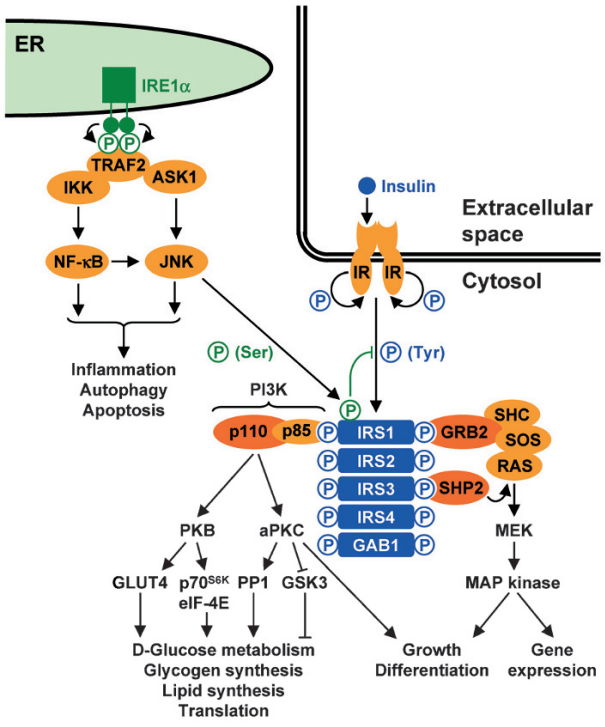

Figure 7. Insulin signaling [213–215] and ER-stress-induced insulin resistance. The insulin receptor (IR) activates itself by autophosphorylation on tyrosine residues after binding to insulin and then phosphorylates tyrosine residues (represented by a blue P in the center of a blue circle) in several IR substrates, including IRS1, IRS2, IRS3, IRS4, and GAB1. SH2 domain containing proteins bind to and are activated by tyrosine phosphorylated IR substrates, resulting in activation of gene expression, growth, differentiation, D-glucose metabolism, glycogen and lipid synthesis, and stimulation of translation. Upon ER stress, IRE1α activates itself by phosphorylating serine residues (represented by a green P in the center of a green circle) in its activation segment [296], resulting in recruitment of TRAF2 to serine phosphorylated IRE1α [179]. TRAF2 recruits the protein kinases IKK [180] and ASK1 [11] to IRE1α, which activate the transcription factor NF-κB and the JNK protein kinases, respectively. Activated JNKs phosphorylate IR substrates at serine residues [217]. This serine phosphorylation inhibits tyrosine phosphorylation of these proteins, inhibiting IR signaling [221–223]. As a consequence, ER-stressed cells become resistant to insulin. p85, 85-kDa regulatory subunit of PI3K; and p110, 110-kDa catalytic subunit of PI3K.

matory responses, cell growth, and of pro- and anti-apoptotic genes [189]. Activation of IκB kinase (IKK) in a complex with IRE1α and TRAF2 and phosphorylation of IκBα by IKK contributes to proteolytic destruction of IκBs and activation of NF-κB (Fig. 7) [180]. NF-κB is also activated by ROS, counteracts ROS accumulation, and may contribute to the antioxidant response mounted during the UPR. However, the effects of ROS on NF-κB are complex, because oxidation of NF-κB decreases its DNA binding affinity [190], and oxidation of IKK inhibits IκB phosphorylation by IKK [191].

Several escape mechanisms for translational attenuation exist. These include cap-independent translation mediated by internal ribosomal entry sites, regulation of selection of the translation initiation codon by several upstream open reading frames (uORFs) in the 5' untranslated region (UTR), and leaky scanning mechanisms (reviewed in [18, 192]). Currently, the only known mRNA that utilizes one of the mechanisms to escape translational attenuation during ER stress is the mRNA for the bZIP transcription factor ATF4. *ATF4* mRNA encodes two uORFs in its 5′ UTR, of which the second overlaps with the *ATF4* ORF [193, 194]. Heterodimers of ATF4 and NRF2 contribute to the antioxidant response activated by the UPR [195, 196]. A second major role of ATF4 in the UPR is activation of the pro-apoptotic bZIP transcription factor CHOP/CHOP-10 [197], of NIPK/TRB3 [198], of ERAD *via* activation of *HERP* [174], and of gluconeogenesis *via* phosphoenolpyruvate carboxykinase (PEPCK) [199]. Activation of ATF3 by ATF4 down-regulates expression of gluconeogenic genes [200], i.e., fructose bisphosphatase and PEPCK [201]. ATF4 is also responsible for activation of GADD34/MyD116 late in the UPR [202]. GADD34 is a regulatory subunit of protein phosphatase 1 (PP1) that targets PP1 towards dephosphorylation of eIF2α [202].

Attenuation of metabolism

D-Glucose starvation is a potent activator of the UPR [203], but it is not known to what extent ATP depletion, because of decreased glycolytic rates, and decreased synthesis of dolicholpyrophosphate-linked oligosaccharides are responsible for this effect. Activation of gluconeogenic genes by ATF4 [194, 199] and repression of cholesterogenesis by ATF6 to preserve carbon units for gluconeogenesis [204] suggests that inhibition or altered *N*-linked glycosylation is the major cause for ER stress in D-glucose starvation. Starvation also alters the activity of ER luminal chaperones. GRP94 is activated by depletion of ER luminal adenine nucleotide pools [35, 36]. ADP-ribosylation of BiP increased in amino acid starved mouse hepatoma cells [31, 205]. In yeast, ER stress stimulates eIF2α phosphorylation by Gcn2p [170], a protein kinase activated by amino acid starvation. Mammalian GCN2 is required for G_1 arrest in response to ER stress [188]. Activation of amino acid biosynthetic genes by ATF4 [196] also suggests the existence of a link between ER stress and amino acid or nitrogen starvation. This link between ER function and amino acid metabolism may reflect that elevated secretory activity depletes cellular amino acid pools, because of loss of amino acids in the form of secreted proteins to the extracellular space [196], or that the level of protein folding and transport activity of the ER functions as an intracellular sensor for

cellular amino acid pools. Data from budding yeast provides experimental support for the second interpretation. In diploid budding yeast, enforced expression of Hac1^{i}p repressed two nitrogen starvation responses of this organism, pseudohyphal growth and meiosis [206]. Pseudohyphal growth is an alternative growth form of this organism induced by nitrogen starvation that allows this otherwise immobile organism to forage for nutrients by adopting a growth form similar to the growth form of filamentous fungi [207]. Severe nitrogen and carbon source starvation induces meiosis or sporulation, in which a cell type called an ascus is formed that harbors four stress- and starvation-resistant haploid spores [208]. Deletion of *IRE1* or *HAC1* derepressed pseudohyphal growth [206]. Activation of the UPR by sublethal concentrations of drugs that inhibit protein folding in the ER, inhibited pseudohyphal growth in an *IRE1*- and *HAC1*-dependent manner [206]. In meiosis, Hac1^{i}p repressed a set of genes controlling entry into meiosis, the early meiotic genes (EMGs) [206, 209]. A basal level of *HAC1* mRNA splicing was observed in exponentially growing cells in rich media [206]. Induction of nitrogen starvation inhibited *HAC1* mRNA splicing rapidly within less than 5 min. Addition of nitrogen sources to nitrogen-starved cells was sufficient to reactivate *HAC1* splicing. These observations show that Hac1^{i}p is synthesized at low levels in a nitrogen-rich environment and that Hac1^{i}p synthesis stops rapidly when cells encounter nitrogen starvation. In nitrogen starvation Hac1^{i}p is rapidly lost from the cell, because of its very short $t_{1/2}$ of 1–2 min [210], as is expected for an inhibitor of nitrogen-starvation responses. Repression of EMGs by Hac1^{i}p required their characteristic promoter element upstream repressing site 1 (URS1, TCGGCGGCT) [211], the URS1-binding protein Ume6p [211], and the catalytic deacetylase activity of the Rpd3p-Sin3p histone deacetylase [211]. Yeast and filamentous fungi do not have a PERK homolog. URS1 and Ume6p also regulate expression of genes involved in carbon and nitrogen metabolism [212]. Repression of these genes by spliced Hac1p during ER stress may result in down-regulation of metabolism, and in turn, of mRNA translation rates, thus substituting for translational attenuation by phosphorylation of eIF2α by PERK.

Insulin resistance

Insulin stimulates D-glucose uptake, D-glucose metabolism, and protein synthesis after binding to the insulin receptor (IR) (Fig. 7, reviewed in [213–215]). Binding of insulin to the IR induces a conformational change in the IR, activating its protein tyrosine kinase domain, resulting in tyrosine autophosphorylation of the IR and tyrosine phosphorylation of IR substrate (IRS) 1, 2, 3, and 4, and of several SRC homology 2 (SH-2) domain containing (SHC) proteins (Fig. 7). Tyrosine-phosphorylated IRS and SHC proteins are anchoring points for proteins containing SH-2 domains, including recruitment of both SH-2 domains in the regulatory subunits of phosphatidylinositol (PI) 3-kinase (PI3K). Activated PI3K catalyzes the formation of PI-3,4-bisphosphate and PI-3,4,5-trisphosphate and recruitment of phosphoinositide-dependent kinases (PDK) 1 and 2 and several protein kinase B (PKB/AKT) isoforms to the plasma membrane. When co-localized at the plasma membrane, PDKs phosphorylate and activate PKB1, -2, and -3. Activated PKB controls many cellular events, including D-glucose transport, protein and glycogen synthesis, cell proliferation and survival by phosphorylation of numerous substrates. Insulin stimulates protein synthesis *via* activation of the protein serine/threonine kinase mTOR by PKB and PDKs. mTOR phosphorylates the serine-threonine protein kinase p70^{S6K} and an inhibitor of translation initiation, eIF-4E binding protein, to stimulate protein synthesis. Activation of RAS through SHC and IRS1 proteins, RAF and the MAP kinases ERK1 and ERK2 also mediates the proliferative and mitogenic effects of insulin. Inhibition of IRS1 proteins by phosphorylation at serine residues, i.e., Ser312 in human IRS1, causes insulin resistance. Serine phosphorylation of IRS proteins inhibits recruitment of PI3K to IRS proteins [216], inhibits tyrosine phosphorylation of IRS proteins by the IR [217, 218], and promotes degradation of IRS1 [219]. At least two protein kinases have been implicated in serine phosphorylation of IRS proteins, p70^{S6K} [220] and JNK [221–223]. JNK increases IRS1 serine phosphorylation in response to free FAs, stress, inflammation, and the adipokine TNF-α *via* TNFR signaling and activation of JNK [224, 225]. IRS1 serine phosphorylation and insulin resistance caused by ER stress was mediated by activation of JNK by IRE1α [217]. Insulin resistance caused by ER stress contributes to translational and metabolic attenuation, and thus to the protective response to ER stress.

Antioxidant response

A second substrate for the protein kinase activity of PERK is the cap'n'collar bZIP transcription factor NRF2 [119, 120]. Phosphorylation of NRF2 by PERK disrupts a cytosolic complex of NRF2 with the anchor protein KEAP1 [119, 120] and induces translocation of NRF2 to the nucleus. Binding of NRF2 to KEAP1 restricts NRF2 to the cytosol and also promotes its ubiquitination by the E3 ubiquitin ligase Cul3 and its subsequent proteasomal degradation [226]. Whether oxidation of cysteine residues in KEAP1 contributes

to activation of NRF2 in response to ER stress is unclear [227, 228]. NRF2 activates transcription of genes containing an antioxidant response element (ARE) as a heterodimer with other bZIP transcription factors, i.e., ATF4 [195, 196], *c*-JUN [229], JUN-B [229], and JUN-D [229]. Genes activated by NRF2 encode enzymes involved in phase II metabolism of xenobiotics, i.e., the A1 and A2 subunits of glutathione *S*-transferase, NAD(P)H:quinone oxidoreductase, γ-glutamylcysteine synthetase, heme oxygenase 1, and phenolic sulfotransferases, which are localized at the ER membrane [230]. This antioxidant response counteracts increased formation of ROS in ER-stressed cells [50, 51]. ROS interfere with oxidative protein folding by inhibiting ER resident oxidoreductases [231].

ROS may also function as signaling molecules in the UPR. ROS activate several transcription factors, including NF-κB, heat shock transcription factor 1 (HSF1), and NRF2 [228, 232]. ROS also regulate the activity of protein kinases and protein phosphatases. Oxidization of catalytic thiol groups inhibits JNK phosphatases and may activate JNK signaling in the UPR [233] and may contribute to insulin resistance and activation of inflammatory signaling in the UPR. ROS or reactive nitrogen species activate RyR by oxidizing the thiol groups of several cysteine residues [232], which, depending on the level of thiol oxidation, reversibly or irreversibly activates the RyR and Ca^{2+} efflux from the ER, thus increasing ER stress and promoting Ca^{2+} signaling in the UPR. Increased cytosolic Ca^{2+} levels stimulate mitochondrial ROS production, thus generating a positive feedback loop that stimulates ER luminal Ca^{2+} release and ROS production. Through activation of NF-κB, JNK, and ER luminal Ca^{2+} release, ROS may contribute to inflammatory and apoptotic signaling in the UPR.

ER expansion

Secretory cell types, cells subjected to ER stress, or expressing folding-incompetent proteins display an enlarged ER [3]. Formation of a heterodimer of the bZIP transcription factors Opi1p and $Hac1^{i}p$ was proposed to inhibit transcriptional repression of phospholipid biosynthetic genes, i.e., *INO1*, by Opi1p in yeast [73]. In cells with an Opi^{-} phenotype caused by elevated phosphatidic acid levels, induction of *INO1* by inositol starvation was independent of *IRE1* or *HAC1* [234]. Nevertheless, inositol starvation triggered *HAC1* splicing, and *INO1* induction by ER stress was *IRE1* dependent [73]. Furthermore, *ire1*Δ and *hac1*Δ strains are inositol auxotrophs [73], suggesting that Ire1p and Hac1p inhibit Opi1p. Taken together, these observations suggest that Ire1p, $Hac1^{i}p$, or ER stress affect the composition of the ER membrane, i.e., stimulate an increase in its phosphatidic acid content to tether Opi1p to the ER membrane to inactivate Opi1p. Indeed, the phosphatidic acid content of the ER membrane of inositol starved *ire1*Δ and *hac1*Δ cells was decreased [234]. Furthermore, the inositol auxotrophy of *hac1*Δ cells is rescued by overexpression of Scs2p [235]. The simplest explanation for these observations is that ER stress interferes with the enzymatic activity of ER membrane-bound enzymes involved in phospholipid biosynthesis, i.e., phosphocholine cytidylyltransferase [85] and diacylglycerol:cholinephosphotransferase [85]. A heterodimer formed between ATF6 and SREBP2 represses transcription of cholesterogenic genes, including HMG-CoA synthetase, squalene synthetase, and the low-density lipoprotein receptor [204] to counteract ER stress caused by cholesterol accumulation in the ER membrane and to conserve carbon units for gluconeogenesis during glucose starvation [204].

Apoptosis and inflammatory signaling

Initiation of apoptosis

Prolonged or severe ER stress is widely considered to trigger apoptosis. The entire process of apoptosis takes between several hours to days [236]. Initial events leading to activation of executioner caspases are fast and take as little as 10 min to complete [236]. Further, once activated, the actions of the executioner caspases are irreversible, because of proteolysis of essential proteins, such as nuclear lamins, cytoskeletal proteins, and of DNA degradation. Whether a cell lives or initiates apoptotic cell death pathways in response to intracellular damage is controlled by pro- and anti-apoptotic BCL-2 family proteins. Anti-apoptotic family members are BCL-2, BCL-x_L, BCL-w, MCL1, A1/BFL-1, BOO/DIVA, BHRF1, and NR-13. Pro-apoptotic family members can be divided into three classes, multidomain proteins such as BAX, BAK, BOK/MTD, and BCL-x_S, single BCL-2 homology 3 (BH3) domain-only proteins like BIK/NBK, BIM/BOD, BLK, BMF, HRK, BAD, BID, BBC3/PUMA, NOXA, and SPIKE [237, 238], and proteins with a less well conserved BH3 domain, called BNIPs and BNIP-like proteins [239]. Two models have been proposed to explain how and when cells initiate apoptosis in response to intracellular damage (Fig. 8) [237, 238]. In the direct binding model BH3-only proteins bind to and directly activate BAK/BAX [237, 238]. Of all BH3-only proteins only caspase-8-truncated BID (tBID) and BIM have this activity. Both are bound to and inactivated by anti-apoptotic BCL-2 proteins. Activa-

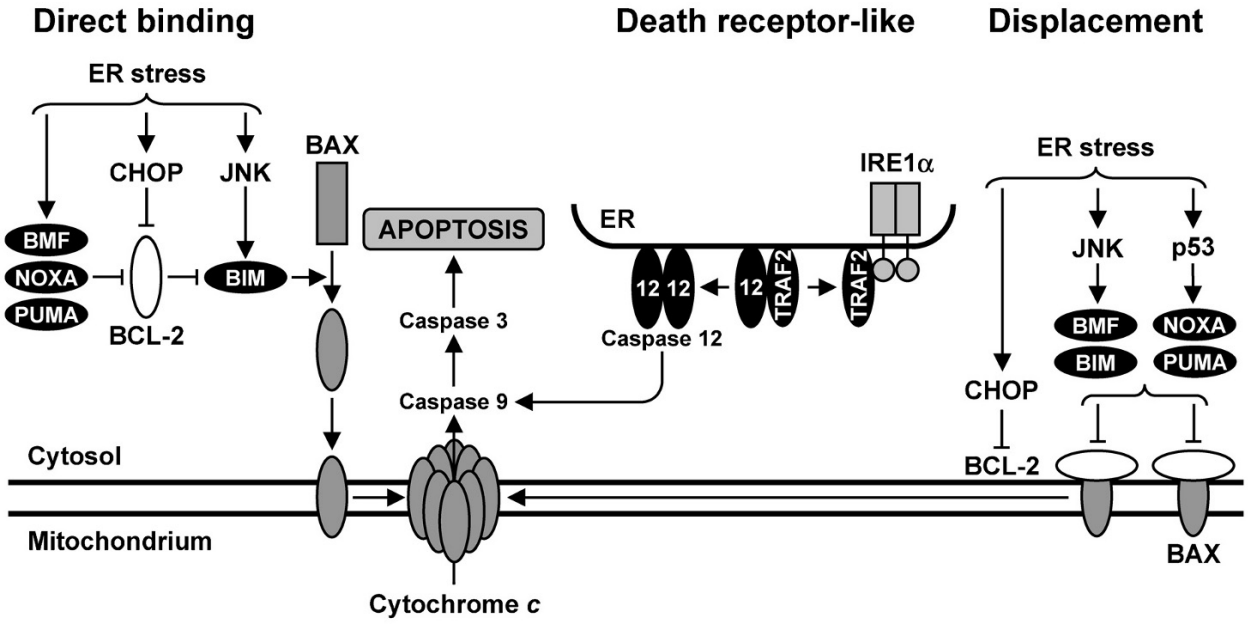

Figure 8. Models describing how the decision between an apoptotic and a protective response to ER stress is made. In the direct binding model (left) [237, 238] BH3-only proteins such as tBID and BIM physically interact with and activate BAK/BAX. BCL-2 exerts its anti-apoptotic function by sequestering and inactivating tBID and BIM. As long as the stoichiometric balance between BCL-2 and BH3-only proteins is maintained, cells do not initiate apoptosis. Intracellular damage, for example ER stress, perturbs the balance between BCL-2 and tBID and BIM in favor of the pro-apoptotic BH3-only proteins by increasing expression of additional BH3-only proteins [244–246], releasing BH3-only proteins from cellular stores such as the cytoskeleton [247, 248], protecting BH3-only proteins from proteasomal degradation by dephosphorylation [244], and inhibiting expression of BCL-2 *via* CHOP [252]. In the displacement model (right) [237, 238] anti-apoptotic BCL-2 proteins such as BCL-2, MCL1, and BCL-x_L bind to and inactivate BAK/BAX at the mitochondrial membrane. BH3-only proteins, activated as described above, sequester anti-apoptotic BCL-2 from BAK/BAX, liberating and allowing BAK/BAX to oligomerize and to form a pore in the mitochondrial membrane. Inhibition of *BCL-2* expression by CHOP and clearance of BCL-2 over time from ER-stressed cells contributes to activation of BAK/BAX in the displacement model. In both the direct binding and displacement model, tipping the balance in favor of the pro-apoptotic BH3-only proteins takes time (cf. text). If in this time window a successful protective response to ER stress is mounted, ER-stress signaling stops, short-lived pro-apoptotic proteins, such as CHOP [250], are cleared from the cell, the balance between anti-apoptotic BCL-2 and pro-apoptotic BH3-only proteins is restored, and the cell survives ER stress. If, on the other hand, the cell fails to mount a protective UPR in this time window, the balance between anti-apoptotic BCL-2 and pro-apoptotic BH3-only proteins is perturbed in favor of the pro-apoptotic BH3-only proteins, and the cell initiates apoptosis. BAK/BAX then oligomerize in the mitochondrial membrane to form a pore through which cytochrome *c* is released into the cytosol, which ultimately activates a caspase cascade that initiates execution of apoptosis. As a consequence, the cell dies irrespective of whether it has or has not mounted a phenotypically protective UPR in the execution phase of apoptosis. In the death receptor-like pathway (middle) [254] phosphorylated IRE1 sequesters TRAF2 from procaspase-12, resulting in oligomerization and activation of this caspase. The function of this pathway may be to overrule the decision-making process in response to intracellular damage described above to trigger apoptosis in response to severe, sudden, or particular forms of ER stress.

tion of other BH3-only proteins by transcriptional induction, post-translational modification, increased turnover and decreased synthesis of anti-apoptotic BCL-2 family members [237, 238], liberates tBID and BIM from their inhibitory interactions with anti-apoptotic BCL-2 proteins and results in activation of BAK/BAX. In the displacement model anti-apoptotic MCL1 and BCL-x_L sequester BAK/BAX at the mitochondria. Activated BH3-only proteins displace BAK/BAX from MCL1 or BCL-x_L [237, 238]. Once activated, multidomain pro-apoptotic BAX and BAK oligomerize and insert themselves at the mitochondrial membrane releasing cytochrome *c* from mitochondria (Fig. 8) [237, 238]. Cytochrome *c*, APAF1, and caspase-9 form the apoptosome that activates caspase-9. Caspase-9 initiates a proteolytic caspase cascade leading to activation of the executioner caspase, caspase-3. Release of DIABLO/SMAC and of HTRA2/OMI from mitochondria inhibits IAPs, which are caspase inhibitors. BAX and BAK also cause release of Ca^{2+} from the ER by oligomerizing and inserting themselves into the ER membrane [240, 241]. Translation of human IAP2 is induced *via* an internal ribosomal entry site [242], and transcription of IAP2 and X-linked IAP is induced by ER stress [243].

ER stress perturbs the balance between pro- and anti-apoptotic Bcl-2 proteins. The BH3-only proteins BIM [244], PUMA [245, 246] and NOXA [246] are transcriptionally induced by ER stress. ER stress-induced dephosphorylation of BIM by protein phosphatase 2A protects BIM from ubiquitination and proteasomal

degradation [244]. Phosphorylation of BIM and BMF by JNKs releases these proteins from dynein and myosin motor complexes associated with the cytoskeleton and augments BAX-mediated apoptosis [247, 248]. All UPR signaling pathways are bifunctional, i.e., they activate protective and apoptotic signaling pathways. How then does a cell make the decision between life and death? If apoptosis is triggered by prolonged or severe ER stress and survival by short periods of ER stress or moderate ER stress, this decision has to be made by molecules that sense ER stress, i.e., ATF6, PERK, and IRE1. There are two possible explanations for how these signaling pathways can make this decision. First, a phase shift from a protective UPR to an apoptotic UPR, i.e., manifested by changes in efferent ATF6, PERK, or IRE1 signaling in response to prolonged or severe ER stress, may make this decision. In this model moderate ER stress selectively activates protective signaling pathways, whereas severe or prolonged ER stress activates apoptotic-signaling pathways. Experimental evidence supports that all stress sensors activate protective and apoptotic signaling pathways at the same time [249, 250]. Separation of these signaling activities or a phase shift from protective to apoptotic, or possibly from protective to protective and apoptotic signaling have not been reported. In the second model, apoptotic signaling activities of the UPR perturb the balance between pro- and anti-apoptotic BCL-2 proteins, but tilting this balance towards apoptosis requires time. Association of IRE1α with BAX and BAK is required for efferent IRE1 signaling, i.e., splicing of *XBP1* mRNA and activation of JNKs [249], but may also provide an additional reservoir for BAK/BAX to tilt the balance between pro- and anti-apoptotic Bcl-2 proteins in favor of the anti-apoptotic Bcl-2 proteins to prolong the time window available to mount an effective protective UPR. ATF6 [251], ATF4 [194, 197], and XBP1 [251] all induce expression of CHOP. CHOP, in turn, represses transcription of *BCL-2* [252]. BCL-2 has a $t_{1/2}$ of ~7 h in HeLa cells [253], but its stability is regulated by post-translational modifications including phosphorylation [253]. Depletion of BCL-2 and subsequent activation of BAX/BAK will take some time (~6–24 h), providing for a time window in which a protective UPR can be mounted. If an effective protective response has been mounted before BCL-2 levels fall below a critical threshold concentration, the UPR is turned off by inactivation of the stress sensors by binding to BiP, CHOP levels decline because of its short $t_{1/2}$ [250], BCL-2 levels recover, and the cell survives the ER stress insult. If an effective UPR has not been mounted before BCL-2 levels fall below the critical threshold level, the cell initiates apoptosis and dies (Fig. 8). A detailed investigation of regulation of the BCL-2 protein interaction network and its modulation by ER stress will be necessary to test if this simple model can explain the decision making process between life and death in response to ER stress.

Activation of apoptosis by the IRE1·TRAF2·ASK1 signaling complex is similar to activation of apoptosis by the death receptor pathway [254], and probably serves to overrule the BCL-2 protein decision-making process when cells experience extreme or possibly particular forms of ER stress, i.e., ER stress caused by viral infections. ASK1 activates JNKs, and phosphorylation of pro-apoptotic BH3-only proteins such as BIM and BMF (Fig. 8) [247, 248]. Second, IRE1 sequesters TRAF2 from ER membrane-bound procaspase-12 [255], resulting in clustering and activation of this caspase at the ER membrane [255] and cytochrome *c* independent execution of apoptosis (Fig. 8) [256]. In humans, caspase-4 may substitute for caspase-12, because humans lack a functional *CASPASE 12* gene [257, 258]. Association of TRAF2 with IRE1 is dependent on a functional protein kinase activity of IRE1 [179], suggesting that TRAF2 specifically interacts with phosphorylated IRE1.

Inflammatory signaling

To repair tissue damage caused by ER stress-induced cell death, the UPR mounts an inflammatory response. Inflammation increases the permeability of microvessels, promotes attachment of circulating cells close to the damaged site, induction of the growth of new tissue and blood vessels, and stimulates apoptosis. Several inflammatory cytokines that stimulate apoptosis (TNF-α), chemotaxis (MCP-1) to the damaged site, and interleukins (IL-2, IL-6, IL-8) are induced by the UPR [180, 259]. Activation of JNK by IRE1α activates the AP-1 transcription factor and expression of inflammatory genes [260], i.e., TNF-α, IL-6, IL-8 and MCP-1 (Fig. 7) [261, 262]. The IRE1α·TRAF2·IKK complex induces degradation of IκBα, activation of NF-κB, and transcription of inflammatory genes such as TNF-α, TNF-β, IL-2, IL-6 and IL-8 [180, 259] and acute phase genes, i.e., serum amyloid A precursor, complement factors B and C4 [259]. PERK-mediated translational attenuation of cap-dependent translation shifts the balance between IκBs and NF-κB towards free NF-κB and contributes to activation of the NF-κB transcription factor [263–266]. Activation of the type II transmembrane transcription factor CREB-H by the proteases S1P and S2P activates expression of acute inflammatory response genes such as serum amyloid P-component and C-reactive protein [267].

Conclusions

In the past few years our understanding of molecular processes and of the physiological role of the UPR has vastly increased. Nevertheless, many important questions remain to be answered. In this review I have attempted to highlight areas that require further research, such as activation of the ER stress sensors, regulation of lipid and sterol biosynthesis by the UPR, and the decision-making process between life and death in response to ER stress. Addressing these open questions promises an interesting and fruitful future for research into the ER and its stress response.

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