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ER-resident co-chaperones in mammalian cells

A critical analysis of experimental data published on

structure, localization, regulation and function

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Abbreviations

19S RP ..... *19S recognition particle*
AAT *α1-antitrypsin*
ACTH ..... *Adrenocorticotropic hormone*
AD ..... *Alzheimer's Disease*
ADAMTS9 *A disintegrin-like and metalloprotease domain with thrombospondin type 1 motifs 9*
ADP ..... *Adenosine diphosphate*
ADPKD ..... *Autosomal dominant polycystic kidney disease*
ADPLD ..... *Autosomal dominant polycystic liver disease*
ApoB ..... *Apolipoprotein B*
Apobec 1 ..... *Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1*
AQP2 ..... *Aquaporin 2*
ARPKD ..... *Autosomal recessive polycystic kidney disease*
ATF4 ..... *Activating Transcription Factor 4*
ATF6 ..... *Activating transcription factor 6*
ATP ..... *Adenosine triphosphate*
Aβ ..... *Amyloid beta protein*
Bak ..... *Bcl-2 homologous antagonist killer*
Bcl-2 ..... *B cell lymphoma 2*
BiP ..... *Binding immunoglobulin protein*
CAML ..... *Calcium-modulating cyclophilin ligand*
CD4 ..... *Cluster of differentiation 4*
CD63 ..... *Cluster of differentiation 63*
CDKAL1 ..... *CDK5 regulatory subunit associated protein 1-like 1*
CFTR ..... *Cystic Fibrosis Transmembrane Conductance Regulator*
CHIP ..... *Carboxy-terminus of Hsc70 interacting protein*
CHOP ..... *C/EBP Homologous Protein*
CK2 ..... *Protein kinase 2*
CT ..... *Cholera toxin*
DGAT1 ..... *Diacylglycerol acyltransferase1*
DHA ..... *Docosahexaenoic acid*
DHFR ..... *Dihydrofolate reductase*
DNA ..... *Deoxyribonucleid acid*
DOG ..... *Deoxyglucose*
DTT ..... *Dithiothreitol*
EDEM ..... *ER degradation-enhancing α-mannosidase-like protein*

EGTA ..... *Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid*
eIF2α ..... *Eukaryotic translation initiation factor 2α*
EMT ..... *Epithelial-mesenchymal-transition*
ENaC ..... *Epithelial sodium channel*
EndoMT ..... *Endothelial-mesenchymal transition*
ER ..... *Endoplasmatisches Retikulum/Endoplasmic reticulum*
ERAD ..... *ER associated degradation*
ERFAD ..... *ER flavoprotein associated with degradation*
Ero1α ..... *Endoplasmic reticulum oxidoreductase 1 α*
ERp57 ..... *ER-resident protein 57*
FENIB ..... *Familial encephalopathy with neuroserpin inclusion bodies*
FGN ..... *Fibrillary glomerulonephritis*
FRET ..... *Fluorescence Resonance Energy Transfer*
G/F rich region ..... *Glycine/phenylalanine rich region*
GFP ..... *Green fluorescent protein*
GPS ..... *G protein-coupled receptor proteolysis site*
GRP170 ..... *Glucose-regulated protein 170*
GRP78 ..... *Glucose-Regulated Protein 78*
GRP94 ..... *Glucose-regulated protein 94*
GST ..... *Glutathione-S-transferase*
HA ..... *Hemagglutinin*
HBV.L ..... *L envelope protein of HBV*
HBV.S ..... *S envelope protein of HBV*
HCC ..... *Hepatocellular carcinoma*
His ..... *Polyhistidine*
Hsp40 ..... *40 kilodalton heat shock protein*
Hsp70 ..... *70 kilodalton heat shock protein*
Hsp72 ..... *Heat shock 70 kDa protein 1*, *Heat shock protein 72*
Hsp73 ..... *Heat shock 70 kDa protein 8*
IDD ..... *Intrinsically disordered domain*
IFALD ..... *Intestinal failure associated liver disease*
IP3R ..... *Inositol 1,4,5-trisphosphate receptors*
IP3R1 ..... *Inositol 1,4,5-trisphosphate receptor 1*
IRE1 α ..... *Inositol-requiring enzyme 1 α*
ITI ..... *Inter-alpha-trypsin inhibitor family proteins*
ITIH4 ..... *Inter-α-trypsin inhibitor heavy chain 4*
IVC ..... *Invariant chain of the human class II major histocompatibility complex*

JDP ..... *J-domain protein*
JHDM1D ..... *Jumonji C domain containing histone demethylase 1 homolog D*
KCl ..... *Potassium chloride*
Keap1 ..... *Kelch-like ECH-associated protein 1*
LAMP-3 ..... *Lysosomal-associated membrane protein 3*
LDLR ..... *Low-densitiy lipoprotein receptor*
LOH ..... *Loss of heterozygosity*
MAM ..... *Mitochondria-associated membranes*
MC2 ..... *Melanocortin 2*
MEndoT ..... *Mesenchymal-endothelial transition*
MET ..... *Mesenchymal-epithelial transition*
MHV.M ..... *M envelope protein of the mouse hepatitis coronavirus*
MRAP ..... *Melanocortin 2 receptor accessory protein*
MSI ..... *Microsatellite instabilities*
mTORC2 ..... *Mechanistic target of rapamycin complex 2*
NBD ..... *Nucleotide binding domain*
NEF ..... *Nucleotide exchange factor*
NGF ..... *Nerve growth factor*
Nrf2 ..... *Nuclear factor erythroid 2-related factor 2*
OS-9 ..... *Osteosarcoma amplified 9 protein*
OST ..... *Oligosaccharyltransferase complex*
OxPAPC ..... *Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine*
OxPAPS ..... *Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoserine*
PC1 ..... *Polycystin 1*
PDI ..... *Protein disulfilde-isomerase*
PERK ..... *Protein kinase RNA-like ER kinase*
PKD1 ..... *Polycystic kidney disease 1*
PKD2 ..... *Polycystic kidney disease 2*
PLD ..... *Polycystic liver disease*
PLSC ..... *Periodontal ligament stem cells*
PPA ..... *Preproapelin*
ppcecA ..... *Preprocecropin A*
PPL ..... *Preprolactin*
PPS ..... *Prestatherin*
PpαF ..... *Prepro-α-factor*
PRKCSH ..... *Protein Kinase C Substrate 80K-H*
PrP ..... *Prion Protein*

Ptc2 ..... *Serine/threonine phosphatase of type 2C*
RAMP ..... *Ribosome associated membrane proteins*
RIDD ..... *Regulated IRE1-dependent decay of mRNA*
RNA ..... *Ribonucleid acid*
ROS ..... *Reactive oxygen species*
SBD ..... *Substrate binding domain*
SDF2 ..... *Stromal cell derived factor 2*
SDF2L1 ..... *Stromal cell derived factor 2 like 1*
SDS-PAGE ..... *Sodium dodecyl sulfate polyacrylamide gel electrophoresis*
SERCA ..... *Sarcoplasmic/endoplasmic reticulum calcium ATPase*
Sig-1R ..... *Sigma 1 receptor*
siRNA ..... *Small interfering RNA*
SPR ..... *Surface Plasmon Resonance*
SR ..... *Signal recognition particle receptor*
SRP ..... *Signal recognition particle*
SubAB ..... *Subtilase cytotoxin*
SV40 ..... *Simian virus 40*
sXBP1 ..... *Spliced XBP1*
TA ..... *Tail-anchored*
TRC 40 ..... *Transmembrane domain recognition complex*
Trx ..... *Thioredoxin*
TTR ..... *Transthyretin*
UDP ..... *Uridine 5'-diphosphate*
UDP-GT ..... *Uridine 5'-diphospho-glucuronosyltransferase*
UGGT ..... *UDP-glucose:glycoprotein glucosyltransferase*
UPR ..... *Unfolded Protein Response*
uXBP1 ..... *Unspliced XBP1*
VP1 ..... *SV 40 viral particle*
WRB ..... *Tryptophan-rich basic protein*
XBP1 ..... *X-Box binding protein 1*
ZAAT ..... *Z variant of α1-antitrypsin*
ZEB1 ..... *Zinc-finger E-box–binding homeobox 1*
β-GCase ..... β-glucocerebrosidase

1. Zusammenfassung

Das endoplasmatische Retikulum (ER) spielt eine wichtige Rolle bei vielen zellulären Prozessen. Zum einen stellt das ER einen wichtigen Kalzium-Speicher innerhalb der Zelle dar. Zum anderen werden im ER von Säugerzellen Steroide und Lipide synthetisiert. Außerdem werden im ER Transmembranproteine und sekretorische Proteinen, die etwa 30% der gesamten Proteinmenge der Zelle ausmachen, gefaltet. Das Sec61 Translokon ist der Kanal in der ER Membran, durch welchen Proteine in das ER gelangen. Es konnte gezeigt werden, dass das Sec61 Translokon ebenfalls als Kalzium-Kanal in der ER Membran fungiert. Neu synthetisierte Proteine werden im ER von Chaperonen gefaltet und in ihre endgültige Konformation gebracht. Können Proteine beispielsweise aufgrund einer gestörten zellulären Kalzium-Homöostase, einem Mangel an Nährstoffen, Hypoxie oder Mutationen in proteinkodierenden Genen nicht richtig gefaltet werden, kommt es zu einer Anhäufung falsch- oder ungefalteter Proteine im ER und es entsteht ER-Stress. In Folge kommt es zu einer zellulären Stressantwort, der „Unfolded Protein Response“ (UPR). Im Rahmen der Unfolded Protein Response werden zunächst vermehrt Chaperone produziert, um eine Faltung der im ER angesammelten Proteine zu ermöglichen. Um die Proteinmenge im ER zu reduzieren wird zudem die Protein-Translation herunterreguliert und ungefaltete oder falsch-gefaltete Proteine werden aus dem ER in das Zytosol transportiert, wo die Proteine durch das Proteasom abgebaut werden. Wird der ER-Stress durch diese Mechanismen nicht bewältigt, leitet die Zelle die Apoptose ein.

Eine wichtiges Chaperon im ER ist das Binding immunoglobulin protein/Glucose-Regulated Protein 78 (BiP/GRP78), ein Chaperon der Hitzeschockprotein 70 (Hsp70) Familie. Neben seiner Lokalisation im ER, wurde BiP auch an der ER-Membran, im Zytosol sowie an der Plasmamembran von Zellen und im Extrazellularraum gefunden. Proteine der Hsp70 Familie binden an hydrophobe Domänen von ungefalteten oder falsch gefalteten Proteinen und können so verhindern, dass diese Proteine Aggregate bilden. Außerdem helfen Proteine der Hsp70 Familie als Chaperone dabei, die gebundenen (Substrat-) Proteine unter Adenosintriphosphat (ATP) -Verbrauch zu falten. Hierfür besitzen die Hsp70 Proteine eine ATPase Domäne. Im ATP-gebundenen Zustand binden Hsp70 Proteine schwächer an ihre jeweiligen Substrate und es kommt zu höheren Substratassoziations- und -dissoziationsraten. Wird ATP zu Adenosindiphosphat (ADP) gespalten, kommt es zu einer Konformationsänderung im Hsp70 Protein, wodurch eine stärkere Substratbindung erreicht wird.

Eine wichtige Klasse an Proteinen, die die ATPase Aktivität von Hsp70 Proteinen steigern können, sind Proteine der Hitzeschock 40 (Hsp40) Familie. Die Proteine der Hsp40 Proteinfamilie besitzen eine konservierte J-Domäne, mit der sie an die Hsp70 Proteine binden können. Die Hsp40 Proteine werden auch als Co-Chaperone bezeichnet.

Derzeit sind acht Mitglieder (ERdj1 bis ERdj8) der Hsp40 Familie bekannt, die im ER von Säugerzellen lokalisiert sind. ERdj1 bis ERdj7 können an BiP binden und dadurch die ATPase-Aktivität von BiP stimulieren. Für ERdj8 wurde dies bisher noch nicht experimentell untersucht. Außerdem werden für alle acht Co-Chaperone zahlreiche weitere Funktionen im ER postuliert. So regulieren die Co-Chaperone ERdj1, ERdj2 und ERdj6 die Translation und ERdj2 kann den Import von einigen Proteinen ins ER regulieren. Die Co-Chaperone ERdj3, ERdj4, ERdj5 und ERdj6 spielen zudem eine Rolle beim Transport von ungefalteten oder falsch gefalteten Proteinen aus dem ER ins Zytosol und beeinflussen auch den nachfolgenden Abbau der Proteine im Zytosol. Die Co-Chaperone ERdj1, ERdj3, ERdj5 und ERdj6 haben wichtige Funktionen in Hinblick auf den Kalzium-Haushalt der Zelle.

Während ERdj3, ERdj4, ERdj5 und ERdj6 durch ER-stress hochreguliert werden, ist dies für ERdj1 und ERdj2 nicht der Fall. Ob ERdj7 oder ERdj8 im Rahmen von ER-Stress hochreguliert werden, wurde bisher nicht untersucht. Für ERdj2, ERdj4, ERdj5 und ERdj6 liegen experimentelle Daten vor, welche zeigen, dass die Co-Chaperone die Unfolded Protein Response unter normalen Bedingungen unterdrücken und nach Phasen von ER-stress wieder herunterregulieren können. Aufgrund der zahlreichen zentralen Funktion der Co-Chaperone steht ihre Dysfunktion oder eine Dysregulation im Zusammenhang mit verschiedenen Krankheitsbildern. So sind Mutationen im ERdj2 kodierenden Gen mit der Entwicklung polyzystischer Lebererkrankung assoziiert, während ERdj4 aktuell bereits als Biomarker für die fibrilläre Glomerulonephritis verwendet wird.

Da ERdj8 erst im Jahr 2020 beschrieben wurde, gibt es zu diesem Co-Chaperon derzeit nur wenige Daten. Auch in Hinblick auf das Co-Chaperon ERdj7 gibt es verhältnismäßig wenige Daten. Es gibt jedoch zahlreiche Daten zu Struktur, Lokalisation, Funktion und Regulation der Co-Chaperone ERdj1-ERdj6. Bisher fehlte es an einer vollständigen und kritisch evaluierten Zusammenfassung aller publizierten Experimente zu ERdj1-ERdj8. Dieser Umstand war die Motivation für die vorliegende Doktorarbeit. Im Rahmen der vorliegenden Arbeit wurde eine systematische Literaturrecherche in PubMed durchgeführt. Es wurde lediglich ein Sprachfilter angewandt, der die angezeigten Publikationen auf Publikationen in deutscher oder englischer Sprache begrenzte. Die angezeigten Publikationen wurden dann in einem ersten Schritt auf Titel und Abstract gescreent. Übersichtsarbeiten und Publikationen, die sich nicht auf Säugerzellen oder Säugetiere bezogen, wurden von vornherein aus der Sammlung ausgeschlossen. Nach dem „Titel- und Abstract Screening“ erfolgte für alle Orginalpublikationen ein Volltext-Screening. In den so selektierten Orginalpublikationen wurden alle gezeigten Experimente hinsichtlich ihres experimentellen „Designs“, der verwendeten Kontrollen, des erzielten Ergebnisses und ihrer Aussagekraft analysiert. Nach Studium aller zu einem Protein gehörigen Publikationen,

wurden die Ergebnisse in Hinblick auf die gezeigten experimentellen Daten vergleichend betrachtet, diskutiert und eigene Interpretationen der Experimente sowie hypothetische Modelle erarbeitet. Besonderer Fokus wurde bei der Literaturarbeit auf Daten zu der subzellulären Lokalisation und Topologie der acht Co-Chaperone, ihrer Funktion in Bezug auf Translation, Translokation und Degradation von Proteinen sowie ihrer Funktion in Bezug auf den zellulären Kalzium Haushalt gelegt. Große Teile der im Rahmen dieser Dissertation erhobenen Daten und Schlussfolgerungen wurden in einer Originalarbeit und vier Übersichtsarbeiten veröffentlicht.

2. Abstract

The ER (Endoplasmic reticulum) of mammalian cells is an important intracellular calcium storage and the place where lipids are generated. Furthermore, the maturation and folding of transmembrane proteins as well as secretory proteins, which constitute around one third of all synthesized proteins within the cell, takes place within the ER. The Sec61 translocon constitutes the main channel for protein translocation across the ER membrane. Upon translocation into the ER lumen, proteins associate with chaperones that assist their substrate proteins in reaching their final conformation. Under conditions of nutrient deficiency, hypoxia or disturbed calcium homeostasis, protein folding and maturation can be impaired. ER-stress develops as a consequence of the increased burden of ER luminal unfolded or misfolded proteins. The cellular response to ER-stress is called the Unfolded Protein Response (UPR). During the UPR, general protein translation is downregulated and transcription and translation of chaperones is upregulated in order to maintain ER luminal proteostasis. Terminally misfolded proteins are retrotranslocated to the cytosol for proteasomal degradation. If these mechanisms are insufficient to restore ER homeostasis, apoptosis is initiated by the cell.

An important ER-resident chaperone is the Hsp70 family member BiP/GRP78. HSP70 chaperones bind to hydrophobic domains of unfolded or misfolded proteins, promote folding and prevent aggregation of their substrates. In order to promote folding of substrate proteins, an interaction of Hsp70 chaperones with Hsp40 co-chaperones is necessary. Hsp40 co-chaperones possess a conserved J-domain. The J-domain binds to Hsp70 chaperones and stimulates the ATPase activity of the Hsp70 chaperones. Currently, eight members of the Hsp40 family (ERdj1 to ERdj8) are known to be localized within the ER of mammalian cells. ERdj1 to ERdj7 have been shown to bind to BiP and stimulate the ATPase activity of BiP. As ERdj8 has only recently been discovered in 2020 there is only little data on the protein and there is no information on a possible interaction with BiP so far. Apart from their function as co-chaperones of BiP, ERdj1, ERdj2 and ERdj6 were shown to regulate protein translation

and in the case of ERdj2 it was shown that the co-chaperone also regulates protein translocation. For ERdj3, ERdj4, ERdj5 and ERdj6 a role in degradation of terminally misfolded proteins has been proposed. Furthermore, ERdj1, ERdj3, ERdj5 and ERdj6 are involved in the regulation of cellular calcium homeostasis. Due to their various central functions within the cell, dysfunction of the ERdj co-chaperones can result in different diseases. For example, mutations in the ERdj2 protein have been associated with Polycystic liver disease (PLD), while ERdj4 is a known biomarker for fibrillary glomerulonephritis (FGN).

With regard to ERdj7 and ERdj8, only little data exists as compared with ERdj1 to ERdj6. For these co-chaperones there is a multitude of published experimental data on structure, localization, function and regulation. Also, there is a large amount on publications on diseases associated with the co-chaperones. Until now, there has not been a complete and critical summary and analysis of all published experimental data regarding ERdj1 to ERdj8. This was the motivation for this thesis. In order to create a complete and critical analysis of all published data on ERdj1 to ERdj8, a systematic literature search was done on PubMed. The published experimental results were carefully analysed with regard to their experimental design, the controls that were applied and their significance. Afterwards, the published experimental data was compared with special focus on topology and localization of the co-chaperones, their functions regarding protein translation, translocation, degradation and cellular calcium homeostasis. Most results of this thesis have already been published in four reviews and one original paper.

Originalarbeit:

Lea Daverkausen-Fischer, Myriam Motyl-Eisemann, Margarethe Draga, Martin Scaal, Felicitas Pröls (2020) Protein expression pattern of the molecular chaperone Mdg1/ERdj4 during embryonic development. Histochem Cell Biol.154(3):255-263. doi: 10.1007/s00418-020-01881-x.

Übersichtsarbeiten:

Lea Daverkausen-Fischer and Felicitas Pröls

Dual topology of co-chaperones at the membrane of the endoplasmic reticulum. Cell Death Discov. (2021) 7(1):203. doi: 10.1038/s41420-021-00594-x.

Lea Daverkausen-Fischer and Felicitas Pröls

The Function of the Co-chaperone ERdj4 in Diverse (Patho-)Physiological Conditions. Cell Mol Life Sci. (2021) 79(1):9. doi: 10.1007/s00018-021-04082-4.

Lea Daverkausen-Fischer, Margarethe Draga and Felicitas Pröls
Regulation of Translation, Translocation, and Degradation of Proteins at the Membrane of the Endoplasmic Reticulum. International Journal Molecular Sciences (2022) 23(10):5576. doi: 10.3390/ijms23105576

Lea Daverkausen-Fischer and Felicitas Pröls
Regulation of calcium homeostasis and flux between the endoplasmic reticulum and the cytosol. Journal Biological Chemistry (2022) 298(7):102061. doi: 10.1016/j.jbc.2022.102061

3. Introduction

3.1. Protein transport into the ER

Parts of this section “Protein transport into the ER” are published in

- Daverkausen-Fischer L, Draga M, Pröls F.
  Regulation of Translation, Translocation, and Degradation of Proteins at the Membrane of the Endoplasmic Reticulum. International Journal Molecular Sciences (2022) 23(10):5576. doi: 10.3390/ijms23105576

- Daverkausen-Fischer L, Pröls F.
  Regulation of calcium homeostasis and flux between the endoplasmic reticulum and the cytosol. Journal Biological Chemistry (2022) 298(7):102061. doi: 10.1016/j.jbc.2022.102061

In order to enter the ER, substrate proteins have to be targeted to the ER membrane for translocation. Import into the mammalian ER is mediated via the Sec61 translocon that is comprised of the Sec61α, Sec61β and Sec61γ subunits [1]. The Sec61α subunit is the largest subunit, containing ten transmembrane domains [2]. The Sec61β and Sec61γ subunits span the ER membrane only once [2]. Inside the ER, the Sec61 translocon forms a plug-like structure that keeps the translocon in a closed state when no translocation is in progress and opens the translocon upon initiation of translocation [3-5]. The Sec61 translocon also possesses a lateral gate that engages with signal peptides or transmembrane domains of proteins at the beginning of the translocation process [3,5]. The Sec61 translocon can associate with various accessory factors to mediate import of proteins as well as to enable post-translational modifications such as signal peptide cleavage or glycosylation [6]. Apart from mediating protein import into the ER, the Sec61 translocon has been associated with retrograde transport of proteins, viral particles and bacterial toxins from the ER into the cytosol of mammalian cells [7,8]. Furthermore, it was shown that the Sec61 translocon constitutes a calcium channel within the ER membrane that becomes leaky for calcium at the end of the protein translocation phase [9].

Two different mechanisms of translocation into the ER via the Sec61 translocon are known, co- and post-translational translocation [10]. In eukaryotes most proteins are translocated co-translationally [11]. These proteins contain an N-terminal signal peptide - which is a short hydrophobic amino acid stretch – or a signal anchor which serves as a membrane anchor after the protein has been targeted to the ER membrane [11]. The signal peptide or

signal anchor is recognized by the signal recognition particle (SRP) in the cytosol [11]. The ribosome nascent chain complex is then targeted to the Sec61 translocon at the ER membrane and recognised by the signal recognition particle receptor (SR) [10,11]. The signal peptide or signal anchor is inserted into the Sec61 translocon and translocation can be initiated [10,12].

Apart from co-translational translocation, proteins can also be translocated post-translationally [13]. This mode of translocation is mainly used by small presecretory proteins that are too short to engage with the SRP before translation is finished [13]. Furthermore tail-anchored (TA) proteins, which – as described in mammals - contain their hydrophobic membrane anchor at the C-terminus are known to be translocated in a post-translational way [13]. It is estimated that over 200 mammalian proteins are translocated in a post-translational mode [13]. Targeting of post-translationally translocated proteins to the ER membrane can be mediated by different factors. Firstly, some TA-proteins can be targeted to the ER membrane by the SRP after translation has been completed [14]. Secondly, the proteins can be targeted to the ER membrane by a cytosolic transmembrane domain recognition complex (TRC40) [13]. This complex recruits its target proteins to a receptor at the ER membrane, which is composed of tryptophan-rich basic protein (WRB) and calcium-modulating cyclophilin ligand (CAML) [13]. To prevent the aggregation of the proteins in the cytosol, cytosolic Hsp70 and Hsp40 proteins bind to and shield the aggregation prone sites of the newly synthesized proteins while they are directed to the ER membrane for translocation [13].

3.2. Protein folding within the ER

3.2.1. Role of BiP in controlling cellular homeostasis

After a protein has successfully entered the ER, it has to acquire its final tertiary structure, a process that is assisted by ER luminal chaperones [15]. One important ER luminal chaperone is BiP, a member of the Hsp70 family [16,17]. BiP assists in folding of client proteins, a process that requires ATP [15]. BiP contains a substrate binding domain (SBD) and a nucleotide binding domain (NBD) [15,18]. The SBD is composed of a β sandwich and a helical structure that acts as a lid which can close the binding pocket of the SBD after a substrate has bound [19]. A change in the affinity of BiP for substrate proteins is mediated by conformational changes following ATP hydrolysis [15]. When BiP is bound to ATP, BiP has low affinity for substrate proteins while in the ADP bound state affinity for substrates is high [15]. As soon as folding of a substrate protein has been completed, the substrate protein is actively released from BiP [20]. The nucleotide exchange factor (NEF) mediates exchange of ADP for ATP to enable reiterative rounds of chaperoning [15] (see Figure 1).

Within the ER, BiP is not only involved in protein folding but also protein degradation, ER-stress signalling and calcium homeostasis [21]. With regard to calcium homeostasis, it was shown that BiP functions as a calcium binding protein within the ER and is essential for the maintenance of calcium homeostasis [22]. Furthermore, BiP controls calcium efflux from the ER by gating the Sec61 translocon and keeping it in a closed state [23]. Also, BiP was shown to control calcium efflux from the ER via Inositol 1,4,5-trisphosphate receptors (IP3R)[24,25]. IP3Rs are ER resident calcium channels that mediate calcium efflux from the ER. Three subtypes of IP3Rs, IP3R1, IP3R2 and IP3R3, have been identified. There are differences in the activation, the regulation as well as the sensitivity to binding agents between the three subtypes [26]. In HeLa cells, BiP was shown to bind to the IP3R1, thereby stimulating calcium efflux via promotion of IP3R1 tetramer formation [24] (see Figure 1a). The interaction between BiP and IP3R1 depends on a functional ATPase activity of BiP as BiP mutants that are able to bind to ATP but unable to change their conformation upon ATP binding do not promote calcium release from the ER via IP3R1 [24]. Upon treatment of HeLa cells with inductors of ER-stress (tunicamycin, thapsigargin and dithiothreitol (DTT)), less BiP is shown to interact with IP3R1 [24]. The effect was more pronounced upon treatment of cells with tunicamycin and DTT than upon treatment with thapsigargin [24]. Since thapsigargin specifically inhibits calcium import into the ER, these results suggest that upon ER-stress calcium release from the ER might be impaired due to reduced ER luminal calcium levels [24].

BiP also regulates calcium efflux via IP3R3 [25]. At mitochondria-associated membranes (MAMs) BiP was found to interact with the chaperone protein sigma 1 receptor (Sig-1R) [25] (see Figure 1b). The mammalian Sig-1R is an integral protein of the ER membrane and was

found to be enriched in MAMs where it regulates calcium efflux from the ER via IP3R3 [25,27]. Upon ER luminal calcium depletion, which can either be achieved by ATP-induced activation of the IP3R or by application of thapsigargin, BiP is released from Sig-1R and forms a complex with IP3R3 channels [25]. Association of Sig-1R with IP3R3 stabilizes the calcium channels and prevents their degradation [25].

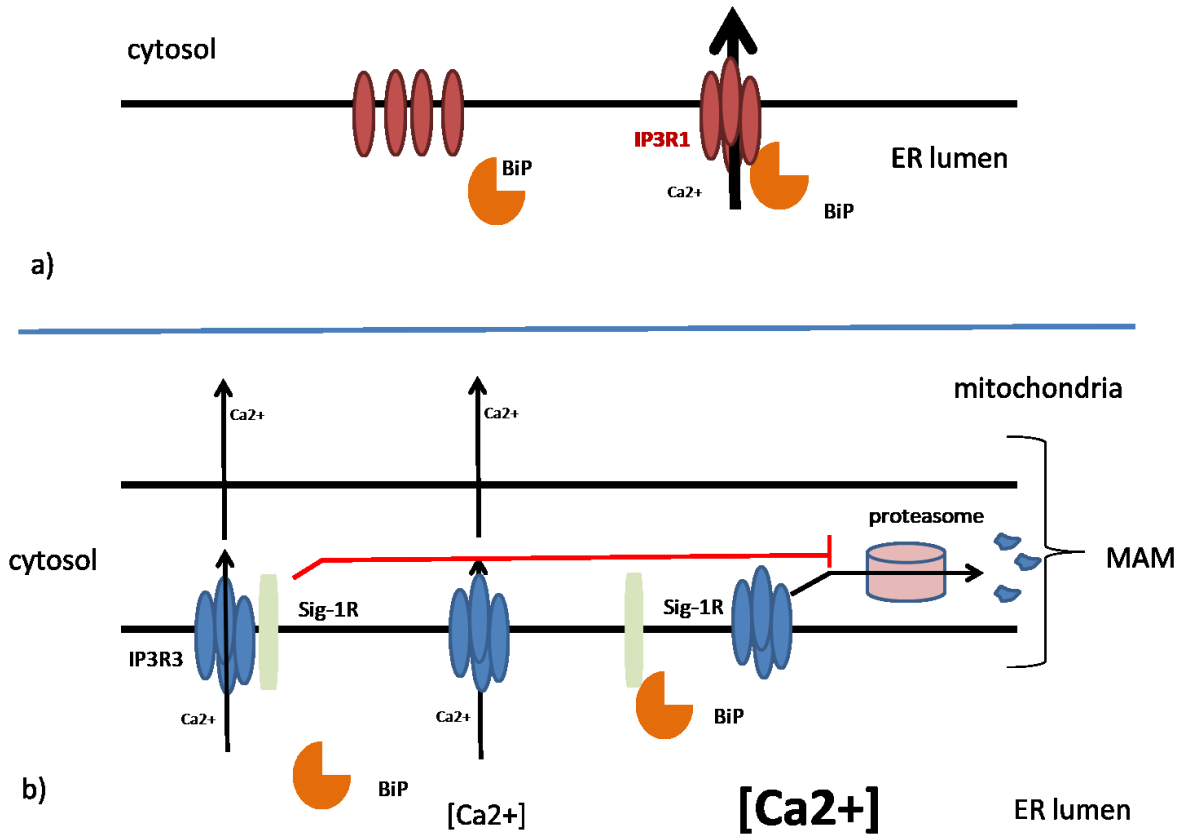

Fig. 1 BiP regulates calcium homeostasis via IP3R1 and IP3R3 by two different mechanisms.
a) BiP can specifically bind to IP3R1 and stimulates tetramer formation [24].
b) Upon normal luminal calcium concentrations BiP is bound to Sig-1R at MAMs. Calcium depletion from the ER causes BiP to dissociate from Sig-1R, which subsequently binds to IP3R3. Association of IP3R3 with Sig-1R stabilizes the IP3R3 channels and prevents their degradation [25].

Apart from its ER luminal location, a subpopulation of BiP is present as an ER-transmembrane protein [28]. An additional BiP pool, described in mouse and human cell lines, is located in the cytosol and emerges due to alternative splicing [29,30]. The cytosolic isoform of BiP lacks the signal peptide and its expression was shown to be enhanced under ER-stress conditions [30]. Either in its cytosolic form or as an ER transmembrane protein, BiP suppresses ER-stress induced apoptosis by complex formation with caspase 7 and caspase 12 [28,29]. The complexing of BiP with caspase 7 was shown to depend on a functional ATPase domain of BiP [28].

The cytosolic isoform of BiP can also stimulate PERK signaling [30]. In HeLa cells, stable transfection with the cytosolic isoform of BiP resulted in increased phosphorylation of PERK [30]. Upon ER-stress, higher amounts of phosphorylated eukaryotic translation initiation factor 2α (eIF2α) and higher levels of Activating Transcription Factor 4 (ATF4) were detected in HeLa cells transfected with the cytosolic BiP isoform, [30]. It was further found that the cytosolic isoform of BiP can bind to the cytosolic isoform of ERdj6, which was shown to inhibit PERK activity via the cytosolic domain of PERK [30,31].

In 1997, a "highly homologous protein to BiP" was localized at the cell surface of lymphoma cells of malignant cutaneous T cell lymphoma [32]. This "highly homologous protein" proved to be BiP and, in human rhabdomyosarcoma cells, the location of BiP to the cell surface was shown to be induced by thapsigargin [33]. Also, in NG108-15 glioblastoma cells, cell surface expression of BiP was reported [34]. In NG108-15 cells, translocation of BiP to the plasma membrane could be suppressed by treatment with brefeldin A, indicating that translocation of BiP is mediated through the Golgi apparatus [34]. In liver and pancreatic cell lines, cell surface localization of BiP was also reported to be Golgi-dependent [35]. Golgi dependent transport of BiP to the plasma membrane depends on the activation of the tyrosinkinase SRC, which is located at the Golgi membrane [36]. Activation of SRC results in dispersion of KDEL receptors from the Golgi [37]. KDEL-receptors are required for binding to proteins carrying a KDEL sequence that have translocated from the ER to the Golgi [38]. Upon binding of these KDEL-tagged proteins to the KDEL-receptors, the proteins are transported back to the ER [38]. This mechanism results in ER retention of KDEL-tagged proteins[38]. In HeLa cells it was shown that binding of IRE1α to SRC results in KDEL receptor dispersion in the Golgi apparatus, which enables BiP to escape ER retention [36]. Recently, it was described in colon cancer and lung cancer cell lines that BiP can also translocate to the cell surface in a Golgi-independent mechanism utilizing endosomal transport [35]. As a cell surface protein, BiP plays an important role in pro-survival as well as pro-apoptotic signalling [21]. Furthermore, a role as a receptor for viral entry has been proposed [21].

Apart from its localization within the ER lumen, at the ER- and plasma membrane and in the cytosol, BiP is also secreted into the extracellular space [39,40]. Solid tumor cell lines (PC-3 and HRT-18) were shown to secrete high amounts of BiP into the extracellular space where BiP was shown to stimulate pro-survival signalling [39]. In pancreatic beta cells, secreted BiP was shown to stimulate pro-apoptotic signalling by binding cell surface localized BiP [41]. In colon cancer cells dimerization of secreted BiP and cell surface BiP was shown to stimulate cell proliferation [42]. Moreover, the extracellular BiP pool plays a role in the differentiation of bone marrow mesenchymal stem cells, cancer cell proliferation, cytoskeleton remodelling

and angiogenesis [43]. In colon cancer cells, the amount of secreted BiP is controlled by its acetylation status [40].

3.2.2. Interaction between Hsp70 and J-domain proteins

Like all members of the Hsp70 family, the ATPase activity of BiP is stimulated by a group of proteins, the J-domain proteins (JDPs) [15,44] (see Figure 2). Hydrolysis of ATP to ADP enhances the affinity of Hsp70 proteins for substrates [15]. Binding to Hsp70 and stimulation of the ATPase activity is mediated by a highly conserved tripeptide within the J-domain, which is also called the HPD motif due to its amino acid sequence (histidine (H), proline (P) and aspartic acid (D))[45,46]. The J-domain is a highly-conserved region of around 70 amino acids, present in JDPs from yeast, bacteria and eukaryotes [47]. As JDPs are no client proteins of Hsp70 themselves but stimulate the function of Hsp70 chaperones, these proteins are also termed co-chaperones [48]. During interaction with Hsp70 chaperones, the J-domain of the JDPs was found to interact with the substrate binding domain as well as the nucleotide binding domain of Hsp70 chaperones [44].

JDPs can be classified into type I, type II and type III family members, dependent on specific structural features. Type I JDPs contain a J-domain, a glycine/phenylalanine rich region (G/F region) as well as a zinc finger domain. Type II JDPs lack the zinc finger domain but possess the J-domain as well as the G/F region. Type III JDPs only possess the J-domain but lack a G/F region and the zinc finger region. It was proposed that apart from the J-domain, the G/F region of type I and II family members is also important for binding of JDPs to Hsp70 chaperones [49]. Also, experimental data point to a role of the G/F rich region in affecting substrate binding specificity of Hsp70 chaperones [44].

Within the ER lumen, there is a specific set of JDPs, the so called ERdj proteins. These ERdj proteins act as co-chaperones for BiP [15]. Eight members of these ERdj proteins, termed ERdj1 – ERdj8, have been characterized by now [50-57]. For ERdj1-ERdj7 it was shown that the co-chaperones stimulate ATPase activity of BiP thereby contributing to proper protein folding within the ER [56,58-63]. With regard to ERdj8, stimulation of ATPase domain of BiP has not been shown so far.

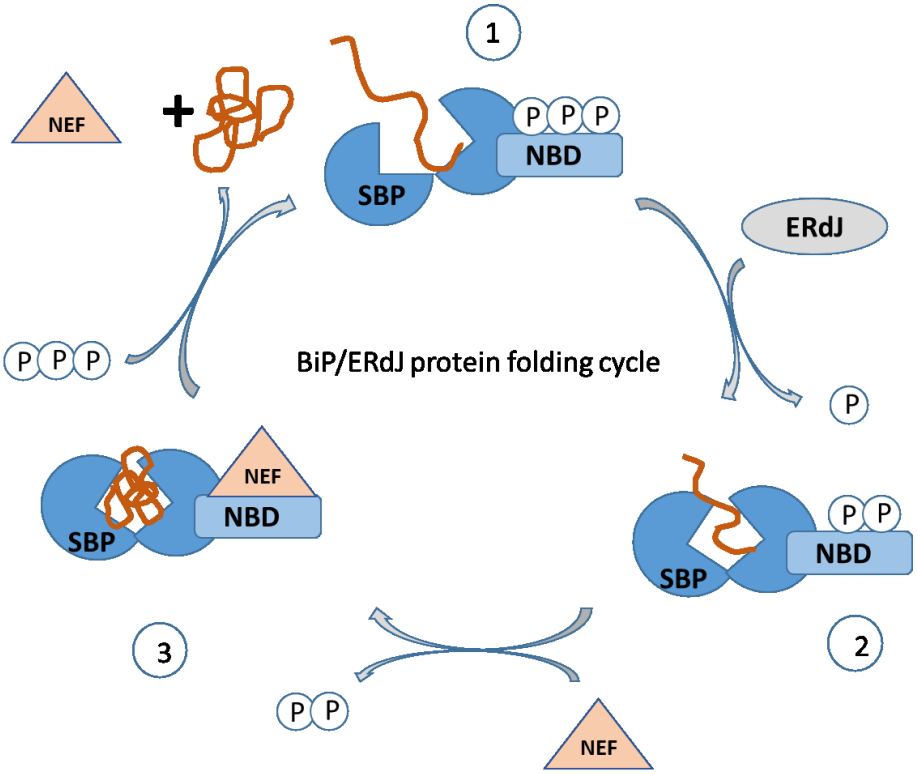

Fig. 2 Folding of substrate proteins mediated by BiP in cooperation with co-chaperones
(1) BiP has low affinity for substrate proteins in its ATP-bound state[15]. (2) Binding of ERdj proteins to BiP stimulates ATP hydrolysis to ADP [56,58-63]. BiP has increased affinity for substrate proteins in its ADP-bound state [15]. After completion of the folding process, the nucleotide exchange factor (NEF) exchanges ADP for ATP which results in the dissociation of the substrate protein (3) [15]. The figure has been modified from Daverkausen-Fischer L, Prols F. Regulation of calcium homeostasis and flux between the endoplasmic reticulum and the cytosol. *J Biol Chem* 2022: 102061[64].

3.3. Degradation of misfolded proteins via the ERAD and cotranslocational degradation pathways

Terminally misfolded proteins that accumulate in the ER lumen have to be degraded. One important degradation pathway is the ER associated degradation pathway (ERAD) [65]. Misfolded proteins are recognized and targeted to a retrotranslocon channel [65]. After retrotranslocation into the cytosol, the misfolded protein is ubiquitinated and targeted to the proteasome for degradation [65]. There is also a continuous ERAD-mediated turnover of a small amount of misfolded or unfolded wildtype proteins **[66].**

Apart from ERAD, another degradation pathway exists. During co-translocational degradation proteins, whose translocation is in progress, are pulled out of the Sec61 channel at the onset of ER-stress [67]. Co-translocational degradation is supposed to lower the protein burden within the ER and, by clearing the translocons of translocating substrates, it enables translocation of proteins, which are upregulated during ER-stress in order to restore homeostasis in the ER lumen [67,68]. In the following, mechanisms and proteins of the ERAD pathway will be explained while the role of co-chaperones involved in co-translocational degradation will be discussed later on.

In order to prime proteins for ERAD, substrate proteins have to be recognized as terminally misfolded [65]. Many proteins are co- or posttranslationally modified by addition of a pre-formed lipid-linked oligosaccharide consisting of nine mannose and three glucose moieties [69]. This modification, termed N-glycosylation, is achieved by the oligosaccharyltransferase complex (OST), which is associated with the ER [70]. As glycosylation of proteins takes place in the lumen of the ER, the achieved glycosylation pattern points to successful translocation of substrate proteins across the ER membrane. After transfer of the glycan to the newly synthesized protein, the outermost and the penultimate glucose residues are trimmed by glucosidase I and glucosidase II respectively [71]. The resulting mono-glycosylated glycan can then be bound by two lectin-like chaperones of the ER, calreticulin and calnexin [71]. While calnexin is a type I ER membrane protein, calreticulin is an ER luminal protein [72,73]. The two chaperones are involved in folding of glycosylated proteins and can transiently associate with the mono-glycosylated glycan of these proteins [74]. After the ultimate glucose redidue has been trimmed by glucosidase II and if the protein has achieved its final structure, the substrate protein can escape the chaperoning cycle and travel to the Golgi [71]. If the protein is still misfolded, a glucose residue is transferred to the glycan by the Uridine 5'-diphosphate (UDP)-glucose:glycoprotein glucosyltransferase, which allows for de novo binding of the protein to calnexin or calreticulin [71]. When the protein remains misfolded even after transition of repetitive cycles of chaperoning, mannose residues are trimmed by ER α1,2-mannosidase I and ER

degradation-enhancing α-mannosidase-like 1-3 proteins (EDEM 1-3) [71]. Trimming of mannose residues prevents glucose transfer by UDP-glucose:glycoprotein glucosyltransferase to the glycans [71]. EDEM 1 is a protein with a dual topology, existing as an ER luminal protein as well as a type II ER membrane protein [75], with its C-terminus facing the ER lumen. The two EDEM1 pools were shown to have a different set of interaction partners [75]. While luminal EDEM1 preferentially binds to luminal substrates, membrane EDEM1 preferentially binds to membrane proteins [75]. EDEM2 and EDEM3, on the other hand, have only been identified as luminal proteins[75]. After the mannose residues have been trimmed by mannosidases, the glycans can be recognized by the ERAD associated proteins osteosarcoma amplified 9 protein (OS-9) and XTP3-B in mammals [69,71]. OS-9 and XTP3-B recruit the substrate protein to adaptor protein SEL1L, which provides the link between the misfolded protein and the retrotranslocation machinery [69], which will be discussed in the following section. Apart from glycosylated proteins, also nonglycosylated proteins that are misfolded are degraded via the ERAD pathway [65]. For some of these proteins such as a nonglycosylated mutant of NHK it was shown that they are recruited to SEL1L by BiP and its Hsp40 cochaperones using the nonglycosylated ERAD pathway [65,76]

Having been recognized as misfolded, a protein has to be retrotranslocated into the cytosol[65]. After discovery of the ERAD pathway, it was long believed that the Sec61 translocon constitutes the retrotranslocon channel as it was shown that translocated proteins can slip out of the translocon into the cytosol [7]. In addition, it was shown that bacterial toxins utilize the Sec61 translocon for retrotranslocation into the cytosol [8]. Recent data show that mutations in the Sec61 translocon result in retrotranslocation deficits [77,78]. Furthermore, the 19S recognition particle (19S RP) of the proteasome was shown to bind the Sec61 translocon in yeast[79]. Therefore, the Sec61 translocon seems to be involved in the retrotranslocation of proteins into the cytosol during ERAD and co-translational degradation. Yet, an ubiquitin-gated protein-conducting channel formed by the protein Hrd1 is considered to constitute the main retrotranslocon channel[80,81].

After retrotranslocation into the cytosol, the misfolded substrate proteins become ubiquitinated by so called E3 ligases of which more than 20 are known to be present in mammalian cells and to be engaged with the degradation of substrates. The four major E3 ligases in mammals are Hrd1, gp78, TEB4 and carboxy-terminus of Hsc70 interacting protein (CHIP) [82]. After ubiquitination, the substrate proteins are delivered to the 26S proteasome for degradation. The proteasome consists of the 20S proteolytic core particle and the 19S RP [77]. After substrate degradation the proteasomal subunits disassemble [77].

3.4. Calcium homeostasis within the ER

- Daverkausen-Fischer L, Pröls F.
  Regulation of calcium homeostasis and flux between the endoplasmic reticulum and the cytosol. Journal Biological Chemistry (2022) 298(7):102061. doi: 10.1016/j.jbc.2022.102061

Calcium is an important second messenger involved in a broad spectrum of intracellular signaling pathways including metabolism, cell proliferation and death, protein phosphorylation, gene transcription, neurotransmission, muscle contraction, and secretion [83]. Apart from physiological signaling, calcium is also involved in pathological processes as for example in cancer development [84]. The endoplasmic reticulum is an important cellular calcium store with luminal calcium concentrations of 100µM up to 1 mM [85]. Calcium concentrations in the cytosol on the other hand are only around 100nM [85]. During cellular calcium signaling, calcium flux across membranes take place to transfer the calcium signal from one organelle to another [86]. Therefore, a variety of calcium pumps and transporters exist at the plasma membrane as well as on membranes of intracellular organelles [86]. Within the membrane of the ER, sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) is mainly responsible for calcium import into the ER [87]. Calcium export from the ER is mainly mediated by the family of IP3Rs [88,89]. However, it was also shown that calcium efflux from the ER can take place via the Sec61 translocon at the end of protein translocation and that Sec61 gating is mediated by BiP [9,23]. Calcium depletion from the ER can elicit ER-stress as is shown after thapsigargin treatment, an inhibitor of SERCA [90]. It was shown that upon thapsigargin treatment ERdj3, ERdj5 and ERdj6 are upregulated [31,91,92]. For each of the three ERdj proteins functions in calcium homeostasis have been proposed. While ERdj3 and ERdj6 seem to be involved in gating the Sec61 translocon in cooperation with BiP [93], ERdj5 can reduce intramolecular disulfide bonds within the SERCA thereby activating calcium import into the ER [94]. Apart from ERdj3, ERdj5 and ERdj6, ERdj1 also plays a role in calcium signaling as the ERdj1/BiP complex at the plasma membrane serves as a receptor for ligands that can induce a rise in intracellular calcium concentrations via G Protein Coupled Receptor Signaling [95,96]. However, no data is available on whether ERdj1 is upregulated upon ER calcium deprivation. Having identified calcium homeostasis as a common theme of four of the known ERdj proteins, we examined the original data published on this topic and discussed the respective functions of the ERdj proteins also taking into account the association of calcium signaling with redox homeostasis and ATP. Furthermore, we discussed possible implications of altered calcium signaling on disease with special attention to diabetes.

Open questions:

- As it was shown that cooperation of BiP with a co-chaperone is necessary for closure of the translocon [97] and the evidence that BiP closes the translocon in cooperation with ERdj3 and ERdj6 [93], it would be interesting to examine whether overexpression of ERdj3 and ERdj6 HPD mutants also results in a decrease of calcium efflux from the ER.
- In our review, we discussed the diabetic phenotype of ERdj6 knockout mice and stated that it is not known whether ERdj5 knockout mice also display a diabetic phenotype [64,98]. In fact, ERdj5 knockout mice display a phenotype with many features of Sjörgen's Syndrome [99]. In the ERdj5 knockout mouse model, body weight and basal glucose levels were compared with wildtype mice and no differences could be observed [99]. However, levels of insulin, glucagon and glucose tolerance were not investigated in the ERdj5 knockout mice [99] which would be interesting to investigate in the future. Especially, when taking into account that ERdj5 is involved in the maturation of insulin by breakage of non-native disulfide bonds [100].

3.5. ER-stress and the unfolded protein response

Parts of this section “ER-stress and the unfolded protein response” are published in

- Daverkausen-Fischer L, Pröls F.
  The Function of the Co-chaperone ERdj4 in Diverse (Patho-)Physiological Conditions. Cell Mol Life Sci. (2021) 79(1):9. doi: 10.1007/s00018-021-04082-4.

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Protein and ionic homeostasis in the ER are well balanced and essential for cellular survival. Disturbance of ER homeostasis results in ER-stress [101]. In order to regain homeostasis, the cell has developed an ER-stress response, termed the unfolded protein response (UPR) [101]. During the UPR three different ER-stress sensors that are located in the ER membrane are activated [101]. If ER homeostasis cannot be restored, apoptosis is induced at a late stage of ER-stress [101].

3.5.1. The inositol-requiring enzyme 1 (IRE1) pathway

Inositol-requiring enzyme 1 (IRE1) is a transmembrane kinase, which also possesses endoribonuclease activity[102]. There are two different IRE1 proteins in mammals, IRE1α, which is ubiquitously expressed, and IRE1β, which is only present in the gut [101]. In an inactive state, the luminal domain of IRE1 is bound to BiP [103]. When increasing amounts of unfolded proteins accumulate in the ER lumen upon ER-stress, BiP dissociates from IRE1[103]. This results in oligomerization of IRE1 and autophosphorylation of the cytosolic IRE1 domain [104]. Recently, a new model was established by Credle et al.[105]. The group performed structural and mutational analyses in yeast [105]. It was found that unfolded proteins can directly activate IRE1 by binding to the highly conserved groove of IRE1 [105,106]. Therefore, the release of BiP from IRE1 might not be required for IRE1 activation. Instead, it could constitute a regulatory mechanism under conditions of high levels of ER-stress. An IRE1 mutant that cannot bind to BiP is constitutively active at a low level [105]. Consequently BiP binding could switch off the constitutive IRE1 activity[105].

Upon IRE1α activation, the mRNA of X-Box binding protein 1 (Xbp1) is spliced resulting in the transcription factor spliced XBP1 (sXBP1) (Figure 3) [107]. It was found that IRE1α, but not IRE1β, exists in a complex with the Sec61 translocon in HEK293 cells [108]. During the UPR, splicing of Xbp1 mRNA takes place at the Sec61 translocon when unspliced Xbp1 (uXbp1) mRNA bound to a ribosome is guided to the translocon by the SRP[108]. Therefore, association with the Sec61 translocon was proposed to be a mechanism that ensures sufficient uXbp1 splicing by IRE1α even though its low abundance within the ER membrane [108]. Indeed, IRE1 mutants that could not bind to the translocon were not able to sufficiently splice uXbp1[108]. Spliced XBP1 serves as a transcription factor that upregulates several

genes associated with the ERAD pathway as well as the folding machinery in the ER lumen (see Figure 3) [109].

The activated endoribonuclease domain of IRE1 degrades a subset of coding and non-coding cytosolic mRNAs to further lower the protein burden of the ER. This mechanism is called regulated IRE1-dependent decay of mRNA (RIDD) [110] (see Figure 3). Termination of IRE1 signaling was shown to occur via dephosphorylation of IRE1 by a serine/threonine phosphatase of type 2C (Ptc2) in yeast [111]. In mammalian cells monomerization of IRE1α at the Sec61 translocon was shown to be necessary for termination of IRE1 signaling[112,113].

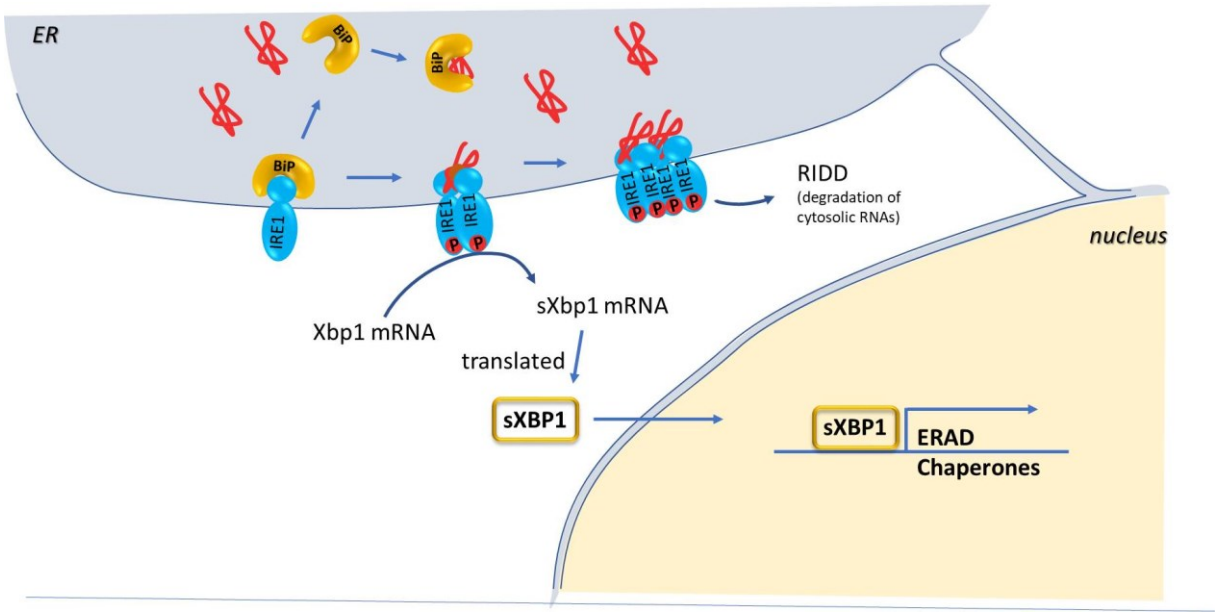

Fig. 3 Schematic drawing to illustrate the ER-stress signaling cascade mediated by IRE1
IRE1 senses ER-stress by the release of BiP and binding of unfolded proteins to its luminal domain[103,105]. Activated IRE1α oligomerizes and subsequent autophosphorylation activates the intrinsic endonuclease activity, which splices the prevailing Xbp1 mRNA into its spliced form sXbp1 [104,107]. Spliced Xbp1 mRNA is efficiently translated into sXBP1 protein, a transcription factor that travels to the nucleus to induce transcription of chaperones and components of the ERAD pathway [109]. Oligomerization of IRE1α also activates regulated IRE1-dependent decay of mRNA (RIDD), the degradation of mRNA molecules in the cytosol [110].

3.5.2. The protein kinase RNA-like ER kinase (PERK) pathway

The second stress sensor in the membrane of mammalian cells is protein kinase RNA-like ER kinase (PERK) [114]. PERK is also a transmembrane kinase [114]. Similar to IRE1, the luminal domain of PERK is bound to BiP under normal conditions [103]. Upon accumulation of misfolded proteins in the ER lumen, BiP dissociates from PERK and PERK oligomerizes and autophosphorylates [112](Figure 4). This in turn results in phosphorylation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and the eukaryotic initiation factor 2α (eIF2α) [114,115]. Following its phosphorylation, Nrf2 dissociates from its binding partner Kelch-like ECH-associated protein 1 (Keap1)[115]. This results in trafficking of Nrf2 to the nucleus where it acts as a transcription factor for genes encoding detoxifying enzymes, cellular

transporters, folding proteins as well as proteasomal subunits (Figure 4)[115,116]. Phosphorylation of eIF2α on the other hand, results in downregulation of general protein translation in the cytosol, while the translation of a selected "stress" repertoire is upregulated[112]. One of these proteins, the activating transcription factor 4 (ATF4), controls transcription of genes playing a role in the transport of amino acids, synthesis of gluthathione and maintenance of redox homeostasis [101,116,117]. During ER-stress, ATF4 also upregulates transcription of the transcription factor C/EBP Homologous Protein (CHOP), which can either induce apoptosis or, by forming a complex with ATF4, activates the transcription of GADD34, which results in dephosphorylation of eIF2α and restoration of general protein translation (Figure 4) [112,118]. Since the luminal domains of IRE1 and PERK are highly homologous, activation of PERK might also be achieved by binding of unfolded proteins to the luminal domain as was proposed by Credle et al. for IRE1 activation [105].

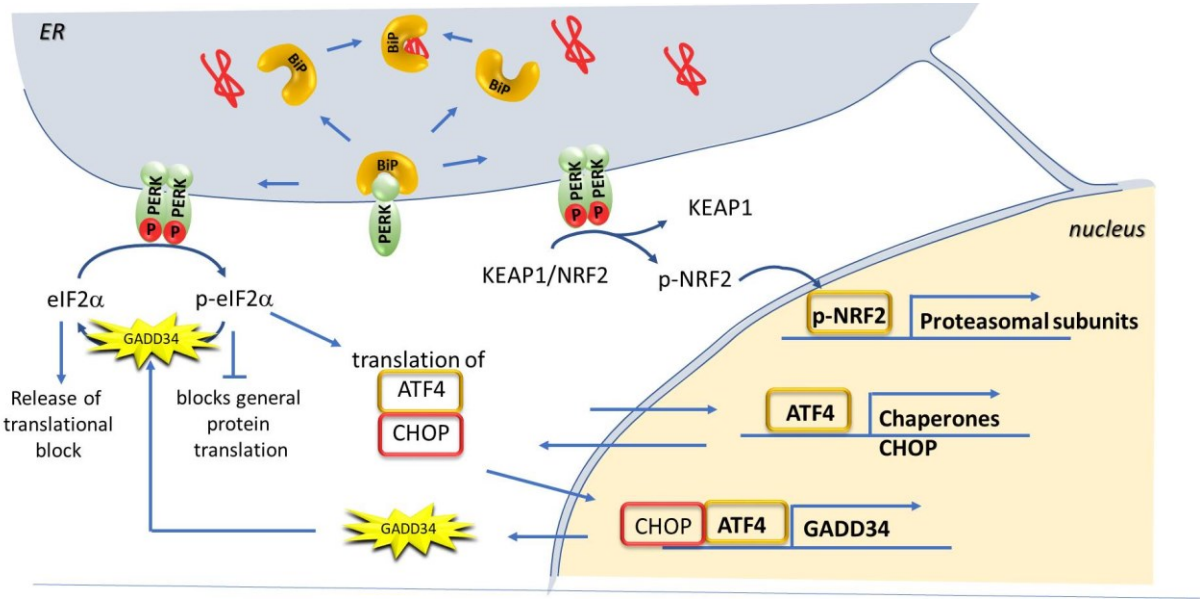

Fig. 4 Schematic illustration of the ER-stress signaling cascade mediated by PERK
PERK signaling is silenced by its binding to BiP [103]. Upon release of BiP, PERK oligomerizes and autophosphorylizes to induce two signaling cascades to activate the transcription factor NRF2, which induces the transcription of proteasomal components [112]. On the other hand, the translation initiation protein eIF2α becomes phosphorylated thereby inhibiting general protein translation by simultaneously promoting translation of the transcription factor ATF4 [112,114]. ATF4 induces transcription of chaperones and of CHOP [112]. CHOP/ATF4 heterodimers activate the transcription of GADD34, which dephosphorylates eIF2α thereby resuming general protein translation [112,118].

3.5.3. The activating transcription factor 6 (ATF6) pathway

The third ER-stress sensor residing in the ER membrane is activating transcription factor 6 (ATF6) [119]. As shown for IRE1 and PERK, ATF6 is inactive when bound to BiP [120]. Accumulation of misfolded proteins in the lumen of the ER results in dissociation of BiP from ATF6 and in trafficking of ATF6 to the Golgi complex where the N-terminal part of ATF6 is cleaved off and travels to the nucleus to induce the transcription of BiP and other chaperones [112,119,120]. ATF6 can also dimerize with transcription factor sXBP1 to increase upregulation of several ERAD components and co-chaperones (Figure 5) [121].

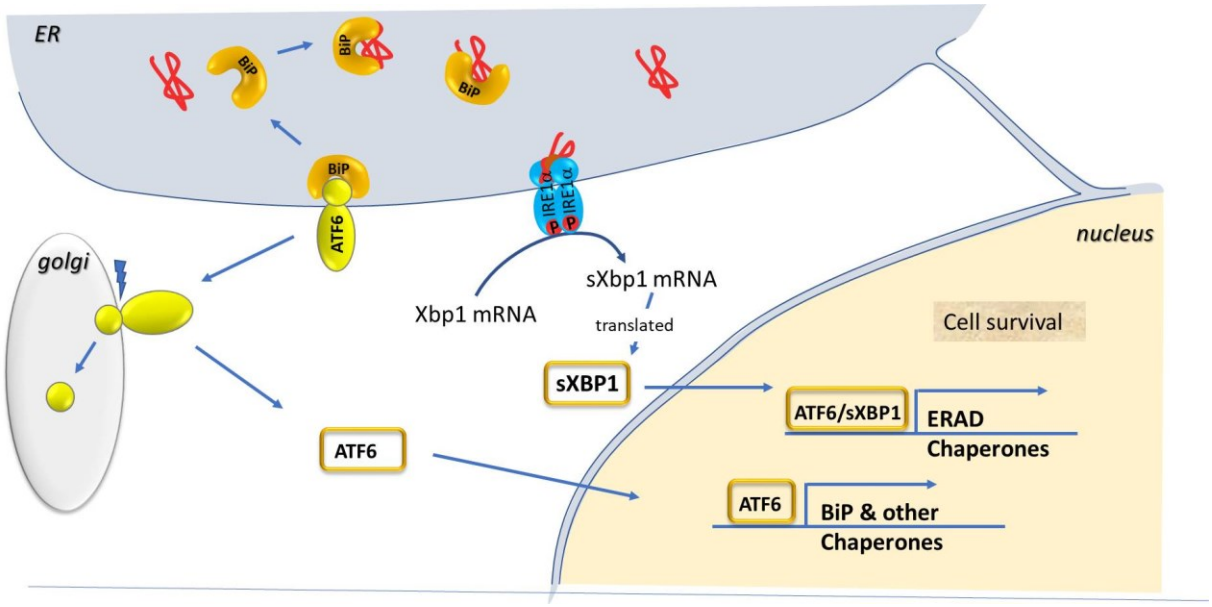

Fig. 5 Schematic illustration of the ER-stress response mediated by ATF6
ATF6 is silenced by its binding to BiP [120]. ER-stress conditions lead to dissociation of BiP, releasing ATF6, which travels to the Golgi where its N-terminal part is cleaved to travel as a transcription factor to the nucleus [112,119,120]. ATF6 upregulates the transcription of chaperones and – when dimerized with XBP1 – induces components of the ERAD pathway [112,121].

3.6. Question and aim of the thesis

Since their discovery in the 1990s, a large amount of experimental data on structure, localization, function and regulation of ERdj1 to ERdj8 has been published. However, until now there has not been a complete and critically evaluated review on all published experimental data. Thus, the creation of such a review was the aim of the presented thesis. During analysis of the literature and experimental data published, special focus was placed on the subcellular localization and topology of the eight ERdj poteins and on the function of the eight ERdj with regard to protein translation, translocation and degradation as well as with regard to cellular calcium homeostasis. Further focus was directed at the role of ERdj proteins in diseases.

4. Materials and methods

4.1. Systematic literature search on PubMed

Before a systematic literature search was done on PubMed, all synonyms for the eight co-chaperones were collected and an appropriate search term was created. It was only searched for publications in English or German language, no other filters were applied. For ERdj1 to ERdj5, the search terms produced 29 to 219 publications. For ERdj6, the respective search term yielded 162 results, a number which would have gone beyond the scope of this work. ERdj6 was, therefore, excluded from the systematic research but included in the reviews, in which specific aspects were depicted to analyze the function and role of all co-chaperones in the ER. The respective search terms for ERdj7 and ERdj8, on the other hand, did only yield few papers, which included too little information for a convincing comparison. A reason for this might be that ERdj7 and ERdj8 have only recently been discovered. Accordingly, the systemic literature search focused on ERdj1 to ERdj5. Publications were first screened by title and abstract. In a second step a full text screening was performed. Reviews were excluded and papers focusing on non-mammalian cells or organisms were also excluded. The numbers of excluded papers and the reasons for exclusion are listed in Table 1. The last update on the literature search was done on 14t^{h} of September 2021. The included publications were carefully read and all experimental data were analyzed with respect to controls, limitations of experimental methods and the conclusions that can be drawn according to the experimental design and results. Results and conclusions drawn and depicted in this work were strictly limited to the experiments shown in the publications. After collection and organization of the experimental data regarding ERdj1 to ERdj5, the co-chaperones were compared with each other with respect to their subcellular localization and topology. Furthermore, regulation of calcium homeostasis and regulation of translation, translocation and degradation of substrates were identified as common themes of some of the ERdj proteins and individual roles of the ERdj proteins in this context were examined in detail. The co-chaperones ERdj6 to ERdj8 were only analyzed in the context of these common topics. The results of the literature search are published in four reviews [64,122-124].

In this section, the literature search is summarized for ERdj1 to ERdj5. In the Results section, the data for each ERdj protein are summarized individually. In a second part, specific aspects are addressed. The corresponding reviews are briefly summarized and attached. The discussion addresses some of the open questions which are discussed with novel, up-to-date literature.

4.1.1. ERdj1

For ERdj1 the search term: “ERdj1 OR Mtj1 OR Mtj-1 OR Mtj1p OR Htj1 OR Htj-1 OR DnaJ-like protein 1[tw] OR Erj1 OR Erj1p” was used. The search yielded 29 results. After title and abstract had been screened, 10 papers were assessed as irrelevant and excluded. Further article screening classified another 3 papers as irrelevant so that they were excluded. In the end 16 papers were included of which 1 was related to diseases associated with ERdj1.

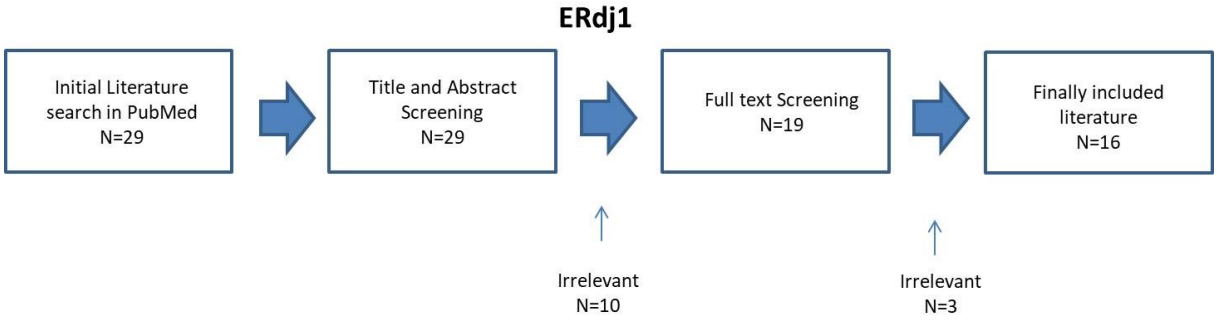

Fig. 6 Schematic presentation of literature search on PubMed for ERdj1

4.1.2. ERdj2

For ERdj2 the search term: “ERdj2 OR ERj2 OR Sec63 OR Sec63p” was used. The search yielded 219 results. After title and abstract had been screened, 160 papers were assessed as irrelevant and excluded. Further article screening classified another 19 papers as irrelevant so that they were excluded. In the end 40 papers were included of which 20 papers were related to diseases associated with ERdj2.

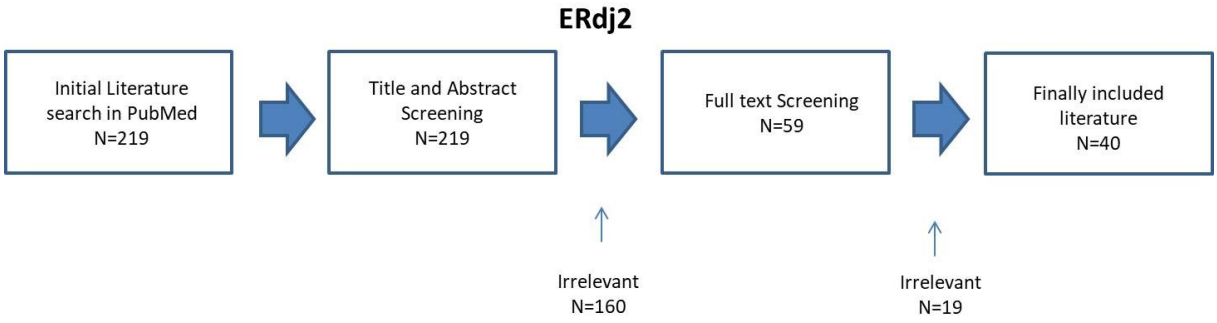

Fig. 7 Schematic presentation of literature search on PubMed for ERdj2

4.1.3. ERdj3

For ERdj3 the search term: “ERdj3 OR Erj3 OR Erj3p OR Dnajb11 OR Hedj OR Hdj9 OR ABBP-2 OR PWP1- interacting protein 4[tw]” was used. The search yielded 77 results. After title and abstract had been screened, 23 papers were assessed as irrelevant and excluded. Further article screening did classify three more papers as irrelevant so that in the end 51 papers were included of which 17 papers were related to diseases associated with ERdj3.

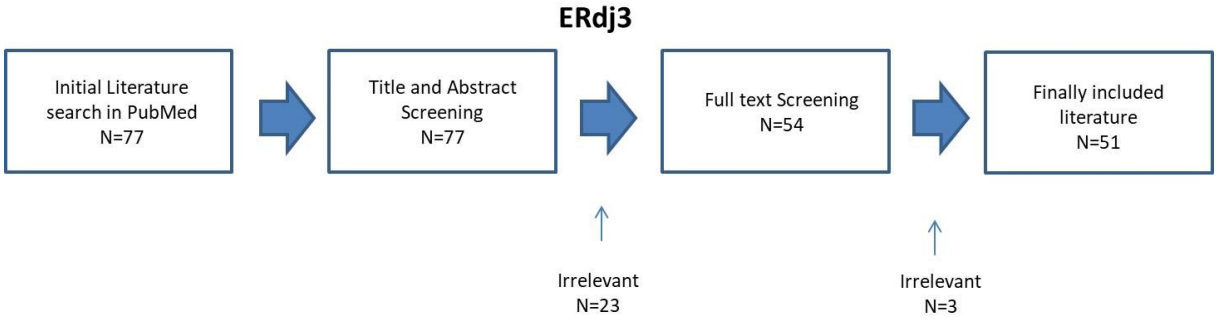

Fig. 8 Schematic presentation of literature search on PubMed for ERdj3

4.1.4. ERdj4

For ERdj4 the search term: “ERdj4 OR Mdg1 OR Mdj7 OR Dnajb9” was used. The search yielded 189 results. After title and abstract had been screened, 131 papers were assessed as irrelevant and excluded. Further article screening did classify nine more paper as irrelevant so that in the end 49 papers were included of which 24 papers were related to diseases associated with ERdj4.

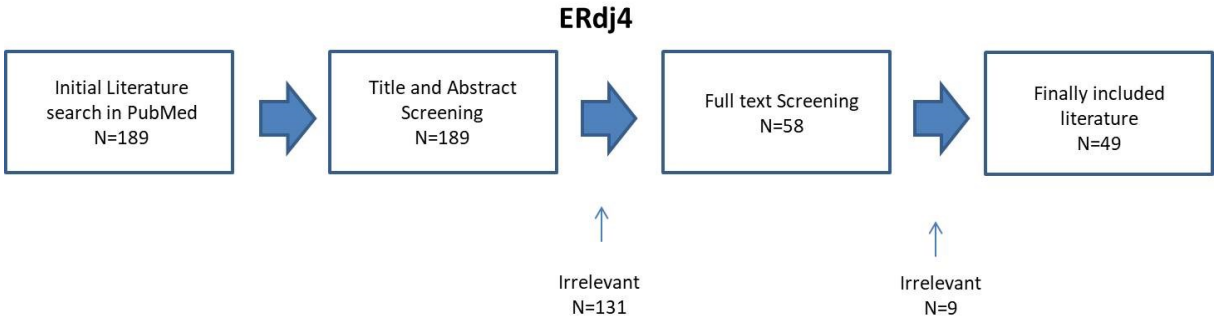

Fig. 9 Schematic presentation of literature search on PubMed for ERdj4

4.1.5. ERdj5

For ERdj5 the search term: “ERdj5 OR DNAJC10 OR Macrothioredoxin OR JPD1 OR MTHr OR Erj5 OR ERdj-5 OR ERj5p” was used. The search yielded 76 results. After title and abstract had been screened, 32 papers were assessed as irrelevant and excluded. Further article screening did classify six more papers as irrelevant so that in the end 38 papers were included of which 19 papers were related to diseases associated with ERdj5.

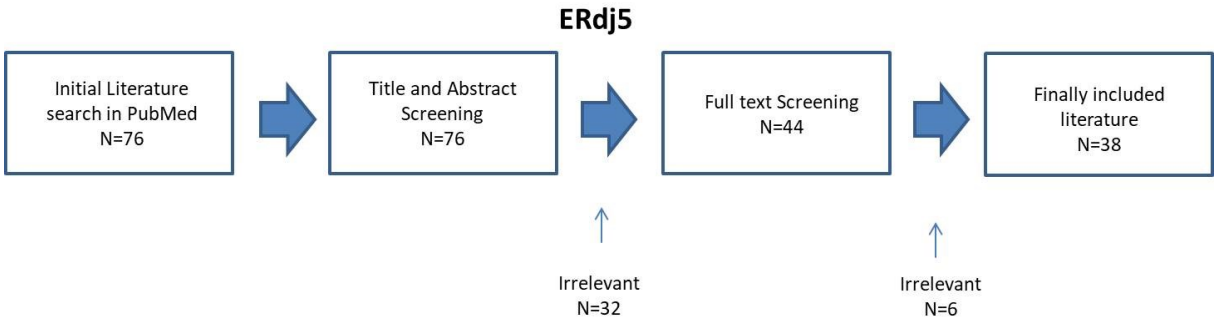

Fig. 10 Schematic presentation of literature search on PubMed for ERdj5

Table 1 Reasons for exclusion of papers during the systematic literature search

Co-chaperone	Reason for exclusion of publications
ERdj1	Review (2); Paper based on non-physiological (drug treatment) conditions (2); Paper based on another protein (3); Paper did not include relevant information (5); Paper focused on yeast (1)
ERdj2	Review (26); Paper based on another protein (23); Paper did not include relevant information (18); Paper focused on a non-mammalian organism (111); Paper was not available (1)
ERdj3	Review (9); Paper based on non-physiological (drug treatment) conditions (3); Paper based on another protein or gene (3); Paper did not include relevant information (9); Paper focused on a non-mammalian organism (1); Paper was not available (1)
ERdj4	Review (8); Paper based on non-physiological (drug treatment) conditions (20); Paper based on another protein or gene (4); Paper did not include relevant information (51); Paper focused on a non-mammalian organism (53); Paper was not available (4)
ERdj5	Review (8); Paper based on non-physiological (drug treatment) conditions (2); Paper did not include relevant information (24); Paper focused on a non-mammalian organism (3) Corrigendum (1)

Numbers in brackets: Number of publications excluded for the respective reason

5. Results

5.1. ERdj1

5.1.1. Structure

The co-chaperone ERdj1 [125], also known as ERj1p [126], MTJ1 [50], Mtj1p [127] or HTJ1 [128], is a protein of 552 amino acids and was first identified in 1995 in murine lung carcinoma cells [50]. Early experimental studies presented immunoblots using two different antibodies – one against the C-terminus of ERdj1 and the other against a fusion protein between ERdj1 and Glutathione-S-transferase (GST), GST-ERdj1. In the experiments, proteins with molecular weight of approximately 62kDa, 34kDa, 41kDa and 42kDa were detected in murine melanoma cells [50]. The existence of the 62kDa protein is in accordance with the predicted molecular weight of ERdj1, which is 59kDa (Accession: NP_001177746). Computational analysis based on genomic sequences derived from Mus musculus as well as Homo sapiens predict ERdj1 isoforms 42kDa (Accession: XP_016872025), 46kDa (Accession: XP_011517916) and 34kDa (Accession: XP_011237249) in size that could represent the smaller proteins detected in the immunoblot experiments [50].

When synthesized in the absence of microsomes, ERdj1 is slightly larger than when synthesized in the presence of microsomes suggesting that ERdj1 possesses an N-terminal signal peptide that is cleaved after translocation of ERdj1 into the ER lumen [127]. According to sequence analysis, ERdj1 contains a J domain (aa 56-129), which is flanked by two predicted transmembrane segments identified by hydropathy plots [50]. Computational analysis of the ERdj1 sequence predicts a potential cleavage site immediately after the first predicted transmembrane segment (see Figure 11) [58]. This potential cleavage site is in accordance with experimental results showing that after proteinase K treatment a 16kDa fragment persists, which was shown to be the J-domain and the second transmembrane segment [127]. Taken together, these data indicate that a signal peptide which might constitute the first transmembrane segment is cleaved from ERdj1 upon integration into the ER membrane resulting in the mature ERdj1 protein, which possesses only one single transmembrane segment separating the luminal J-domain from the cytosolic domain of ERdj1 (see Figure 11) [127]. Based on computational data, an additional topology was suggested for ERdj1 with the C-terminal region facing the ER lumen due to a second transmembrane domain [58]. If this suggested topology existed, the C-terminal domain of ERdj1 should be protected from proteinase K digestion. However, experimental data did not reveal a protein band of the expected size after proteinase K treatment of ERdj1 [127].

In addition to its J-domain and transmembrane segment, ERdj1 contains two domains, called the SANT1 (aa327-377) domain and the SANT2 (aa 493-545) domain, which mediate protein-protein interactions [58,128]. While ER luminal proteins are retained in the ER lumen by the KDEL sequence, type I membrane proteins were shown to be retained at the ER membrane by a cytosolic retention motif containing two lysine residues[129,130]. Computational data analysis revealed that ERdj1 contains such an ER retrieval signal (KKQA) at its C-terminal, cytosolic site [58]. Proteins containing a KDEL retention sequence bind to the KDEL receptor within the lumen of the Golgi [131]. The lysine residues of the cytosolic retention motif of type I transmembrane proteins on the other hand were shown to bind to the coatomer [129]. The coatomer is involved in the budding of vesicles from the Golgi [132]. Those vesicles are directed back to the ER after the coatomer has bound to the di-lysine containing retention motif [133]. A ribosome binding domain exists between amino acid 177 and amino acid 193 at the N-terminus of the cytosolic domain [126,134]. Furthermore, ERdj1 contains four potential phosphorylation sites [135]. A schematic representation of the ERdj1 structure is depicted in Figure 11.

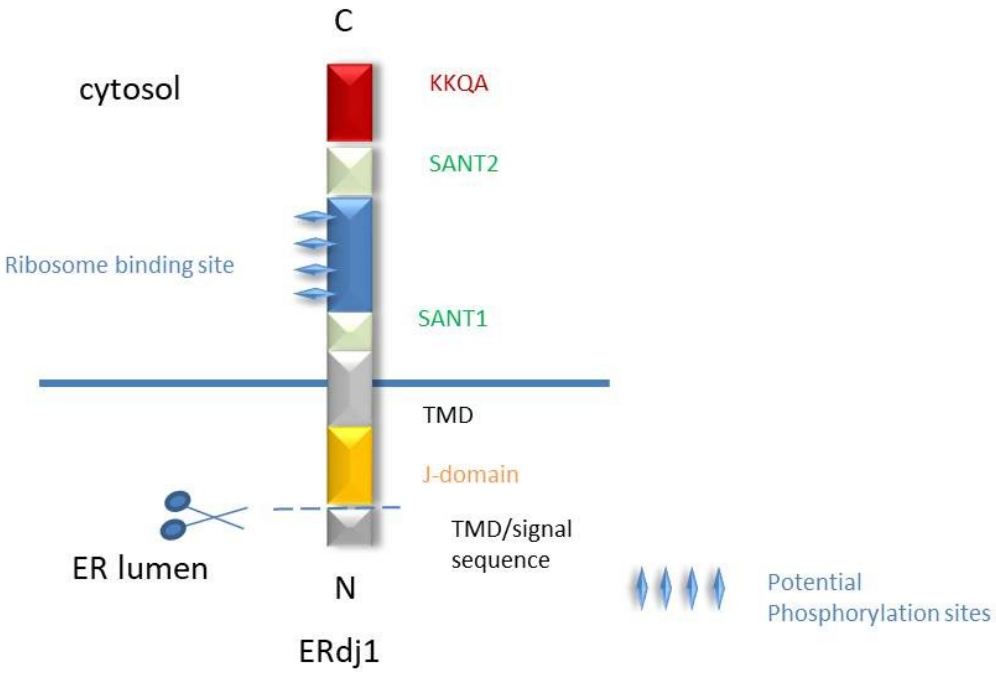

Fig. 11 Schematic structure of ERdj1
The N-terminal signal peptide is cleaved from ERdj1 upon translocation into the ER lumen [127]. The luminal N-terminal J-domain of ERdj1 is followed by a transmembrane domain [50]. The cytosolic part of ERdj1 contains two protein binding domains (SANT1 and SANT2 domain) and an ER retention sequence (KKQA) [58,128]. Furthermore, the cytosolic domain of ERdj1 contains four potential phosphorylation sites and a ribosome binding site [126,135]. The figure shows the protein domains of ERdj1 without representation of the actual proportions between the different domains.

5.1.2. Localization

With respect to subcellular localization, ERdj1 was detected in different cell compartments by different groups [50,96,127,136-138]. Brightman et al. detected the full length ERdj1 as well as smaller isoforms 41kDa and 42kDa in size in the heavy microsomal fraction of murine melanoma cells [50]. The localization of ERdj1 in ER-derived microsomes was confirmed by immunocytochemistry experiments [136] and by immunoaffinity purification in dog pancreas microsome extracts[127].

ERdj1 is also present at the plasma membrane of macrophages and liver cells, where it forms a complex with BiP [137,139]. Coimmunoprecipitation experiments show that - within the plasma membrane of endothelial cells - the BiP/ERdj1 complex can be located in lipid rafts upon ligand binding to BiP. Within the lipid rafts, the complex might play a role in transferring signals from the extracellular to the intracellular space [96,138].

Smaller ERdj1 isoforms of 34kDa, 41kDa and 42kDa were detected in the nuclear fractions of murine melanoma cells in immunoblot experiments [50]. The presence of a nuclear ERdj1 pool is further supported by immunocytochemistry experiments in hamster ovary (COS-7) cells [136]. In these experiments a truncated variant of ERdj1, that only contains the cytosolic part of the protein, was localized to the nucleus[136].

5.1.3. Function

Interaction with Hsp70 proteins

The J-domain of ERdj1 was shown to associate with BiP in the ER while association of full-length ERdj1 with BiP could be demonstrated in the plasma membrane of macrophages [58,127,137,140]. As a conserved feature of the Hsp 70/Hsp 40 interaction [45], ATPase assays and pull-down assays identified the HPD motif within the J-domain of ERdj1 to mediate binding to BiP [58,140,141]. Also, a basic arginine residue seven amino acids upstream of the HPD motif was found to be important for binding of ERdj1 to BiP [140]. While mutations in the histidine of the HPD motif abandoned binding of ERdj1 to BiP, mutation in the arginine residue reduced binding to about 50% [58,140]. The dissociation constant (K_D value) of ERdj1-BiP binding was calculated to be 0.12 µM [142].

In BiP-ERdj1 binding assays using polyhistidine (His)-tagged BiP as well as the J-domain of ERdj1, binding of BiP to ERdj1 could be demonstrated in the presence and absence of ATP as well as in the presence of ADP [58]. In the experiments both proteins were allowed to bind to each other before a metal-chelating resin was added and the bound complexes were eluted [58]. Surface plasmon resonance spectroscopy as well as pull down assays could only detect significant interaction between BiP and ERdj1 in the presence of ATP but not in the

absence of ATP [127,140]. In the experimental setting the J-domain of ERdj1 was immobilized and BiP was allowed to pass the bound J-domain in the presence or absence of ATP [127,140]. The interaction between BiP and the J-domain of ERdj1 in the presence of ADP was not examined in these studies. ERdj1 and BiP not only bind to each other but the J-domain of ERdj1 was also shown to stimulate ATPase activity of BiP as was shown by ATPase assays [58].

Conflicting experimental data exist with regard to the question whether or not the J-domain of ERdj1 can also stimulate the ATPase activity of the bacterial Hsp70 homologue, DnaK. Whereas Chevalier et al. provide experimental evidence that the J-domain of ERdj1 can indeed stimulate the ATPase activity of DnaK, data presented by Nicoll et al. did not confirm this [58,140]. These conflicting results could be due to different experimental approaches. While Chevalier et al. used ATPase assays to show a low stimulation of the in vitro ATPase activity of DnaK [58], Nicoll et al. determined the capability of ERdj1 to complement the endogenous DnaJ in E.coli in in vivo complementation assays [140]. Nicoll et al. used a chimeric protein in which the J domain of E.coli DnaJ was replaced with the J domain of ERdj1 [140] while Chevalier et al. examined the stimulation of DnaK ATPase activity by using the isolated J-domain of ERdj1 [58]. This indicates that even though the isolated J domain of ERdj1 can stimulate the ATPase activity of DnaK in vitro, this is not necessarily the case under in vivo conditions.

Inhibition of protein translation at the ribosome

Using sucrose gradient centrifugation of detergent microsomal extracts, ERdj1 was detected in fractions that also contain ribosomal proteins and the Sec61 translocon [127]. Furthermore, in HeLa as well as in COS-7 cells, RNase treatment resulted in a significant increase of antibody bound to ERdj1 protein indicating a close proximity of ERdj1 to ribosomes [141]. Using in vitro ribosome binding assays, it was further shown that the first 21 N-terminal amino acids of the cytosolic domain of ERdj1 associate with non-translating eukaryotic ribosomes [126,127,141]. The binding site was identified within the 28S subunit of the 60S subunit and close to the tunnel exit [126,134]. The affinity of ERdj1 to ribosomes is high with a dissociation constant of 30pM [127,141]. High potassium chloride (KCl) concentrations disrupt the association of ERdj1 with ribosomes [127].

Apart from binding to the ribosome, ERdj1 also interacts with emerging proteins at the tunnel exit of ribosomes in a BiP-independent manner [126]. In vitro translation assays show that the cytosolic domain of ERdj1 can inhibit translation of preprolactin (PPL) and luciferase at the ribosome [126,127]. Translation is inhibited at its initiation since ERdj1 is not able to inhibit translation of preprolactin when added after translational initiation [126]. Cryo-EM mapping

analysis revealed that the determined binding site of ERdj1 to the emerging protein does presumably not directly interfere with factors involved in translational initiation [134]. It rather seems to be an ERdj1-induced conformational change within the ribosome that is responsible for the observed inhibition of translation [134]. Translational inhibition as well as ribosome binding is facilitated by a highly charged amino acid stretch (RKKRERKKK) found at the N-terminus of the cytosolic domain. Within this sequence the four amino acids RKKR seem to be most important for translational inhibition at the ribosome [126]. Strikingly, this polybasic amino acid stretch resembles a region within the signal recognition particle [134]. Within the SRP, the sequence is responsible for the arrest of elongation upon binding of the emerging protein to the SRP [143]. When the ribosome nascent protein complex has been successfully recruited to the SRP receptor (SR) at the ER membrane elongation continues [143].

Increasing amounts of BiP are associated with ribosomes in the presence of ERdj1 in an ATP-dependent manner [126]. Therefore, ERdj1 might be required for the recruitment of BiP to the ribosome-ERdj1 complex [126,127]. Binding of BiP to ERdj1 abolishes the protein translational arrest [126,127]. Whether BiP and ribosomes bind to ERdj1 simultaneously or sequentially is not known. Dudek et al. demonstrate that ERdj1 can bind to the ribosome and BiP at the same time and that binding of BiP to ERdj1 results in the reinitiation of protein translation [126]. Contrary, experimental data obtained from surface plasmon resonance indicate that binding of ERdj1 to ribosomes is prevented by binding of BiP to ERdj1[141]. The experiments suggest binding of ERdj1 to BiP is a perquisite to prevent an interaction between ERdj1 and ribosomes as an ERdj1 HPD mutant preincubated with BiP could still bind to ribosomes [141]. However, the ability of BiP to bind or hydrolyze ATP is not necessary for the prevention of ERdj1-ribosome binding [141]. In conclusion, ERdj1-mediated inhibition of protein translation is abolished by BiP whether or not binding of BiP to ERdj1 and binding of ribosomes to ERdj1 occurs simultaneously or sequentially [126,141].

Formation of regulatory protein-protein interactions

Two proteins that interact with the SANT2 domain of ERdj1 have been identified by yeast two hybrid systems, Western blots and dot-plot analysis [125,128]. ERdj1-SANT2 interacts with the protease inhibitor α1-antichymotrypsin (AAT) (amino acids 140-400) and the protease inhibitor inter-α-trypsin inhibitor heavy chain 4 (ITIH4) (amino acids 588-930) [125,128].

Association of ERdj1 with α1-antichymotrypsin inhibits complex formation of α1-antichymotrypsin and the digestive enzyme chymotrypsin. Consequently, binding of ERdj1 to α1-antichymotrypsin increases the enzymatic activity of chymotrypsin [128]. Experimental data

from yeast two hybrid systems suggest that the SANT1 domain of ERdj1 can also interact with α1-antichymotrypsin [128]. What still remains unclear is whether binding of the SANT1 domain to α1-antichymotrypsin also affects chymotrypsin activation. Unfortunately, the authors did not assess the relevance of this interaction.

ITIH4, a component of Inter-alpha-trypsin inhibitor family proteins (ITI), is an atypical acute phase protein associated with malignant and inflammatory diseases [144]. ITIH4 can be cleaved by various proteases giving rise to an active peptide that can then form a complex with the respective protease [145]. Complexing of ITIH4 with the protease was shown to inhibit the proteolytic activity of the protease[145]. Association of the SANT2 domain of ERdj1 with ITIH4 was shown to decrease processing of ITIH4 by the protease kallikrein[125]. Therefore, interaction of ERdj1 with ITIH4 might increase the proteolytic activity of kallikrein (see Figure 12).

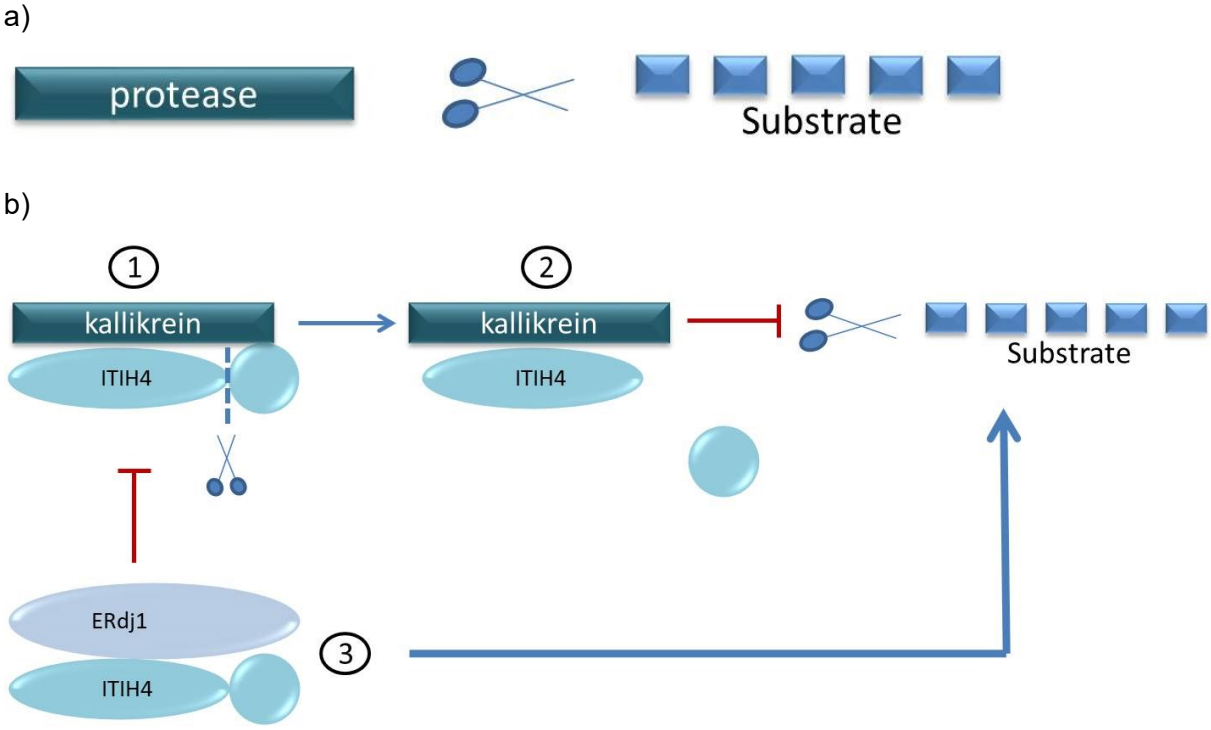

Fig. 12 Association of ERdj1 with ITIH4 might increase proteolytic activity of proteases
a) Proteases normally break down their substrate proteins by cleavage of intramolecular peptide bonds. b) Binding of ITIH4 to several proteases results in cleavage of ITIH4 (1) giving rise to an active peptide that inhibits proteolytic activity of the associated protease[145] (2). Binding of ERdj1 to ITIH4 was shown to inhibit cleavage of ITIH4 by the protease kallikrein[125] (3). Thereby association with ERdj1 decreases the inhibitory capacity of ITIH4 on proteolytic activity of proteases.

Potential function as a nuclear transcription factor

Computational analysis revealed that the cytosolic domain of ERdj1 possesses a DNA binding domain which can also be found in transmembrane tethered transcription factors [136]. Different experimental studies assessed the localization of a recombinant truncated protein consisting of the cytosolic domain of ERdj1 using immunocytochemistry [126,136]. When monkey or murine fibroblasts were transfected with plasmids encoding the cytosolic domain of ERdj1, the truncated derivate of ERdj1 was found in the nucleus [126,136]. Further experimental data show that the cytosolic domain of ERdj1 can bind to importin β, a protein involved in translocation of proteins into the nucleus [126]. Supporting the hypothesis that ERdj1 can act as a transcription factor are the protein bands 34kDa and 42kDa in size that reacted with an ERdj1 antibody and could be detected in the nuclear fraction of murine melanoma cells [50]. A drawback in the given experiment by Brightman et al., which shows immunoreactivity in the nuclear fraction, is the lack of compartment specific markers verifying the purity of the differential subcellular fractions in the respective Western blot. Also, the cytosolic domain of ERdj1 would only be able to act as a transcription factor if the protein were cleaved at the ER or plasma membrane. Until now, there is no experimental evidence demonstrating the occurrence of a cleavage event at all. Also, no cleavage site in the N-terminal region of the cytosolic domain has been predicted by computational analyses. Taken together, no clear evidence is presented that ERdj1 is in fact localized in the nuclear compartment in vivo.

The ERdj1/BiP complex at the plasma membrane

ERdj1 and BiP form a complex at the plasma membrane of macrophages and liver cells [96,137,139]. At the plasma membrane, the ERdj1/BiP complex has been identified to be a cell surface receptor for α2-macroglobulin [95]. Under normal conditions, binding of α2-macroglobulin to BiP results in an increase of intracellular calcium concentrations [95]. Upon downregulation of ERdj1 using RNA interference, less BiP is found in the plasma membrane fraction in human endothelial cells [138]. Probably as a consequence of impaired translocation of BiP to the cell surface, less α2-macroglobulin was shown to bind to the cell surface upon ERdj1 downregulation [137]. Consequently, downregulation of ERdj1 inhibits α2-macroglobulin-mediated increase of the intracellular calcium concentrations [137]. There is experimental evidence that at the plasma membrane, the ERdj1/BiP complex is involved in G-protein coupled signaling after α2-macroglobulin stimulation [96]. After stimulation of macrophages with α2-macroglobulin, BiP and ERdj1 coimmunoprecipitate with Gαq11, a subunit of trimeric G proteins, located in lipid rafts within the plasma membrane [96]. Downregulation of either BiP or ERdj1 using RNA interference decreased the amount of BiP, ERdj1 and Gαq11 in the

plasma membrane by approximately 50% following α2-macroglobulin stimulation [96]. This might be the reason for the defective calcium influx into ERdj1 deficient cells.

Treatment with oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) was shown to stimulate translocation of BiP from the ER to the plasma membrane of endothelial cells [138]. Furthermore, OxPAPC and oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoserine (OxPAPS) were detected as ligands for the ERdj1/BiP complex in endothelial cells [138]. Oxidized phospholipids as OxPAPC enhance endothelial cell barrier by promoting the formation of adherent and tight junctions as well as by remodeling the cortical cytoskeleton [146]. VE-cadherin is an important protein for the formation of cell-cell interactions, while the protein cortactin controls contractility of actinmyosin [147,148]. In endothelial cells the amount of VE-cadherin and cortactin at the plasma membrane increases after OxPAPC stimulation [138]. The increased amount of cortactin at the plasma membrane correlates with an increase in actin filaments in the cell periphery of endothelial cells after OxPAPC stimulation, which results in reinforcement of the endothelial cell barrier [138]. Downregulation of ERdj1 by RNA interference can abolish the barrier-protective effects of OxPAPC in mouse models of acute lung injury as well as in cell culture experiments [138]. Downregulation of ERdj1 decreases the accumulation of BiP and cortactin at the plasma membrane in endothelial cells. Also, ERdj1 downregulation prevents the OxPAPC-mediated increase in actin filaments in the cell periphery. Taken together, these results highlight that the ERdj1/BiP complex plays a crucial role in barrier enhancement during inflammation mediated by oxidized phospholipids [138].

Adipokines are secreted from white adipose tissue and mediate cell-signaling [149]. Visceral adipose tissue–derived serine proteinase inhibitor (vaspin) is an adipokine that is involved in insulin resistance and obesity [139,149]. Vaspin has been identified to be an additional ligand of the ERdj1/BiP complex at the plasma membrane of liver cells [139]. Upon receptor binding, vaspin ameliorates ER-stress and triggers signaling pathways that seem to regulate glucose metabolism [139].

5.1.4. Regulation

So far, little is known about the regulation of ERdj1. Northern blots of murine tissues showed the existence of two transcripts, one of 3.2kb and the other of 6.5kb in size [50]. This experimental result is confirmed by two ERdj1 entries in the nucleotide database, one entry encompasses about 5kb (Accession: NM_001190817.1), the other about 2,1kb (Accession: NM_0223654.4) in size. Both transcripts encode the same protein. Both transcripts are present in murine lung, liver, brain, spleen, heart and kidney [50]. High levels of the 2.1kb sized transcripts can be found in lung, liver and muscle. High levels of the 5kb sized transcript can

only be detected in the lung [50]. The implication of the existence of two different transcripts for ERdj1 and whether they might contain different regulatory domains has not yet been assessed in detail.

The cytosolic domain of ERdj1 has four potential protein kinase 2 (CK2) phosphorylation sites [135]. The amount of phosphorylation was shown to depend on the concentration of ERdj1. According to dot plot analysis as well as CK2 phosphorylation experiments, the degree of phosphorylation at the different sites varies: highest phosphorylation levels are reported for the CK2 site at position aa 477-481 with the amino acid sequence SSDEE [135]. The functional relevance of CK2 phosphorylation is not clear. Yet, it was shown that phosphorylation of ERdj1 by CK2 does not inhibit binding of ERdj1 to ribosomes [135].

Unfortunately, the examined literature did not contain any experiments about whether ERdj1 mRNA or protein levels are upregulated during ER-stress. However, there seems to be unpublished data indicating that ERdj1 is not induced during ER-stress [92].

5.1.5. ERdj1 in disease

Little is known about the implications of ERdj1 in diseases. The pattern of BiP and ERdj1 expression in malignant melanoma was assessed in one study [150]. Two different patterns of BiP expression were observed. One pattern showed a continuous loss of BiP expression from superficial to deeper layers of the skin. The second pattern showed a regain of BiP expression in deeper layers of the skin. The “regain pattern” was associated with poorer patient survival [150]. This might also be due to an anti-apoptotic effect of BiP.

With regard to ERdj1 expression, three distinct patterns could be differentiated in malignant melanoma cells: i) no expression of ERdj1, ii) a weak/blush ERdj1 expression, and iii) a strong ERdj1 expression. Statistical analysis showed that patients with strong ERdj1 expression had the greatest probability of survival whereas patients with weak/blush ERdj1 expression showed the poorest survival rates [150].

The results are difficult to understand as high ERdj1 levels as well as ERdj1 deficiency result in better patient outcomes than the presence of little ERdj1 which points against a dosage dependent effect of ERdj1 protein. ERdj1 levels in healthy skin are not shown to enable comparison of the protein levels in healthy and cancer tissue. It would further be of interest to characterize the subcellular localization of ERdj1 in the malignant melanoma skin samples and to know whether there are spatial differences in ERdj1 distribution. So far, these questions have not been addressed.

5.2. ERdj2

5.2.1. Structure

Mammalian ERdj2, also known as ERj2, Sec63 [151] and Sec63p [51] was first discovered in HeLa cell extracts and dog pancreatic microsomes [51,152]. The gene encoding ERdj2 is localized on chromosome 6 [152]. ERdj2 consists of 760 amino acids [51] and according to the protein data base in humans, the predicted size of the translated protein is 87,8kDa (Accession: NP_009145.1). Sequence analysis reveals that the protein possesses a J-domain and three potential transmembrane segments, two of which flank the J-domain [51]. ERdj2 lacks an N-terminal signal peptide [51]. At its C-terminal region, mammalian ERdj2 sequence possesses a motif of unknown function that is also found in U5 small nuclear ribonucleoproteins [153]. Furthermore, ERdj2 possesses four potential phosphorylation sites [154].

With respect to membrane orientation, proteinase K assays show that neither the C-terminus nor the N-terminus of ERdj2 are resistant to proteinase K [51]. However, the J-domain of ERdj2 is resistant to proteinase K treatment [51]. Accordingly, only the transmembrane domains located between amino acid residues 93-109 and between amino acid residues 221-239 span the ER membrane[51]. Taking together the existing data, the C- and the N-terminus of ERdj2 face the cytosol, while the J-domain is located within the ER-lumen giving rise to a U-shaped conformation of the full protein. Yet, all published papers examined by us present the N-terminus of ERdj2 within the ER lumen and the C-terminus in the cytosol [6,58,142,154,155]. In the course of my literature search I did not discover any experimental data that support this orientation of ERdj2 in the ER membrane of mammalian cells. Chevalier et al. [58] cited a paper by Feldheim et al. [156] as the source for the proposed topology. However, the cited paper did not assess topology of Sec63 but of Sec66 in yeast [156]. Still, there is an earlier paper by Feldheim et al. that proposed a topology of yeast Sec63 with the N-terminus being located within the ER lumen [157]. However, the experiments published by the group only assessed the topology of the C-domain and the J-domain of Sec63 in detail. The topology of the N-domain was only predicted according to computational analysis [157]. Recently, cryo-EM mapping of the translocon complex in yeast confirmed the existence of three transmembrane domains in yeast ERdj2 [158]. So far, the topology of the mammalian ERdj2 has not been clarified and similar cryo-EM mapping experiments as have been performed in yeast would be interesting to conduct in mammalian cells.

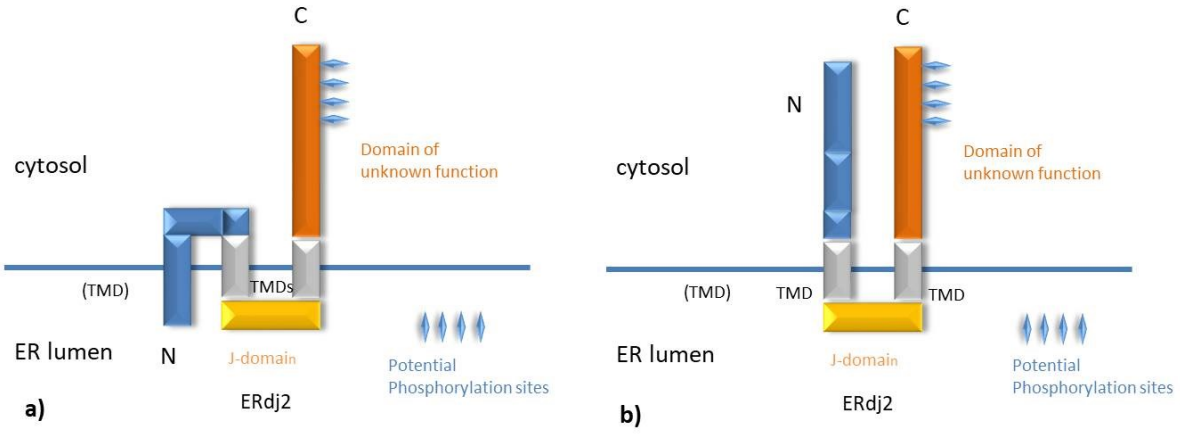

Fig. 13 Schematic structure of ERdj2
Sequence analysis predicts that ERdj2 possesses three transmembrane domains (see a)) [51]. However, according to proteinase K digests, the C-terminus as well as the N-terminus of ERdj2 is facing the cytosol while the J domain is located within the ER lumen [51]. These results suggest that ERdj2 spans the ER membrane only twice (see b)) [51]. Within its cytosolic C-terminal part, ERdj2 contains a domain of unknown function also found in U5 small nuclear ribonucleoproteins [153]. ERdj2 further possesses four potential phosphorylation sites [154]. The figure shows the protein domains of ERdj2 without representation of the actual proportions between the different domains.

5.2.2. Localization

Rat and bovine tissue blots show highest amounts of ERdj2 protein in pancreas and liver and lower levels in testis, kidney and brain [151]. In mouse uterus, a nearly four-fold increase in ERdj2 mRNA levels were detected during early pregnancy at implantation sites and was also associated with decidualization, a process during pregnancy when endometrial cells grow and proliferate [159].

Within mammalian cells, ERdj2 is localized within the ER membrane where it resides as an integral membrane protein [51,59,151]. The concentration of ERdj2 in canine pancreatic microsomes was determined to be 1.98 µM [59]. In bovine liver cells small amounts of the protein were found in the mitochondrial pellet of these cells[151]. However, no compartment specific markers were shown so that the purity of the mitochondrial fraction cannot be judged [151]. In cholangiocytes, Western blot analysis showed predominant localization of ERdj2 in the ER with minor portions in the nuclear fraction [160]. In the experiments, histon 3.1 was used as a nuclear marker protein and PDI was used as an ER marker [160]. The potential nuclear ERdj2 pool has however not been assessed in other experiments. It has also to be noted that PDI is not exclusively localized within the ER lumen [161,162] and therefore is a suboptimal ER marker.

5.2.3. Function

Protein-protein interactions

In the presence of ATP, the J-domain of ERdj2 binds to BiP and stimulates the ATPase activity of BiP [59]. With a K_D value of 5 µM, ERdj2 has a lower affinity for BiP than ERdj1 (K_D value of 0.12µM) [142].

Apart from its association with BiP, ERdj2 also associates with the translocon subunits Sec61α, Sec61β, Sec61γ and the translocon-associated protein Sec62 in the ER membrane of mammalian cells [59,151,155] . With respect to the interaction between Sec62 and ERdj2, experimental data from pulldown assays and Surface Plasmon Resonance Spectroscopy (SPR) show that the C-terminal residues 734-760 of ERdj2 interact with the N-terminal residues 11-155 of human Sec62 and that the usually weak binding of the two proteins is strengthened by CK2 mediated phosphorylation of ERdj2 [59,151,154,155]. Interestingly, different coimmunoprecipitations using microsomal extracts revealed binding of Sec62 to ERdj2 but not vice versa [59,151]. In the experiments that failed to show coimmunoprecipitation of ERdj2 with Sec62, antibodies directed against the C-terminus of Sec62 were used [59,151]. A possible interpretation of these results could be that binding of the precipitating antibody induces a conformational change at the N-terminus of Sec62 thereby disabling its binding to ERdj2. If this were the case, only Sec62 that does not bind ERdj2 would be precipitated.

The cytosolic domain of ERdj2 contains two sequences, which are also found in helicases[153]. In helicases, these sequences are responsible for RNA unwinding suggesting that ERdj2 could act as a ribosome receptor [153]. However, a direct interaction of ERdj2 with ribosomes could not be confirmed so far. Differential centrifugation experiments even failed to show that ERdj2 is localized in the same cellular fraction as ribosomes [127,151]. Interestingly though, the ERdj2 binding partner Sec62 was found to bind ribosomes [59,151]. The N-terminal domain of Sec62 contains a polybasic amino acid stretch that was also found in other proteins like ERdj1, for which binding to ribosomes has been confirmed [155]. Sec62 seems to be in close vicinity to ribosomes as Sec62 exhibits increased sensitivity to antibodies after RNAse treatment of permeabilized HeLa and MDCK cells [155]. In vitro experiments further demonstrate that the N-terminal region of human Sec62 (Sec62N) inhibits protein translation by binding to ribosomes [155]. Both subunits of the ribosome seem to be necessary for binding of Sec62 to the ribosome. Since Sec62 is found in the same cellular fractions as ribosomal proteins and also has been demonstrated to bind to ERdj2, it is surprising that ERdj2 is not found in ribosomal fractions [59,151,155]. Using SPR, it was shown that binding of Sec62 to ribosomes results in the displacement of ERdj2 from Sec62[163]. This is according with data, which show a higher affinity of Sec62 for ribosomes (Kd=0.13nM) than for ERdj2 (Kd=5nM) [2,155]. The experimental results argue for two different functional pools of Sec62 [155]. Firstly,

Sec62 can be bound to ERdj2 in the ER membrane. Secondly, Sec62 can dissociate from ERdj2 and associate with ribosomes which results in the inhibition of translation. Similar to ERdj1, Sec62N can also interact with emerging proteins, suggesting that ERdj2 binds near the tunnel exit of the large ribosomal subunit [134,155]. Remarkably, ERdj1 and Sec62 fulfill similar functions. Pre-incubation of ribosomes with ERdj1 prevents Sec62 from binding to ribosomes, confirming that ERdj1 and Sec62 bind to the same ribosomal site [155]. Association of Sec62 with ribosomes prevents the initiation of protein translation in a comparable manner as association of ERdj1 with ribosomes does [155].

Regulation of protein translocation into the Endoplasmic Reticulum

Proteins that are meant to enter the ER have to be translocated across the ER membrane during (co-translational) or after (post-translational) translation [13]. Proteins are translocated across the ER membrane via the Sec61 translocon [13]. BiP inactivation using subtilase cytotoxin (SubAB) as well as small interfering RNA (siRNA) mediated downregulation of Sec62 reduces translocation efficiency of several proteins supporting the idea that BiP and Sec62 play a role in regulating protein translocation across the ER membrane [164-166]. ERdj2 downregulation using siRNA or knockout of ERdj2 also results in the impaired translocation of several substrate proteins [165,167,168]. The role of ERdj2 in translocation has been assessed by different groups and the obtained data will be summarized in the following.

With regard to post-translational translocation in yeast, it is well established that ERdj2 and Sec62 act as accessory factors promoting post-translational translocation of secretory proteins [13]. Based on the cryo EM structure of the yeast Sec61 complex, it was recently proposed that ERdj2 and Sec62 play a role in opening the lateral gate of the translocon to enable signal peptide integration and translocon opening during post-translational translocation [169]. With regard to mammalian cells the effect of ERdj2 on post-translational translocation has only been investigated to a small extent. In mammalian cells, only a slight defect in post-translational translocation of the small secretory protein preprocecropin A (ppcecA) was observed upon ERdj2 downregulation [167]. Futhermore the post-translational translocation of the two small presecretory proteins preproapelin and prestatherin was impaired upon downregulation of ERdj2 [168]. Also, a transient association of TA-proteins with ERdj2 has been observed during early stages of translocation even though post-translational translocation of TA-proteins was not impaired when ERdj2 was downregulated [164,167,170,171]. All in all, the effect of ERdj2 on post-translational translocation in mammals has to be examined in more detail in the future.

With respect to co-translational translocation, ERdj2 controls the translocation of many proteins including aquaporin 2 (AQP2), two derivates of Prion Protein (PrP), the invariant chain of the human class II major histocompatibility complex (IVC), and the co-chaperone

ERdj3 as was demostrated in HeLa and NIH/3T3 cells [167]. Still, there are also co-translationally translocated proteins that are ERdj2-independent during their translocation [167]. The factors that determine whether a protein relies on ERdj2 during co-translational translocation have been extensively discussed in one of our recent reviews[124]. Accordingly, BiP and Sec62 dependency seems to be determined by similar factors as dependency on ERdj2 [166]. However, it was shown that some proteins are solely dependent on BiP or Sec62 whereas others only depend on ERdj2 for their proper translocation [164,166]. Thus, ERdj2, Sec62 and BiP can work together but can also act independently of each other during protein translocation.

Overexpression of ERdj2 in human liver cell line HuH-7 and human embryonic kidney cells HEK293 results in decreasing levels of multi-spanning membrane proteins in the ER membrane, as was shown for the S envelope protein of HBV (HBV.S), the L envelope protein of HBV (HBV.L), the M envelope protein of the mouse hepatitis coronavirus (MHV.M), and the cluster of differentiation 63/lysosomal-associated membrane protein 3 (CD63/LAMP-3)[172]. ERdj2 overexpression did not affect protein levels of soluble or single-pass membrane proteins [172]. Since transcript levels of HBV.S and CD63/LAMP-3 were only slightly reduced in ERdj2 overexpressing cells, the decrease in protein levels was not due to a decrease in transcription but occurred at the posttranscriptional level. Mutational analyses revealed the HPD site, and accordingly BiP-interaction, to be important in regulating protein levels of multi-spanning membrane proteins indicating that BiP and ERdj2 cooperate in reducing the levels of multi-spanning membrane proteins [172]. The mechanism how levels of multi-spanning membrane proteins are affected by ERdj2 and BiP has not been elucidated until now.

Determining protein fate at the translocon

Binding of ERdj2 to target proteins not only facilitates their translocation across the ER membrane but can also target translocation-incompetent proteins to their degradation. Stable association of ERdj2 with a preprolactin mutant with an inserted zink finger domain was observed under in vitro conditions although preprolactin is usually translocated independently of ERdj2 [167,173]. Even though the preprolactin mutant was associated with ERdj2, translocation of preprolactin was not successful [173]. The prion protein contains an intrinsically disordered domain (IDD) [166], which might confer steric hindrance to the protein. Translocation of prion protein into the ER requires its association with ERdj2. Association of prion protein with ERdj2 results in complete translocation into the ER, while in the case of the preprolactin mutant, association with ERdj2 results in translocational arrest [173]. ERdj2-dependency of the prion protein mainly results from the presence of a positively charged domain positioned directly next to the signal peptide [166]. While the authors suggest that the association of ERdj2

to the preprolactin mutant might be due to steric hindrance, ERdj2-dependency could also be based on the two additional, positively charged histidine residues within the zink finger domain [173,174]. Yet, the two positive histidine residues are not located in direct vicinity of the signal peptide, which rather points to a combined effect of positive charges and steric alterations that confers ERdj2 dependency to the preprolactin mutant. All in all, the presented experimental data point to a role of ERdj2 in determining the fate of proteins that are delayed during translocation into the ER lumen [173].

ERdj2-mediated silencing of the IRE1α signaling during prolonged ER-stress

IRE1α is activated during ER-stress [112]. Upon persistent ER-stress, IRE1α signaling is alleviated [113]. Accordingly, binding of BiP to IRE1α decreases at the onset of ER-stress and increases when ER-stress endures [103,113]. In ERdj2 deficient HEK 293 cells, IRE1α stays activated even under conditions of enduring ER-stress indicating that ERdj2 attenuates IRE1α signaling during later stages of ER-stress [113]. IRE1α activity was determined by its degree of phosphorylated IRE1α, the prevailing levels of sXBP1 and the amount of IRE1α clustering [113]. Transfection of ERdj2 deficient cells with wildtype ERdj2 suppresses IRE1α signaling upon persistent ER-stress [113]. A J-domain defective ERdj2 mutant was not able to suppress IRE1 signaling [113]. This indicates that the J-domain mediated interaction between BiP and ERdj2 is important for suppression of IRE1 signaling in HEK293 cells [113]. ERdj2 is found in a complex with the Sec61 translocon and IRE1α in HEK293 cells [113]. Coimmunoprecipitation assays revealed that the Sec61 translocon acts as a linker between ERdj2 and IRE1α [113]. In vitro experiments show that a Sec61/ERdj2 binding deficient IRE1α mutant can bind BiP in the absence but not in the presence of ATP [113]. This is probably due to low substrate affinity of ATP bound BiP. However, there is no difference in the amount of coimmunoprecipitated BiP with the wildtype IRE1/Sec61/ERdj2 complex in the presence or absence of ATP [113]. These results suggest that stimulation of BiP ATPase activity by ERdj2 in the presence of ATP results in stronger binding of BiP to IRE1α [113]. All in all, the presented data suggest a role of ERdj2 in suppressing IRE1α signaling under conditions of prolonged ER-stress, possibly by recruiting BiP to IRE1.

5.2.4. Regulation

So far, little is known about the regulation of mammalian ERdj2. ERdj2 mRNA levels are not upregulated upon thapsigargin or tunicamycin treatment [92]. Recently, the transcription factor Sox9, which is involved in the development of the bile duct, was shown to control ERdj2 transcription [175]. Knockdown of Sox9 in primary biliary epithelial cells resulted in a significant reduction of ERdj2 mRNA levels [175]. Translation of ERdj2 protein was shown to be downregulated by microRNA 206 [176]. Furthermore, computational analysis and in vitro

phosphorylation assays suggest the existence of four potential phosphorylation sites within the ERdj2 protein: S574, S576, T582 and S748 [154]. In vitro peptide filter assays show that S574 and S576 are highly phosphorylated by CK2 [154]. S748 was shown to be phosphorylated in 40% of cases while no significant phosphorylation of T582 by CK2 could be detected [154]. Phosphorylation of ERdj2 by CK2 strengthens binding of ERdj2 to Sec62 [154]. Other effects of ERdj2 phosphorylation have not been reported yet.

5.2.5. ERdj2 in disease

Polycystic liver disease (PLD)

Mutations in the gene encoding the Protein Kinase C Substrate 80K-H (PRKCSH) have been shown to cause autosomal dominant polycystic liver disease (ADPLD) [177]. PRKCSH is the beta subunit of protein glucosidase II, which is important for maturation of glycosylated proteins in the ER lumen [178]. When a cohort of 66 ADPLD patients was analyzed, in 57 of the cases no mutations in the PRKCSH gene could be detected [179]. Further analysis of these 57 genomes revealed heterozygous sequence variants in the ERdj2 gene in 8 of the 57 individuals [179]. Association of ERdj2 mutations with autosomal dominant polycystic liver disease (ADPLD) has first been described in 2004 [179]. So far, more than 20 different mutations in the ERdj2 gene have been detected in patients suffering from polycystic liver disease [180]. Depending on the study, mutation frequency varies between 5,7%[180], 9%[181] and 12%[179]. Secondary structure modeling suggests that some of the described mutations in ERdj2 result in significant conformational changes in the mature protein [180]. Interestingly, ERdj2 mutations are also found in patients suffering from liver cysts but do not meet all the criteria for PLD diagnosis, which suggests a general role for ERdj2 in cyst formation in the liver [182]. However, mutation frequencies are higher and mutations were found to be more deleterious in patients suffering from PLD than in patients with only few liver cysts [182]. While ERdj2 protein levels in cyst tissue was shown to be independent of the mutational state in most cases [160], a reduction of ERdj2 protein levels due to a mutation in the ERdj2 gene was reported in one patient suffering from PLD [183]. These obviously contradictory results could be due to different mutations examined. Waanders et al. examined cyst tissue of patients with an in-frame deletion [160]. Janssen et al. screened chromosomes of the healthy population for this mutation and did not find a statistically significant difference in mutation frequency between healthy individuals and patients suffering from ADPLD suggesting this mutation not to be causative for ADPLD [183]. They found loss of heterozygosity (LOH) and accompanying reduction of ERdj2 protein levels in one of fourteen cysts of a patient carrying a truncating mutation [183]. Based on this finding, the authors suggested that a somatic second-hit mutation

can result in LOH and, consequently, in reduced levels of ERdj2 protein [183]. However, as LOH was only found in one of fourteen cysts of the affected individual, the question remains how heterozygous mutations in the other thirteen cysts promote cyst development. The induction of transheterozygosity by driver genes is discussed as a possible mechanism for developing ADPLD[184]. A similar mechanism is discussed in the development of kidney cysts. A more severe presentation of autosomal dominant polycystic kidney disease (ADPKD) was observed in individuals carrying heterozygous mutations in the polycystic kidney disease 1 gene (PKD1) and polycystic kidney disease 2 gene (PKD2) [185].

Even though the exact mechanism how mutations in the ERdj2 gene trigger cyst formation is still unresolved, there is some experimental data on possible mechanisms. In murine kidney tissue, knockdown of ERdj2 using the Cre/loxP system results in development of kidney cysts, possibly due to a corresponding reduction of polycystin 1 (PC1) protein levels [186]. Polycystin 1 is an integral membrane protein that is encoded by the polycystic kidney disease gene (PKD1) [187]. Mutations in PKD1 are known to cause autosomal dominant polycystic kidney disease [187]. Polycystin 1 contains a G protein-coupled receptor proteolysis site (GPS) [187]. Cleavage of the GPS is required for proper functioning of the protein and cleavage-deficient polycystin 1 mice develop cysts [187]. The development of kidney cysts in ERdj2 knockout mice was prevented by overexpression of polycystin 1. In fact, ERdj2 was shown to form a complex with polycystin 1 in kidney cells [188].

In kidney cells, ERdj2 knockdown selectively activates the IREα branch of the UPR and induces Xbp1 splicing [113,188,189]. Combined downregulation of ERdj2 and XBP1 in mice causes a more severe renal cystic phenotype than isolated downregulation of ERdj2 [188]. Comparison of the polycystin 1 levels in ERdj2 knockout mice and ERdj2/XBP1 double-knockout mice showed that while knockout of ERdj2 prevents cleavage of polycystin 1, ERdj2/XBP1 double-knockout mice showed an even more pronounced reduction of polycystin 1 cleavage[188]. Overexpression of XBP1 in ERdj2 knockout mice on the other hand was able to increase cleavage of polycystin1 possibly by upregulation of other XBP1 target genes [188]. Furthermore, it was found that concomitant postnatal knockout of ERdj2 and XBP1 results in the development of interstitial inflammation and fibrosis in kidneys of mutated mice and that re-expression of XBP1 rescues the pathological phenotype in these mice[190]. In line with these results is the finding that knockdown of the transcription factor Sox9, which downregulates the transcription of ERdj2, induces cyst formation in mouse livers[175]. Closer examination showed that knockdown of Sox9 resulted in increased proliferation, reduced cilium formation and disturbance in biliary epithelial cell polarity, effects that could partially be reversed by overexpression of ERdj2 [175]. Therefore, downregulation of ERdj2 mediated by

Sox9 could present an additional mechanism, which causes the development of hepatic cysts.

Other signaling pathways affected by ERdj2 include the Wnt signaling pathway. In kidney cells and cholangiocytes, ERdj2 knockout using Crispr/Cas9 results in disturbed cilium formation and reduced Wnt signaling suggesting a role of the Wnt signaling pathway in the development of liver cysts [191]. Indeed, it was shown that ERdj2 interacts with the protein nucleoredoxin, which is involved in the Wnt/ β-catenin signaling pathway [163]. ERdj2 contains multiple interaction sites between amino acid residues 210 and 733 for association with the C-terminal region of nucleoredoxin, an interaction that is favored under oxidizing conditions [163]. Under reducing conditions, nucleoredoxin binds to Disshelved 1, thereby inhibiting Wnt/β-catenin signaling [163]. Therefore, interaction of ERdj2 with nucleoredoxin in wildtype cells might constitute an indirect mechanism to activate the Wnt signaling pathway.

In conclusion, impaired maturation of polycystin 1, increased IRE1 signaling as well as impaired Wnt signaling might constitute mechanisms that promote cyst formation in ERdj2-deficient cells.

Cancer

First evidence that ERdj2 mutations can cause malignancies was obtained in 2001 when microsatellite instabilities (MSIs) in the ERdj2 gene were found in colorectal cancers [192]. 48.8% of the cancer tissues analyzed showed MSI in the ERdj2 gene [192]. Since then, association of ERdj2 with many other cancers including gastric cancer, hepatocellular carcinoma (HCC) and breast cancer have been reported [193-196](Table 2).

Table 2 Cancer types associated with ERdj2

Cancer	Association with ERdj2	Reference
Colorectal cancer	• 48,8 % MSI in the ERdj2 gene	[192]
Hereditary nonpolyposis colorectal cancer (HNPCC)-associated small bowel cancer	• 56% frameshift mutations	[197]
Gastric cancer	• 37.5% MSI in the ERdj2 gene • 46.7% MSI in the ERdj2 gene	[193] [194]
Lynch syndrome associated hepatocellular carcinoma (HCC)	• HCC mouse models show that low ERdj2 levels are associated with increased proliferation and decreased apoptosis • 8.1 fold increase in ERdj2 mRNA levels in HCC tissue compared to surrounding liver tissue	[195,198]
Invasive micropapillary carcinoma (IMPC) of the breast	• Report of one patient carrying a missense mutation in the ERdj2 gene	[196]

Neurodegenerative diseases

Analysis of differentially expressed mRNAs and miRNAs in the tissue of four brains of patients suffering from Alzheimer's Disease (AD), showed that ERdj2 was downregulated in Alzheimer's Disease brains [176]. ERdj2 translation can be suppressed by the microRNA miRNA 206 [176]. In Alzheimer's Disease brains miRNA 206 was shown to be upregulated [176] which might be the cause for reduced levels of ERdj2 protein. Downregulation of ERdj2 might lead to impaired protein translocation into the ER, resulting in impaired protein homeostasis, which is a known feature of Alzheimer's Disease.

5.3. ERdj3

5.3.1. Structure

ERdj3, also known as ERj3p [52], HEDJ [60], hDj9/mDj9 [199], ABBP-2 [200]or DNAJB11 [201] was first discovered as a homolog of yeast Scj1p in dog pancreas microsomes in 1999 [52]. ERdj3 is a protein of 358 amino acids with a calculated mass of 38.2 kDa [52]. Different ERdj3 mRNA transcripts 1.6kb, 2.0kb and 2.2kb in size were detected in human tissues [60,92,200]. Within the PubMed nucleotide database even more ERdj3 mRNA transcripts are documented of which some seem to be non-coding mRNA transcripts. It can be assumed that the different transcripts encoding the ERdj3 protein contain different regulatory elements. A respective analysis has not been performed so far.

As schematically shown in Fig.14, ERdj3 contains a cleavable, N-terminal signal peptide of 2.3 kDa, which is followed by the J-domain (aa17-88) [60,202] (NP_001015021), a glycine/phenylalanine-rich region (aa88-129), and a cysteine rich region (aa160-201) [60,202]. The cysteine rich region domain is enclosed by two domains (aa129-160 and aa201-251), which are expected to fold into one functional domain with a central substrate binding hydrophobic pocket [60,202]. Under oxidizing conditions, as they prevail in the ER, the cysteines within the cysteine rich region form intramolecular disulfide bridges [203]. ERdj3 also contains two potential glycosylation sites at position 5 and 261 but lacks the KDEL sequence of its yeast homolog Scj1p [60,204]. The first N-terminal glycosylation site is located within the signal peptide suggesting that only the glycosylation site at position 261 is glycosylated [60]. Indeed, glycosylation of ERdj3 in microsomes was confirmed by EndoH treatment [60].

Originally, ERdj3 was thought to be present as a dimer formed by its C-terminus (aa251-329) [202]. Different experiments have demonstrated that ERdj3 forms as a multimer [201,205,206]. Gel filtration experiments, analytical ultracentrifugation and electron microscopy was used to show that in medium secreted from HEK293 cells, ERdj3 forms a diamond-shaped tetramer [201]. The tetramer proved to be dimers of two dimers [201]. Whereas phenylalanine at position 326 seems to be essential for dimer formation, two beta sheets within the cysteine-rich domain seem to be important for tetramer formation [201]. A recent study revealed that within the ER, ERdj3 forms a dimer, which is bound to Stromal cell derived factor 2 like 1 (SDF1L). When ERdj3 is not bound to SDF1L, ERdj3 forms a tetramer and is secreted into the extracellular space [207]. Another group reported the existence of ERdj3 as a monomer [203]. Whether this is due to experimental conditions or due to the existence of different ERdj3 pools is not clear.

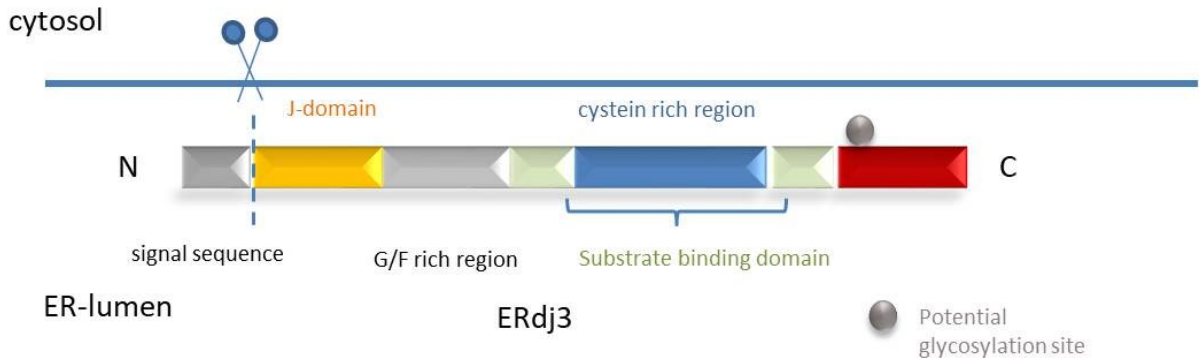

Fig. 14 Schematic structure of ERdj3
The cleavable signal peptide of ERdj3 is followed by the J-domain [60,202,204]. Following the J-domain, ERd3 contains a G/F rich region and a cysteine rich region which is enclosed by two domains that fold into a functional domain forming a hydrophobic pocket which is supposed to be important for binding of client proteins [60,202]. Furthermore, ERdj3 contains a glycosylation site [204]. The figure shows the protein domains of ERdj3 without representation of the actual proportions between the different domains.

5.3.2. Localization

ERdj3 mRNA could be found in all human tissues examined with highest transcript levels in liver, placenta, kidney, heart and testis [60,92].

ERdj3 was found to be present in dog pancreas microsomes where the calculated concentration of ERdj3 is 0.29 µM, which is in the same range as ERdj1 [52,204]. Immunostaining experiments confirmed the ER-localization of tagged and untagged ERdj3 in various cell lines [60,208,209]. According to proteinase K digestion ERdj3 is a luminal, ER-resident protein [60]. In the ER, ERdj3 mainly forms large protein complexes and only a minor fraction of ERdj3 is present as a free pool [210]. It was also shown that ERdj3 is membrane-associated within the ER but that its membrane association is weaker than that of BiP [60,92].

Apart from its localization in the ER, ERdj3 is also secreted to the extracellular space as was shown in HEK293- and Huh7-cells [211]. Secretion to the extracellular space is inhibited by co-expression of the ERdj3 interaction partner, SDF2L1, in HEK 293 cells [207].

Experimental data suggest that small amounts of ERdj3 might also be located in the nucleus or cytoplasm of cells. Apolipoproteins are part of lipoproteins, which constitute a transport for triglycerides and cholesterol in the blood. There are different lipoproteins (chylomikrones, VLDL, IDL, LDL and HDL) which differ by their densities as well as their associated apolipoproteins. ApolipoproteinB (ApoB) is a main apolipoprotein of chylomicrones, VLDL, IDL and LDL lipoproteins. Two isoforms of ApoB exist, ApoB 100 and the smaller isoform ApoB 48. While ApoB 100 is mainly associated with VLDL and LDL, ApoB 48 is associated with chylomicrones. Within the nucleus ApoB mRNA editing enzyme catalytic polypeptide 1 (Apobec1) exchanges one nucleotide within the ApoB mRNA so that a stop codon is introduced. By this mechanism the smaller isoform of ApoB, ApoB 48, is

translated [212]. In murine liver cells, ERdj3 was shown to regulate ApoB mRNA editing, which predominantly takes place in the nucleus [200]. It was shown that downregulation of ERdj3 suppressed mRNA editing of ApoB [200]. Regulation of ApoB mRNA editing by ERdj3 might be achieved by binding of ERdj3 to Apobec1 which was demonstrated in cell lysates [200].

Evidence for a cytosolic ERdj3 fraction came from data showing that ERdj3 is able to bind the cytosolic/nuclear Hsp70 chaperones Hsp72 and Hsp73 [200]. ERdj3 was further shown to stimulate the ATPase activity of cytosolic localized yeast Hsp70 chaperone Ssa1 [213]. Interestingly, ERdj3 was also localized in the cytoplasm of cells of tissue from oral cavity squamous cell carcinoma as was shown by immunohistochemical stainings [214].

5.3.3. Function

Interaction with Hsp70 chaperones

ERdj3 was discovered as a novel Hsp40 co-chaperone in BiP-binding assays performed in dog pancreas microsomes [52]. As was shown for ERdj1 and ERdj2, ERdj3 binds to BiP in the presence of ATP and the J-domain of ERdj3 stimulates BiP's ATPase activity [60]. Binding to BiP as well as stimulation of BiP's ATPase activity is abolished by mutation of the HPD site within the J-domain of ERdj3 [92]. Experiments using Kar2, the yeast homolog of BiP and Ssa1, the yeast homolog of the cytosolic Hsp70 chaperones Hsp72 and Hsp73, revealed that ERdj3 cannot activate the ATPase activity of Kar2 in yeast [213]. Yet, a cytosolic variant of ERdj3 can activate the ATPase activity of cytosolic Ssa1 when expressed in yeast [213]. Also, ERdj3 was shown to stimulate ATPase activity of cytosolic Hsc70 in HepG2 cell extracts [200]. These findings are in accordance with the finding that ERdj3 does bind to the cytosolic Hsp72/73 in HepG2 S-100 cell extracts [200]. Interestingly, in most reports, ERdj3 binds and activates the ATPase activity of ER-localized BiP [60,92,204,209,215]. Both, binding of ERdj3 to cytosolic chaperones as well as to BiP was demonstrated in in vivo experiments [200,209]. These different results indicate that within the cytosol, ERdj3 binds to cytosolic Hsp70 chaperones while within the ER lumen, ERdj3 bind to BiP.

Substrate binding

ERdj3 can bind substrates in the absence of BiP [92]. Binding of ERdj3 to substrates seems to be ATP independent as equal amounts of ERdj3 are bound to substrate proteins in the presence and the absence of ATP [205]. Known substrates of ERdj3 include Immunoglobulin heavy and light chains [92,210], thyroglobulin [203] and denatured luciferase [205] as well as beta-glucocerebrosidase [216] , amyloid protein [211], transthyretin [211], Z variant α1-antitrypsin (ZAAT) [217,218] and Shiga toxin [219]. All substrate proteins and interaction partners of ERdj3 identified so far are summarized in Table 3.

Different domains of ERdj3 are required for substrate binding. Firstly, substrate binding is impaired by deletion of the hydrophobic substrate binding pocket of ERdj3 that encloses the cysteine rich domain [202]. Secondly, substrate binding is also strongly impaired after pretreatment of ERdj3 with DTT, which reduces the intramolecular disulfide bridges within the cysteine rich region [203]. Moreover, mutations within the two beta sheets of the cysteine rich region or deletion of either the C-terminal dimerization domain or the phenylalanine at position 326 results in a reduction of substrate binding [201].

As has been described above, ERdj3 was shown to bind to different substrates in a BiP-independent manner [92]. Accumulating data indicate that binding of ERdj3 to substrates and the transfer of the substrate to BiP is a two-step-process. Evidence for this was obtained in MDCK cells, in which the mobility of BiP significantly decreased upon ERdj3 overexpression [209]. This effect was lost when overexpressing a BiP mutant, which was not able to bind to client proteins [209]. The effect was also lost when an ERdj3 J-domain mutant was overexpressed supporting the idea that ERdj3 delivers client proteins to BiP via a direct interaction with BiP [209]. Fluorescence Resonance Energy Transfer (FRET) analysis shows that binding of different nucleotides, substrates, or co-chaperones induces conformational changes in BiP [215]. Depending on the binding partner, the substrate binding domain and the nucleotide binding domain are at different distances to each other [215]. Similarly, the lid of BiP is either in an open or a closed state depending on the binding partner of BiP [215]. Binding of ERdj3 to BiP has been shown to affect its conformational state and thus can prime BiP for substrate binding [215]. When the delivery of substrate to BiP is impaired by mutations in the J-domain of ERdj3, binding of ERdj3 to the substrate is prolonged thereby possibly determining the further fate of the substrate [92,201]. This idea is encouraged by data showing that in the presence of the ERdj3 HPD mutant, binding of BiP to substrate is weaker and shorter than in the presence of wildtype ERdj3 [92].

There is experimental evidence that release of ERdj3 from substrates is mediated by BiP [92]. Mutations in the J domain of ERdj3 prevent the release of ERdj3 from its substrate [205]. Binding of ERdj3 to BiP goes along with stimulation of the ATPase activity of BiP [52,60]. In the absence of ATP, BiP does not induce the release of ERdj3 from substrate [205]. It was further shown that BiP mutants that cannot hydrolyze ATP to ADP cannot release ERdj3 from substrate indicating that the ability of BiP to hydrolyze ATP is required for ERdj3 substrate release [205]. The results indicate that after stimulation of ATP hydrolysis by ERdj3 or upon exchange of ADP for ATP, ERdj3 is released from its substrate [205]. This assumption is supported by pulse-chase experiments showing that ERdj3 is only bound to heavy chains at the beginning of the folding process whereas BiP remains bound to the substrate until folding is completed [92]. Experiments in HEK293 cells show that immunoglobulin light chains are

released faster from ERdj3 than immunoglobulin heavy chains [206]. Therefore, specific features of the target protein seem to further determine the binding strength and the release rate of substrates from ERdj3.

Table 3 Protein interaction partners of ERdj3

Interaction partner	Experimental conditions/Cell type	Reference
Multiprotein complex (together with Grp94, BiP, SDFL1, PDI, UDP-GT, ERp57, cyclophilin B)	Mouse lymphoma cell line (Ag8(8))	[210]
Apolipoprotein B mRNA editing enzyme , catalytic polypeptide 1 (apobec1)	In vitro, S100 extracts of human liver carcinoma cell line (HepG2)	[200]
Binding immunoglobulin protein (BiP)	In vitro, kidney fibroblast like cell line derived from green meercats (COS-1), Lysates of mardin-darby canine kidney cells (MDCK), Human embryonic kidney cells (HEK293)	[92,201,204,209,220]
Shiga toxin	Microsomes from green meercat kidney cells (Vero cells)	[60]
Sec61	MDCK cells	[209]
Stromal cell derived factor 2 like 1 (SDF2L1)	dog pancreas microsomes	[204]
Stromal cell derived factor 2 (SDF2)	HEK293 cells	[220]
Part of ribosome: RAMP complexes	Canine pancreas microsomes	[221]
Calreticulin	Human liver cells (Huh 7.5)	[217]
Cholera toxin A1 subunit (CTA1)	In vitro	[222]
Salmonella Leucin-rich repeat protein	In vitro, human cervical cancer cells (HeLa)	[223]
Glucocerebrosidase	HeLa cells, fibroblasts	[216]

Heat shock protein 72	S100 extracts of human liver carcinoma cell line (HepG2)	[200]
Heat shock protein 73	S100 extracts of human liver carcinoma cell line (HepG2)	[200]
Unassembled IgG heavy chains	Mouse lymphoma cell line (Ag8(8)), COS-1 cells	[92,202,205,206]
NS-1 non-secreted kappa light chain	COS-1 cells	[92,206]
Transthyretin (TTR)	HEK 293T cells	[201,211]
Temperature-sensitive mutant of the vesicular stomatitis virus glycoprotein (VSV-G ts045)	COS-1 cells	[92]
Thyreoglobulin	In vitro	[203]
Denatured luciferase	In vitro	[205]
Amyloid precursor protein	HEK 293T cells	[211]
α 1 antitrypsin	Huh 7.5 cells, HEK 293 cells	[217,220]
Endonuclein	In vitro	[224]
A disintegrin-like and metalloprotease domain with thrombospondin type 1 motifs 9 (ADAMTS9)	HEK293 cells	[225]
Transmembrane glycoprotein K1 of the Kaposi sarcoma-associated herpesvirus	HEK293 cells	[226]
Proinsulin	Mouse pancreatic β cells (NIT-1)	[227]
Protogenin	HEK293 cells In vitro	[228]

Abbreviations used in this table are listed in the list of abbreviations.

Controlling substrate degradation

There is plenty of evidence that ERdj3 mediates retrotranslocation of unfolded proteins from the ER into the cytosol, which is an early step in delivery of proteins to degradation pathways [65]. There is experimental evidence that ERdj3 is involved in the transport of Shiga toxin out of the ER and into the cytosol via the Sec61 translocon [219]. In MDCK cells, a direct interaction between ERdj3 and the Sec61 translocon was shown for the first time, which might be the reason for the low mobility of ERdj3 in the ER [209]. It was found that

retrotranslocation of cholera toxin is also mediated by ERdj3 [222]. Furthermore, ERdj3 downregulation impairs the transport of Simian Virus 40 major capsid protein VP1 from the ER to the cytosol [229].

There is also evidence that the epithelial sodium channel (ENaC) is an ERAD target of ERdj3 [230]. ENaC is present in the lumen and within the membrane of the ER [230]. When the channel is injected into Xenopus oocytes together with ERdj3, the current that passes through the channel is reduced [230]. When a FLAG-tagged ENaC channel is injected, less ENaC is expressed at the surface in the presence of ERdj3 indicating that ERdj3 probably mediates the degradation of the channel within Xenopus oocytes [230]. Inhibition of the proteasome by MG-132 treatment in ERdj3-overexpresing Xenopus oocytes prevents reduction of the current passing the ENaC channel [230] further supporting the notion that ERdj3 targets ENaC to the degradation pathway.

The fact that inhibition of the proteasome affects degradation pathways within the ER lumen implies that a feedback signaling from the cytosol to the ER lumen exists. In fact, initial data provide possible mechanisms for a feedback communication between the cytosol and the ER. In human HeLa cells, two cytosolic Hsp40 cochaperones, DNAJB12 and DNAJB14, which are anchored at the ER membrane, have been shown to be involved in retrotranslocation of SV40 VP1 from the ER to the cytosol [229]. Furthermore, experiments in yeast showed that the 19S regulatory particle (19S RP) of the proteasome associates with the Sec61 translocon [77]. These data point to possible molecular mechanisms enabling a feedback from the cytosol to the ER lumen possibly via the Sec61 translocon.

In yeast it was shown that a functional J-domain is required for the delivery of substrates to the ERAD pathway. Interestingly, it is not the luminal ERdj3 but the cytosolic form that can substitute for the two cytosolic Hsp40 co-chaperones – Hlj1 and Ydj1 – and that can interact with the yeast Hsp70 chaperone Ssa1 in order to deliver the yeast membrane protein Step6 to the ERAD pathway [213].

Gaucher's disease is a lysosomal storage disease based on mutations in β-glucocerebrosidase [216]. ERdj3 was shown target wildtype as well as mutant β-glucocerebrosidase to degradation pathways even though mutant β-glucocerebrosidase would be functional if it was folded in the right way [216]. Therefore, ERdj3 downregulation leads to increasing numbers of folded and active β-glucocerebrosidase mutants [216]. Analyses of these β-glucocerebrosidase mutants show that increased amounts of calnexin are attached to them when ERdj3 is downregulated [216]. The ER-resident chaperone calnexin promotes folding of glycosylated substrates within the ER lumen [71]. So the inhibition of β-glucocerebrosidase degradation by ERdj3 downregulation results in more β-glucocerebrosidase being targeted to the folding pathway [216](see Figure 12 b)).

Folding and Maturation

Besides being involved in substrate degradation, ERdj3 is also important for folding and maturation of client proteins. In HeLa cells two proteins, stromal cell derived factor 2 (SDF2) and stromal cell derived factor 2 like 1 (SDF2L1), were found to act as cofactors for ERdj3 to prevent protein aggregation within the ER [220]. Association of ERdj3 with SDF2L1 was confirmed in HEK293 cells as well as in dog pancreas microsomes and association of ERdj3 with SDF2 was confirmed in HEK293 cells [204,220]. Both proteins can be stabilized by overexpression of ERdj3 [220]. Transient expression of the two proteins prevents aggregation of an α-1-antitrypsin mutant [220]. Alpha-1-antitrypsin is a secreted protease inhibitor and mutations in the protein can cause α-1-antitrypsin deficiency [231]. Alpha-1-antitrypsin deficiency results in the development of pulmonary emphysema as well as liver fibrosis and liver cirrhosis. The Z variant α-1-antitrypsin (ZAAT) is the most common α-1-antitrypsin mutant in which a single amino acid is substituted at position 342 [231]. ERdj3 associates with both, the wildtype α-1-antitrypsin protein and ZAAT in the ER of human liver cells [217,232]. Association of ERdj3 with wildtype α-1-antitrypsin and ZAAT can be observed in the cell lysate as well as in the medium of cells, indicating that the complexes do not only form in the ER but are also secreted [232]. Pulse chase experiments show that ERdj3 downregulation results in increased intracellular ZAAT degradation suggesting that ERdj3 delays degradation of ZAAT [217] (see Figure 15 c)). Accordingly, the delay in degradation leads to increased levels of intracellular ZAAT [217]. This has also been shown in experiments in which ERdj3 was overexpressed and increased protein levels and aggregation of intracellular ZAAT were demonstrated [217,218]. Apart from preventing proteasomal degradation of ZAAT, ERdj3 is further involved in the prevention of disposal of ZAAT by a lysosomal/exosomal/autophagy degradation pathway [217].

As was described before, ERdj3 binds to target proteins and delivers them to BiP for folding [205]. Mutation of the HPD site within ERdj3, which impairs binding of ERdj3 to BiP, leads to enhanced binding of aggregation prone proteins to ERdj3 but also to SDF2L1 [220]. This shows that when BiP is not available for acceptance of the target proteins a complex between ERdj3 and SDF2L1 prevents aggregation of substrate proteins that stay bound to ERdj3 and SDF2L1 under these conditions [220]. This hypothesis is further supported by the fact that SDF2L1 is upregulated during ER-stress when the protein load within the ER might exceeds the folding capacity of the ER [233]. Prevention of protein aggregation by co-expression of the ERdj3-SDF2L1 complex was shown for α1-antitrypsin mutants (in HEK 293 cells) [207], denatured GSH S-transferase (in vitro) [207] and κLC (in HEK293 cells) [207]. Whether overexpression of SDF2L1 alone is sufficient to prevent aggregation of misfolded cargo is not clear. While one group showed that for NHK QQQ, a non-glycosylated α-1-antitrypsin

mutant, overexpression of SDF2L1 alone is sufficient to significantly decrease protein aggregation in HEK293 cells [220], another group could not confirm this [207].

As outlined above, in the case of β-glucocerebrosidase, ERdj3 downregulation results in increased folding and maturation of the substrate protein while in the case of ZAAT, ERdj3 downregulation increases degradation of the substrate [216,217] (see Figure 15). These contradictory results can be explained by the fact that similar to its association with β-glucocerebrosidase, ERdj3 also competes with the calnexin/calreticulin pathway for α1-antitrypsin binding [216,217,232]. When ERdj3 is downregulated, increasing amounts of calreticulin are associated with ZAAT [217]. When glycosylated proteins cannot be correctly folded by the calnexin/calreticulin cycle, mannosidase residues of the substrate are cleaved, which results in recruitment of EDEM1 and cleavage of an additional mannosidase residue [71]. This additional cleavage event by EDEM1 enables binding of ERAD components OS9 and SEL1 to the misfolded protein [71]. OS9 and SEL1 target misfolded substrates to the retrotranslocation channel for degradation via ERAD [71]. While increased engagement of β-glucocerebrosidase with calnexin results in more functional protein, in the case of ZAAT increased association with calreticulin is also accompanied by increased association with EDEM1 [216,217]. The different effects might be the consequence of differing degrees of misfolding or other differences between the proteins such as that β-glucocerebrosidase is a lysosomal protein while ZAAT is a protein meant to be secreted. The competing association of ZAAT and β-glucocerebrosidase with ERdj3/BiP and calnexin/calreticulin and the different outcomes are depicted in Figure 15.

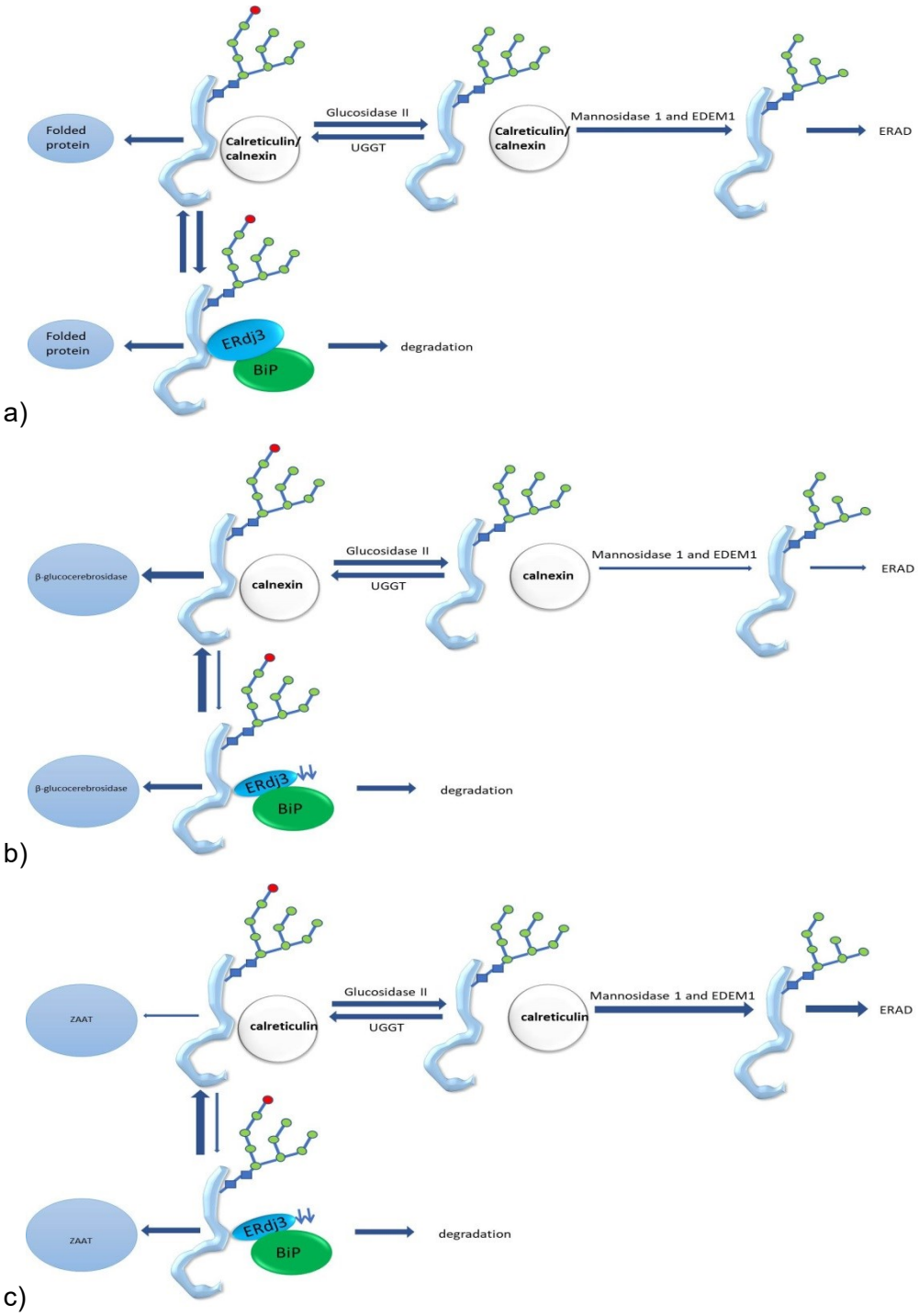

Fig. 15 Competition between the ERdj3/BiP and the calnexin/calreticulin pathways can affect the fate of substrate proteins.

a) Calnexin and calreticulin assist ER luminal glycoproteins to gain their final structure [71]. During their folding process, substrates undergo repetitive cycles of de- and re-glucosylation [71]. In the end, the substrate either achieves its final structure or the substrate undergoes mannose trimming by ER α1,2-mannosidase I and EDEM1 which targets the substrate to the ERAD pathway [71]. b) and c) For β-glucocerebrosidase and ZAAT, it was shown that downregulation of ERdj3 results in increased association of the proteins with the calnexin/calreticulin pathway [216,217]. b) In the case of β-glucocerebrosidase, association of the protein with calnexin upon downregulation of ERdj5 results in an increased amount of folded substrate [216]. c) In the case of ZAAT more

substrate protein is associated with EDEM1 and is subsequently degraded when increased amounts of ZAAT are associated with calreticulin upon downregulation of ERdj3 [217]. Red circles represent glucose residues while green circles represent mannose residues. Blue squares represent N-acetylglucosamine.

Controlling extracellular proteostasis

ERdj3 is secreted into the extracellular space, where it is involved in controlling extracellular proteostasis [211]. In HEK293 and Huh7 cells, ERdj3 was shown to be secreted into the extracellular medium and secretion increased after thapsigargin treatment [211]. Also, increasing amounts of ERdj3 are found in mice blood serum after feeding the mice with a high fat diet, which induces ER-stress [211]. Also, the ER-stress sensor ATF6 seems to be involved in controlling the secretion rate of ERdj3 [211]. While activation of the IRE1 as well as the ATF6 pathway increases ERdj3 mRNA and protein levels in HEK293 cells, only activation of ATF6 affects ERdj3 secretion into the medium in HEK293 cells [211]. ERdj3 can either be secreted on its own and bind to extracellular, aggregation prone proteins such as amyloid beta protein (Aβ) or mutated transthyretin (TTR) or it can be secreted as a complex bound to its substrate [211]. While secretion of ERdj3 alone is increased upon ATF6 activation, the secretion of ERdj3 bound to misfolded substrate (ERdj3/substrate complex) is reduced upon activation of ATF6 [211]. A possible explanation for this observation is the ATF6-mediated upregulation of BiP [121]. Increased levels of BiP probably result in increased levels of trimeric BiP-ERdj3-substrate complexes within the ER resulting in refolding of substrates rather than in its secretion [211]. This hypothesis is supported by the fact that BiP overexpression can reduce the co-secretion of WT ERdj3/substrate complexes, whereas overexpression of BiP does not have an effect on co-secretion of an ERdj3 J-domain mutant bound to a substrate protein [211]. In vitro experiments show that recombinant ERdj3 can prevent aggregation of amyloid beta protein [211]. In cells treated with toxic prion proteins, a misfolded prion conformer, ERdj3 treatment has a cytoprotective effect. These results indicate that ERdj3 is also crucial for maintaining proteostasis in the extracellular space [211]. Recently, ZAAT was identified as another protein that can be secreted in a complex with ERdj3 [232]. Secretion was significantly increased upon calreticulin downregulation [232]. This result also supports the thesis that substrates are secreted in a complex with ERdj3 when substrates cannot be delivered to the folding pathway. Immunoprecipitation of α1-antitrypsin in detergent treated or untreated medium showed that more α1-antitrypsin immunoprecipitated after detergent treatment [232]. These results indicate that ERdj3 and ZAAT are secreted to the medium encoated in membrane-formed vesicles. These vesicles were identified as exosomes [232]. Whether this is also the case for other substrate proteins has not been investigated so far.

Controlling neurogenesis during embryonic development

ERdj3 was found to be a ligand of protogenin, a member of the immunoglobulin superfamily [228]. Binding of ERdj3 to protogenin was demonstrated in yeast-two-hybrid assays as well as by fluorescence microscopy in HEK 293T cells [228]. Transfection of embryonic carcinoma cells P19 with ERdj3 resulted in a reduction of differentiated neuronal cells [228]. The effect was abolished by addition of an antibody against protogenin indicating that ERdj3 exerts its inhibitory effect via a direct interaction with protogenin [228]. The inhibitory effect of ERdj3 on neuronal differentiation was confirmed in vivo by electroporesis of ERdj3 into the chick neuronal tube [228]. Addition of ERdj3 containing medium to protogenin and radil (an intracellular ligand of protogenin) overexpressing AD293 cells (a transformed human embryonic kidney cell line) results in increased levels of cell migration and increased amounts of activated β1- and α5-integrins at the cell surface [234]. Those effects were suppressed upon downregulation of radil using siRNA, indicating that after ERdj3 has bound to protogenin, signaling cascades are activated that involve radil and that promote cell migration probably by activation of integrins [234].

Sec61-dependent regulation of ER luminal calcium homeostasis

Downregulation of ERdj3 in HeLa cells revealed the impact of ERdj3 on calcium metabolism [93]. ERdj3 downregulated HeLa cells showed increased calcium leakage from the ER into the cytosol [93]. Normalization of calcium efflux from the ER was achieved by simultaneous downregulation of Sec61α and ERdj3 indicating that ERdj3 is important for gating the Sec61 translocon and keeping it in a closed state [93]. The role of ERdj3 in calcium metabolism, also in the context of other ER-resident co-chaperones is reviewed in Daverkausen-Fischer L, Prols F. Regulation of calcium homeostasis and flux between the endoplasmic reticulum and the cytosol. J Biol Chem 2022: 102061.

5.3.4. Regulation

ERdj3 transcription and translation is increased during ER-stress. Treatment with tunicamycin or thapsigargin, known inducers of ER-stress, increase ERdj3 mRNA levels in HepG2 cells as well as ERdj3 protein levels, as shown in HeLa cells and renal glomerular endothelial cells [92,235]. During ER-stress, the transcription factor sXBP1 is rapidly translated due to unconventional splicing of the prevailing cytosolic levels of Xbp1 mRNA [107]. As shown in ER-stressed murine lymphoma cells and murine splenic B cells, sXBP1 protein binds to the ERdj3 promoter and promotes transcription of ERdj3 [236]. Downregulation or knockout of XBP1 in mouse embryonic fibroblasts and murine lymphoma cells results in reduced transcription and translation of ERdj3 in these cells [109,236].

The co-chaperone ERdj6 also seems to control the protein levels of ERdj3 [93]. Downregulation of ERdj6 was shown to result in increasing ERdj3 protein levels [93]. This effect was not due to cellular stress that might have been elicited by downregulated ERdj6 levels as was shown by unchanged BiP and CHOP mRNA levels as well as sXbp1 levels [93]. Since ERdj3 mRNA levels remained unaffected in ERdj6 deficient cells [93], ERdj6 is thought to destabilize ERdj3 protein. Besides this destabilizing effect of ERdj6 on ERdj3 protein, reduced ERdj3 protein levels were also obtained upon downregulation of Sec61α [93]. Under physiological conditions, ERdj3 is transported co-translationally into the ER lumen via the Sec61 translocon [167]. Thus, decreased ERdj3 protein levels could be due to defective translocation upon downregulation of the Sec61α subunit [93]. However, if this were the case, a decrease in the level of most ER luminal proteins should be expected as most proteins are translocated into the ER lumen via the Sec61 translocon.

Secretion of ERdj3 to the extracellular space is also increased during ER-stress as was already discussed [211]. Taken together, the current experimental data show that ERdj3 transcription and translation as well as ERdj3 secretion is upregulated during ER-stress indicating that ERdj3 is required to reestablish intra- and extracellular homeostasis undER-stressful conditions [109,211,236].

5.3.5. ERdj3 in disease

ERdj3 was shown to be involved in viral and bacterial infection. As already discussed in an earlier section, ERdj3 has been reported to mediate transport of bacterial toxins and viral particles out of the ER which is an important step during viral and bacterial infection [219,222,229]. During dengue virus infection, ERdj3 downregulation using shRNA was shown to reduce virus production in infected human cells [237]. ERdj3 downregulation did not affect the assembly of viral particles but rather reduced levels of viral RNA and viral protein [237]. Upon dengue virus infection, ERdj3 was shown to be localized in dengue virus replication complexes and viral RNA was shown to associate with ERdj3 by coimmunoprecipitation [237]. These results indicate that ERdj3 is involved in the replication of dengue virus [237]. Moreover, it was shown that ERdj3 overexpression results in higher cell viability of Vero cells treated with Vero toxin [208]. Coimmunoprecipitation of ERdj3 with the Salmonella bacterial protein SlrP was demonstrated in vitro as well as in HeLa cells transfected with ERdj3 and SlrP [223]. Association of ERdj3 with SlrP was found to impair binding of ERdj3 to misfolded substrate substrate proteins [223]. Binding of ERdj3 to BiP or stimulation of BiP's ATPase activity by ERdj3 was not impaired by complexing of ERdj3 with SlrP [223]. In HEK 293 cells as well as in BJAB lymphoblastoid cells, ERdj3 was shown to bind to K1, a viral glycoprotein of the Kaposi sarcoma-associated herpesvirus (KSHV) [226]. Simultaneous downregulation of ERdj3 and the cytosolic chaperone Hsp90 was shown to increase apoptosis in cells transfected with K1

indicating that the two proteins together have an anti-apoptotic effect possibly also by downregulating the viral load in the cytosol [226].

Apart from its role in bacterial and viral infection, ERdj3 overexpression can prevent extracellular aggregation of amyloid-β peptides and transthyretin the aggregation of which can lead to the development of Alzheimer's disease or TTR amyloidosis, respectively [211]. With regard to amyloid-β peptides, it was shown that overexpression of ERdj3 results in decreased levels of Aβ40 and Aβ42 and this effect was even more pronounced upon simultaneous overexpression of BiP [238]. Amyloid-β peptides are generated from amyloid precursor protein (APP) by proteolysis mediated by secretases [238]. ERdj3 overexpression was shown to impair proteolysis of APP, thereby preventing the generation of amyloid-β peptides [238]. ERdj3 mediated ERAD was not involved in the reduced levels of amyloid-β peptides upon ERdj3 overexpression [238].

Furthermore, mutations in the gene coding for ERdj3 have been reported in families suffering from autosomal dominant polycystic kidney disease (ADPKD) [239]. The disease pattern in patients with an ERdj3 mutation differed from that of typical ADPKD patients [239,240]. Cysts remained smaller and kidneys were not enlarged [239]. Furthermore, the patients showed chronic intestinal fibrosis [239]. Due to the atypical presentation of patients carrying mutations in the ERdj3 gene, the term DNAJB11 (synonym for ERdj3) nephropathy was suggested rather than ADPKD [240]. In order to assess the function of ERdj3 in kidney cells, ERdj3 was knocked out in renal cortical tubular epithelial cells using the CRISPR/Cas9 system [239]. It was found that maturation and surface expression of Polycystin-1 was impaired in the ERdj3 knockout cells [239]. Also, the regional distribution of the secretory protein Uromodulin differed in ERdj3 knockout cells compared to wildtype tubular epithelial cells [239] indicating that ERdj3 is required for correct trafficking of these proteins to their functional sites. A biallelic ERdj3 mutation was discovered in a fetus that was prenatally diagnosed with polycystic kidney disease [241]. This might point to a role for ERdj3 in the development of autosomal recessive polycystic kidney disease (ARPKD), a form of polycystic kidney disease that is diagnosed in childhood and is more severe but also rarer the ADPKD [241].

Interestingly, ERdj3 protein levels in the urine are increased in rats upon injection of tunicamycin [235]. In passive Heymann nephritis and puromycin aminonucleoside nephrosis, two different disease patterns associated with podocyte injury, ERdj3 secretion into the urine could be observed and urinary ERdj3 levels correlated with the onset of proteinuria [235]. Therefore, ERdj3 might serve as a urinary biomarker reflecting glomerular ER-stress [235].

Cystic fibrosis is a disease that is caused by a mutation in the chloride channel CFTR[242]. The most common mutation is Δ508CFTR [242]. The mutated chloride channel is misfolded in

the ER and subsequently degraded by ERAD [243]. Downregulation of ERAD can result in a functional chloride channel at the plasma membrane [244]. Recently, ERdj3 has been identified as an interaction partner of CFTR using ProtoArray [244]. However, the implications of this interaction have not been assessed by now but a similar mechanism as shown for ERdj3/β-glucocerebrosidase can be assumed.

With regard to cancer, increased levels of ERdj3 were found in hepatocellular carcinoma (HCC) cell lines in comparison to normal hepatocytes [218,245]. In HepG2 and Huh7 cells it could be shown that ERdj3 overexpression results in increased proliferation, migration and invasiveness of the cancer cells while ERdj3 downregulation has the opposite effect [218]. Also, ERdj3 overexpression facilitates epithelial-mesenchymal-transition (EMT) in HCC cells, which is a crucial process during HCC development [218]. Taken together, these results indicate that overexpression of ERdj3 might cause the transformation of tissue and increase the invasiveness by increasing EMT, proliferation and migration rates [218]. High ERdj3 levels would thus cause or at least correlate with a lower survival rate in patients as was shown by one group [218]. As already described in a previous chapter the Z variant α1-antitrypsin (ZAAT) can cause α1-antrypsin deficiency [217]. Alpha-1-antrypsin deficiency is a known risk factor for the development of hepatocellular carcinoma (HCC) [246]. As ERdj3 can inhibit ZAAT degradation, ERdj3 could promote HCC progression through an ZAAT dependent mechanism that has to be assessed in more detail in future experiments [218]. In tissue samples of oral cavity squamous cell carcinoma ERdj3 protein levels are elevated in the cytoplasm as compared with adjacent non-cancerous tissue [214]. There was however no correlation between ERdj3 protein levels and patient survival rates [214].

When mRNA expression levels were compared between patients suffering from diabetes mellitus type 2 and healthy individuals, it was shown that ERdj3 expression is upregulated in the patients suffering from diabetes mellitus type 2 [245]. However, it has to be noted that only a small sample size was used [245]. Therefore, the up- or downregulation of ERdj3 in diabetes mellitus type 2 should be reassessed using a larger sample size.

ERdj3 was detected in synovia from patients suffering from osteoarthritis, chronic pyrophosphate arthropathy and rheumatoid arthritis [247]. ERdj3 protein levels were significantly increased in synovia from patients suffering from rheumatoid arthritis as compared with patients suffering from osteoarthritis or chronic pyrophosphate arthropathy and ERdj3 protein levels correlated with the extent of inflammation in the synovial tissue indicating that ERdj3 might be involved in regulation of inflammation during synovitis [247].

As ERdj3 levels affect degradation rates of α1-antrypsin, ZAAT and β-glucocerebrosidase[216,217], ERdj3 is probably also involved in the pathomechanisms resulting in α1-antrypsin deficiency and Gaucher's disease.

5.4. ERdj4

5.4.1. Protein expression pattern during embryonic development

- Daverkausen-Fischer L, Motyl-Eisemann M, Draga M, Scaal M, Prols F. Protein expression pattern of the molecular chaperone Mdg1/ERdj4 during embryonic development. *Histochem Cell Biol* 2020; **154**(3): 255-63.

ERdj4 was first discovered in differentiating rat endothelial cells in which ERdj4 mRNA levels were upregulated, suggesting a role for ERdj4 in angiogenesis [53]. Additionally, ERdj4 protein levels were shown to be elevated in mesenchymal stem cells that have the ability to differentiate into endothelial cells of the umbilical vein [53,248]. Furthermore, increased ERdj4 staining could be observed in the tissue of chronic dermal wounds [249] and expression of ERdj4 was found to protect against metastasis in tumor cells [250]. Angiogenesis, wound healing and metastasis all involve the switching of epithelial or endothelial cells to mesenchymal cells called epithelial-mesenchymal transition (EMT) or endothelial-mesenchymal transition (EndoMT) respectively [251-253]. The opposite process in which mesenchymal cells are transformed into epithelial or endothelial cells is called mesenchymal-epithelial transition (MET) or mesenchymal-endothelial transition (MEndoT) [254,255]. During embryonic development EMT as well as MET play important roles, for example during somite formation from the presomitic mesoderm, generation of the splanchno- and somatopleura from the lateral wall mesoderm or formation of sclerotome from the ventromedial somite [253,256].

This made us ask how ERdj4 is expressed during embryonic development. Firstly, we analyzed ERdj4 levels and distribution in developing mesoderm in chick embryo tissue by immunohistochemistry [257]. Secondly, we examined ERdj4 levels and distribution in chick embryo tissues from the developing nervous system as well as the developing digestive tract [257]. We observed that in mesenchymal cells, ERdj4 is distributed in a salt and pepper pattern without any clear polarization [257]. However, in the epithelial layers, ERdj4 is distributed in a polarized manner with strong expression at the apical and basal layers of the epithelium [257]. The distribution of ERdj4 in epithelial layers became more evenly distributed with increasing age of the embryo [257]. We hypothesize that the polarized expression pattern of ERdj4 in epithelial cells could serve as an inhibitory mechanism to prevent epithelial-mesenchymal transition [257]. A possible mechanism might be the ERdj4 mediated regulation of folding and secretion of cell-cell or cell-matrix receptors that are important for maintaining the epithelial state of the cells [257].

5.4.2. Functions of ERdj4

- Daverkausen-Fischer L, Prols F. The function of the co-chaperone ERdj4 in diverse (patho-)physiological conditions. *Cell Mol Life Sci* 2021; **79**(1): 9.

With regard to topology, localization and function of ERdj4, important new findings were published in the last three years. Different subcellular ERdj4 pools within the ER [61,258-260], the nucleus [53,261] and the cytoplasm [66,199] have long been discussed for ERdj4. However, it was only recently in 2020 that a dual topology was suggested for ERdj4 that allows the protein to be present as a luminal ER resident protein as well as an integral ER membrane protein anchored in the ER membrane via its signal peptide and facing the cytosol [262]. The presence of two different pools allows ERdj4 to accomplish different functions in the ER and the cytosol respectively [262]. Within the ER, ERdj4 acts as a co-chaperone for BiP and mediates folding but also degradation of substrate proteins [66,230,244,263]. As a cytoplasmic protein ERdj4 can be found in association with the mechanistic target of rapamycin complex 2 (mTORC2) which is involved in regulating energy-generating as well as energy-consuming pathways [262]. Association with ERdj4 was shown to downregulate mTORC2 kinase activity [262]. Apart from new evidence for a cytosolic ERdj4 pool, recent experiments suggest an additional extracellular ERdj4 pool [257,264,265]. Regarding ERdj4 function, we suggested a role for ERdj4 in regulating EMT during embryogenesis of the chick [257]. Recently, this hypothesis was confirmed in breast cancer cells [266]. The transcriptional repressor zinc-finger E-box–binding homeobox 1 (ZEB1) was shown to promote EMT in ovarian cancer [267]. Downregulation of ZEB1 using shRNA on the other hand resulted in reduced levels of EMT and metastasis in ovarian cancer [267]. Kim et al. showed that ERdj4 promotes degradation of ZEB1 thereby preventing EMT in breast cancer cells [266]. The marked relevance of ERdj4 is depicted by the number of new publications found on PubMed. In the last three years from 2020 to 2022, 45 new publications on ERdj4 can be found on PubMed. The fast advances in research regarding ERdj4 made us decide to compose a comprehensive review discussing the current knowledge on ERdj4 structure, localization, regulation and function.

5.4.3. ERdj4 in disease

In the review:

- Daverkausen-Fischer L, Prols F. The function of the co-chaperone ERdj4 in diverse (patho-)physiological conditions. *Cell Mol Life Sci* 2021; 79(1): 9.

We already discussed the role of ERdj4 in cellular glucose metabolism [268], in folding and degradation of cystic fbrosis transmembrane conductive regulator (CFTR) [244] and the role of ERdj4 as a biomarker for fibrillary glomerulonephritis (FGN) [269]. An important role of ERdj4 in diagnosing patients with FGN is supported by reports on cases of fibrillary glomerulonephritis that could be identified as FGN solely based on detection of ERdj4 in the respective tissue [270,271]. Even though higher ERdj4 protein levels are found in kidney tissue from patients suffering from FGN, there is no increase in ERdj4 transcription in these tissues [272]. Also, anti-apoptotic functions of ERdj4 [259,273-276] which might play a role in cancer development were discussed in our recent review [123]. A multitude of publications is published on ERdj4. This section aims to complement the published data not included in our review. The data presented in the following section are predominantly of correlative nature and a possible ERdj4-based disease-associated mechanism needs to be confirmed by experimental studies.

Hepatic diseases

In a model of Intestinal failure associated liver disease (IFALD), which can be caused by prolonged parenteral nutrition, ERdj4 mRNA levels were shown to be upregulated correlating with severity of the disease [277]. Also, treatment with growth hormone increased ERdj4 mRNA in rat livers [278].

Infectious diseases

In the context of infectious diseases, ERdj4 mRNA levels were shown to be upregulated in macrophages of mice infected with Brucella virus [279]. Furthermore, upregulation of ERdj4 results in reduced post-translational translocation of the preS subunit of Hepatitis B large envelope protein which is important for the function of the viral protein [260].

Neurological diseases

It was further found that ERdj4 gene expression is upregulated in the spinal chord of patients suffering from sporadic amyotrophic lateral sclerosis [280] and one case of a patient suffering from Parkinson carrying a mutation in the ERdj4 gene is reported in the literature [281]. Familial encephalopathy with neuroserpin inclusion bodies (FENIB) is a neurological disease [282]. The disease is associated with neuronal inclusions that contain mutated neuroserpin, a neuronal protease inhibitor [282]. In a mouse model of the disease, it was shown that ERdj4 mRNA is upregulated in mice 80 weeks of age [282].

Cancer

With regard to cancer, it was shown that ERdj4 gene expression is upregulated in a variety of cancer types including Kidney Renal Clear Cell Carcinoma, Kidney Renal Papillary Cell Carcinoma, Head and Neck Squamous Cell Carcinoma, Kidney Chromophobe, Lung Adenocarcinoma and Colorectal Adenocarcinoma among others [273]. It was shown that ERdj4 can inhibit celecoxib induced apoptosis in gastric adenocarcinoma cells in cooperation with BiP [275]. ERdj4 was shown to directly interact with tumor suppressor p53 by its J-domain in the nucleus of cells [273]. Interestingly, it was further shown by another group that downregulation of ERdj4 mRNA and protein levels in chorion carcinoma cells resulted in upregulation of p53 protein levels [283].The mechanism how ERdj4 can regulate p53 protein levels is not known but interaction of ERdj4 with p53 seems to be crucial for the antiapoptotic function of ERdj4. In AML cells, inhibition of the long noncoding RNA SNHG5 results in reduced levels of ERdj4 [284]. Consequently, the cells are more sensitive to chemotherapy treatment[284].

5.5. ERdj5

5.5.1. Structure

Human and mouse ERdj5 [54], also known as DNAJC10 [285], Macrothioredoxin [286], JPD1 [62] or Erj5 [140] consists of 793 amino acids [54,62] . The protein contains an N-terminal signal peptide followed by a J-domain of 66 amino acids (see Figure 16) [54,62]. The J-domain is followed by six thioredoxin-like domains, which are arranged in one plane in the three-dimensional structure of ERdj5 [287]. Four of the six thioredoxin-like domains Trx1, Trx2, Trx3 and Trx4 seem to have reducing capacity, while Trxb1 and Trxb2, which are located between Trx1 and Trx2, lack the CXXC motif, characteristic for reducing thioredoxin-like domains [287]. Analysis of the full-length crystal structure of ERdj5 revealed that the thioredoxin-like domains are arranged in two clusters separated by a cleft. The N-terminal cluster contains Trx1, Trxb1, Trxb2 and Trx2 while the C-terminal cluster contains Trx3 and Trx4 [287]. Maegawa et al. [288] described another crystal structure of ERdj5 in 2017, which differs from the originally described structure with respect to the orientation of the C-terminal cluster towards the N-terminal cluster [288]. Interestingly, the C-terminal and N-terminal clusters do not seem to be in a fixed orientation [288]. High-speed atomic force microscopy showed that the C-terminal cluster can move rapidly and can adopt various conformations in relation to the N-terminal cluster [288]. The C-terminus of ERdj5 contains a KDEL motif, which is an ER-retention sequence [54,62]. ERdj5 has one potential glycosylation site at amino acid position 530 [62]. ERdj5 is slightly smaller when isolated from HeLa cells after EndoH treatment as compared with ERdj5 isolated before EndoH treatment [62]. Therefore it can be deduced that ERdj5 is glycosylated at this site in vivo [62]. No G/F rich region or cysteine rich region is present within the ERdj5 sequence [54].

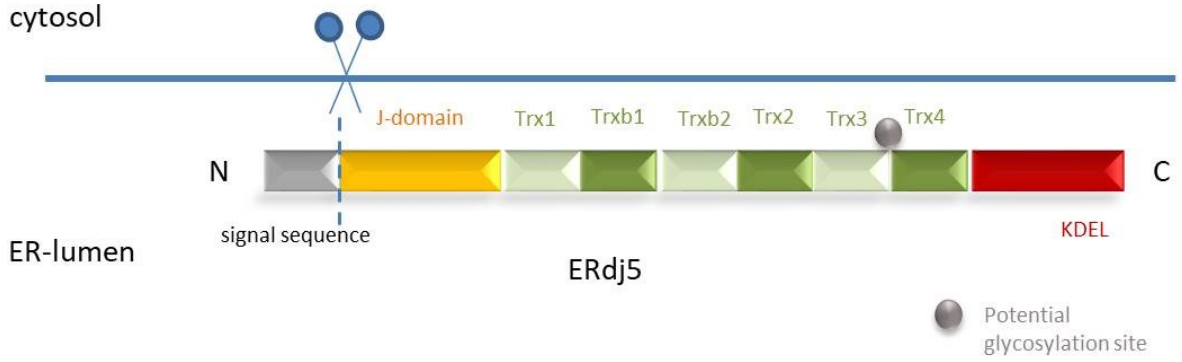

Fig. 16 Schematic structure of ERdj5
ERdj5 possesses an N-terminal signal peptide that is followed by the J-domain [54,62]. Furthermore, ERdj5 contains six thioredoxin-like domains (Trx1-4 and Trxb1-2) of which Trx1-4 have reducing capacity [287]. At its C-terminus ERdj5 contains an ER retention motif, the KDEL sequence [54,62]. The figure shows the protein domains of ERdj5 without representation of the actual proportions between the different domains.

5.5.2. Localization

ERdj5 mRNA is ubiquitously expressed in human tissue with high expression in pancreas, testis, liver, prostate, spleen and heart [54]. In murine tissue, ERdj5 mRNA is also expressed ubiquitously with the highest expression rates in heart, liver, kidney and testis [62]. Northern blot analysis of human tissue revealed three major mRNA bands 4.4kb, 3.4kb and 2.4kb in size, which were recognized by hybridization with ERdj5 cDNA [54]. In human fetal brain ERdj5 splicing variants 3.5kb, 3.3kb and 3kb in size could be identified by another group [286]. In murine tissues two different mRNA bands 4.4kb and 3.4kb in size could be identified [62]. The existence of these multiple different ERdj5 mRNAs could be due to alternative splicing events but the implications of the presence of multiple mRNAs have not been assessed until now.

Within the cell, ERdj5 is localized in the ER lumen, as was shown in various cell lines [54,62,289,290]. In HEK293 cells, Green fluorescent protein (GFP)-tagged ERdj5 co-localized with the ER-marker pDsRed2-ER [54], in NIH3T3 cells hemagglutinin (HA)-tagged ERdj5 co-localized with HA-tagged ER marker Protein disulfide-isomerase (PDI) [62]. Co-localization of ERdj5 and PDI was also demonstrated in HT1080 cells [291]. In COS-7 cells, mCherry-tagged ERdj5 partially co-localized with GFP-tagged BiP [290]. However, it has to be noted that BiP or PDI are both not exclusively localized in the ER and therefore their use as ER markers is suboptimal [21,161,162]. Yet, the reticular pattern observed in SK-K-SH cells overexpressing HA-tagged ERdj5 further points to the localization of ERdj5 in the ER compartment [289]. No change in ERdj5 protein size was observed after treatment of HeLa cells with proteinase K [62]. Accordingly, ERdj5 seems to be exclusively located in the lumen of the ER [62]. ERdj5 is mobile throughout the ER lumen as was demonstrated by rapid recovery of mCherry signal after photobleaching of COS-7 cells [290].

5.5.3. Function

BiP binding and regulation of BiP's ATPase activity

Using surface plasmon resonance, yeast two hybrid assays as well as pull-down assays different groups could show that ERdj5 binds to BiP via its J-domain in an ATP dependent manner [54,62,66,100,292]. The presence of ATP is mandatory since ERdj5 does not bind to BiP in the presence of ADP or in the absence of ATP [54,62]. Furthermore, mutation of the HPD site within the J-domain of ERdj5 abolishes the ability to bind to BiP [62,66,76] and to stimulate BiP's ATPase activity [293]. Exchange of the J-domain of DnaJ protein for the J-domain of ERdj5, resulted in a reversal of the thermosensitivity of an E.coli strain [140]. These results show that the J-domain of ERdj5 can compensate for loss of the J-domain of DnaJ [140]. Consequently, it

can be assumed that the J-domain of ERdj5 can also stimulate the ATPase activity of DnaK [140].

Regulation of apoptosis

The role of ERdj5 in apoptosis in different cell types and in response to different agents has been investigated by different groups. Depending on cell type and agent, adverse results were obtained which will be presented in the following section. While most experiments suggest a pro-apoptotic function of ERdj5, there is also experimental data that demonstrates an anti-apoptotic function of ERdj5 under certain conditions.

In Huh7 cells, ERdj5 downregulation using siRNA results in higher levels of viable cells upon tunicamycin treatment than under control conditions [294]. Similarly, in SHSY5Y cells, FACS analysis as well as cell growth assays demonstrated that overexpression of ERdj5 increases the number of apoptotic cells upon tunicamycin, thapsigargin and bortezomib treatment [295]. In periodontal ligament stem cells (PLSC) overexpression of ERdj5 also decreased the amount of viable cells [285]. These results indicate that ERdj5 has a pro-apoptotic function in Huh7 cells upon tunicamycin treatment, in PLSC cells and in SHSY5Y cells upon treatment with tunicamycin, thapsigargin or bortezomib.

Increased levels of reactive oxygen species (ROS), pro-apoptotic protein bax, cleaved caspase 3 and cleaved caspase 9 were detected in ERdj5 overexpressing periodontal ligament stem cells (PLSC) upon treatment with hydrogen peroxide [285]. At the same time, protein levels of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) were reduced in these cells [285]. In SHSY5Y cells, overexpression of Bcl-2 could reduce the pro-apoptotic effect of ERdj5 after tunicamycin and thapsigargin treatment [295].

High amounts of phosphorylated PERK and eIF2α were shown to correlate with cell viability in SHSY5Y cells [295]. Reduced levels of phosphorylated PERK and eIF2α can be detected in ERdj5 overexpressing SHSY5Y cells upon tunicamycin treatment [295]. In hydrogen peroxide treated PLSCs, overexpression of ERdj5 also results in a reduction of phosphorylated PERK and eIF2α [285,295]. Levels of PERK downstream proteins ATF4, GADD34, CHOP as well as BiP were reduced in ERdj5 overexpressing SHSY5Y cells treated with thapsigargin indicating a defective ER-stress signaling in these cells [295]. ERdj5 was shown to coimmunoprecipitate with PERK in the colon cancer line HCT 116 [296]. Isolated expression of ERdj5 J-domain was sufficient to reduce phosphorylated levels of eIF2α upon thapsigargin treatment [295] suggesting that a functional interaction between BiP and ERdj5 is necessary to negatively regulate PERK signaling. Phosphorylation of eIF2α results in translational arrest during the UPR [112]. Interestingly, additional treatment with cyclohexamide, an inhibitor of translation could reverse the pro-apoptotic effect of ERdj5 in SHSY5Y cells [295].

These results suggest that ERdj5 exerts its pro-apoptotic effect by abolishing translational inhibition mediated by PERK during ER-stress.

Upon thapsigargin treatment overexpression of ERdj5 resulted in higher luminal calcium levels than in control cells [295] hinting at an additional role of calcium signaling in promoting apoptosis.

Jumonji C domain containing histone demethylase 1 homolog D (JHDM1D) controls the methylation status of histones and thereby regulates epigenetic gene regulation [297]. JHDM1D overexpression protects PLSCs against apoptosis induced by hydrogen peroxide [285]. Interestingly, reduced levels of ERdj5 mRNA and protein could be detected in JHDM1D overexpressing cells, supporting the role of ERdj5 in promoting apoptosis upon hydrogen peroxide treatment [285]. The results indicate that the ERdj5 gene, being a target of JHDM1D, might be epigenetically regulated. However, even though a pro-apoptotic function of ERdj5 was proposed by different groups, results by other groups suggest an anti-apoptotic function of ERdj5 [99,294,298].

In TUNEL assays, higher levels of apoptotic cells could be detected in the salivary glands of ERdj5 knockout mice seven months as well as twelve months of age [99]. Doxorubicin treated Huh7 cells show higher levels of apoptosis when ERdj5 is downregulated while ERdj5 overexpression did not have an effect on apoptosis in doxorubicin treated SHSY5Y cells [294,295]. In SHSY5Y cells as well as in melanoma cell lines A375 and SK-MEL110, ERdj5 downregulation increased susceptibility to ferenetide- and velcade-mediated apoptosis in melanoma cell lines [298]. This suggests that doxorubicin-, ferenetide- and velcade- mediated apoptotic pathways probably differ from apoptotic pathways mediated by thapsigargin, tunicamycin, hydrogen peroxide and bortezomib. Also, the results hint at a cell-type specific effect as ERdj5 affected doxorubicin mediated apoptosis in Huh7 cells but not in SHSY5Y cells. Supporting the hypothesis that different apoptotic signaling pathways are induced depending on treatment, it was shown that in contrast to thapsigargin treatment, there is only a minor increase in phosphorylated eIF2α levels upon ferenetide treatment [99]. Consequently PERK signaling seems to play a minor role in ferenetide mediated apoptosis. Here, it would be interesting to know the role of PERK signaling in valcade- and doxorubicin mediated apoptosis. In summary, cell treatment but also cell type might determine whether ERdj5 has a pro- or anti-apoptotic function.

ERAD

It has been shown that ERdj5 binds to aggregation prone sequences of target proteins and passes them to the degradation pathway [66,263]. Multiple experiments showed that ERdj5 binds to and promotes degradation of a set of glycosylated and non-glycosylated ERAD substrates (wild type surfactant protein C and mutant surfactant protein C (non-glycosylated)

[66], mutant insulin (non-glycosylated) [66], wildtype insulin (non-glycosylated) [66], NHK variant of α1-antitrypsin (glycosylated) [76], NHK-QQQ variant of α1-antitrypsin (non-glycosylated) [76], mutant tyrosin kinase (glycosylated) [287], rhodopsin (glycosylated) [289] and J chains (glycosylated) [100]). A mutant of fibulin-3 (glycosylated) that causes autosomal dominant macular dystrophy has also been shown to bind to ERdj5 [299]. Whether this association affects degradation of the mutant protein has not been assessed so far [299].

The C-terminal cluster of ERdj5 was identified as a binding site of the ERAD substrate NHK [287]. In HEK 293 cells, expression of mutant surfactant protein C results in upregulation of ERdj5 [66], which can be considered as an attempt of the cellular machinery to cope with increasing amounts of misfolded proteins [66].

ERdj5 mutants lacking the J-domain are still able to bind to mutant and wildtype surfactant protein C [66]. Binding of HPD mutated ERdj5 to non-glycosylated NHK, however, was decreased suggesting that interaction between ERdj5 and non-glycosylated NHK depends on BiP while interaction between ERdj5 and surfactant protein C is independent of BiP [76]. Also, a mutant of rhodopsin causing the disease retinitis pigmentosa bound less strongly to ERdj5 when the HPD site was mutated [289] indicating that even though the interaction of ERdj5 with BiP is not generally necessary for substrates to bind to ERdj5 it might be important for binding of a subset of substrates to ERdj5. Whether or not binding of a substrate to ERdj5 is dependent on BiP does not only depend on its glycosylation status as rhodopsin is a glycosylated protein while non-glycosylated NHK is not.

The ERdj5-mediated degradation of some substrates requires BiP and the stimulation of its ATPase activity [66]. This was shown by overexpression of ERdj5, mutated at the HPD site [66]. In the presence of this HPD-site mutant, degradation of non-glycosylated mutant surfactant protein C was impaired [66]. Overexpression of a HPD-site ERdj5 mutant was also shown to decelerate degradation of non-glycosylated NHK while degradation of glycosylated NHK was not affected [76]. The results fit in with the observation that non-glycosylated NHK cannot be transferred to ERdj5 when the HPD site is mutated [76]. Glycosylated NHK seems to be degraded via the calnexin/calreticulin/ERdj5/EDEM1 pathway and can still be transferred to ERdj5 when the HPD site is mutated [76] indicating that glycosylated NHK is transferred to ERdj5 not via BiP but via another protein. However, when endogenous ERdj5 is downregulated using siRNA, glycosylated NHK degradation is reduced by approximately 50% probably due to the fact that under these conditions glycosylated NHK cannot be transferred to ERdj5 by any protein, due to downregulation of endogenous ERdj5, and therefore cannot be degraded. The fact that degradation is not completely inhibited points at an additional degradation pathway for glycosylated NHK. When both, endogenous ERdj4

and ERdj5 are downregulated using siRNA, degradation of glycosylated NHK is clearly decelerated [76]. These results suggest that apart from calnexin/calreticulin/ERdj5/EDEM1 dependent degradation, ERdj4 is also important for the degradation of glycosylated NHK [76]. However, degradation of the glycosylated mutant of rhodopsin was shown to be impaired upon mutation of the HPD site within ERdj5 [289]. This result indicates that an interaction between ERdj5 and BiP is required for the degradation of some glycosylated substrates as rhodopsin [289] but not for all as was shown for glycosylated glycosylated NHK [76].

Overexpression of ERdj5 was found to prevent formation of high molecular weight complexes of J-chains and also the formation of NHK dimers, which would probably impede retrotranslocation of the substrates to the cytosol [100]. A major function of ERdj5 relies on its reducing activity within the ER [100]. It was shown that ERdj5 can mediate reduction of the disulfide bridges in wildtype insulin [100] Thus, it is very likely that ERdj5 targets substrates to the ERAD pathway by reducing intra- or intermolecular disulfide bonds, thereby enabling misfolded proteins to pass the retrotranslocon [76]. This hypothesis is supported by data showing that degradation of the cysteine-less mutant of NHK and the cysteine less protein ribophorin is not promoted by ERdj5 [76,100]. Also, ERdj5 mutants with defective reductase activity cannot promote degradation of NHK [100,287]. It has further been shown that the structural flexibility of ERdj5 seems to be important for proper substrate degradation [288].

Coimmunoprecipitation as well as yeast-two hybrid experiments show that ERdj5 associates with components of the ERAD machinery. In cell lysates of HEK 293 cells, ERdj5 coimmunoprecipitates with p97/Cdc48 [66]. P97/Cdc48 is a protein containing ATPase function that provides force for protein export from the ER [78]. It is involved in unfolding of ERAD substrates and in delivering proteins to the proteasome for degradation [300]. However, the nature of association between the two proteins remains unclear, as ERdj5 is a luminal ER protein and p97/Cdc48 is localized at the cytosolic side of the ER membrane [54,62,300]. Possibly, they are associated via the respective target proteins that are retrogradely transported across the membrane.

Furthermore, binding of the C-terminal cluster of ERdj5 (aa 558 – 793) to the soluble EDEM1 pool was shown by coimmunoprecipitation experiments as well as yeast-two-hybrid systems [75,100,287]. EDEM1 specifically binds to thioredoxin domain 4 of ERdj5 [76]. Yet, the association of ERdj5 with EDEM1 is not dependent on the reductase activity of ERdj5 as an ERdj5 mutant without a functional thioredoxin domain still binds to EDEM1 [100]. However, ERdj5 did not bind to an EDEM1 mutant lacking two of the intrinsically disordered domains (IDDs) that are important for EDEM1 turnover implying that ERdj5 might bind directly to these regions [301]. Another possibility would be that the IDDs confer a conformational flexibility to

EDEM1 that facilitates binding to ERdj5 [301]. EDEM1 is involved in ERAD of glycosylated proteins by accepting terminally misfolded glycoproteins from calnexin and calreticulin [302]. For NHK it was shown by pulse-chase experiments that NHK is transferred from calnexin to the ERdj5/EDEM1 complex [287]. An ERdj5 mutant, that could not bind to EDEM1, was unable to accelerate degradation of glycosylated NHK whereas the degradation of non-glycosylated NHK-QQQ mutant was not impaired [76]. This result indicates that association of ERdj5 with EDEM1 is not necessary for degradation of non-glycosylated proteins even though many more substrates have to be examined.

Gel filtration experiments as well as co-immunoprecipitation experiments identified the ERAD-associated membrane adaptor protein Sel1 as another ERdj5 binding partner [292]. Sel1 is involved in the ERAD pathway and mediates dislocation of misfolded proteins from the ER [303]. It was shown that ERdj5 in cooperation with BiP promotes transfer of glycosylated NHK to Sel1L [76]. Within the ER lumen the protein ER flavoprotein associated with degradation (ERFAD) was identified as an additional interaction partner of ERdj5 [304]. ERFAD did also coimmunoprecipitate with ERAD components Os9 and Sel1 suggesting that ERFAD provides a link between ERdj5 and these ERAD components [304].

Protein folding

Its thioredoxin like domains convey reductase activity to ERdj5 [100]. Mass spectrometry revealed that many different ER proteins but also secreted proteins and non ER proteins were shown to form mixed disulfide bridges with ERdj5 [291,305]. Some of the identified interaction partners could be confirmed by subsequent Western blot analysis [291]. Among the confirmed interaction partners are BiP, ERp57, Endoplasmic reticulum oxidoreductase 1 α (Ero1α), the Low-density lipoprotein receptor (LDLR) and the UDP-glucose:glycoprotein glucosyltransferase (UGGT) [291]. While interaction between ERdj5 and BiP was shown to be abolished upon mutation of the HPD site, ERdj5 still formed mixed disulfides with many of the other examined proteins [291]. With regard to misfolded proteins, the reduction of disulfide bridges probably enables retrotranslocation of these proteins into the cytosol for degradation. However, as was shown for LDLR, the maturation and folding process of wildtype proteins also involves formation of disulfide bridges that are not present in the final protein [306]. These non-native disulfide bridges have to be rearranged to enable the generation of the correct protein structure [306]. ERdj5 was proposed to mediate protein maturation by cleavage of these non-native disulfide bridges [291]. With regard to LDLR it was shown that ERdj5 mediates trafficking of the LDLR to the Golgi apparatus and that this function is dependent on an interaction of ERdj5 with BiP as well as on the reductase activity of ERdj5 [291].

Calcium homeostasis

Acting as an oxidoreductase in the ER, ERdj5 is largely involved in controlling calcium homeostasis in the ER [94,290]. This topic is extensively reviewed in

- Daverkausen-Fischer L, Prols F. Regulation of calcium homeostasis and flux between the endoplasmic reticulum and the cytosol. *J Biol Chem* 2022: 102061.

5.5.4. Regulation

The upregulation of ERdj5 mRNA and protein levels in response to various agents has been experimentally assessed by different groups. In HEK293 cells, ERdj5 mRNA levels were shown to be upregulated by treatment with tunicamycin, reducing agent DTT, thapsigargin, calcium ionophore, Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and deoxyglucose (DOG). There was no significant upregulation of ERdj5 mRNA in response to heat shock [54]. Upregulation of ERdj5 mRNA in response to tunicamycin and thapsigargin treatment was much more pronounced in HEK 293 cells than in NIH3T3 cells pointing to a cell specific effect [62]. In A375 melanoma cell lines as well as in SHSY5Y cells ERdj5 mRNA levels are upregulated in response to fenretinide treatment, an agent that promotes the generation of reactive oxygen species (ROS) [298]. A relevant increase in ERdj5 protein levels upon fenretinide treatment could however only be demonstrated in SHSY5Y cells [298]. Thapsigargin treatment also results in an increase of ERdj5 protein levels in SHSY5Y cells [298]. The n-3 polyunsaturated fatty acid docosahexaenoic acid (DHA), an agent that acts on membranes making them more vulnerable to oxidative stress, was shown to increase ERdj5 protein levels in colon cancer cells [296]. The data show that ERdj5 transcription and translation is upregulated in cells stressed by imbalanced calcium levels, misfolded proteins or by oxidative stress. It was shown that during ER-stress caused by tunicamycin ERdj5 is upregulated via the IRE1 pathway, as downregulation of IRE1 in C.elegans was shown to reduce levels of dnj-27, the C.elegans homolog of ERdj5 [307]. Downregulation of ATF6 did not have an effect on ERdj5 protein levels while downregulation of the C.elegans homolog of PERK, pek-1, resulted in an upregulation of dnj-27 levels [307].

In Chinese hamster ovary cells, elevated ERdj5 mRNA levels were also demonstrated upon high ammonium concentrations in the media [308]. Surprisingly, blood serum from ERdj5 knockout mice contains more ammonium than serum from wildtype mice suggesting a feedback mechanism to control ammonium concentrations by ERdj5 [293]. The mechanism by which ERdj5 controls ammonium levels is not known yet. A possible mechanism is depicted in Figure 17. It could be that ammonium ions regulate transcription of the ERdj5 gene resulting in increased levels of ERdj5. ERdj5 itself might serve a negative regulator of ammonium.

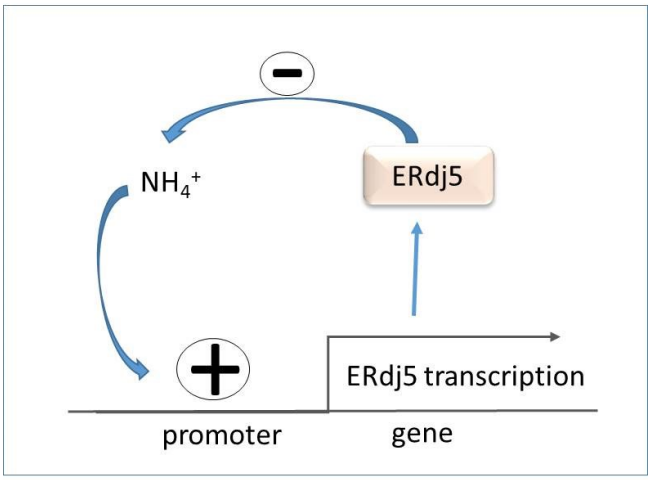

Fig. 17 Possible feedback mechanism between ERdj5 and ammonium.
While ammonium ions might regulate transcription of the ERdj5 gene, ERdj5 itself might serve as a negative regulator of ammonium ion concentrations.

The protein CDK5 regulatory subunit associated protein 1-like 1 (CDKAL1) is involved in protein translation and low levels of CDKAL1 have been associated with the development of diabetes mellitus type 2 [309,310]. Mutations in the CDKAL1 gene have been shown to decrease the amount of secreted insulin in humans [310]. Interestingly, it was shown that downregulation of CDKAL1 reduced ERdj5 protein levels and increased cytosolic calcium levels in GH3 cells (a cell line derived from a rat anterior pituitary tumor) [309]. It is not known whether regulation of ERdj5 by CDKAL1 is mediated on the transcriptional or the translational level [309]. The authors claim that downregulation of ERdj5 mediated by CDKAL1 results in decreased levels of activated SERCA. Reduced levels of activated SERCA result in reduced levels of calcium import into the ER thereby raising cytosolic calcium levels [309].

5.5.5. ERdj5 in disease

Elevated levels of ERdj5 protein are found in HCC cells as compared to cells from healthy liver, which is why a potential role of ERdj5 as a biomarker for HCC has been discussed [294]. Also, ERdj5 mRNA was shown to be upregulated in prostate carcinoma tissue compared with normal prostate tissue [311]. In breast cancer cell lines, ERdj5 expression was downregulated which was associated with reduced probability for overall survival and relapse free survival of patients [312]. Also staining intensity for ERdj5 protein was fainter in breast cancer tissue than in tissue of healthy controls [312]. Similarly, expression of ERdj5 was shown to be downregulated in glioma cells [313]. In contrast to the results obtained in breast cancer patients, in glioma high ERdj5 expression rates were associated with a poor survival [313]. Similarly, high expression levels of ERdj5 were shown to be associated with poor survival in prostate cancer compared with normal ERdj5 expression levels [311]. These adverse results

further highlight the cell-type dependent role of ERdj5 with regard to apoptosis. In the literature it was proposed that ERdj5 could serve as a proliferation marker due to high ERdj5 expression levels in proliferative tissues like small intestine, bone marrow and colon [294]. However, the results from breast cancer cell lines show that the level of ERdj5 alone is not a suitable biomarker for the invasiveness of tumors and a variety of different cancer cell lines have to be examined to understand tumor-specific signatures.

Sjörgen Syndrome (SS) is a chronic autoimmune disease that mainly affects the salivary and lacrimal gland and results in reduced liquid production [314]. The detection of specific antibodies in blood samples of patients suffering from an autoimmune disease is an important diagnostic tool. In the serum of patients suffering from Sjörgen syndrome, Ro- and La- antibodies (as for example anti-Ro52 or anti-Ro60) directed against antigens from the Ro/La system can be detected on a regular basis [315,316]. In human tissue, staining for ERdj5 is stronger in salivary glands of patients suffering from Sjörgen Syndrome (SS) than of healthy patients, which is probably due to a compensatory mechanism as outlined below [99]. ERdj5 staining intensity in whole tissue but also in inflammatory lesions and in ductal epithelium correlates with disease severity [99]. ERdj5 staining is stronger in patients who are positive for SSA-Ro antibodies [99]. No difference in ERdj5 staining intensity can be observed between patients who are positive for the SSA-La antibody and patients who are negative for the indicated antibody [99]. Effects of ERdj5 knockdown were examined in mice and ERdj5 knockout mice were suggested as an animal model for Sjörgen Syndrome [99]. ERdj5 knockout mice present with increased levels of ER-stress in their salivary glands and female knockout mice were shown to produce less saliva than wildtype mice [99,293]. Furthermore, more inflammatory areas and more B- and T-lymphocytes can be detected in salivary glands of ERdj5 knockout mice than in wildtype mice [99,317]. In female ERdj5 knockout mice, higher levels of anti-Ro52 and anti-Ro60 antibodies can be detected at seven months of age while increased levels of anti-La antibodies can be detected at an age of 12 months [99]. At seven and twelve months of age ERdj5 knockout mice were demonstrated to have more apoptotic cells in their salivary glands and various interleukins are upregulated in the salivary glands as well as in the serum upon ERdj5 knockout [99]. Initially, neither general protein levels nor levels of α amylase were shown to be altered in the salivary gland and saliva of ERdj5 knockout mice as was examined by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [293]. To further investigate the differences in the proteome of ERdj5 knockout and wildtype mice, Liquid Chromatography Mass Spectrometry was performed [317]. Here, it could be observed that kallikrain 1b22 was upregulated in ERdj5 knockout mice [317]. Kallkrain1b22 is a protease that can induce Sjörgen Syndrome in rats when injected subcutaneously [318]. Kallikrain 1b22 cleaves and thereby inactivates nerve growth factor (NGF) which according

to some studies has anti-inflammatory properties [317]. Therefore, it is hypothesized that kallikrain1b22 upregulation promotes inflammation by increased cleavage of NGF [317]. However, the detailed mechanisms how signaling pathways promote upregulation of kallikrain and inflammation in ERdj5 knockout mice have to be examined in the future.

Rhodopsin is a protein of rod cells that absorbs light [289]. Mutations in rhodopsin can result in Retinitis pigmentosa, which can finally result in blindness of patients [319]. A common rhodopsin mutation resulting in retinitis pigmentosa is the P23H mutation causing the formation of a non-native disulfide bridge in the mature protein [289,319]. ERdj5 was found to mediate degradation of mutant rhodopsin through its reductase activity and in cooperation with BiP [289]. Overexpression of ERdj5 in a mouse model of retinitis pigmentosa could prevent loss of visual function [320].

Furthermore, ERdj5 seems to play a role in viral and bacterial infection [292,321-323]. In COS-7 cells transfected with viral proteins of the Newcastle Disease Virus, ERdj5 overexpression results in increased membrane fusion, which is an important step during entry of viruses into the host cell [323]. ERdj5 overexpression was shown to increase the amounts of free thiols in the viral fusion protein, which is thought to result in conformational changes in the fusion protein that allows virus entry [323]. Similarly, overexpression of ERdj5 was shown to reduce intermolecular disulfide bond in the simian virus 40 major capsid protein VP1, resulting in conformational changes of the viral protein as was detected by electron microscopy [322]. ERdj5 was shown to mediate retrotranslocation of VP1 and cholera toxin from the ER into the cytosol during SV40 or cholera infection respectively [292,322]. Retrotranslocation of cholera toxin is mediated by ERdj5 in cooperation with BiP as ERdj5, mutated at its HPD site, cannot facilitate cholera toxin retrotranslocation [292]. Retrotranslocation of cholera toxin allows the toxin to modify activation of chloride channels resulting in the typical clinical manifestation of cholera infection [292]. Retrotranslocation of VP1 to the cytosol, on the other hand, allows the protein to travel to the nucleus where transcription of viral genetic information takes place [322]. Downregulation of ERdj5 in dengue virus infected human cells resulted in reduced levels of viral particles [237]. ERdj5 was shown to affect post-entry steps during dengue virus infection but the exact role of ERdj5 in dengue virus infection has not been assessed until now [237].

In C.elegans models of Alzheimer disease, Parkinson and CAG repeat disorders (for example Huntington disease), overexpression of dnj-27, the C.elegans homolog of ERdj5, was found to decrease amounts of aggregates formed by amyloid-β peptides, α-synuclein or the polyglutamine containing proteins respectively [307]. Downregulation of dnj-27 on the other hand was found to increase the amount of aggregates formed by these proteins [307]. These results suggest that ERdj5 can be protective against neurodegenerative diseases by preventing the formation of toxic protein aggregates.

6. Comparision of topology, localization and function between ER-resident co-chaperones

6.1. Dual topology of ER-resident co-chaperones

- Daverkausen-Fischer L, Prols F. Dual topology of co-chaperones at the membrane of the endoplasmic reticulum. *Cell Death Discov* 2021; **7**(1): 203.

Dual topology proteins have been mostly examined in bacteria [324] but a subset of dual topology proteins have been reported to be present in mammalian cells [325-327]. Incomplete signal cleavage was reported for the ERAD associated protein EDEM1 resulting in two different EDEM1 pools [75]. EDEM1 can be present as an ER luminal protein as well as an ER membrane anchored protein facing the ER lumen [75]. Therefore, signal peptide cleavage efficiency seems to regulate the ratio between membrane anchored and luminal pools of the protein [75]. Signal peptide or transmembrane domain integration into the Sec61 translocon on the other hand can also determine the topology of newly synthesized proteins [328]. Examination of the current data on topology and localization of ERdj proteins yielded the result that many of the ERdj proteins seem to be located in different compartments of the cell [122]. A recent report by Sun et al. suggests that the presence of ERdj4 in the cytosol as well as in the ER lumen is due to a dual topology of ERdj4 with one pool being present as an ER membrane protein facing the cytosol and another pool residing in the ER lumen as a soluble protein [262]. The two different ERdj4 pools were shown to have different functions in the cell [262]. This model probably relies on a dual mode of signal peptide integration into the Sec61 translocon. While the ER luminal ERdj4 pool depends on a loop-wise integration of the signal peptide, the cytosolic membrane anchored pool relies on a head-on signal integration [328]. This in turn indicates that ERdj4 can exhibit a dual topology at the ER membrane during maturation. The new data on ERdj4 presented by Sun et al. [262] made us wonder whether other ERdj proteins can also exhibit a dual topology at the ER membrane possibly giving rise to different cellular protein pools. Further we asked whether there are data suggesting insufficient cleavage of the signal peptides in ERdj proteins. To assess these questions, we compared the available experimental data including cell fractionation experiments, proteinase K assays and carbonate extraction methods. Furthermore, we exploited the computational programs SignalIP as well as DeepLoc to gain predictional data on signal cleavage probability and subcellular localization of ERdj proteins. Also, we assessed the signal peptides of the respective proteins and compared their hydrophobicites. As a result we propose that not only ERdj4 can exhibit a dual topology at the ER membrane but that ERdj3

and ERdj6 can also exhibit dual topologies at the ER membrane [122]. Different subcellular pools of proteins can have important implications on a protein's function as different subcellular pools allow the protein to have different interaction partners and to fulfill different cellular functions [122]. However, the indications for a dual topology of ERdj proteins have to be validated experimentally in the future.

Open questions:

- ERdj1 was shown to translocate into the ER lumen independently of ERdj2 or BiP while ERdj3 is dependent on BiP and ERdj2 for translocation [167]. For ERdj1, there is no ambiguous data regarding the topology of the protein [122]. However, for ERdj3 there is ambiguous data regarding the mode of signal peptide insertion and subcellular localization [122]. Therefore, it would be interesting to assess whether the translocation of other ERdj proteins is dependent on ERdj2 and BiP. Furthermore it would be interesting to assess whether translocation efficiencies of the ERdj proteins change under conditions of ER-stress.

- In the cytosol, ERdj4 binds to mTORC2 and modulates protein synthesis and insulin signaling while within the ER, ERdj4 serves as a co-chaperone of BiP and is involved in the ERAD pathway [66,262]. ERdj3 is involved in the ERAD pathway when located in the lumen [217,230]. In the nucleus, ERdj3 was proposed to bind to apobec-1 and control ApoB100 mRNA editing [200]. Therefore the co-chaperones seem to have different functions depending on their localization and topology. In future studies topology of the co-chaperones should be assessed in more detail also with regard to the functions of the different subcellular ERdj pools.

6.2. Regulation of Translation, Translocation, and Degradation of Proteins by ER-resident co-chaperones

- Daverkausen-Fischer L, Draga M, Prols F. Regulation of Translation, Translocation, and Degradation of Proteins at the Membrane of the Endoplasmic Reticulum. Int J Mol Sci 2022; 23(10).

First steps in the synthesis and maturation of membrane as well as secretory and ER luminal proteins involve the translation of a protein from the respective mRNA by ribosomes and translocation across the ER membrane. Proteins can be translocated across the ER membrane co- or post-translationally [329]. Within the ER lumen the protein undergoes folding and post-translational modifications giving rise to the mature protein structure [330]. When translocation of a protein is impaired or if a protein cannot be folded correctly due to mutations, the protein has to be retrotranslocated to the cytosol for its degradation [67,330]. During ER-stress, translation can be arrested by the ER membrane protein PERK [114]. Also, ERAD of misfolded proteins is stimulated by IRE1 dependent upregulation of essential ERAD components [109]. Analyzing the published experimental data, we evaluated what is currently known on ERdj functions with regard to controlling cellular proteostasis. We found that ERdj1 and ERdj2 can regulate protein translation by direct (in the case of ERdj1) or indirect (via Sec62 in the case of ERdj2) association with ribosomes [126,127,134,141,155]. Furthermore we found that ERdj2 and ERdj6 can affect protein translocation [67,164,167,168]. ERdj2 is required for proper translocation of a subset of co-translationally translocated proteins in mammalian cells [165,167]. ERdj6 on the other hand was proposed to mediate co-translocational degradation by clearing translocons of translocation incompetent proteins [67], a thesis that has been supported by a recent publication [331]. Summarizing, ERdj2 is involved in “pulling” translocating proteins into the ER lumen, while ERdj6 is involved in “pulling” proteins out into the cytosol for degradation which displays an interesting opposing role of ERdj2 and ERdj6.
Four of the co-chaperones, ERdj3, ERdj4, ERdj5, and ERdj6, have been reported to bind to aggregation prone sequences in substrate proteins and to be involved in the ERAD pathway mediating the degradation of a subset of misfolded substrates [66,76,216,263]. In the presented review, all published data on ERdj protein involvement in cellular proteostasis were compared and discussed. Open questions were highlighted and methods to investigate these open issues were suggested.

7. Discussion

7.1. Topology and subcellular localization of ERdj proteins

The topogenesis of membrane proteins has been a scientific field of interest for many years reaching back to the 1980s and beyond. How proteins possessing transmembrane domains orientate within a membrane is determined by different features. In the following section, I want to summarize what is currently known about the mechanisms that enable membrane proteins to adopt dual topologies in prokaryotes as well as in eukaryotes. Also, I am going to highlight the link between dual topology and dual localization of proteins. Thereafter, I shortly present a subset of proteins that are known to possess a dual topology/dual localization in eukaryotic cells. The most important ambiguous results regarding the topology of ERdj proteins will be discussed, also in the context of a recent paper. In the end, important open questions will be summarized and experimental procedures to address these issues will be suggested.

The topology of a membrane protein can have important implications as the topology of a protein determines which domains of a protein are located within the cytoplasm and which domains are located luminally. Therefore, the topology of a protein determines potential binding partners and also the function of a protein [260,262]. Some of the features that determine the topology of membrane proteins have striking similarities with features that determine whether the signal peptide inserts itself in a head-on orientation or in a loop-orientation into the translocon during protein translocation [328]. As for transmembrane domains, positive charges flanking the signal peptide were shown to be determinant for the mode of signal peptide integration and translocation efficiency of proteins [332]. It was proposed that BiP, ERdj2 and Sec62 mediate flipping of “weak” signal peptides that prefer a head-on orientation within the translocon due to positively charged amino acid residues at the C-terminal end of the signal peptide and therefore enable the proteins to translocate across the ER membrane [166]. Signal peptides can serve as signal anchors or reverse-signal anchors that anchor proteins to the ER membrane [328]. Therefore, the mode of signal peptide insertion in a head-on orientation or a loop orientation can determine the topology of a given protein [328]. Interestingly, the strength of a signal peptide does not only determine the mode of integration into the Sec61 translocon or whether the signal peptide is dependent on accessory factors for translocation but it also influences whether a protein has dual localizations in the ER lumen and the cytosol which can be a consequence of a dual mode of signal peptide insertion [333] (see Figure 18).

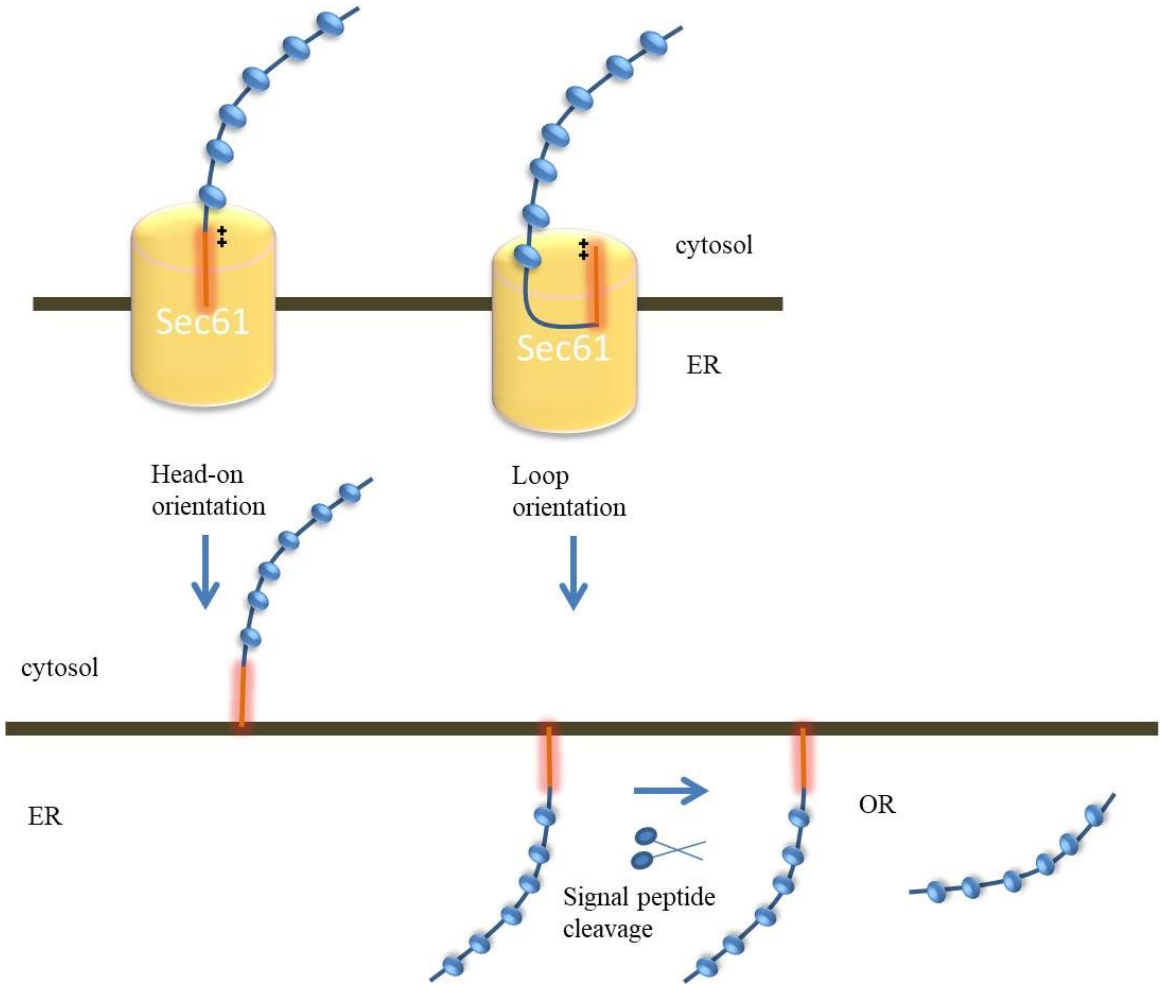

Fig. 18 Mode of signal peptide insertion can affect topology of membrane proteins
Proteins possessing positively charged amino acids in the C-terminal region of their signal peptide or close to the C-terminal region favor to insert in a head-on orientation during translocation [332,334]. If the signal peptide serves as a reverse signal anchor, a type I ER membrane protein emerges that is anchored in the ER membrane via its signal peptide [328]. Proteins that possess positively charged amino acids in or close to the N-terminal region of their signal peptide prefer a loop orientation during signal peptide insertion [332,334]. As a result the C-terminal part of the newly synthesized protein is translocated into the ER lumen [332]. Depending on the protein the signal peptide can either serve as a signal anchor giving rise to a type II ER membrane protein facing the ER lumen or the signal peptide is cleaved off the protein giving rise to an ER luminal protein as was shown for EDEM1 [75].

Apart from signal peptide integration, there are different mechanisms that can facilitate one protein being present in the cytosol as well as in the ER lumen [333]. Alternative splicing events were discussed to be responsible for the presence of a cytosolic BiP pool [30]. Also, inefficient recognition of the first start codon by the ribosome has been described and can result in different subcellular localizations of one protein [333,335,336]. As was shown for a preprolactin construct, translational initiation from a subsequent start codon results in a subpopulation not being targeted to the ER even though it possesses a signal peptide [333]. It might also happen that translational initiation from a subsequent start codon results in a protein pool lacking the signal peptide or having a signal peptide with impaired function.

Inefficient recognition by the SRP could be another mechanism that could allow for two pools of the same protein, one residing in the cytosol and one residing in the ER lumen (see Figure 19).

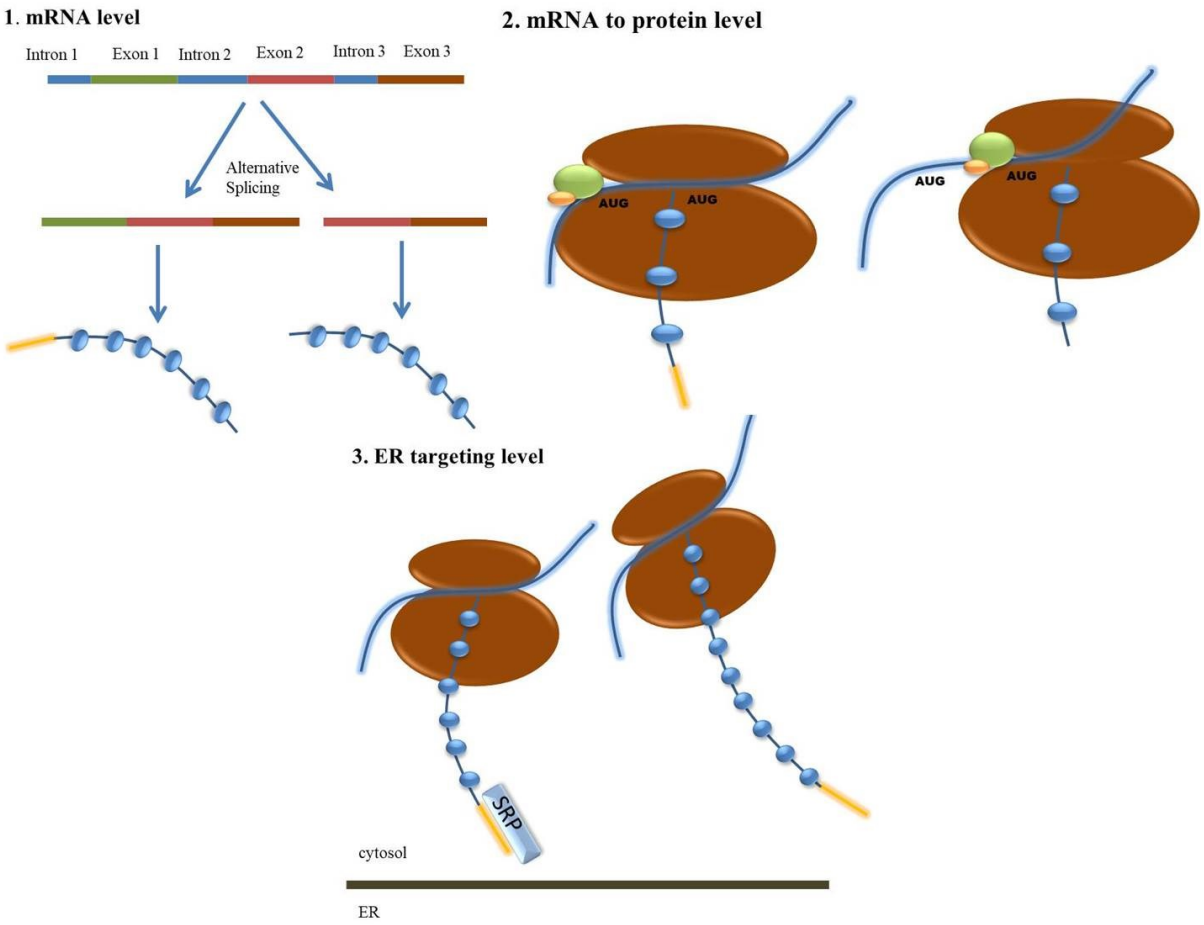

Fig. 19 Mechanisms that result in a dual localization of proteins
Different mechanisms can result in a dual localization of the same protein with a pool residing in the ER and another pool being present in the cytosol. On the mRNA level, alternative splicing events can result in two different mRNAs giving rise to two different protein pools: One containing the signal peptide and being targeted to the ER and the other lacking the signal peptide therefore residing in the cytosol. This mechanism has been shown to be responsible for the generation of a cytosolic BiP pool [30]. On the mRNA to protein level, leaky scanning of the ribosome can result in translational initiation from different start codons resulting in different protein pools with different subcellular localizations [333,335,336]. Furthermore, inefficient recognition of a signal peptide by the SRP could result in two protein pools: One being targeted to the ER via interaction with the SRP and one pool residing in the cytosol.

To investigate the topology and localization of proteins, different experimental procedures can be employed. Proteins can be fused to reporters like GFP or hemagglutinin and subsequently be visualized by fluorescence. This method is used to determine the subcellular localization of a protein [53,61,209]. Proteinase K digests can be performed to discriminate between ER luminal protein pools or protein domains that are protected from

proteinase K treatment and cytosolic protein pools or protein domains that are digested by proteinase K [51,60,127] . N-linked glycosylation takes place as a post-translational modification in the lumen of the ER, so that glycosylation is a sign for successful translocation into the ER lumen. Therefore, EndoH treatment, that removes oligosaccharides from a glycosylated protein, can be used to examine whether a protein has been translocated into the ER lumen or not [60,62]. Also, non-glycosylated proteins can be fused to glycosylation sites, overexpressed in eukaryotic cells and subsequently subjected to EndoH treatment to discriminate between glycosylated and non-glycosylated forms and accordingly between ER luminal or cytosolic localization [55]. Another experimental method to investigate subcellular localization of a given protein is the identification of potential interaction partners that can exclusively be found in a specific cellular compartment by coimmunoprecipitation. However, it has to be noted that coimmunoprecipitation of two proteins does not necessarily has to be due to a direct interaction between the two proteins but can also be mediated by a third protein that connects the two proteins. Also, cell fractionation can be used to identify the localization of proteins [50]. Here it is important that appropriate marker proteins are used. For example, PDI has often been used as a marker for ER localized proteins [62]. Over time, it was shown that PDI can be localized in various intracellular compartments making the protein an inappropriate marker protein [161,162]. Apart from experimental procedures, computational tools such as DeepLoc can also be used to predict subcellular localization of proteins. However, computer-based predictions need always to be validated experimentally. With regard to ER luminal proteins, another interesting question to be asked is whether the respective protein exists as a free floating luminal protein or whether it is anchored in the ER membrane by its signal peptide. To assess this question, carbonate extraction experiments can be done that are used to discriminate between integral membrane proteins that remain in the pellet after carbonate extraction or membrane associated proteins that will be dissolved in the supernatant. Furthermore, proteins can be translated in the absence and presence of microsomes. If a protein is shorter after translocation into the microsome this suggests that the signal peptide is cleaved upon translocation into the ER lumen. Also, computational programs such as Signal IP can be used to predict the probability of signal peptide cleavage for a given protein.

However, there are limitations to most of these methods. Limitations of the carbonate extraction method include the fact that proteins having less hydrophobic transmembrane domains can be extracted from inner mitochondrial membranes by carbonate extraction even though they constitute integral membrane proteins [337]. When proteins are tagged and visualized by immunofluorescence as well as by immunoblotting a lot of noise is generated by the protein pool being in the prevailing localization that impedes documentation of other

smaller subcellular protein populations [333]. To make small cytoplasmic protein pools visible, the noise of the predominant protein pool has to be reduced and the signal by the smaller cytosolic protein pool has to be amplified [333]. Adressing this problem, a reporter assay was generated by one group that only allowed the cytosolic protein pool to generate luciferase luminescence thereby circumventing the "noise" generated by the corresponding predominant ER-luminal protein pool [333]. The experimental methods that can be used to examine topology and localization of proteins and the limitations of each method are summarized in Table 4.

Table 4 Experimental methods that can be used to examine the topology and localization of proteins

Experimental method	
Immunocytochemistry of tagged proteins	- Information about the subcellular localization of a given protein - Limitation: Noise generated by predominant protein pool
Proteinase K treatment and subsequent immunoblotting	- Used to differentiate between ER luminal protein pools or protein domains and cytosolic protein pools or protein domains - Limitation: Noise generated by predominant protein pool
EndoH treatment and subsequent immunoblotting	- Cleaves oligosaccharides from proteins that have been subjected to N-linked glycosylation - Detection of a smaller protein band after treatment suggests the existence of an ER luminal protein pool - Method can also be used to assess whether a protein is glycosylated in the ER lumen - Limitation: Noise generated by predominant protein pool
Coimmunoprecipitation	- Information about potential binding partners - Limitations: o Coimmunoprecipitation of two proteins does not necessarily have to be due to a direct interaction but can also be mediated by a "linker" protein o Appropriate marker proteins for different cell

	compartments have to be used
Cell fractionation and subsequent immunoblotting	- Information about subcellular localization of a protein - Limitation: Appropriate marker proteins for different cell compartments have to be used to prove the purity of the fraction
Carbonate extraction	- Is used to differentiate between integral membrane proteins and membrane-associated proteins - Limitation: Integral membrane proteins with faintly hydrophobic transmembrane domains can behave like membrane-associated proteins
Computational analysis (DeepLoc/SignalIP)	- Predicts probability of certain subcellular localizations - Predicts probability of signal sequence cleavage - Limitation: Only prediction

Dual topology of membrane proteins has originally been studied in E.coli. It was found that there is a variety of small membrane proteins that can adopt dual topology in bacteria [324]. A set of different mechanisms can result in a dual topology. Firstly, positively charged lysine and arginine residues flanking transmembrane segments have been found to determine the membrane orientation of transmembrane domains [324,338]. The “positive-inside rule” was first stated in 1990 [339]. The rule was deduced from the observation that in bacteria, luminal loops of transmembrane proteins possess less positive charges than cytoplasmic loops [339,340]. The difference between the sum of arginine and lysine residues in the lumen and the sum of arginine and lysine residues in the cytoplasm is called the K+R bias [339]. Proteins with a small K+R bias are proposed to have a greater tendency to adopt a dual topology within a membrane [324]. Indeed, introducing only single arginine or lysine charges into these proteins can result in a re-orientation of the respective protein within the membrane [324]. Gene duplications have been found in proteins that adopt a dual topology, resulting in the same protein being encoded by two genes [324,341]. The two emerging proteins can have opposite K+R biases and therefore only differ in their membrane orientation [324]. Apart from gene duplication events and the K+R bias, it was also proposed that the lipid composition of

membranes can influence the orientation of prokaryotic membrane proteins [342,343].

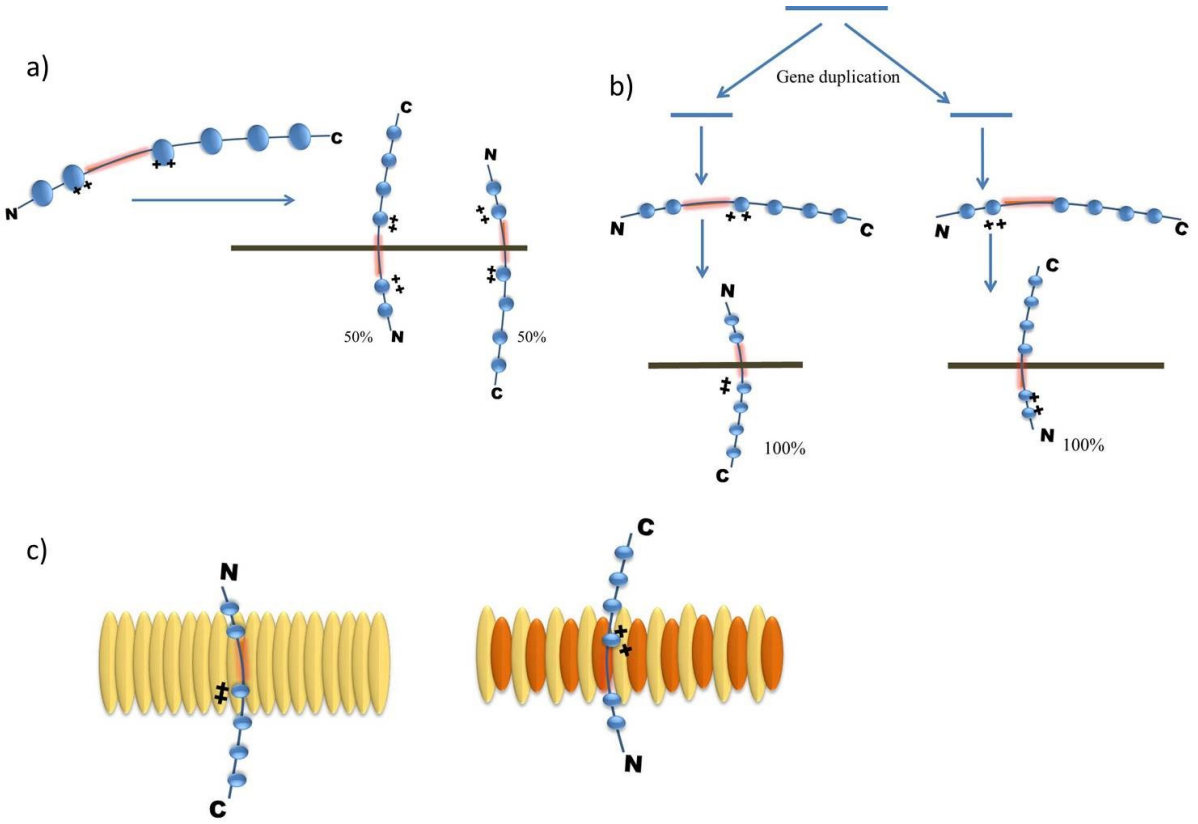

Fig. 20 Mechanisms that result in a dual topology of membrane proteins
Different mechanisms can result in a dual topology of a prokaryotic protein. a) Proteins can adopt a dual topology due to their low K+R bias [324]. b) Gene duplication events can result in two different protein pools that only differ in their K+R bias and therefore adopt a dual topology [324,341]. c) Proteins can also adopt a dual topology depending on membrane composition [342,343].

Apart from their detailed examination in prokaryotes, dual-topology proteins have also been examined in eukaryotic cells. For the ER resident protein calreticulin, it was shown that the protein has a dual localization: In the ER lumen it serves as a chaperone, while functions in the cytosol include among others regulation of integrin function by binding to the cytosolic tails of integrin proteins and regulation of the translation of a subset of mRNAs [344]. The proportion of the cytosolic protein pool can be modified by exchange of the signal peptide. Exchange of the calreticulin signal peptide for the “strong” signal peptide of prolactin reduced the fraction of cytosolic calreticulin [344]. For various proteins it was shown that the ratio between cytosolic and ER luminal localized protein pools depends on the cell type and accordingly to different sets of accessory proteins [333]. A fraction of the preS subunit of the Hepatitis B large envelope protein was shown to translocate post-translationally giving rise to a dual topology with the preS subunit being partially located within the ER lumen and partially located in the cytoplasm [345]. Post-translational translocation of the preS subunit was shown to be dependent on BiP in HEK 293 cells [260]. This example shows that proteins can adopt a

dual topology in a post-translational way and that accessory proteins are playing a role in the generation of a dual topology [260,345]. Furthermore the melanocortin 2 (MC2) receptor accessory protein (MRAP) was shown to exhibit a dual topology in COS cells forming antiparallel homodimers [346]. Interestingly, a positively charged region located close to the transmembrane domain is important for this dual topology as deletion of the region abolishes the dual topology exhibited by the wildtype protein [347]. MRAP is important for trafficking of the adrenocorticotropic hormone (ACTH) receptor MC2 to the plasma membrane [348]. This function is also lost upon loss of the dual topology of MRAP highlighting the relevance of protein topology with regard to protein function [347]. Other proteins exhibiting a dual topology in eukaryotic cells are diacylglycerol acyltransferase1 (DGAT1), a protein involved in triglyceride synthesis [326], fatty acyl-CoA reductase 1, a protein involved in the synthesis of fatty acids [349], aquaporin-1 [350], ductin [351] and p-glycoprotein [352]. In the case of p-glycoprotein it was found that charged residues flanking transmembrane domain 4 are responsible for the dual topology of the protein [353]. Deletion of N-terminally located charged residues in the ductin protein, however did not affect the topology of ductin [351].

With regard to ERdj3, ERdj4 and ERdj6 there is also experimental data pointing to different localizations and dual topologies of these proteins. In the review:

- Daverkausen-Fischer L, Prols F. Dual topology of co-chaperones at the membrane of the endoplasmic reticulum. *Cell Death Discov* 2021; **7**(1): 203.

we discussed the experimental data that point to a dual topology of ERdj3, ERdj4 and ERdj6 [122]. For ERdj4, experimental data point to three different protein pools. Two ER membrane anchored pools, one facing the ER lumen and the other the cytosol and a third free floating protein pool within the ER lumen [61,258,259,262]. The fact that a subpopulation of ERdj4 is present as an integral membrane protein is supported by carbonate extraction experiments that could detect a fraction of endogenous ERdj4 in the pellet after extraction [354]. The existence of ERdj4 in this dual topology would however require a dual mode of signal peptide integration into the translocon. This would imply that the signal peptide of ERdj4 is rather weak. To assess this, it would be interesting to investigate whether ERdj4 can translocate across the ER membrane independently or whether translocation is dependent on accessory proteins like BiP, ERdj2 or Sec62. As ERdj4 is hardly expressed under non-stress conditions, a very sensitive assay must be used. As ERdj4 levels are largely elevated undER-stress conditions, it would further be of interest which of the subcellular pools is increased and whether different stressors induce elevated levels of ERdj4 in different subcellular compartments. Two groups have performed proteomic analysis in HeLa cells in which ERdj2 was downregulated [165,355]. Here, ERdj4 could not be identified as a target of ERdj2. It has to be noted, that the experiments were not conducted under conditions of ER-stress so that

under the experimental conditions ERdj4 was only barely expressed. Furthermore proteomic analysis can only detect differences in the amount of whole cellular protein. If a protein depends on ERdj2 for translocation but can also be present in the cytosol without being degraded, the total amount of protein would not change upon downregulation of ERdj2 but there would rather be a shift between different subcellular pools.

In the case of ERdj3, translocation was shown to depend on ERdj2, Sec62 and BiP [165,167]. Exchanging the signal peptide of ERdj3 for that of prolactin conferred increased translocation efficiency to ERdj3 [165]. Deletion of the J-domain of ERdj3 made translocation of ERdj3 independent of BiP and ERdj2 [165]. The J-domain of ERdj3 is in close vicinity to the signal peptide and contains two positively charged amino acids and four alpha helices [165]. Deletion of the positively charged residues resulted in increased ERdj2 and BiP independency during translocation of ERdj3. However, the alpha helical domains within the J-domain also seem to affect ERdj2 and BiP dependency as a mutant lacking positively charged residues but containing two alpha helical domains still exhibited a certain level of ERdj2 and BiP dependency during translocation [165]. According to the data it can be stated that the signal peptide of ERdj3 is rather weak and translocation across the membrane requires accessory proteins as ERdj2, Sec62 and BiP. ERdj3 should therefore have the potential to adopt a dual topology. In cell free assays, a major part of ERdj3 was shown to be sensitive to proteinase K treatment, which could indicate the presence of a cytosolic ERdj3 pool. However, the authors attributed the results to the inefficiency of the translocation assay employed [92,204]. This assumption was confirmed in HED-3 cells, in which the complete ERdj3 protein pool was shown to be resistant to proteinase K treatment. Accordingly, these data suggest that the entire ERdj3 pool is located within the ER lumen (at least in unstressed cells) [60]. Inactivation of BiP results in the accumulation of cytosolic pre-ERdj3 that still contains the signal peptide [165] indicating that in stressed cells ERdj3 is predominantly localized in the cytosol. In HeLa cells, this cytosolic ERdj3 pool is readily degraded by the proteasome as was detected by comparison of protein levels before and after treatment with a proteasome inhibitor [165]. This implies that even though a cytosolic pool of ERdj3 can be present when translocation is impaired, this pool is readily degraded and therefore might not play a physiological role in the cell. There is ambiguous data on whether within the ER ERdj3 only exists as an ER luminal protein or whether it can also exist as an integral membrane protein [52,60,92]. Carbonate extraction experiments show that a fraction of ERdj3 is present as an integral membrane protein [258] even though the predominant pool shows signal peptide cleavage upon translocation [92]. However, whether ERdj3 indeed exists as a transmembrane protein in cells is still not clear and has to be examined in future experiments. The major pool of ERdj3 seems to be located in the lumen of the ER, but a minor pool might also be present

as an integral membrane protein whereas the cytosolic pool seems to exist transiently when translocation or proteasomal degradation is impaired.

Also the subcellular localization of ERdj6 was addressed in our reviews and the existence of a cytosolic next to the ER pool is discussed. A recent paper by Pauwels et al. [331] re-addressed the dual topology and localization of ERdj6. The group examined in vitro translation and translocation of ERdj6 in cell free assays. In an autoradiogram, two different ERdj6 bands could be detected after proteinase K treatment. The slower migrating one represented the preprotein with signal peptide and the faster migrating band represented the signal peptide cleaved form of ERdj6. Signal peptide cleavage was observed in 80% of the cases. However, when HEK293 cells were transfected with ERdj6, only one protein band could be detected representing the cleaved ERdj6 protein pool [331]. Accordingly, the authors questioned the existence of an additional ER luminal, uncleaved, membrane-bound pool of ERdj6 [331]. However, another group could identify different ERdj6 pools in cells upon ER-stress induced by thapsigargin [55]. In unstressed cells all ERdj6 was translocated into the ER lumen and the signal peptide was cleaved [55]. After eight hours of thapsigargin treatment, an additional ERdj6 band appeared that constituted an uncleaved luminal protein pool [55]. After 24 hours of thapsigargin treatment a small fraction of ERdj6 could be found in the cytosol probably anchored in the ER membrane by the signal peptide [55]. The different results could be due to differences in the experimental settings used by the two groups. Firstly, the experiments done by Pauwels et al. [331] were conducted upon treatment with CADA, an inhibitor of translocation while Rutkofski et al. [55] conducted their experiments upon treatment with thapsigargin, an inhibitor of SERCA. Secondly, different ERdj6 constructs were used. Pauwels et al. [331] used an ERdj6 construct containing the ERdj6 signal peptide and the first 62 amino acids of ERdj6 fused to the human cluster of differentiation 4 (CD4) protein while Rutkofski et al. [55] used an ERdj6 protein with artificially introduced glycosylation sites. Thirdly, the cell type varied in the two experiments. Pauwels et al. [331] used HEK293 cells while Rutkofski et al. [55] used MEFs. Indeed, there is experimental evidence that the translocation efficiency can vary between cell types as was shown for the Prion Protein [333]. Pauwels et al. also noted that uncleaved, proteinase K resistant protein pools do not necessarily represent membrane bound protein pools but could also represent uncleaved luminally located, free floating protein pools [331]. To assess this, carbonate extraction or alkaline flotation assays should be performed [331].

It also still remains an open issue whether a cytosolic pool of ERdj6 exists. In the cell free translation and translocation assays, proteinase K treatment showed that most but not the entire pool of ERdj6 was sensitive to proteinase K treatment which supports the presence of a cytosolic ERdj6 pool [331]. In NIH3T3 cells, the existence of a cytosolic ERdj6 pool after 24

hours of thapsigargin treatment was confirmed by proteinase K treatment [55]. Also ERdj6 was shown to inhibit PERK signaling by binding to the cytosolic domain of PERK [31]. According to the recently published data, no statement can be made regarding the existence of a cytosolic ERdj6 pool in HEK293 cells [331].

In conclusion, ERdj3, ERdj4 and ERdj6 have the potential to be present as dual topology proteins within the cell. How different pools of the proteins are affected by stress and the functions of the different pools have to be assessed in future studies.

7.2. Open questions regarding ERdj involvement in cellular proteostasis

In the review:

- Daverkausen-Fischer L, Draga M, Prols F. Regulation of Translation, Translocation, and Degradation of Proteins at the Membrane of the Endoplasmic Reticulum. *Int J Mol Sci* 2022; **23**(10).

we already discussed how ERdj1 and ERdj2 can inhibit translation by either direct or indirect association with ribosomes [124]. Prebinding of ERdj1 to BiP inhibits ERdj1 binding to ribosomes [141]. However, it was also shown that the amount of ERdj1 bound to ribosomes is not affected by the presence or absence of BiP when ERdj1 was simultaneously co-incubated with BiP and ribosomes indicating that BiP cannot dissociate preformed ERdj1/ribosome complexes [126]. Still, in the presence of BiP translational inhibition mediated by ERdj1 is abolished [126]. It was further shown that the amount of BiP found in the ribosomal pellet increased in the presence of ERdj1 indicating that ERdj1 recruits BiP to ribosomes [127]. It has been suggested that ERdj1 might act as an additional ER-stress sensor that is bound to BiP in unstressed cells and dissociated from BiP under conditions of ER-stress resulting in translational arrest [126]. To further examine this hypothesis, it would be interesting to assess whether the amount of BiP bound to ERdj1 decreases under conditions of ER-stress. A putative phosphorylation of ERdj1 does not affect the ERdj1/ribosome interaction [135]. The implication of phosphorylation of ERdj1 on its association with BiP has not been assessed. It would be interesting to assess whether phosphorylation incompetent ERdj1 mutants can still recruit BiP to ribosomes.

Also, translational inhibition mediated by ERdj1 has only been assessed under in vitro conditions so far. Therefore, it would be interesting to see whether ERdj1 can inhibit translation under in vivo conditions and whether binding of ERdj1 to ribosomes increases undER-stress conditions.

Analogous to ERdj1, the ERdj2 interaction partner Sec62 was shown to bind to ribosomes [155]. Binding of ribosomes to Sec62 displaced Sec62 from ERdj2 [155]. ERdj2 is a substrate for the protein kinase CK2 and phosphorylation of ERdj2 was shown to strengthen interaction between ERdj2 and Sec62 [154]. Therefore, it would be interesting to investigate whether phosphorylation of ERdj2 prevented dissociation of Sec62 from ERdj2 in the presence of ribosomes. To assess this, surface plasmon resonance could be employed. Sec62 could be immobilized on a sensor chip and be incubated with phosphorylated ERdj2 to allow pre-binding of Sec62 with ERdj2. Afterwards, buffer or buffer containing ribosomes could be passed over the sensor chip. If pre-binding of Sec62 to phosphorylated ERdj2 prevented binding of Sec62 to ribosomes, no response should be detected after passing ribosomes over the chip.

Whether phosphorylation of ERdj2 is important for translocation of proteins in mammalian cells has not been addressed so far. In yeast, it was shown that phosphorylation incompetent ERdj2 had a negative effect on the translocation of substrate proteins [356]. In mammalian cells, binding of Sec62 to ribosomes inhibits translation [155]. However, Sec62 also mediates translocation of a subset of substrate proteins across the ER membrane [165,167]. In order for a protein to translocate, translation has to be on-going, a process that seems to be controlled by ERdj2. It can be assumed that Sec62 inhibits translation until the translocon complex has been assembled. Phosphorylated ERdj2 could then bind to Sec62 and displace Sec62 from the ribosome allowing translation to continue and translocation to begin. To test this hypothesis various questions have to be experimentally addressed. Firstly, could phosphorylated ERdj2 displace Sec62 from ribosomes? To assess this, ribosomes could be incubated with Sec62. Afterwards the pellet and supernatant fractions should be separated and the amount of bound Sec62 could be determined by immunoblotting. Subsequently, Sec62 should again be incubated with ribosomes and after some time phosphorylated or non-phosphorylated ERdj2 should be added. Pellets and supernatant fractions should again be separated and the amount of Sec62 and ERdj2 in the ribosomal pellet should be assessed. If phosphorylated ERdj2 were able to displace Sec62 from ribosomes, there should be less Sec62 bound to ribosomes in the presence of phosphorylated ERdj2 than in the presence of non-phosphorylated ERdj2 or under control conditions. Another method to assess the question would be to use Surface Plasmon Resonance (SPR). Ribosomes could be passed over a sensor chip containing immobilized Sec62. Afterwards non-phosphorylated or phosphorylated ERdj2 should be allowed to pass over the chip to evaluate if pre-binding of ribosomes prevents interaction of Sec62 with ERdj2.

In order to validate the model that phosphorylation of ERdj2 affects protein translocation in mammalian cells, protein translocation assays could be done using phosphorylation incompetent ERdj2 mutants as were already performed by Wang et al. in yeast [356].

While ERdj1 seems to recruit BiP to ribosomes, it could be that Sec62 recruits ERdj2 to the translocon to assist in translocation. In order to test this hypothesis it would be interesting to investigate whether the amount of ERdj2 associated with the translocon increased in the presence of Sec62 or whether the amount of ERdj2 associated with the translocon decreased upon downregulation of Sec62. BiP and ERdj2 are both involved in the assistance of translocation of a subset of protein. Therefore, ERdj1 and Sec62 could work together to assemble the translocon complex. In this context, it would be interesting to assess whether ERdj1 is involved in protein translocation and if this were the case whether this is dependent on the phosphorylation status of ERdj1. Interestingly, the question has recently been assessed by one group [355]. It was shown that when ERdj1 was depleted, protein levels of

172 proteins were altered [355]. While 80 proteins were quantitatively upregulated, the remaining 92 proteins were quantitatively downregulated [355]. This data could hint at a role of ERdj1 in protein translocation. However, a protein can regulate the levels of other proteins also by mechanisms other than translocation. For ERdj1 a role as a nuclear transcription factor has been proposed [136]. This could be a possible mechanism how ERdj1 regulates the levels of other proteins. In order to further assess the mechanism by which ERdj1 affects the levels of proteins, protein translocation assays could be done comparing the amount of translocated proteins in the presence and absence of ERdj1. A theoretical model of how ERdj1 and ERdj2 might regulate translation and translocation under conditions of ER-stress as well as under unstressed conditions is presented in Figure 21.

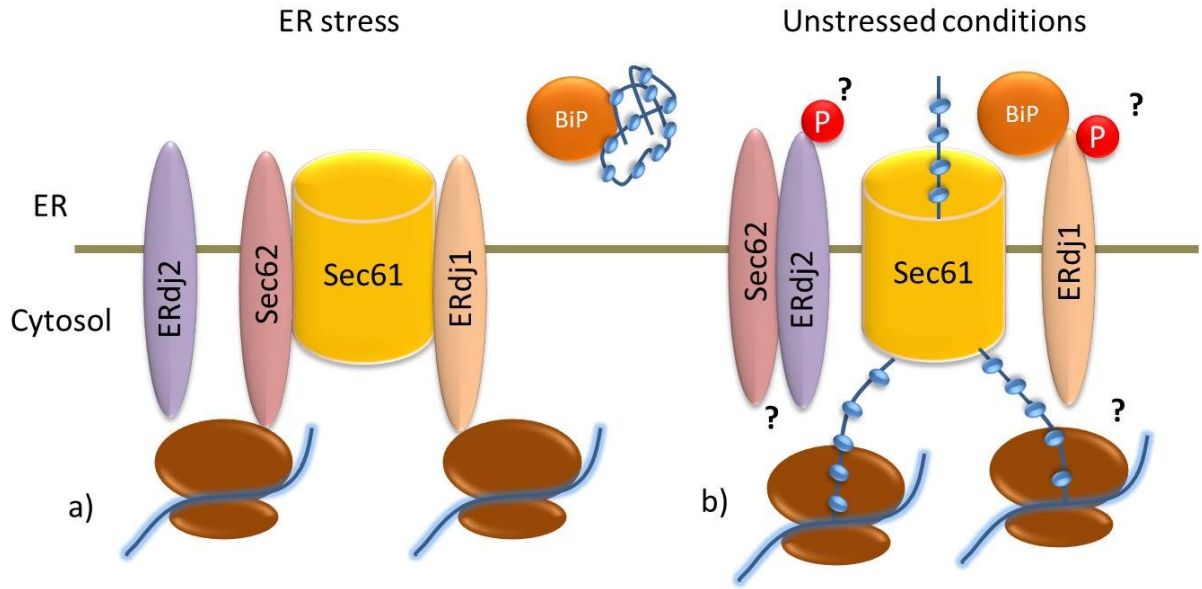

Fig. 21 Assembly of the translocon complex mediated be ERdj1 and Sec62
ERdj1 and ERdj2 are involved in the regulation of protein translation and translocation [126,167]. a) Under conditions of ER-stress BiP is occupied with misfolded proteins and assists their folding. Under these conditions less BiP should be bound to ERdj1 allowing ERdj1 to bind to ribosomes and inhibit protein translation. Similarly, Sec62 should bind to ribosomes and inhibit translation. Translational inhibition by ERdj1 and Sec62 decreases the protein burden within the ER lumen b) After ER-stress has been overcome, BiP should bind to ERdj1 releasing the translational arrest mediated by ERdj1. Furthermore, BiP assists in translocation of newly synthesized proteins at the Sec61 translocon. Binding of ERdj2 to Sec62 might also release the translational arrest mediated by Sec62. After translation has been initiated, Sec62 and ERdj2 assist in protein translocation.
Question marks highlight uncertain ties. Whether or not ERdj2 can displace Sec62 form ribosomes and whether phosphorylation of ERdj2 is involved in displacement of Sec62 from ribosomes is not known. Also, it is not known whether phosphorylation of ERdj1 affects binding of ERdj1 to BiP. Whether the release of translational arrest mediated by ERdj1 goes along with a release of ERdj1 from ribosomes or whether only a conformational change occurs has also not been investigated.

For ERdj6, a role in co-translocational degradation has been proposed [67]. However, it is still unclear whether co-translocational degradation is mediated by the ERdj6 pool located

within the ER lumen or by the cytosolic protein pool. It was shown that mutations within the J-domain of ERdj6 impair co-translocational degradation implying that interaction with an Hsp70 chaperone is important for co-translocational degradation [67]. As ERdj6 was found in the same fractions as the Sec61 translocon but also as cytosolic HSP70, it was proposed that cytosolic ERdj6 extracts substrates from the translocon in cooperation with cytosolic chaperones [67]. However, there is new convincing data that it is the ERdj6 luminal pool that affects translocation efficiency [331]. Pauwels et al. [331] showed that when the entire ERdj6 pool was located within the ER lumen (by using PPL-ERdj6, a preprolactin signal peptide fused to ERdj6), protein levels of translocation deficient substrates were reduced to a larger extent than in WT ERdj6 transfected cells [331]. Yet, this effect could also be interpreted to be due to a titration effect since within the ER lumen ERdj6 might displace ERdj2 from the translocating substrates.

7.3. Negative feedback-loops during the UPR mediated by ERdj proteins

While activation of the UPR during ER-stress can be beneficial to clear the ER of misfolded cargo by promoting ERAD and reducing the protein load within the ER, a sustained activation of the UPR can result in cell death [357]. To ensure cellular survival, a feed-back mechanism is required to switch off the UPR. For ERdj2 and ERdj4 it was shown that they can recruit BiP to the IRE1 luminal domain thereby switching off IRE1 signaling during ER-stress or keeping IRE1 in a silenced state [113,358]. For ERdj4 a role in negative regulation of the UPR was also predicted by gene ontology enrichment analysis [359]. With regard to ERdj6, it was shown that ERdj6 can bind to the cytosolic domain of PERK and switch off PERK signaling as was measured by reduced levels of phosphorylated eIF2α, phosphorylated PERK and ATF4 in ERdj6 overexpressing cells [31,91]. Interestingly, cytosolic ERdj6 fractions have been shown to be upregulated during ER-stress suggesting that this is a mechanism to switch off PERK signaling at the end of a stress period, thereby re-initiating protein translation [55]. To examine whether the luminal ERdj6 pool is also involved in negatively regulating PERK signaling it would be interesting to investigate whether an ERdj6 translocation efficient PPL-ERdj6 construct can also inhibit PERK signaling. Furthermore, it would be interesting to assess whether the regulatory role of ERdj6 is dependent on a functional J-domain. If this were the case, ERdj6 might recruit BiP or cytosolic Hsp70 chaperones to PERK. Similar to ERdj6, ERdj5 was shown to downregulate levels of phosphorylated eIF2α and PERK in PDLSCs [285,295]. ERdj5 was also shown to associate with PERK in total cell lysates of colon cancer cells [296]. So far, the domains required for interaction of ERdj5 protein and PERK are not known. It was, however, shown that overexpression of the ERdj5 J-domain was sufficient to reduce levels of phosphorylated eIF2α and to protect cells from thapsigargin mediated apoptosis [295]. This could indicate that ERdj5 is involved in recruitment of BiP to the luminal domain of PERK. Another explanation would be that ERdj5 interacts with PERK via its J-domain. As there are no ambiguous data on the localization of ERdj5 and neither the topology of ERdj5, it is probable that ERdj5 regulates PERK signaling from within the ER lumen maybe in an analogous way as was described for ERdj2 and ERdj4 with regard to IRE1 signaling. Therefore, it would be interesting to investigate whether levels of BiP bound to PERK differ in the presence or absence of ERdj5. Interestingly, in CHO cells lacking ERdj5 or ERdj6, PERK signaling was not increased under unstressed conditions [358]. However, in CHO cells lacking ERdj2 or ERdj4 significant activation of the IRE1 pathway was reported in comparison to wildtype cells under unstressed conditions [358]. This implies that ERdj2 and ERdj4 are involved in keeping IRE1 in a silenced state also under unstressed conditions while ERdj5 and ERdj6 might be involved in switching off PERK signaling after activation during the UPR.

All in all, ER resident co-chaperones in mammalian cells are involved in a multitude of cellular signaling pathways and regulate many different cellular functions in many different cellular compartments. There is increasing data on the involvement of the co-chaperones in various diseases. However, there are still several open questions that have to be addressed in the future to gain an even deeper understanding of how ER resident co-chaperones maintain cellular proteostasis and homeostasis and how the ER-resident co-chaperones are connected to disease.

8. Literature

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9. Supplement

9.1. Table of figures

9.2. Tableindex

10. Pre-release

1. Daverkausen-Fischer L, Motyl-Eisemann M, Draga M, Scaal M, Prols F. Protein expression pattern of the molecular chaperone Mdg1/ERdj4 during embryonic development. *Histochem Cell Biol* 2020; **154**(3): 255-63.

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