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Endoplasmic Reticulum

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Endoplasmic Reticulum
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Contributors
Petek Ballar, Burcu Erbaykent Tepedelen, Han-Jung Chae, Hyung-Ryong Kim, Mohammad Fazlul Kabir, Angel Catala, Christian A.M. Wilson, Hilda M. Alfaro-Valdes, Francesca Burgos-Bravo, Diego Quiroga-Roger, Nathalie Casanova-Morales, Gerardo Hebert Vázquez-Nin, Nayeli Torres-Ramírez, Rosario Ortíz-Hernández, Echeverría Om, Ml Escobar

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IntechOpen Book Series

Physiology

Volume 2

Angel Catalá was born in Rodeo (San Juan), Argentina. He studied chemistry at the Universidad Nacional de La Plata, Argentina, where he received a PhD in Chemistry (Biological Branch) in 1965. From 1964 to 1974, he worked as an assistant in Biochemistry at the School of Medicine, Universidad Nacional de La Plata, Argentina. From 1974 to 1976, he was a fellow of the National Institutes of Health (NIH) at the University of Connecticut, Health Center, USA. From 1985 to 2004, he served as a Full Professor of Biochemistry at the Universidad Nacional de La Plata, Argentina. He is a member of the National Research Council (CONICET), Argentina, and Argentine Society for Biochemistry and Molecular Biology (SAIB). His laboratory has been interested for many years in the lipid peroxidation of biological membranes from various tissues and different species. Professor Catalá has directed 12 doctoral theses and published more than 100 papers in peer-reviewed journals, several chapters in books, and 12 edited books. He received awards at the 40th International Conference on Biochemistry of Lipids in Dijon, France, in 1999. He is a 2006 and 2012 winner of the Bimbo Pan-American Nutrition, Food Science and Technology Award. He is also a 2006 Bernardo Houssay award recipient in pharmacology, in recognition of his meritorious works of research. Dr. Catalá belongs to the editorial board of the *Journal of Lipids*, *International Review of Biophysical Chemistry*, *Frontiers in Membrane Physiology and Biophysics*, *World Journal of Experimental Medicine*, *Biochemistry Research International*, *World Journal of Biological Chemistry*, *Oxidative Medicine and Cellular Longevity*, *Diabetes and the Pancreas*, *International Journal of Chronic Diseases & Therapy*, and *International Journal of Nutrition*. He is coeditor of *The Open Biology Journal*.

Book Series Editor and Editor of Volume2: Angel Catalá
Facultad de Ciencias Exactas
Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA-CCT La Plata-CONICET)
Universidad Nacional de La Plata, La Plata, Argentina

Scope of the Series

Modern physiology requires a comprehensive understanding of the integration of tissues and organs throughout the mammalian body, including the expression, structure, and function of molecular and cellular components. While a daunting task, learning is facilitated by our identification of common, effective signaling

pathways employed by nature to sustain life. As a main example, the cellular interplay between intracellular Ca2 increases and changes in plasma membrane potential is integral to coordinating blood flow, governing the exocytosis of neurotransmitters and modulating genetic expression. Further, in this manner, understanding the systemic interplay between the cardiovascular and nervous systems has now become more important than ever as human populations age and mechanisms of cellular oxidative signaling are utilized for sustaining life. Altogether, physiological research enables our identification of clear and precise points of transition from health to development of multi-morbidity during the inevitable aging process (e.g., diabetes, hypertension, chronic kidney disease, heart failure, age-related macular degeneration; cancer). With consideration of all organ systems (e.g., brain, heart, lung, liver; gut, kidney, eye) and the interactions thereof, this Physiology Series will address aims of resolve (1) Aging physiology and progress of chronic diseases (2) Examination of key cellular pathways as they relate to calcium, oxidative stress, and electrical signaling & (3) how changes in plasma membrane produced by lipid peroxidation products affects aging physiology.

Contents

Preface

The endoplasmic reticulum is a network of membranes localized in all eukaryotic cells that performs a variety of essential cellular functions. Although this structure has received increased attention in recent years, it requires further investigation of its many properties and roles. The purpose of this book is to concentrate on recent developments on endoplasmic reticulum. The articles collected in this book are contributions by invited researchers with a long-standing experience in different research areas.

This book presents up-to-date, expert reviews of the fast-moving field of endoplasmic reticulum. The book is divided in two sections: 1. Introduction and 2. Endoplasmic Reticulum Properties and Functions.

In Chapter 1, Dr. Catala describes the properties and functions of the endoplasmic reticulum. In Chapter 2, Dr. Wilson et al. describe the mechanical properties of chaperone BiP, the master regulator of the endoplasmic reticulum. In Chapter 3, Dr. Ballar Kirmizibayrak and Tepedelen summarize endoplasmic reticulum-associated protein degradation (ERAD). In Chapter 4, Dr. Han-Jung Chae et al. discuss endoplasmic reticulum stress and autophagy. Finally, in Chapter 5, Dr. Vázquez-Nin et al. describe endoplasmic reticulum stress during mammalian follicular atresia.

We hope that the material presented here is understandable and useful to a broad audience, including not only scientists but also people with a general background in the biological sciences.

I would like to express my gratitude to Ms. Anita Condic, the Author Service Manager, and IntechOpen for their efforts in the publishing process.

Angel Català
Facultad de Ciencias Exactas,
Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas
(INIFTA-CCT La Plata-CONICET),
Universidad Nacional de La Plata,
Argentina

Section 1

Introduction

Chapter 1

Introductory Chapter: Endoplasmic Reticulum-Knowledge and Perspectives

Angel Catala

1. Introduction

The endoplasmic reticulum is one of the most studied and fascinating organelles. It is found in all eukaryotic cells and performs a variety of functions. The organelle was designated by this name by Keith Porter in 1953 on the basis of studies carried out with the electron microscope in cells in tissue culture. Porter was able to differentiate the exoplasm, an adjacent region devoid of organelles, from the neighboring endoplasm. In the endoplasm, he examined a system of interrelated tubules, a reticulum, for this reason, the name "endoplasmic reticulum" (ER).

The collaboration between Keith Porter and George Palade showed that ER exists in all eukaryotic cells and that it consists of different but continuous domains, the smooth and rough ER, whose abundance fluctuates between different types of cells. Palade observed on the surface of the rough ER the ribosomes that synthesized secretory proteins. The secretory proteins would cross an intracellular membrane, instead of the plasma membrane. The verification of this concept led to the discovery of the secretion pathway and the conception of intracellular protein binding to various organelles.

2. Brief history of the endoplasmic reticulum

The history of the endoplasmic reticulum began in 1945 when Porter, Claude, and Fullam [1] observed vesicle-like bodies in cell culture studies using electron microscopy. These elements had a size that varied between 100 and 150 mμ. The most important characteristics of this new cytoplasmic system were described: (1) its reticular disposition and (2) the vesicular nature of the component elements. In later articles, Porter and his colleagues explained the chosen concentration of the vesicular elements of the reticulum in the endoplasm and their insufficiency or absence in the supposedly ectoplasm periphery of the cytoplasm [1–3], a result that subsequently led to the choice of the name "endoplasmic reticulum," designation used in a subtitle in 1948 [3] and finally used in an article published by Porter and Kallman in 1952 [4]. In addition to the reticular arrangement and the endoplasmic position implicit in the name, Porter's studies recognized a number of other significant characteristics for the new cytoplasmic constituent, namely, the usual continuity of the system throughout the endoplasm of normal cells, the extraordinary polymorphism of its components, and the disintegration of the whole system in cytolysis in a set of isolated vesicles.

3. My participation in studies with endoplasmic reticulum

Ten years after my first experience with polyunsaturated fatty acids (PUFA) [5], in 1974, I participated in a project that demonstrated in a reliable way the mechanism of action of stearoyl-CoA desaturase. As an international fellow of the National Institutes of Health (NIH), I started under the direction of Prof. Philip Strittmatter in a project with the objective of analyzing the physical, chemical, and catalytic properties of a desaturating system of fatty acids reconstructed in egg lecithin or vesicles of dimyristoyl lecithin, devoid of detergent. This initial characterization of the mechanism included data on the substrate specificity of the desaturase, the interaction of the substrate with the enzyme, and the possible functions of the phospholipid in the transport of electrons, the binding of the substrate, and the desaturation stage that limits the speed. The ER is the main site for the synthesis of sterols and phospholipids that constitute most of the lipid components of all biological membranes. In addition, many enzymes and regulatory proteins involved in lipid metabolism reside in the ER. The ER, therefore, plays an essential role in the control of the lipid composition of the membrane [6] and the lipid homeostasis of the membrane in all cell types. Stearoyl-CoA desaturase is a microsomal oxidase system required for the biosynthesis of oleic acid. Three protein components of this system (cytochrome b5 reductase, cytochrome bs, and terminal oxidase) were resolved, and an enzymatically active desaturase was reconstituted from the purified components. As a result of these studies, an article was published in J. Biol. Chem. under the title "Microsomal stearoyl-CoA desaturase mechanism of rat liver: studies of substrate specificity, enzyme-substrate interactions and function of the lipids." Undoubtedly, these studies have opened new paths in the fatty acid desaturation reaction [7]. Stearoyl-CoA desaturase (SCD) is an enzyme of the endoplasmic reticulum (ER) that catalyzes the biosynthesis of monounsaturated fatty acids (MUFAs) from saturated fatty acids that are synthesized again or derived from the diet. The SCD along with NADH, the flavoprotein cytochrome b5 reductase, and the electron acceptor cytochrome b5 as well as the molecular oxygen introduced a simple double bond in a spectrum of acyl-CoA fatty substrates interrupted with methylene (**Figure 1**).

The preferred substrates are palmitoyl- and stearoyl-CoA, which are then converted into palmitoleoyl- and oleoyl-CoA, respectively [7]. These products are the most abundant monounsaturated fatty acids (MUFAs) and serve as substrates

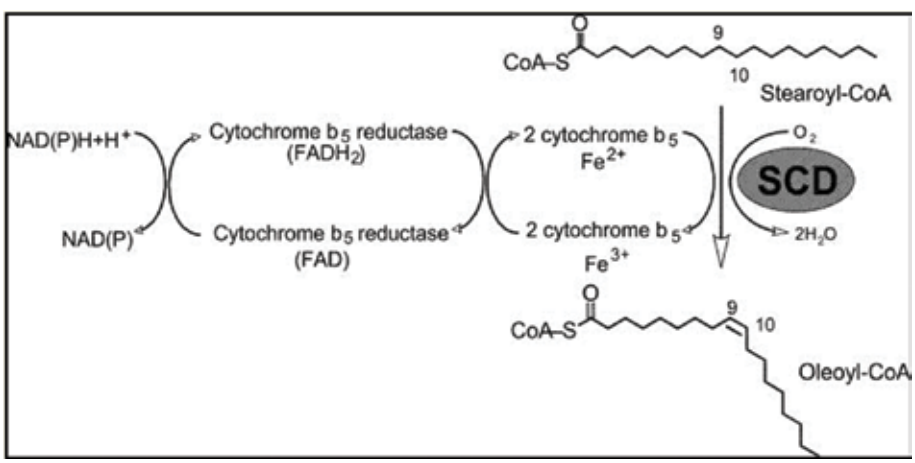

Figure 1.
The pathway of electron transfer in the desaturation of fatty acids by stearoyl-CoA desaturase (SCD).

for the synthesis of various kinds of lipids, including phospholipids, triglycerides (TG), cholesteryl esters, wax esters, and alkyldiacylglycerols. Apart from being the components of lipids, MUFAs have also been implicated to serve as mediators in signal transduction and cellular differentiation, including neuronal differentiation [8]. Recently, oleate has been shown to regulate food intake in the brain [9], and MUFAs may also influence apoptosis and mutagenesis in some tumors [10]. Thus, given the multiple roles of MUFAs, variation in stearoyl-CoA desaturase activity in mammals would be expected to influence a variety of key physiological variables, including cellular differentiation, insulin sensitivity, metabolic rate, adiposity, atherosclerosis, cancer, and obesity.

4. General remarks, conclusions, and perspectives

It has been fascinating to follow the field of endoplasmic reticulum research during almost six decades. Quantitative proteomics and lipidomics analysis are now available for measurement of the main components of the endoplasmic reticulum. From my experience, it is impossible to predict which aspects in endoplasmic reticulum research will dominate in the future.

Acknowledgements

This book is dedicated to the memory of Emeritus Professor Dr. Rodolfo R. Brenner,* the main guide in my research on lipid metabolism: "It will remain forever in the memory of those who had the privilege of knowing him and receiving his teachings." The outstanding scientist was a pioneer in the study of fatty acid desaturases (enzymes that play a prominent role in lipid metabolism and are located in the endoplasmic reticulum).

* On July 3, 2018, the distinguished scientist, Dr. Rodolfo R. Brenner, passed away. He was a Senior Investigator Emeritus of CONICET and the Head Professor Emeritus of UNLP. He held the position of Established Academic of the National Academy of Exact, Physical and Natural Sciences, of the National Academy of Sciences of Buenos Aires, and of the National Academy of Pharmacy and Biochemistry, as well as the Academic of the Medicine Academy of Córdoba, Argentina.

Author details

Angel Catala†
Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas, (INIFTA-CCT La Plata-CONICET), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina

*Address all correspondence to: catala@inifta.unlp.edu.ar

†The author is Member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Argentina.

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Section 2

Endoplasmic Reticulum Properties and Functions

Chapter 2

Mechanical Properties of Chaperone BiP, the Master Regulator of the Endoplasmic Reticulum

Hilda M. Alfaro-Valdés, Francesca Burgos-Bravo, Nathalie Casanova-Morales, Diego Quiroga-Roger and Christian A.M. Wilson

Abstract

Immunoglobulin heavy-chain-binding protein (BiP protein) is a 75-kDa Hsp70 monomeric ATPase motor that plays broad and crucial roles maintaining proteostasis inside the cell. Its malfunction has been related with the appearance of many and important health problems such as neurodegenerative diseases, cancer, and heart diseases, among others. In particular, it is involved in many endoplasmic reticulum (ER) processes and functions, such as protein synthesis, folding, and assembly, and also it works in the posttranslational mechanism of protein translocation. However, it is unknown what kind of molecular motor BiP works like, since the mechanochemical mechanism that BiP utilizes to perform its work during posttranslational translocation across the ER is not fully understood. One novel approach to study both structural and catalytic properties of BiP considers that the viscoelastic regime behavior of the enzymes (considering them as a spring) and their mechanical properties are correlated with catalysis and ligand binding. Structurally, BiP is formed by two domains, and to establish a correlation between BiP structure and catalysis and how its conformational and viscoelastic changes are coupled to ligand binding, catalysis, and allosterism (information transmitted between the domains), optical tweezers and nano-rheology techniques have been essential in this regard.

Keywords: immunoglobulin binding protein (BiP), optical tweezers, nano-rheology, posttranslational translocation, molecular motor

1. Introduction

The endoplasmic reticulum (ER) is involved in protein synthesis and the folding, assembly, transport, and secretion of nascent proteins [1]. One of the most important functions of the ER involves the quality control (ERQC) of nascent proteins, which is accomplished by ER chaperones [2, 3]. Chaperones are proteins that assist other proteins in the folding process, facilitating correct folding pathways or providing microenvironments in which folding can occur [4]. One of the most important chaperones is BiP protein (immunoglobulin heavy-chain binding protein).

BiP, a monomeric ATPase, has been referred to as the master regulator of the ER because of the broad and crucial roles that play in ER processes and functions [5], such as protein synthesis, folding, assembly, and translocation across the ER [3, 6]. Although BiP is still in early stages of study at a molecular level, some research groups have published findings of great value. These findings suggest that this protein could be a key player in various fields, such as in detection and treatment of serious diseases (neurodegenerative diseases, cancer, and heart diseases, among others) [7, 8]. Until now, most of the previous studies have been focused on the function of BiP with classical biochemical approaches and have not taken into account the mechanical properties of this protein. The role played by force on macromolecular structure and function is a subject of recent intensive research. Mechanical processes are a key component of many biological events. The coupling of mechanics and chemistry is one of the most important features of enzymes, which is highly specific and regulated [9]. Enzymes need to couple their chemical reactions to mechanical motion. In this way, an enzyme can work like a molecular motor using the hydrolysis or binding of ATP, converting this chemical energy to mechanical work. Allosterism and conformational changes are examples of how a chemical event could be transduced to mechanical events regulated by catalysis and ligand binding events based on changes in the elastic properties of domains [10]. Exploring this coupling may contribute to the understanding of the mechanical properties of enzymes, such as the mechanochemical mechanism of BiP. Understanding viscoelasticity is crucial because biological materials show different phenomena such as stiffening or softening upon ligand binding because proteins behave as springs [11, 12]. Due to recent technological progress, it is possible to measure changes in viscoelasticity in the folded state of proteins and we could correlate these changes with functionality. All these new approaches help to solve biological problems based on a mechanical description of molecular mechanisms to obtain a complete view of how the proteins perform their function with high efficiency.

2. The ATP-regulated Hsp70 chaperone BiP is the master regulator of the endoplasmic reticulum

Approximately, one-third of proteins produced in mammalian cells are folded and assembled in the ER, including secretory, membrane-bound, and some organelle-targeted proteins [13]. In the ER, proteins are translocated into the lumen where they acquire their functional tertiary and quaternary structure [3], and then correctly folded proteins exit the ER and are transported to intracellular organelles and the cell surface. The success of the maturation of a protein in its passage through the secretory pathway is monitored by the ERQC process, which is highly conserved in most eukaryotic organisms [2, 3]. For this, molecular chaperones proteins interact with partially folded or improperly folded polypeptides, facilitating correct folding pathways or providing microenvironments where folding can occur [4]. However, those proteins that fail to fold properly must be translocated back to the cytoplasm and degraded in the proteasomes through a process known as ER-associated degradation (ERAD) [3]. Two main chaperone systems help to fold the proteins in the ER or target them for ERAD if folding fails: lectins such as calnexin/calreticulin, unique to the ER, and the heat shock protein 70 (Hsp70) system, which has many aspects that are common to all Hsp70s. BiP (also known as glucose-regulated protein 78 kDa, HspA5, or Kar2p in yeast) is the only known conventional Hsp70 chaperone in the ER [14, 15].

BiP binds transiently to newly unfolded synthesized proteins translocated post-translationally into the ER (**Figure 1**). Binding of BiP to the incoming polypeptide

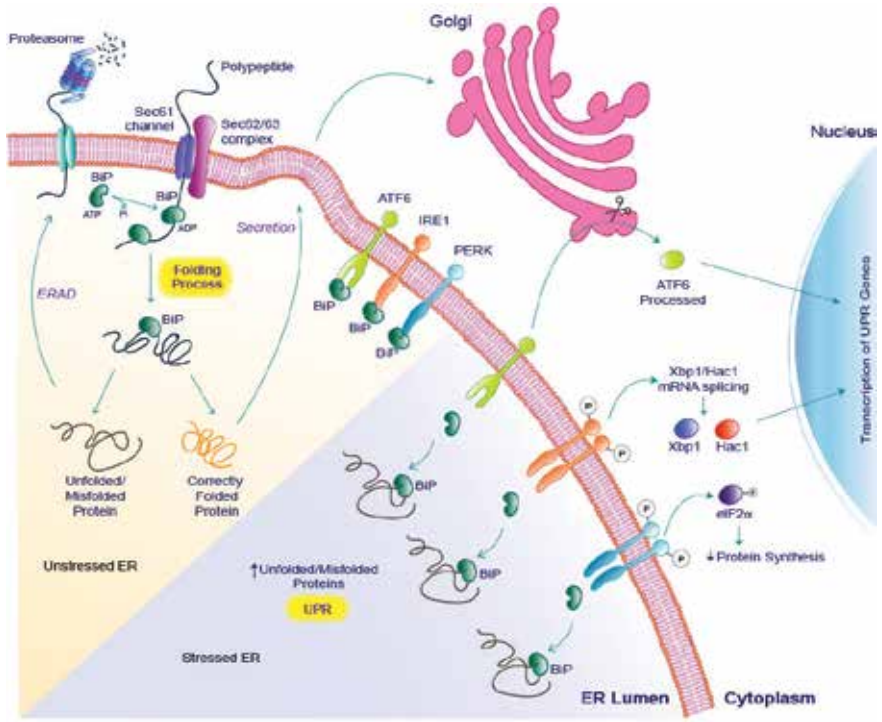

Figure 1.
Hsp70 chaperone BiP is a master ER regulator. Under nonstressed conditions (unstressed ER), BiP binds to hydrophobic regions of unfolded polypeptides fully synthesized to favor their posttranslational translocation into the ER lumen. The high substrate binding affinity of BiP to hydrophobic patches is achieved in the ADP-bound state upon the hydrolysis of ATP to ADP. After the translocation, BiP facilitates correct folding of nascent unfolded proteins or incompletely folded proteins nonglycosylated for their subsequently secretion. The proteins that fail to fold properly are targeted for proteasomal degradation in the cytoplasm through the ER-associated degradation (ERAD) pathway. BiP also interacts with the luminal domains of three ER stress sensors: IRE1, PERK, and ATF6 to maintain them in the ER. However, upon accumulation of unfolded/misfolded proteins in the ER lumen (stressed ER), BiP is released from these molecules to interact with unfolded proteins and favor their correct folding. BiP dissociation from these sensors allows their activation that involves: IRE1 dimerization, autophosphorylation, and splicing of Xbp1 and Hac1 mRNA; PERK dimerization, autophosphorylation, and phosphorylation of eIF2α, which lead to the attenuation of protein translation; and ATF6 transportation to the Golgi where it is processed by proteases. The ATF6 cytoplasmic domain obtained after its processing together with Xbp1 and Hac1 is translocated to the nucleus to activate the transcription of UPR-responsive genes.

contributes to efficiency and unidirectionality of transport due to its role as a molecular motor in the posttranslational translocation (will be discussed below). As a molecular chaperone, binding of BiP to hydrophobic patches exposed on nascent unfolded proteins that enter into the ER lumen or incompletely folded nonglycosylated proteins prevents nascent polypeptide chains from folding incorrectly and their interaction with nascent immature secretable proteins synthesized from membrane-bound polysomes. This prevents immature protein denaturation or degradation and ensures proper folding and its secretion (**Figure 1**).

Any condition perturbing the correct functioning of the ER, leading to an increase in protein synthesis or to the generation and accumulation of misfolded proteins inside the ER, is known as ER stress [16]. Moreover, misfolded proteins can also aggregate into insoluble higher order structure that has been associated with numerous neurodegenerative human diseases [17]. Adaptation to protein-folding stress is mediated by the activation of the unfolded protein response (UPR), which has evolved to detect the accumulation of misfolded proteins and activate a cellular response to maintain homeostasis and a normal flux of proteins in the ER, by increasing its folding capacity [18]. In this context, BiP serves as a

master UPR regulator and plays essential roles in activating three distinct ER stress sensors: IRE1, PERK, and ATF6 (**Figure 1**). Under nonstressed conditions, BiP binds to IRE1, PERK, and ATF6 by their luminal domains to maintain them in the ER. The accumulation of unfolded/misfolded proteins induces dissociation of BiP from IRE1 and PERK to permit their dimerization, trans-autophosphorylation, and activation [19]. Activated IRE1 initiates mRNA splicing of two transcriptional factors (Xbp1 and Hac1) to generate potent transcriptional activation of UPR target genes. PERK activation involves phosphorylation of the translational elongation factor eLF2 to attenuate protein synthesis. The release of ATF6 favors its transport to Golgi where is cleaved to generate the cytosolic domain of ATF6 that translocate to the nucleus to activate transcription of UPR-responsive genes [20]. Therefore, the activation of these sensors results in the attenuation of translation to reduce the workload of the ER, the transcriptional upregulation of genes encoding ER chaperones to increase the folding capacity of the ER, and the overexpression of the ERAD component to favor the degradation of these unfolded proteins by the proteasome [21, 22]. Thus, BiP participates not only in assisting protein folding, assembly and translocation but also in protein degradation and in the stress adaptability of the ER [1]. One big difference between BiP and lectins is that BiP detects only the unfolded regions of the nascent polypeptide chains, whereas lectins can detect both N-linked glycans of the peptides and unfolded regions [23]. However, it is not yet completely clear how BiP binds to its unfolded substrate because usually peptides are used as substrates instead of complete unfolded proteins. Recently, we developed a new method to study this process by specifically unfolding a complete protein substrate for BiP and measuring in optical tweezers the time that BiP remains bound to its substrate [24]. Previously, a work with DnaK (a close homolog of BiP) shows that it binds and stabilizes also partially folded protein structures [25]. BiP has a crucial role during posttranslational translocation, acting as a molecular motor. Molecular chaperones in the cytoplasm and ER lumen are involved in polypeptide translocation across the ER. Proteins enter the ER by a channel protein complex known as the translocon, discovered in yeast in Randy Schekman's laboratory [26]. In eukaryotic cells, the translocation of proteins is carried out by the Sec61 complex [6, 27]. Sec61 complex consists of three subunits, α, β, and γ, in which the pore to transport the polypeptide chain is created by the α-subunit of Sec61 protein. This complex functions as a passive channel that requires accessory proteins to provide a driving force to facilitate the vectorial translocation of the polypeptide chain through the membrane. Those accessory proteins are molecular motors [28]. Motor enzymes use the energy of nucleotide binding/hydrolysis or product release to generate mechanical work. The two mechanisms of translocation across the ER are co-translational translocation and posttranslational translocation [29]. In the co-translational mechanism, which has been well studied in mammalian systems, the signal sequence at the N-terminus of the nascent polypeptide interacts with the signal recognition protein (SRP) in the cytoplasm, keeping the ribosome attached to the Sec61 complex [6]. In this mechanism, the ribosome acts as an "auxiliary protein," since the driving force for translocation is given by GTP hydrolysis during the elongation of the polypeptide chain [30]. However, the driving force delivered by the ribosome is missing for posttranslationally translocated proteins. In this case, the driving force for polypeptide chain translocation comes from BiP protein [30]. Thus, in posttranslational translocation, after the polypeptides are fully synthesized, cytoplasmic molecular chaperones keep them unfolded to be transported through the Sec61 complex. In this mechanism, the channel partners with another membrane-protein complex, the Sec62/Sec63 complex, and with the lumenal chaperone BiP. However, in spite of the crucial roles of BiP during translocation, it is not fully understood

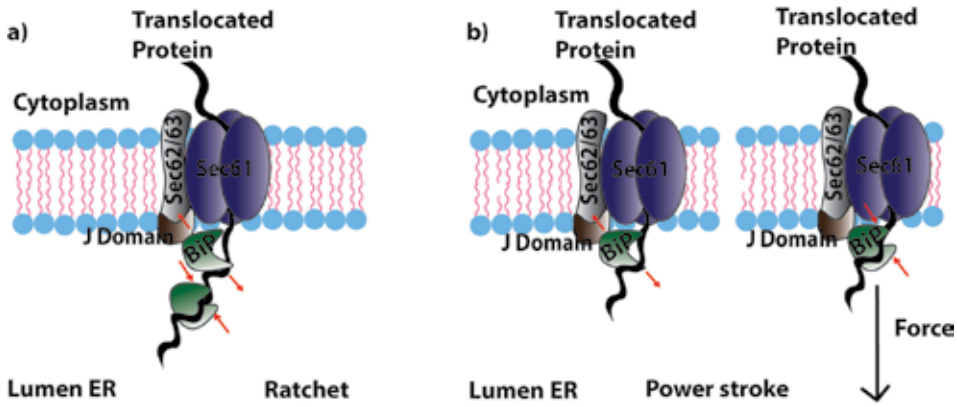

Figure 2.
Schematic representation of the two mechanisms of BiP in translocation. The figure shows how BiP could be involved in the transport process of the protein into the ER. (A) The ratchet theory is shown in which several BiP molecules would be interacting with the incoming chain, and in this way, the chain will not be returned to the cytoplasm. (B) The theory of power stroke is shown, where BiP binds to the polypeptide chain exerting a force greater than that of the thermal bath. Open domains of BiP are represented by red arrows outward and domains closed by red arrows inward.

if the action of BiP is through an active mechanism of pulling (as a power stroke), mediated by the binding/hydrolysis of ATP, or as a ratchet mechanism (**Figure 2**). In the latter, the polypeptide chain enters the channel passively by Brownian motion, and the BiP protein prevents it from returning to the cytoplasm. The hypothesis of the ratchet mechanism has been supported by employing antibodies against the polypeptide chains passing through the ER lumen [31]. Evidence for the translocation mechanism has been obtained using coarse-grained model simulations [32]. This study suggests that Hsp70 chaperones use an "entropic pulling mechanism," applying a force of about 15pN, and proposes that the Hsp70's would use a combination of ratchet and power stroke mechanisms [33]. Translocation in all eukaryotes is likely to be similar to yeast because of the high identity of amino acids between their channels. The channel interacts with the Sec62/Sec63 complex, with BiP acting as a molecular motor to bias the passive movement of a polypeptide in the Sec61 channel. In bacterial posttranslational translocation, the channel interacts with the cytoplasmic ATPase SecA. SecA moves polypeptides through the SecY channel to the periplasm by a "push and slide" mechanism [34]. Archaea probably use both cotranslational and posttranslational translocation, but it is unknown how posttranslational translocation occurs because these organisms lack SecA, Sec62/Sec63 complex, and BiP [6, 30]. In double membrane system, as in chloroplast, it is mediated by translocon at the outer envelope membrane of chloroplasts (TOC) and translocon at the inner envelope membrane of chloroplasts (TIC), which facility the import of translated proteins with assistant of a TIC associated ATP-driven import motor [35]. However, in mitochondria, the import of preproteins is carried out by translocases called as TOM complex (translocon outer mitochondria membrane) and TIM23 complex (translocon at the inner mitochondrial membrane), where proteins with a hydrophobic sorting signal can be released into the inner membrane and hydrophilic proteins are imported into the matrix by one presequence translocase-associated motor (PAM) in which the force is driven by chaperone mtHsp70 [36]. Therefore, the mHsp70 pulls the presequence by power stroke or Brownian ratchet mechanism to finally translocate the presequence at the mitochondria matrix. This suggests that the mechanism of Hsp70 in the import of preprotein in mitochondria and ER could have similar basic mechanism.

2.1 BiP structure and catalysis

The effective application of work depends on the elastic properties of a motor based on the softening and stiffening of some domains [37], and it is important to understand how the information is transmitted through domains by BiP. BiP is formed by two domains: a nucleotide-binding domain (NBD), with ATPase activity, connected by a flexible hydrophobic linker to the substrate-binding domain (SBD) (**Figure 3**). The SBD can be further divided into a compact β-sandwich domain harboring, a cleft for substrate binding, and an α-helical domain at its C-terminal end, the so-called "lid" [38]. Many conformational changes, such as the open and close movement of the lid and the variation in the distance between the SBD and NBD, have been associated with the ATPase cycle of BiP in the ER. Once BiP binds K^+ and ATP, its NBD and SBD come into close proximity to each other and the lid of the SBD opens, which results in a form that binds substrates with low affinity [3]. Also, a number of BiP cofactors have been discovered that assist in controlling the substrate-binding cycle and its localization within the ER. Nucleotide exchange factors (NEFs) help in the transition from the ADP to the ATP bound state for BiP, catalyzing the release of substrate. Hsp70 hydrolysis of ATP to ADP is accelerated by Hsp40 family members or so-called J domain proteins. The J-domain binds to Hsp70 and stimulates its ATPase activity [39]. In addition to controlling the localization and activity of Hsp70's, J-domain proteins may also bind the substrate themselves and help with the initial delivery of the substrate to Hsp70 chaperone. In the mammalian ER, there are seven J-domain proteins (ERdj1–7) that assist with the diverse functions of BiP [40]. After the Mg^{2+}-dependent hydrolysis of ATP, BiP enters a state with low on and off rates for substrates [3]. For elongated/peptide substrates, the lid closes over the bound substrate; for globular substrates, there are direct interactions between the lid and the substrate, but the lid may not close completely [3]. The SBD and NBD become farther apart upon substrate binding and ATP hydrolysis, which is less pronounced for globular substrates. ADP must be exchanged for ATP in order to release the substrate and make BiP available for another round of client binding. Ca^{2+} increases the affinity for ADP, whereas NEFs Grp170 and Sil1 facilitate the nucleotide exchange reaction [3]. Conformational changes in murine BiP during ATPase cycle have been determined by Förster Resonance Energy Transfer (FRET) at the single molecule level, showing that NBD and SBD come into close

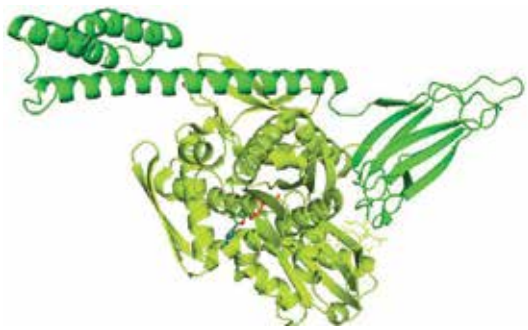

Figure 3.
Structure of ATP-bound BiP in the open conformation. BiP has two domains, NBD (light green) and SBD (dark green). The latter has a subdomain that acts as an α helix lid that covers the binding pocket for polypeptides formed by β sheets. In the ATP-bound BiP conformation, the lid is open. This structure corresponds to protein data bank number: 5E84 and was drawn as a ribbon diagram, using PyMOL molecular visualization system.

contact with a mean distance 58–75 Å [41]. Additionally, by using NMR residual dipolar coupling, spin labeling, and dynamics methods, it has been determined in DnaK that the NBD and SBD are loosely linked and can move in cones of 35° with respect to each other [42]. Moreover, the distance between the base and the lid of the SBD domain in Hsp70 has been calculated to be 77 Å by means of FRET [43]. Also, there is a crystal structure of human BiP bound to ATP that shows similar distances [44]. The conformational changes and movements of BiP are not independent for each domain because an important communication and coupling exists between them.

2.2 BiP allosteric mechanism

Most HSP70 do their work coupling the Mg^{2+}-dependent hydrolisis of ATP to large conformational changes, involving movements of its structural domains (SBD and NBD) and the interdomain linker. So, HSP70 protein function rely on a dynamic ATP dependent cycle in which several conformations are visited, with different substrate binding affinities in them [45–47]. For example in DnaK, ATP binding favors a compact, domain-docked, and linker-bound conformation, which has low ATPase activity [3, 45]. Substrate binding to this state stabilizes a transient domain-undocked conformation, with a linker-bound state, that has high ATPase activity and fast substrate binding and release kinetics but low substrate affinity. Then, when ATP is hydrolyzed to ADP, it is favored a domain-undocked conformation, linker-unbound state, which has high substrate affinity but very slow substrate binding and release kinetics [45–48]. Recently, X-ray structures of ATP-bound DnaK [49] and human BiP [44] have shown that both proteins have big structural similarity, but their functional activity (and between different Hsp70s) varies significantly between them [3]. On this ground, considering the fact that in spite of the structural similarity between different Hsp70, they have different functional activity; it was suggested that an important feature that should modulate Hsp70 function was its allosteric communication between both structural domains, mediated by the interdomain linker [44, 50]. Basically, the allosteric mechanism transmits information on the nucleotide state from NBD to SBD and on the substrate occupancy state from SBD back to NBD [51]. At the beginning, three different ideas explaining how interdomain communication occurs have been suggested. In the *E. coli* Hsp70 DnaK, bound nucleotide is sensed by residues in NBD that are closer to the bound ATP, in particular, a proline residue (Pro143) and a surface-exposed arginine (Arg151), and the communication with the SBD domain is thought to be via this proline, which can likely undergo cis/trans isomerization [52]. Replacement of the arginine completely disrupted the mutual allosteric communication between ATPase domain and substrate binding domain. Moreover, arginine had been shown to be an important residue in allosteric communication in other proteins [53]. Replacement of the proline destabilized the allosteric communication, increasing the rate of spontaneous transition between ATP-like and ADP-like states. Interestingly, all residues of the proposed DnaK sensor-relay system are conserved in BiP [3, 54]. In addition to this putative proline-focused sensor-relay system, threonine in position 37 (Thr37) in NBD plays a particularly important role as a nucleotide sensor in a hamster BiP [55], likely due to a direct interaction of its hydroxyl group with the γ-phosphate oxygen of bound ATP. Once this position was mutated, no more conformational change occurred, while nucleotide binding and hydrolysis were not affected [3]. The third known communication path between NBD and SBD occurs through the conserved hydrophobic linker, which connects both domains.

Upon ATP binding, the linker binds to a cleft in NBD, which is important in transmitting the nucleotide state of NBD to SBD and increases ATP hydrolysis of the NBD once bound to the cleft. Basically, it has been suggested that allostery results from an energetic tug-of-war between domain conformations and formation of two orthogonal interfaces: between the NBD and SBD and between the helical lid and the β subdomain of the SBD [46]. More recently, "soft" amino acid substitutions have been performed in BiP, trying to affect the allosteric communication between SBD and NBD, uncoupling the substrate-binding site with the NBD-SBD interdomain interface. In particular, Val461 was mutated to Phe; Ile526 to Val; Ile437 to Val; and Ile538 to Val. It has been reported that in the presence of ATP, all these "soft" mutations affected the equilibrium between the domain-docked and domain-undocked conformations, suggesting that this residue enables allosteric control of BiP conformational ensemble [45].

Moreover, allosterism in BiP has been studied at the single molecule level with optical tweezer manipulation [24]. The results showed that BiP binds reversibly to the unfolded state of MJ0366 (substrate protein), preventing its refolding, and that this effect depends on both the type and concentration of nucleotides. Additionally, more clues about BiP allosteric mechanism have arisen from BiP ensemble measurements performed with nano-rheological experimental setup, which will be explain later.

Finally, it has been studied how the posttranslational modification of BiP by AMPylation onto Thr518 [56] could affect BiP conformational cycle, modulating in this way the allosteric mechanism of BiP. The results showed that effectively, this modification shifted BiP conformational equilibrium toward the domain-docked conformation in the presence of ATP, stabilizing the domain docking in the absence of ATP and demonstrating posttranslational fine tuning of BiP conformational equilibrium [45].

As a general overview, BiP allosteric mechanism has a high level of complexity, as it has different layers of control. From a structural point of view, there are residues that exert the communication between the SBD and NBD domains, and other residues that are involved in stabilizing conformational ensembles of BiP that affect allosteric communication. Moreover, changes in the mechanical properties of BiP are also involved in the allosteric mechanism regulation, as it has been demonstrated with the nano-rheological studies. Finally, posttranslational modifications also play a role in this chaperone function, as their importance in shuffling conformational ensembles, involved in this ATP and Mg^{2+} dependent cycle, has been demonstrated.

2.3 Mechanical aspects

Considering that translocation through the ER is a crucial process to maintain homeostasis inside the cell, it is essential to have a mechanistic understanding of the role that BiP has in translocation to maintain proteostasis. Therefore, classical biochemical assays, or ensemble studies, have been conducted to study each of these processes without taking into account the measurement of forces and changes in elasticity. Single molecule level studies, called *in singulo* studies, have become very relevant during recent years. These studies have become the gold standard to study biomolecular mechanisms because of their advantages when it comes to obtaining specific information about biological phenomena, and it also permits the application of force in molecules [57]. *In singulo* studies are very direct approaches, following the behavior of an individual molecule in time, thus making it possible to obtain not just the average behavior of many molecules, but rather the

whole distribution and individual behaviors of a population that may not be homogeneous. It is possible to study a single biomolecule by visualizing it or manipulating it, with the most common approaches being single molecule fluorescence and single molecule force spectroscopy [9]. With force spectroscopy, it is possible to mechanically manipulate and apply forces to molecules in a highly specific manner [58]. This technique lets us measure the mechanical stability of particular domains, instead of the whole protein, thus allowing us to determine the energetic coupling between one domain and the other. Techniques such as atomic force microscopy (AFM), magnetic tweezers, and optical tweezers allow the direct application of mechanical forces to biological macromolecules and let us study the conformational changes [9, 59]. One example of single molecule studies with BiP has been the analysis of the conformational cycle of BiP achieved by single molecule and ensemble FRET measurements. In this study, the authors determined that nucleotide binding resulted in concerted domain movements of BiP. Conformational transitions of the lid domain allowed BiP to discriminate between peptide and protein substrates [41]. Also, we recently developed a method to measure how BiP binds to its substrate using optical tweezers [24]. Without single molecule approaches, it is very difficult to learn about how BiP binds to its substrate, since the substrate of BiP is an unfolded peptide, and if we unfold the substrate, we may also unfold BiP. However, by optical tweezers manipulation, we can specifically unfold the substrate without affecting BiP. Another study, by directly pulling DnaK using optical tweezers, the authors were able to study the mechanics and the order of events of unfolding of each domain of this Hsp70 [60, 61]. This study shows that DnaK has more than two mechanical intermediates in each domain.
All the single molecule techniques that exert force on the protein are not able to measure small changes in distance at subnanometer resolution at low forces (below 1–5 pN), and so it is difficult to correlate the elastic properties of the folded protein with ligand binding. A new technique called nano-rheology developed in Giovanni Zocchi's laboratory at the University of California at Los Angeles (UCLA) allows measurement of elasticity in folded proteins [62]. Nano-rheology is a traditional rheology experiment, in which an oscillatory force is directly applied to the protein and where average deformation is measured [63]. This technique exploits sub-Angstrom resolution to study the mechanical properties of the folded state of proteins by applying low force to the proteins in bulk [12]. The universal mechanical property of the folded state is the viscoelastic behavior, meaning, when a protein is subjected to a force, it can behave as an elastic or viscous material, getting stiffer or softer (flexible). Then, stiff and soft here refer to both elastic and viscous mechanical responses; the two are coupled because the structure is viscoelastic [63]. This behavior is relevant for the large conformational changes of protein which often accompany substrate binding in proteins [12, 64]. Using this technique (**Figure 4**), we studied the mechanical properties of BiP, considering the viscoelastic behavior upon ligand binding. We observed that the folded state of the protein behaves like a viscoelastic material, getting softer when it binds nucleotides but stiffer when it binds peptide substrate. The explanation for this mechanical behavior is related to the ATPase cycle of BiP. As shown **Figure 4B**, when the protein is in the presence of ATP, the protein is softer state because the lid is more flexible and the NBD and SBD domains are closer [50, 65]. Also, the protein is in softer state in the presence of ADP, but the structural reason is different. The hydrophobic linker is more flexible, and the domains seem to be in a dynamic distance distribution [3, 6]. Finally, the protein is more rigid or stiffer in the presence of the HTFPAVL peptide substrate. The structural reason is because the lid is close [6]. Additionally, it was observed in presence of peptide the dissociation constant (KD) for ADP decreased

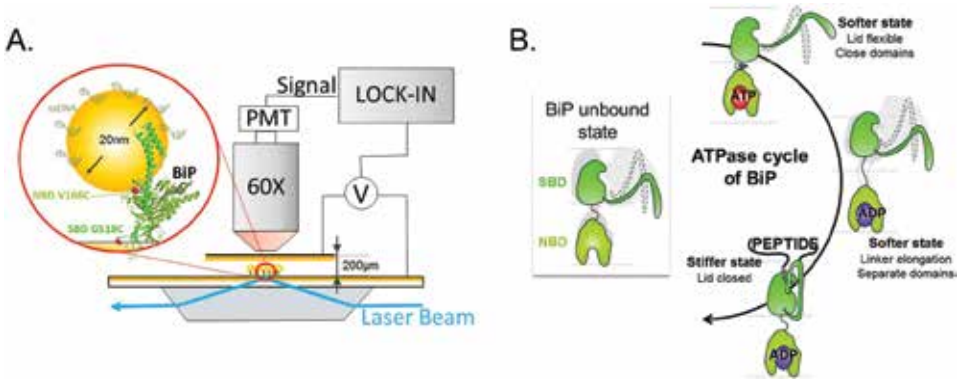

Figure 4.
Mechanical aspects of BiP. (A) BiP nano-rheology setup shows the flow chamber with BiP attached to both gold surfaces, the parallel plates capacitor geometry used for mechanical excitation, and the evanescent wave scattering optics used for read out. BiP was directly tethered between a gold film surface evaporated on a glass slide and 20 nm diameter GNPs, constituting the lower part of a thick flow chamber. BiP attachment proceeds via two exposed cys residues at positions 166 and 518, located in NBD and SBD, respectively. GNPs are covered with ssDNAs on the surface to negatively charge them. (B) Model for mechanical response of BiP in the presence of different ligands in ATPase cycle. The illustration shows BiP unbound state. In ATPase cycle of BiP, the structure is softer in two cases: first, in the presence of ATP, the lid is more flexible and the domains are closer leading to an important rigidity decrease. Second, in the presence of ADP, the domains are separated by the linker elongation. SBD seems to be in a dynamic distance distribution with a general trend toward domain separation. Finally, the structure is stiffer in the presence of peptide because the lid of BiP is closed, then generating a compact state.

1000 times, demonstrating that peptide binding dramatically increases the affinity for ADP which evidences the allosteric coupling between SBD and NBD domains [66].

3. Conclusion

Changes in the conformational state and viscoelastic properties of BiP triggered by ATP binding and/or hydrolysis are essential for allosteric communication between its domains (NBD and SBD), as these could supply the mechanical work to move the peptides through the Sec61 channel, with BiP behaving as a molecular motor. It is still not completely known how BiP applied the force in the peptide that is translocating or if it just uses the water bath. Taking into account the important role of BiP in proteostasis and diseases, an in-depth study of the functioning of the mechanics of BiP with new technology has major relevance to future research and development in science, biomedicine, and health, as well as in technological developments in biotechnology and even education, thus opening up new investigative directions of great potential and impact for science worldwide.

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Conflict of interest

None conflict of interest.

Author details

Hilda M. Alfaro-Valdés[†], Francesca Burgos-Bravo[†], Nathalie Casanova-Morales[†], Diego Quiroga-Roger[†] and Christian A.M. Wilson[*†]
Biochemistry and Molecular Biology Department, Chemistry and Pharmaceutical Sciences Faculty, Mechanobiology and Single Molecule Biochemistry Laboratory, University of Chile, Chile

*Address all correspondence to: yitowilson@gmail.com

†All authors contributed equally to this book chapter

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Chapter 3

Endoplasmic Reticulum-Associated Degradation (ERAD)

Burcu Erbaykent Tepedelen and Petek Ballar Kirmizibayrak

Abstract

The newly synthesized proteins are kept in the endoplasmic reticulum (ER) until their maturation is completed. The accurate protein folding is vital for homeostasis, but this process is error-prone since it is chemically complicated. Aberrant folding may result in aggregates having a toxic gain of function or may lead to a loss of protein function; therefore, protein misfolding can lead to several pathologies. The ER protein quality control mechanism monitors the fidelity of protein folding. Those proteins that fail to fold or assemble properly are subjected to degradation via a process known as ER-associated degradation (ERAD). Besides clearing proteins having folding problems, ERAD is also known to regulate the levels of some physiological proteins including 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMGR) catalyzing the rate-limiting step of cholesterol biosynthesis. ERAD is a complex, multistep process starting with the recognition and targeting of substrates, followed by ubiquitination, retrotranslocation and proteasomal degradation. A large number of ERAD factors functioning in different molecular machineries increases the complexity of mammalian ERAD. ERAD is fundamental for human health and there is increasing evidence linking ERAD with various diseases. Here, the different modules/machineries of the ERAD process together with its tight regulation will be discussed.

Keywords: endoplasmic reticulum-associated degradation (ERAD), protein misfolding, ubiquitin-mediated degradation, proteasomal degradation

1. Introduction

The endoplasmic reticulum (ER) is an extensive network of flattened, membrane-enclosed tubes or sacs that extends throughout the cytosol [1]. ER has important roles in many biochemical processes required for cell survival and normal cellular functions. ER regulates these cellular processes through proteins that are localized in its complex network structures [1–3]. In addition to protein synthesis, significant cellular activities such as protein transport and folding, lipid and steroid synthesis, carbohydrate metabolism, calcium storage and protein quality control processes occur in the ER [1–4].

Approximately one-third of all newly synthesized proteins are targeted to the ER and traffic to other organelles of secretory pathway, plasma membrane or the extracellular space [5]. Protein translocation to the ER occurs through Sec61 complex [6, 7]. As synchronized with translocation, protein is exposed to the ER's oxidizing and calcium-rich environment, which is suitable for protein folding and

co- and post-translational modifications such as glycosylation, disulfide bond formation and glycosylphosphatidylinositol (GPI) anchoring [8]. During this folding process, many proteins such as lectin-type molecular chaperones (e.g., calnexin (CNX) or calreticulin (CLR)), HSP70-like chaperone BiP) and enzymes like protein disulfide isomerases (PDI) work in association with each other [4, 9, 10]. Conformational maturation and folding of the proteins in the ER are instantly controlled through the added N-glycan groups to decide whether the proteins are directed to distant compartments via the secretory pathway or included in the refolding cycle [11, 12].

The folding process is not completely accurate. In mammals, 30% of all newly synthesized proteins are estimated to be incorrectly folded [13]. However, genetic mutations, errors in transcription and translation, toxic compounds and cellular stresses such as defects in cellular redox regulation due to hypoxia, oxidants and reducing agents that interact with disulfide bonds in the ER lumen, glucose starvation and abnormalities in calcium regulation lead to a significant increase in the ratio of incorrectly folded proteins [4, 11, 14]. Adequate removal of these unwanted proteins is crucial for protecting cells from proteotoxicity caused by the formation of protein aggregates through the re-opening of hydrophobic residues as well as by unfolded or misfolded proteins that may compete with their properly folded counterparts for substrate binding or for complex formation with partners. Even though the primary damage of these unwanted proteins is restricted to the cell they reside, the damage gets wider if it is a secretory protein [11]. Therefore, there is a robust control via "Protein Quality Control Mechanisms" for the removal of defective proteins in living cells, and thus, only properly folded proteins are allowed to exit from ER lumen to the secretory pathway [11, 15–18]. When the folding process fails, the terminal mannose residues from the core glycan chain are gradually removed, allowing the proteins to be recognized by mannose-specific lectins and defective proteins are transferred to the 26S proteasome for degradation through the protein quality control mechanism called "ER-associated degradation (ERAD)" [19–21].

In addition to misfolding proteins, ERAD also targets some proteins that might fold into their native structures under the right conditions and also orphan subunits of oligomeric complexes. The chloride channel protein CFTR (cystic fibrosis transmembrane conductance regulator) is the best example, where it is targeted to ERAD as a consequence of its complex and inefficient folding pathway. The low folding efficiency is further decreased upon mutation as seen in CFTRΔF508. CFTRΔF508 is the most common mutation found in cystic fibrosis patients, can fold and function in plasma membrane; thus, degradation of CFTR via ERAD is obtrusive. ERAD also functions in supporting the correct stoichiometry of multimeric protein complexes by degrading components that are produced in excess of the limiting monomer [22]. For example, the unassembled subunits of T cell receptor-like TCRα and CD3δ are also well-known ERAD substrates [23]. These proteins contain charged residues in the intramembrane sections promoting the assembly of complexes. However, when oligomerization is not proper, these residues might initiate degradation via recruiting specific ERAD factors [23].

ERAD also functions in cell homeostasis by regulating the endogenous levels of many enzymes and signal molecules especially those localized to the ER membrane or plasma membrane under physiological conditions [24]. For instance, ERAD plays a homeostatic role in the regulation of HMG-CoA reductase (HMGR), which is the key enzyme of cholesterol metabolism; apolipoprotein B, an essential secreted protein member of triacylglycerol-rich lipoproteins responsible for the export of lipids, triglycerides and cholesterol; hepatic cytochrome P450 enzyme 3A4 metabolizing endo- and xenobiotics; IP3 receptor, an ER-localized protein allowing Ca^{2+} release

by binding seconder messenger inositol 1,4,5-triphosphate (IP3); type II iodothyronine deiodinase, an ER-localized enzyme converting thyroxin (T4) to the biologically active hormone triiodothyronine (T3) and GABA neurotransmitter receptor responsible for the reduction of neuronal excitability and the tumor metastasis suppressor KAI1 levels [22, 25–28].

Some viruses hijack the ERAD system through encoding effectors by serving as adaptors that redirect correctly folded molecules towards degradation. US2 and US11, the human cytomegalovirus gene products, induce degradation of major histocompatibility complex (MHC) class I heavy chain, which enables virus-infected cell to avoid detection by the immune system [29]. Similarly, Vpu is a glycoprotein encoded in the human immunodeficiency virus (HIV-1) genome and binds and targets newly synthesized CD4 for degradation [30], allowing them to evade immunosurveillance. Moreover, toxins like diphtheria, cholera and ricin enter the cell by endocytosis and move to the ER. They use the ERAD system to escape from the ER lumen and gain access to their enzymatic substrates in the cytoplasm [31].

ERAD is a highly complicated and regulated mechanism in which the diversity and combination of components change according to the protein to be destroyed [19–21, 32]. Maturation-defective proteins are removed from the ER lumen or lipid bilayer by retrotranslocation through the ERAD pathway and degraded by proteasome. The ubiquitin system is an integral part of the ERAD and is composed of factors necessary for the recruitment, processing and binding of ubiquitin chains to substrates [24]. In other words, ERAD is composed of steps that include substrate selection, modification with ubiquitin chain, retrotranslocation and 26S proteasomal degradation. Several key molecules such as E1, E2, and E3 enzymes responsible for ubiquitin transfer, channel components responsible for retrotranslocation, chaperones and cofactor proteins function in a synchronized manner during ERAD pathway [12, 19–21].

This critical role of ERAD in the regulation of cell homeostasis is an evident that ERAD disorders will have important effects on cell survival. Furthermore, it has been shown that aberrations in ERAD function play a role in the pathology of nearly 70 diseases such as cystic fibrosis, α1-antitrypsin (AAT) insufficiency, diabetes, neurodegenerative diseases (Parkinson, Alzheimer's and Huntington's diseases), viral infection and albinism [4, 33].

In this section, the knowledge related to the basic mechanism and regulation patterns of the ERAD will be summarized and presented.

2. Molecular mechanisms of ERAD

2.1 Protein folding process and recognition of misfolded proteins

About 30% of the total proteins and all transmembrane proteins of the cell are synthesized in the ER, which acts as a portal for entry into the secretory pathway via the Sec61 channel [7–8]. As being translocated, the N terminal hydrophobic signal sequence of newly synthesized protein is cleaved by a peptidase complex [34]. Co- and post-translational modifications such as disulfide bond formation, initial steps of N-glycosylation, and glycophosphatidylinositol (GPI) anchorage take place in the ER.

The oxidizing environment of ER assists the formation of disulfide bonds, which stabilizes tertiary protein structure and facilitates protein assembly. During the folding process, disulfide bonds are formed through the oxidation of pairs of free thiols on cysteine residues by protein disulfide isomerases (PDIs). PDIs act as

cycles, and after initial oxidation, disulfide bonds are sometimes isomerized by PDI and ERp57, which is a thiol oxidoreductase, in order to stabilize the correct folding of protein [35]. Conversely, the reduction of disulfide bonds of misfolded proteins is necessary for retrotranslocation step of ERAD. Indeed, PDI enables the retrotranslocation of the simian virüs-40 (SV-40) and cholera toxin [36, 37]. ERdj5, an ER oxidoreductase, reduces disulfide bonds and interacts with EDEM (ER-degradation enhancing mannosidase-like protein) and also accelerates the step of retrotranslocation of SV-40 [37]. ERDJ5 also regulates the degradation of disease-causing α1-antitrypsin variant (null Hong Kong) [38].

Folding is aided by molecular chaperones shepherding against misfolding and unfolding. Chaperone-like glycans bind to N-glycans playing a crucial role in protein folding and degradation. It is apparent that N-glycosylation, quality control of protein folding and ERAD are functionally linked. After entering to the ER, a large majority of the newly synthesized polypeptide chain are being N-linked glycosylated. The oligosaccharyltransferase enzyme recognizes the Asn-X-Ser/Thr consensus sequence in the most of the nascent protein molecule and covalently integrates a high mannose containing core glycan groups ($Glc_3Man_9GlcNAc_2$) from dolichol localized on the ER membrane to the protein [39]. Due to the very short half-life of triglycosylated form of protein-bound oligosaccharide, glycan processing starts immediately after the transfer of precursor glycan groups through glucosidase enzymes. Following cleavage of two of three glucose residues, the nascent protein could interact with quality control lectins like CNX and CLR. This interaction is preserved until cleavage of remaining glucose residue. After releasing the glycoprotein from CNX/CLR cycle, final glucose is also trimmed creating unglycosylated substrate. This compromises the interaction of substrate with the lectin chaperones. At this stage, if protein is properly folded, it could exit the ER for their final destination. However, if glycoprotein is still unfolded, it is retained in the ER and reglucosylated by UDP-glucose:glycoprotein glucosyltransferase and rebound with CNX and CLR giving protein more time for proper folding [40, 41]. It is not yet understood the mechanisms involved in the termination of reglycosylation/deglycosylation cycles. However, it is clear that, if the polypeptide chain cannot reach its mature form after repeated folding attempts, terminal mannose residues from the core glycan chain are gradually removed by ER α1,2-mannosidase I (ERMan1). ERMan1 produces $Man_8GlcNAc_2$ isomer by removing a mannose residue from the middle branch of N-glycans. By this trimming, glycoprotein becomes poorer substrates for reglycosylation and exit from the CNX cycle [11].

The hydrophobic patches of properly folded proteins are usually buried within the interior of soluble proteins. However, those patches could be exposed in misfolded proteins. If a protein has exposed hydrophobic surfaces, BiP binds to it in order to hide these aggregation-prone surfaces for proper folding attempts by preventing aggregation. However, if folding does not succeed or delayed, extended chaperone-misfolding protein interaction serve for a sophisticated process where protein is transferred to other chaperones and/or to the ERAD process [27, 42].

It is well accepted that the first step of ERAD is selection of misfolded proteins by chaperones. As early as 1999, it was found that yeast ERAD substrates strikingly differed in their requirement for the ER-luminal Hsp70, BiP [43]. The degradation of soluble substrates such as pαF and a mutant form of the vacuolar protease carboxypeptidase Y* (CPY*) were dependent on BiP, while degradation of transmembrane proteins Pdr5*p, Ste6-166p, Sec61-2p and Hmg2p occurred in a BiP-independent manner. In 2004, it has been shown that substrates with cytosolic domain such as Ste6-166p were degraded BiP-independently, while proteins with luminal defects required BiP, suggesting that depending on the topology of

misfolded lesion (ER lumen, ER membrane and cytoplasm) cytosolic or luminal chaperones function in the recognition and targeting for the degradation [44].

It is possible to study substrate recognition during ERAD using model misfolded proteins. It is clear that de-mannosylation is required for degradation of misfolded glycoproteins since inhibition of this mannose trimming stabilizes misfolded glycoproteins in the ER [45]. Overexpression of ERMan1 accelerates the degradation of N-glycosylated proteins [39, 46]. The resulting Man_8-$GlcNAc_2$ containing glycoprotein after this trimming becomes a substrate for EDEM1 (ER-degradation enhancing mannosidase-like protein 1, Htm1p in yeast)—a mannosidase-related lectin in the ER. It was further proposed that misfolded glycoproteins interact with ERManI and EDEM1 for their ERAD, and lectin-carbohydrate interaction found to be crucial for EDEM substrate recognition [47]. Although ERMan1 was suggested to be a biological timer initiating the ERAD of misfolded proteins [48], recent studies revealed that mannosidases are not solely responsible for intensive demannosylation during ERAD, especially under non-basal conditions. Under ER stress (unfolded protein response active) conditions, the transcriptional elevation of EDEM1 enhances the ERAD efficiency by suppressing proteolytic downregulation of ERMan1 [49]. It appeared that EDEMs also play an important role in demannosylation of substrates [50]. EDEM1 also prevents reglycosylation and promotes retrotranslocation and degradation of some ERAD substrates [51]. On the other hand, while mannosidase homology domain (MHD) of Htm1p is necessary for substrate binding, mammalian EDEM1 binds misfolded proteins independent of MHD domain, and therefore, EDEM1 substrate binding may not require mannose trimming or even glycosylation [52]. Thus, in addition to N-linked oligosaccharide moieties of glycoproteins, EDEM1 can recognize the folding lesions of misfolded proteins. In summary, EDEMs are directly or indirectly involved in demannosylation of glycoproteins and/or serve as receptors that bind and target mannose-trimmed proteins for ERAD (**Figure 1**).

Truncation of terminal mannose from branch C exposes α terminal α1,6-bonded mannose residues functioning as a recognition signal for ERAD lectins such as OS9 (Yos9 in yeast) and XTP3-B (**Figure 2**). Through their mannose-6-phosphate receptor homology (MRH) domain, both proteins primarily recognize α1,6-linked mannose j. Additionally, OS-9 also recognizes α1,6-linked mannose e and c [53].

Several reports suggest that factors (EDEMs, OS9 and XTP3-B) required for substrate recognition and targeting reside within supramolecular complexes and/or interact with important ERAD regulators [54]. For example, EDEM1 interacts with CNX, receives substrates from CNX cycle and facilitates ERAD substrate

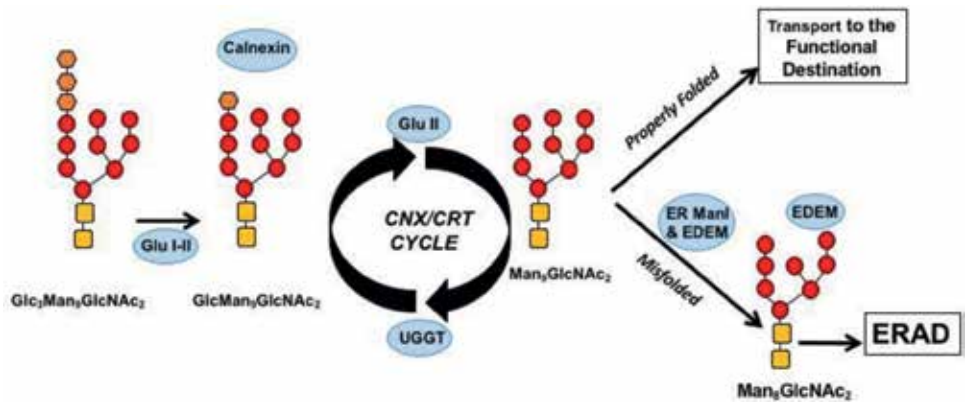

Figure 1.
Protein quality control and targeting misfolding proteins to the ERAD.

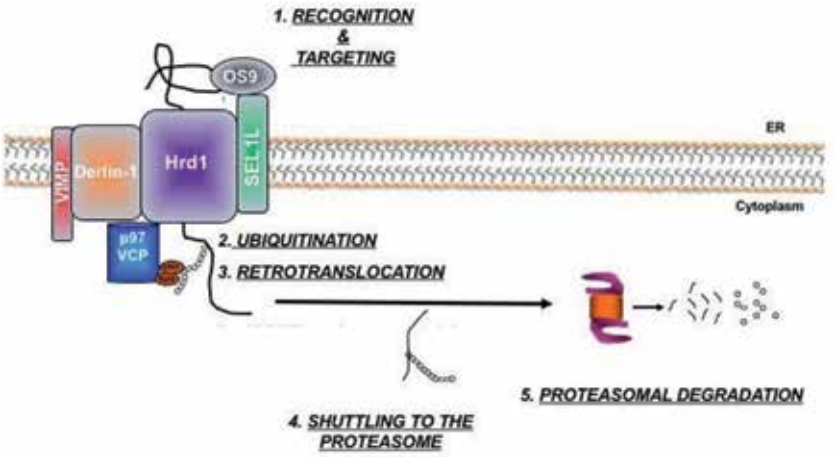

Figure 2.
Schematic representation of ERAD using the Hrd1 complex as model.

degradation such as NHK-α1-antitrypsin mutant [55–57]. EDEM1 also associates with the components of ER retrotranslocation machinery. It is suggested that EDEM1 binds misfolded proteins and uses its MHD domain to target aberrant proteins to the ER-resident glycoprotein SEL1L protein of the Hrd1-SEL1L ubiquitin ligase complex [58]. SEL1L scaffolds several luminal substrate recognition factors and links them to Hrd1. OS9 and XTP3-B also associate with Hrd1-SEL1L complex, which also includes BiP and GRP94 [59, 60]. Furthermore, XTP3-B is proposed to link BiP with Hrd1 complex [60]. According to a hypothesis, these three chaperones (EDEM1, OS9 and XTP3-B) function as oligomers, where one monomer interacts with substrate and another with Hrd1-SEL1L complex [61]. Additionally, EDEM1 also interacts with Derlins, a transmembrane protein, which is a candidate for translocon [62]; furthermore, Derlin2 is shown to enhance the interaction of EDEM1 with a cytosolic AAA-ATPase p97, which couples ATP hydrolysis to the retrotranslocation of misfolded proteins [50].

It is clear that substrate recognition step of ERAD is a complicated mechanism, in which several different enzymes and chaperones having distinct but concerted roles in the ERAD are involved. Moreover, depending on substrates, the number and features of involved proteins vary. For example, concerted roles of EDEM, ERdj5 and BiP in the degradation of misfolded proteins have been suggested [63]. After exiting CNX-CLR cycle, EDEM1 further trims the Man_8-$GlcNAc_2$ glycan structure and ERdj5 reduces disulfate bonds. Concomitantly, ERdj5 activates BiP's ATPase activity. ADP-bound BiP binds to the misfolded protein and holds it in a retrotranslocation component form until it transfers to the retrotranslocation complex [63].

ERAD is also involved in the quality control of non-glycosylated proteins, which is independent of lectin-like proteins. Immunoglobulin light chain (Ig-K-LC), a non-glycosylated ERAD substrate, is degraded in a BiP-dependent manner. Okuda-Shimizu and Hendershot have characterized an ERAD pathway for this non-glycosylated BiP substrate [64] and different protein interaction dynamics seen to play a role in this process. Ig-K-LC has two intramolecular disulfide bonds, and its fully oxidized form does not have ability to pass from the ER to the cytoplasm. BiP interacts with only partially oxidized form of the Ig, preventing the full oxidation of Ig-K-LC and thereby facilitating its release from the ER [64]. Furthermore, a transmembrane UBL domain-containing protein, homoCys-responsive ER-resident protein (HERP), has been implicated as a receptor for non-glycosylated BiP

substrates [64]. HERP interacts with Derlin1, and the partially oxidized Ig-K-LC is transferred from BiP to the HERP-Derlin1-Hrd1 complex and subsequently directed to proteasomal degradation [65]. Besides BiP, ERdj5 as disulfide reductase is also indicated to be important for ERAD of non-glycosylated proteins [63]. The non-glycosylated substrates captured by BiP are transferred to ERdj5 for the cleavage of disulfide bonds. Then, these substrates are transferred to SEL1L by the help of BiP for retrotranslocation [63]. Besides BiP, both OS9 and XTP3-B have been implicated in the ERAD of non-glycosylated proteins [12].

2.2 Ubiquitination

Ubiquitin is a 76 amino acid polypeptide encoded on multiple genes. It is ubiquitously expressed in all eukaryotic cells and highly conserved from yeast to human. Ubiquitin can be covalently conjugated to other proteins as monomers or as chains through a complex, highly regulated process called ubiquitination. Although there are reports for evidence of Ser- and Thr-linked ubiquitination, ubiquitin chain is generally attached on the Lys residue on misfolded protein. Lys-6, -11, -27, -29, 33, -48 and -63 are the residues used for ubiquitin linkage. Both the type of ubiquitination (mono/poly) and the linkages of ubiquitin chains affect the fate, localization, stability and activity of target proteins [9].

Ubiquitination has a regulatory role in almost all cellular processes by altering the fate and function of the proteins. The most well-established role of ubiquitination is targeting proteins for degradation by the 26S proteasome, and the most efficient way of targeting proteins to the proteasome is by tagging them with chains of ubiquitin [66]. This targeting requires modification of proteins with chains of four or more ubiquitins attached through lysine 48 (K48) and the specific recognition of these chains by the 19S cap of the 26S proteasome [67]. Mainly Lys-48 but rarely Lys-11-based polyubiquitin chains are reported to bind onto ERAD substrates [68].

Ubiquitination regulates several critical cellular functions, often by mediating the selective degradation of important regulatory proteins. Antigen presentation, inflammatory response induction and cell cycle progression are few examples. As expected, malfunctioning of ubiquitin-dependent proteolysis has implications for cancer and several inherited diseases, such as Angelman syndrome, Parkinson's disease and Alzheimer's disease [69].

The role of ubiquitination, however, is not limited to proteasomal targeting. The type of residue that the chain is built is critical for the fate of the ubiquitinated protein. Monoubiquitination has effects in protein trafficking, including endocytosis and lysosomal targeting. Polyubiquitin chains conjugated through K48 or other lysines (often K63) also have effect on proteasome-independent mechanisms, such as DNA repair, regulation of transcription factor activity and protein kinase activation [70].

Ubiquitination is a multi-enzyme process. Three enzymes are involved: E1-ubiquitin activating enzyme, E2-ubiquitin conjugating enzyme and E3-ubiquitin ligase. During ubiquitination, E1 forms a thiol-ester bond between its active cysteine and C-terminal glycine of ubiquitin in an adenosine triphosphate (ATP)-dependent manner. Ubiquitin on E1 is now activated and transferred to the active cysteine of E2 by a trans-thiolation reaction. E3 binds both to E2 and substrate and facilitates the formation of an isopeptide linkage between C-terminal glycine of ubiquitin and an internal lysine residue on substrate. Ubiquitin modification is dynamic and could be removed by deubiquitination enzymes (DUBs).

Today only 2 E1 enzymes and 35 E2 enzymes have been identified in mammals, but there are approximately 100 E3 in yeast and at least 600 in humans [71, 72]. E3s

catalyzing the transfer of active ubiquitin moieties on the substrate are responsible for substrate specificity. There are two large families of E3s: (1) HECT [homologous to E6-associated protein (E6AP) C-terminus] domain E3s and (2) RING [really interesting new gene] domain E3s. HECT domain E3s share a 350-residue region harboring a strictly conserved cysteine residue that forms an essential thiol-ester intermediate during catalysis. That is why ubiquitin is transferred to the active-site cysteine of the HECT domain followed by transfer to substrate or to a substrate-bound multi-ubiquitin chain. The RING finger defines the largest family of E3s. RING fingers range from 40 to 100 amino acids and are defined by eight conserved cysteine and histidine residues that coordinate two zinc ions stabilizing a characteristic cross-braced conformation. For RING E3s, current evidence indicates that ubiquitin is transferred directly from E2 to substrate [69, 70].

Ubiquitination step marks ERAD substrates for proteasomal degradation. In yeast, Doa10p and Hrd1p ligases are mainly responsible for ubiquitination of ERAD substrates, but additional E3s shown to contribute to the ERAD under special circumstances [9]. Depending on the topology of misfolded lesion, factors required for ERAD vary. In yeast, three ERAD pathways have been proposed. ERAD-C, ERAD-L and ERAD-M target proteins with lesions in the cytoplasmic, luminal and membrane domains, respectively [44, 73, 74]. ERAD-L substrates use the Hrd1p ubiquitin ligase complex containing Hrd1p, Hrd3p, Usa1p, Der1p, and Yos9p, whereas ERAD-M substrates use Hrd1p and Hrd3p, only in some cases Usa1p [68]. Hrd3p is specifically important for structural integrity of Hrd1p complex. Hrd3p stabilizes Hrd1p, and when it is absent, Hrd1p is auto-ubiquitinated and rapidly degraded. Hrd3p and its mammalian homolog SEL1L also function as an adaptor bridging substrate recognition, ubiquitination and retrotranslocation in Hrd1-mediated ERAD. On the other hand, ERAD-C substrates interact with the Doa10p ubiquitin ligase complex. These three pathways have been identified only in yeast and mammalian has more complicated machinery. Even in yeast, some membrane proteins require both Doa10p and Hrd1p E3s; thus, these pathways could overlap [42].

Although Hrd1p and Doa10p are conserved evolutionary (mammalian homologs: Hrd1 and TEB4, respectively), the number of ERAD E3s in mammals is highly expanded. Besides Hrd1 and TEB4, gp78, RNF5/RMA1, RNF170, RNF185, Trc8, RNF103, RFP2, Fbx2, Fbx6, Parkin, CHIP and UBE4a are other characterized ERAD E3s [9, 27]. Hrd1 and gp78, both homologues to yeast Hrd1p, are the most studied ERAD E3 indicated for degradation of several substrates, some of which are associated with the quality of disease-related proteins. HMG-CoA reductase, apolipoprotein B, cytochrome P450 CYP3A4, CFTRΔF508, z-variant antitrypsin, CD3δ and KAI1 are shown to be degraded via gp78-mediated ERAD, whereas studies have been suggested that Hrd1 is important for the ERAD of GABAb receptor, Nrf2, Pael-receptor mutant tyrosinase, z-variant antitrypsin and gp78 [22, 75–78]. Only a couple of substrates are known for other E3 ligases. It is also interesting that multiple E3s often function in the degradation on same substrate either in parallel or in tandem.

As Hrd1p in yeast, Hrd1 in mammals functions in a multi-protein complex. While it is complex with EDEM1, Derlins, OS9, XTP-3B and SEL1-L have been linked with degradation of glycosylated substrates (**Figure 2**), and another Hrd1 complex utilizing BiP, HERP and Derlin1 functions in the degradation of non-glycosylated substrates. Other ERAD factors have also been shown to interact with Hrd1 including UBXD2 and UBXD8 that interact with p97/VCP and recently identified chaperones such as ubiquilin and BAG6. Similarly, gp78, the second major mammalian ERAD E3 enzyme, functions in multiprotein complex in conjunction with E2 enzyme UBE2G2. Besides its diversity on substrate specificity, gp78 also has

variety of different partners allowing its communication with proteins on both sites of ER membrane. gp78 uses a VIM (VCP-interacting motif) segment to bind p97/VCP [77] and CUE domain recruiting a multiprotein complex composed of Bag6 and its cofactors [79].

After initial E3-mediated ubiquitin attachment, ubiquitin chain extension ("polyubiquitination") occurs by the covalent modification of additional ubiquitin monomers onto a Lys residue in a previously linked ubiquitin. This forms an extended isopeptide-linked polyubiquitin chain. In some selected cases, the cooperative extension of a polyubiquitin chain is by the E4s, ubiquitin chain extension enzymes, that facilitate ERAD [80–82].

2.3 Retrotranslocation and shuttling substrates to the proteasome

The ERAD substrates must be retrotranslocated to the cytosol for proteasomal degradation and the cytoplasmic AAA+ ATPase p97 (VCP or Cdc48p in yeast) is the main retrotranslocation protein providing the mechanical force required for removal of proteins from the ER. It is an essential protein having many roles in diverse biological processes, such as endoplasmic reticulum-associated degradation (ERAD), homotypic membrane fusion, transcriptional control, cell cycle regulation, autophagy, endosomal sorting and regulating protein degradation at the outer mitochondrial membrane [83–85].

p97/VCP has a multidomain structure including N domain, D1 weak ATPase, D2 major ATPase and C domain [86–88]. p97/VCP functions as a homohexamer and D1 domain is responsible for oligomerization independent of nucleotide binding. The change in the conformation of hexameric ring by ATP hydrolysis is persistent with its function in retrotranslocation [88, 89].

The diversity in cellular functions of p97/VCP is dictated by the variety of its partner proteins that interact with its N domain. p97/VCP associates with several E3s like Hrd1 and gp78, DUBs like ataxin3 and YOD1 and ERAD accessory factors such as UbxD2 and VIMP. Moreover, many p97/VCP interacting proteins (Ufd1-Npl4 dimer, gp78 etc.) bind directly to ubiquitin. p97/VCP functions as a segregase using the energy from ATP hydrolysis to segregate ubiquitinated proteins from large immobile complexes of ER to the cytosol. This cytosolic protein is recruited to the ER membrane through its interaction with membrane-embedded ERAD components. There are at least seven different ERAD members that could interact with p97/VCP via certain motifs such as VIM motif (gp78 and SVIP), UBX domains (UBXD2 and UBXD8), SHP boxes (Derlin1 and Derlin2) and uncharacterized cytosolic regions of Hrd1 and VIMP that found to have p97/VCP-binding motif [12, 42, 90].

Retrotranslocation is tightly coupled with both ubiquitination and proteasomal degradation. In most cases, inhibiting ubiquitination prevents both degradation and retrotranslocation. The interaction of p97/VCP/CDC48p with its cofactor Ufd1-Npl4 dimer enhances its affinity to ubiquitin (**Figure 2**). However, it has been also suggested that Hrd1-mediated ERAD requires well-established retrotranslocation machinery, the p97/VCP–Ufd1–Npl4 complex, whereas the gp78 pathway needs only p97/VCP and Npl4 [75].

Many deubiquitinating enzymes (DUBs) in mammalian cells, including Ataxin3, USP13, USP25 and YOD1, are also implicated in the ERAD through physical interaction with ERAD core machinery [72, 91, 92]. Several studies revealed that p97/VCP interacts with DUBs. However, the function of DUBs in the ERAD is still not fully characterized. Otu1p (yeast homolog of YOD1) binds to CDC48p and trims the polyubiquitin chain, resulting oligoubiquitin chains with up to 10 ubiquitin molecules. It has been further suggested that releasing substrates from CDC48p requires DUBs [93]. Consistently, catalytically inactive YOD1 inhibits retrotranslocation of

ERAD substrates [91]. In conclusion, many p97-associated DUBs serve as positive regulators of ERAD.

Several putative retrotranslocation channels have been proposed such as the Sec61 complex, members of Derlin family and polytopic E3s such as Hrd1 and gp78. Sec61 is one of the proposed channel protein mutants, which prevented degradation of some ERAD substrates in yeast [94, 95]. Cholera toxin also translocates from ER by utilizing Sec61 [96]. On the other hand, retrotranslocation of some other ERAD substrates has been suggested to depend on Derlins [97, 98], a family of polytopic transmembrane ER proteins linked to some ERAD substrates. Moreover, Derlin1 recruits p97/VCP [99], a key protein of retrotranslocation, which provides energy for the process. Derlin1 also interacts with some E3s like Hrd1, gp78 and RNF5 forming large complexes on the ER membrane [9]. Recently, Hrd1 ubiquitin ligase has been suggested to be the top candidate for retrotranslocation channel [9]. Auto-ubiquitination of Hrd1p in its RING finger domain triggers conformational change allowing the misfolded luminal domain of a substrate to move across the membrane. Thus, it was suggested that Hrd1 forms an ubiquitin-gated protein-conducting channel [33]. It has also been suggested that proteins might exit the ER via the formation of lipid droplets or lipid droplets serve as an intermediate step for substrates en route to the proteasome [100]. However, studies in yeast suggested that lipid droplet formation is dispensable for ERAD-L and ERAD-M [101].

Once retrotranslocated from ER to the cytosol, ERAD substrates should be rapidly targeted to the proteasome for degradation in order to avoid accumulation of aggregates in the cytosol. Consistently, proteasomal inhibition also stabilizes ERAD substrates in the ER lumen. For the coupling of retrotranslocation with degradation, ubiquitinated substrates must be recognized by cytosolic proteins functioning as ubiquitin receptors. Ubiquitin-binding domain containing proteins has ability to shuttle ubiquitinated proteins from retrotranslocation complex at the ER membrane to the proteasome since these proteins interact both with proteasome and p97/VCP. Indeed, it has been suggested that p97/VCP bridges the ER to the proteasome by forming a complex with mHR23B (homolog of yeast Rad23p)-PNGase [102] (**Figure 2**). In yeast, the substrates are probably transferred from CDC48p to the proteasome indirectly via ubiquitin- and proteasome-binding domains containing shuttling factors Rad23p and Dsk2p [103, 104]. Recently, Bag6/Bat3/Scythe has been characterized as a novel chaperone system with regulatory functions in protein degradation [79]. The chaperone holdase activity of this system keeps some retrotranslocated substrates in a soluble state for proteasome degradation. Bag6, also a partner protein of gp78 E3 enzyme, interacts with proteasome, and proteins like ubiquilin that known as proteasome adaptor proteins suggesting Bag6 might act between p97/VCP and proteasome to hand substrates off from retrotranslocation machinery to the proteasome.

3. Regulation of ERAD

Regulation of ERAD in normal and pathological conditions is also of great importance since hyper-ERAD may cause in loss-of-function phenotypes upon unnecessary degradation of folding intermediates as seen in CFTR and hypo-ERAD may result in gain-of-function phenotypes upon accumulation and/or aggregation of misfolded and unassembled proteins. Several studies suggested different regulation paths for ERAD activity via ubiquitin ligases and their dynamic ERAD complexes, UPR and endogenous ERAD inhibitors.

It is thought that ERAD functions at relatively low levels under basal conditions, but under proteotoxic stress its activity is enhanced. Accumulation of the unfolding

or misfolding proteins in the ER lumen triggers "ER stress" by decreasing free chaperone levels [105]. In response to this cellular stress, the pathway known as the "Unfolded Protein Response (UPR)" is activated and results in specific cellular functions classified as adaptation, alarm and apoptosis [4]. Three transmembrane proteins with luminal domains that sense the changes in the ER environment function as UPR sensor proteins are inositol requiring enzyme-1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase RNA-like endoplasmic reticulum kinase (PERK). PERK is a serine/threonine kinase, and IRE1 possesses both kinase and endoribonuclease domains [27, 50]. These sensors initiate signal transduction by sensing the presence of unfolded proteins in the ER lumen and thus control the UPR pathway [15, 18, 106]. All these transmembrane proteins interact with BIP under basal conditions. However, when unfolded proteins are present, BIP dissociates from the UPR sensor proteins. After dissociation of BIP, PERK and IRE1 dimerize and become activated by auto-phosphorylation, whereas ATF6 become translocated to the Golgi and proteolytically cleaved [27, 50]. Activated PERK phosphorylates translation factor eIF2α attenuating protein synthesis to limit protein load. IRE1 activates XBP-1 that enhances transcription of ERAD factors [27, 50]. On the other pathway, ATF6 upregulates many genes that encode ER-resident chaperones and folding assistants like BIP, CNX, CLR and PDI. To summarize, with the induction of UPR in the cell, the overall translation is inhibited for several hours primarily to slow down the entry of newly synthesized proteins to the ER, the amount of chaperones and ER protein folding capacity is increased for proper folding of accumulated unfolded proteins, and thus, the normal ER function and homeostasis are protected [4, 107]. UPR also enhances ERAD capacity by upregulating some of the ERAD genes to ensure that defective proteins are degraded when the folding attempts fail [21–23]. EDEM proteins, Hrd1, SVIP, OS9 and gp78, are only some of the targets of the ER stress-induced Ire1/Xbp1 pathway [62, 108–111]. If the cellular stress is consistently increasing, UPR induces cell death mechanisms such as apoptosis or autophagy [4, 14, 112].

It has been suggested that large or prolonged variations such as change in Ca^{2+} or redox homeostasis, exposure to pathogens and large-scale accumulation of misfolded proteins may induce UPR to adapt ERAD activity. However, smaller or more transient fluctuations on ER load may be overcome rapidly by post-translational pathways that control stability, localization and assembly of ERAD components [23]. For example, reversible ADP ribosylation adapts BIP response for short-term fluctuations [113]. Reversible palmitoylation changes the sub-organelle distribution of CNX [114, 115]. Moreover, many ERAD factors/enhancers, including EDEM1, ER Man1, HERP, OS9, SEL1L and gp78, have fast turnover. This is important since when protein misfolding crisis is over, ERAD activity should rapidly turn back to the basal levels. Many ERAD factors then rapidly degraded via a process called ERAD tuning [23]. ERAD tuning does not require signal transduction from the ER to the nucleus [23]. Hrd1 was suggested to be a central regulator of ERAD tuning. It has been shown that Hrd1 ubiquitinates gp78 E3 enzyme and enhances its degradation, which in turn causes inhibition of gp78-mediated ERAD. Very recently, Hrd1 was also found to regulate the stability of OS9 [116]. Hrd1 also undergoes auto-ubiquitination to induce its own proteasomal degradation [117]. Another homeostatic control mechanism, in which ERAD activity itself is regulated post-translationally and independent of UPR, is degradation of EDEM1, OS9 and SEL1L by the E2 enzyme UBC6e, a component of Hrd1 supramolecular complex [118].

Another type of ERAD regulation occurs via substrate-specific adaptor, as reported for HMGR. The adaptor proteins, Insig1 or Insig2, bind to HMGR only in the presence of 24,25-dihydrolanosterol, an intermediate molecule in sterol

biosynthesis. Under low sterol levels, HMGR is stable; however, when sterol levels are high, Insig-HMGR interaction become favored, leading delivery of HMGR to E3 complex following by its proteasomal degradation [119]. Likewise, ERAD-mediated degradations of apolipoprotein and IP3R are initiated when lipid levels are low and calcium levels are high, respectively [23].

DUBs are also proposed as factors that regulate ERAD. As explained above, several DUBs have been reported to interact with p97/VCP and function as positive regulators of retrotranslocation. Additionally, some DUBs are linked with the regulation of E3 enzyme stability. For example, USP19, an ER-anchored DUB, rescues HRD1 from proteasomal degradation and thereby regulates HRD1 stability [120]. Similarly, USP19 enhances the stability and activity of another E3 MARCH6 [121].

SVIP (small VCP interacting protein), a VCP-interaction motif (VIM) containing protein, is the first identified endogen ERAD inhibitor. SVIP interacts with p97/VCP and Derlin1 and inhibits the ubiquitination and degradation of gp78-dependent ERAD substrates [111]. Another endogen ERAD inhibitor is SAKS1. SAKS1 binds to the polyubiquitin chain of the substrate and p97/VCP and attenuates the ERAD process [122].

ERAD activity can also be controlled by hormonal regulation. Glucocorticoids have been suggested to ameliorate ER stress by promoting correct folding of secreted proteins and enhancing removal of misfolded proteins from the ER probably through induction of UPR. Recently, androgen-mediated regulation of ERAD has been reported. Androgen treatment upregulated the expression of Os9, p97/VCP, Ufd1, Npl4, Hrd1 and gp78, but downregulated ERAD inhibitor SVIP, which in turn enhanced the proteolytic activity of ERAD in androgen-sensitive prostate cancer cells [123]. Furthermore, the regulation of ERAD by androgen is mediated via AR and is partially or fully independent on the androgen-mediated induction of IRE1α branch [123].

Conflict of interest

The authors declare no conflict of interest.

Author details

Burcu Erbaykent Tepedelen[1] and Petek Ballar Kirmizibayrak[2]

1 Faculty of Science, Molecular Biology and Genetics, Uludag University, Bursa, Turkey

2 Biochemistry Department, Faculty of Pharmacy, Ege University, Izmir, Turkey

*Address all correspondence to: petek.ballar@ege.edu.tr

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Chapter 4

Endoplasmic Reticulum Stress and Autophagy

Mohammad Fazlul Kabir, Hyung-Ryong Kim and Han-Jung Chae

Abstract

In eukaryotic cells, the aggregation of the endoplasmic reticulum (ER)-mediated unfolded or misfolded proteins leads to disruption of the ER homeostasis, which can trigger ER stress. To restore the ER homeostasis, the ER stress activates the intracellular signaling cascade from the ER to the nucleus, referred to as the unfolded protein response (UPR). Autophagy primitively portrayed as an evolutionarily conserved process is involved in cellular homeostasis by facilitating the lysosomal degradation pathway for the recycling and elimination of intracellular defective macromolecules and organelles. Autophagy is tightly regulated by the protective mechanism of UPR. The UPR and autophagy are interlinked, which indicates that the ER stress can not only induce autophagy but also suppress it. Here, we discuss the molecular mechanism of ER stress and autophagy and their induction and inhibition signaling network.

Keywords: ER stress, autophagy, calcium, lysosome

1. Endoplasmic reticulum

The endoplasmic reticulum (ER) is a central membrane-bound organelle constructed from a dynamic network of tubules involved in cellular processes such as protein synthesis, gluconeogenesis, lipid synthesis and processing, and calcium storage and release in the cell and contributes to the generation of autophagosomes and peroxisomes [1]. The extension of ER morphology depends on the cell's activity and lineage; it is organized into subcompartments of different shapes, such as cisternae and tubules. ER appears as two main interconnected compartments, namely, the smooth endoplasmic reticulum (SER) and the rough endoplasmic reticulum (RER), which are abundant in different proportions in different cell lineages [2]. RER is less tubular than the SER, which forms an interrelated network of subdomains of ER; the RER is illuminated with ribosomes on their membranes, which are absent in the SER. RER has appeared in all cells and its density is higher, similar to that of the Golgi apparatus and nucleus because in all cells the nascent polypeptide is cotranslationally inserted into their membranes from the ER membrane. However, SER is present in only certain cell types, such as the liver cells, steroid-synthesizing cells, neurons, and muscle cells. SER is involved in the generation of steroid hormones within the adrenal cortex and endocrine glands and acts as a center for detoxification and protein transportation [3, 4]. A remarkable number of proteins are Ca^{2+}-dependent and need a completely oxidizing environment [5]. In the lumen, the

abundant molecular chaperones bind to the proteins and prevent them from aggregation, which makes the ER an ideal and unique milieu for proper protein folding. In fact, the ER quality control checkpoints allow the existence of only the precisely folded proteins. In addition, the ER facilitates the formation of three-dimensional structures by cotranslational and posttranslational modifications of the proteins [6].

2. ER stress

The ER is a subcellular organelle predominantly known as a protein-folding checkpoint, which has an important role to ensure the proper folding and maturation of newly secreted proteins and transmembrane proteins. Several pathological and physiological conditions, such as perturbation in the cellular ATP level, calcium fluctuation, hypoxia, viral infection, inflammatory cytokines, nutrient deprivation, and environmental toxins, result in the loss of ER homeostasis and a reduction in the protein-folding potential of ER, eventually leading to the accumulation and aggregation of unfolded proteins in the ER lumen, acknowledged as the ER stress [7]. In experimental settings, the ER stress and protein misfolding or aggregation is instigated by treating cells with ER stress-inducing toxic chemicals. Versatile mechanisms of the UPR, under these nonphysiological conditions, are unable to maintain the homeostasis in the ER and the cells finally undergo apoptosis [8].

3. The unfolded protein response (UPR)

The UPR can be viewed as a process that is involved in the sensing of the ER stress and transduces this signal to the regulation of downstream transcription factors that are involved in stress reduction or the induction of proapoptotic programs [9]. The ER stress enacts the UPR as an adaptive response for maintaining protein homeostasis [10, 11]. The UPR is initiated by three ER transmembrane proteins: the inositol-requiring 1α (IRE1α), PKR-like ER kinase (PERK), and activating transcription factor 6α (ATF6α). Under normal conditions, the ER chaperone, luminal domain binding immunoglobulin protein (BiP), binds to these proteins and keeps them inactive; but, when ER stress occurs, the BiP dissociates from these three proteins, UPR arms are activated [12]. This activates the UPR, which has three noteworthy functions: (a) adaptive feedback, which encompasses decreasing the ER workload, in anticipation of further augmentation of unfolded proteins, by the upregulation of molecular chaperones and protein-processing enzymes that maximize the folding efficiency, and an accompanying increase in the ER-associated protein degradation (ERAD) and the upregulation of the autophagy components to aid in the removal of misfolded proteins; (b) feedback control, which includes prevention of the hyperactivation of UPR, when the ER homeostasis is retrieved; (c) cell fate regulation, by the coordination of apoptotic and antiapoptotic signals, in the form of a switch between life and death of ER-stressed cells [12, 13]. The gene targets of the UPR change depending on the type of tissue and the nature of the physiological trigger that induces the ER stress. In distinctive hereditary backgrounds like the mouse and human cells, the different gene expression patterns triggered by the ER stress have been reported [9, 14].

4. The UPR signaling pathway

The UPR and misfolded or unfolded proteins as a prominent characteristic of mammalian cell ER stress were first reported in the 1980s by Kozutsumi et al. [10].

The UPR signaling pathway consists of three main branches involving the proteins IRE1α, PERK, and ATF6α. These proteins present in the ER resident transmembrane are major signaling elements. These three signaling sensors are all confederate with the ER chaperones, such as GRP78 (glucose-regulated protein 78 kDa, the main ER chaperone that is also named as BiP), which regulate their activation by fastening or discharge mechanism [15]. Primarily, IRE1α, PERK, and ATF6α are activated by the interaction with GRP78. GRP78 is well established to bind to the hydrophobic domains of proteins with its C-terminal binding domain, to prevent misfolding and unfolded protein aggregation (**Figure 1**). GRP78 accelerates the oligomerization and autophosphorylation of IRE1α and PERK activates them, while ATF6α is translocated to the Golgi apparatus [16].

4.1 IRE1α

IRE1α is the most conserved ER stress signaling branch, and its activation mechanism has been studied thoroughly. IRE1α is a bifunctional type 1 transmembrane protein kinase containing three domains: an N-terminal luminal domain, a cytosolic endoribonuclease (RNase) domain, and a cytosolic serine/threonine kinase domain [17]. In response to the accumulation of unfolded proteins under the ER stress condition, IRE1α dimerizes and transautophosphorylates leading to activation of

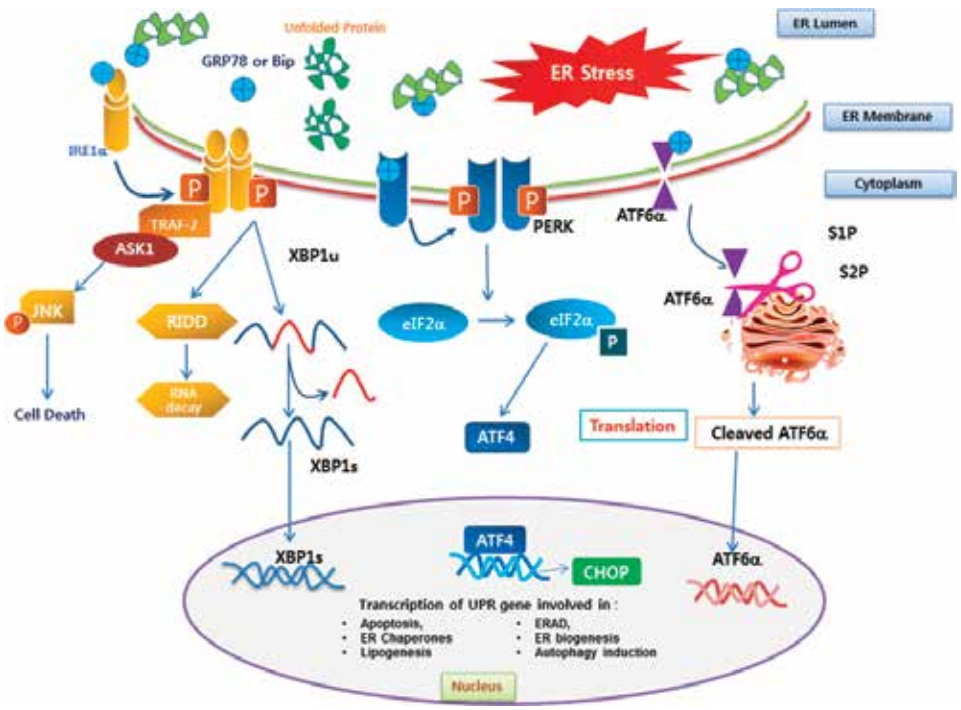

Figure 1.
Schematic overview of UPR signaling. The three sensors of UPR, namely, IRE1α, PERK, and ATF6α, are activated when the misfolded protein aggregates recruit GRP78 or Bip, by dissociating them from the sensors. Activated IRE1α dimerization and phosphorylation induces XBP1 mRNA splicing to generate active XBP1s, which increase the expression of UPR functional gene. UPR also activates another cellular pathway by interacting with Jun N-terminal kinase (JNK), via recruit TRAF2 and ASK1. PERK phosphorylates the downstream translation initiation factor eIF2α, leading to the attenuation of overall protein translation and the activation of ATF4, which activates the expression of CHOP. Under ER stress conditions, the ATF6α is transported to the Golgi apparatus and its cytosolic domain is cleaved by S1P and S2P proteases, which triggers the transcription of the ER chaperones. XBP1, ATF4, and ATF6α transcription factors are translocated to the nucleus where they actuate the expression of target genes.

the cytosolic region RNase domain, resulting in the conformational change that activates the excision of the 26-nucleotide intron from the mRNA encoding the transcription factor XBP1 (**Figure 1**) [18–21]. This splicing event results in a frame shift in the mRNA and leads to the expression of an active and stable form of the transcription factor XBP1. The XBP1 is then translocated to the nucleus where it upregulates target genes that are involved in prosurvival events, such as quality control, maintaining ER homeostasis (via the ER chaperones GRP78, ERDj4, HEDJ, and PDI-P5) and ERAD (ER-associated degradation) [22, 23]. In the ER and Golgi compartment, XBP1 also increases the secretion rate of proteins. In addition, the RNase domain of IRE1α can rapidly cleave a group of mRNAs and microRNAs, degradation through a process known as IRE1α-dependent decay (RIDD) [24, 25]. IRE1α activation is associated with the reduction of levels of a myriad of cytosolic RNAs, ribosomal RNAs, and microRNAs that have significant roles in inflammation, glucose metabolism, and apoptosis. Furthermore, active IRE1α not only promotes UPR but also mediates other pathways, including the mitogen-activated protein (MAPK) kinase pathway, where the activated IRE1α interacts with the adaptor protein tumor necrosis factor receptor-associated protein (TRAF-2) to form the complex IRE1α-TRAF2. This complex interacts with the apoptosis signal-regulated kinase 1 (ASK1) to form the IRE1α-TRAF2-ASK1 complex, which interacts with the ER stress-triggered c-Jun N terminal kinase (JNK) and results in the production of reactive oxygen species and activation of the autophagy and inflammatory pathways that involve the nuclear factor-κB [26–28].

4.2 PERK

PERK is a type 1 transmembrane kinase that is structurally and functionally related to IRE1α and is activated by transautophosphorylation and dimerization [29]. Under the ER stress conditions, PERK phosphorylates the downstream substrate eukaryotic translation initiator factor-2α (eIF2α) at serine 51, which leads to the inhibition of protein synthesis within the ER lumen (**Figure 1**) [30–32]. This blockade reduces the continuous accumulation of unfolded proteins in the ER, thus reducing the ER stress. In addition, it allows the selective translation of the mRNA encoding the transcription factor ATF4, which has a ribosome entry site in its 5′ nontranslated region, enabling its cap-independent translation [33, 34]. ATF4 is translocated to the nucleus where it upregulates the expression of the ER chaperone proteins (GRP78 and GRP94), the genes involved in macroautophagy, amino acid biosynthesis, protein secretion, antioxidant response, and the proapoptotic transcription factor CHOP [34, 35]. In addition to its role in UPR, eIF2α phosphorylation assumes the role of a confluent marker of a particular stress pathway known as the integrated stress response," which is led by the unambiguous kinase that triggered during nutrient deficiency, viral infection, inflammation, and heme deficiency [35].

4.3 ATF6α

A third sensor of the ER stress, ATF6α, is an ER-targeted type 2 transmembrane protein that includes a basic leucine zipper transcription factor domain (**Figure 1**) [36, 37]. Under upregulation of UPR, ATF6α is translocated to the Golgi apparatus for cleavage by the endopeptidases S1P and S2P, thereby releasing the activated form of ATF6α. In response to the ER stress condition and GRP78, GRP94 agglomeration, similar to that of IRE1α and PERK activation, the redox state is involved in the activation of ATF6α [38].

The activation of IRE1α, PERK, and ATF6α has several effects, such as reduced translation, enhanced ER protein-folding capacity, and clearance of misfolded ER

proteins. The UPR stress sensors interact with and activate several transcription factors, which indicate the functional role of the UPR in proteostasis.

5. Autophagy

Autophagy, derived from the Greek words "auto," meaning "self," and "phagy," meaning "to eat," is a lysosomal pathway for cell survival used by eukaryotes, in which the cells digest and recycle their own cytoplasmic contents [39]. In the past three decades, several studies, especially in yeast, have revealed the molecular mechanisms involved in autophagy. Cells attune the number of components or vitiate parts of the organelle to maintain the optimum activity by assisting the minimal basal level of autophagy [40, 41]. In response, the basal autophagy can be activated to play a crucial role in cellular starvation and other cellular stresses, by lysosomal degradation and the exclusion of perennial and misfolded proteins, pernicious cellular substances, and pernicious organelles and infecting pathogens [42, 43]. In addition, autophagy can involve the rearrangement of the cellular membrane to concede parts of the cytoplasm being transported to the compartment, and it also acts as an energy source for the biosynthesis of new macromolecules produced by recycling metabolites of lysosomal proteolysis [44, 45]. Autophagy can maintain the energy homeostasis not only in particular organelle but also in the entire cell, through the increase of metabolic activity [45]. Moreover, autophagy plays critical roles in physiological processes such as cell growth, cell cycle, differentiation, tumor suppression, and programmed cell death including apoptosis and cellular senescence. In these ways, autophagy plays crucial roles throughout the life cycle of the cells [46, 47].

In mammal cells, there are three types of autophagy that have been documented; they are distinguished according to their physiological function and mechanism of cargo sequestration at the known destination lysosomes. These subtypes include macroautophagy, microautophagy, and chaperone-mediated autophagy [43, 48]. Macroautophagy is a major type of autophagy, and it has been the most studied compared to microautophagy and chaperone-mediated autophagy. It uses cytosolic double-membrane sequestering vesicles formed from phagophores, known as the autophagosomes, which transport cytosolic content to the lysosome [43, 49]. In microautophagy, the lysosome itself is a component of the cytoplasm where it engulfs cytoplasmic protein and small components of the lysosomal membrane. Macroautophagy and microautophagy both carry out the nonselective degradation of proteins, lipids, and organelles [50, 51]. In contrast, the chaperone-mediated autophagy does not involve the membrane rearrangement; instead, the protease of the lysosomal matrix acts on the substrate unfolded protein by directly translocating across the lysosomal membrane, which is dependent on LAMP2A (lysosomal-associated membrane protein 2A) and the lysosomal molecular chaperon HSPA8/HSC73/lys-HSC70 (heat shock cognate 70) [52, 53].

6. Molecular mechanism of autophagy

The mechanism of autophagy is a complex process that can be categorized into multiple steps. It involves the formation of double-membrane vesicles containing cellular and external malformed proteins. Long-lived proteins can be induced autophagy, which are ensued by cargo recognition and packaging, an extension of the phagophore membrane, and closure to form the complete autophagosome. Fusion of the autophagosome with the lysosome occurs, which leads to the derogation of the autophagosomal contents, and the breakdown products are finally

eliminated [54–56]. The initiation of autophagy can be observed by TEM (transmission electron microscopy) during the expansion of phagophore and autophagosome. The induction of autophagy, vesicle nucleation, and formation of autophagosomes are regulated by the proteins named as autophagy-related genes (ATGs) [50]. They are highly conserved genes and were originally discovered in yeasts. Mammalian orthologs of the ATGs have also been discovered [57]. Autophagy induction is controlled at the molecular level by the multiprotein complex of unc-51-like autophagy-activating kinase 1 (ULK1, the mammalian homolog of yeast Atg1), ATG13, ATG101a, and RB1 inducible coiled coil 1 (RB1CC1, also known as FIP200) [58, 59]. This complex is regulated by the mammalian target of kanamycin complex 1 (mTORC1), which remains inhibited by mTORC by the phosphorylation of ULK1/2 and ATG13, which suppresses the phosphorylation activity of ULK1/2-ATG13-FIP200 complex [59–61]. Under starvation and other stress conditions, the inhibition of mTORC1 dissociates it from the ULK complex followed by the dephosphorylating of specific residues within the ULK1/2 and ATG13 (phosphorylated by mTORC1) complex, which in turn promotes the induction of the phagophores [61]. Formation of phagophores includes a class III phosphatidylinositol 3-kinase complex (PtdIns3K) consisting of Beclin-1 (ATG6 in yeast), VPS34 (class III PI3K), VPS15 (also known as p150 in mammals), PIK3R4/p150, ATG14, UV radiation resistance-associated gene (UVRAG), and nuclear receptor binding factor 2 (NRBF2) [62–64]. In addition, the nonapoptotic proteins, such as the B-cell lymphoma-2 (BCL2) and BCL2L1/BCL-XL, hold Beclin-1 directly interacting with Beclin-1(BECN-1s) BH3 domain and negatively regulating autophagy inducing the PtdIns3K. The c-Jun protein kinase (JNK1) and death-associated protein kinase (DAPK) phosphorylate BCL2 and are positive regulators involved in the induction of autophagy [65, 66].

The elongation or obstruction of phagophore depends on two diverse ubiquitin-like protein conjugation reactions [67, 68]. The first pathway involves the covalent conjugation reaction of ATG12 to ATG5, with the assist of the E1-like enzyme ATG7 and the E2-like enzyme ATG10. This conjugate ATG12-ATG5 complex interacts with ATG16L in a no covalent reaction to form the multiprotein complex ATG12-ATG5-ATG16L, which performs the E3 ligase reaction of the cytosolic MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3), LC3-I to the membrane-bound lipidated form, LC3-II [50, 69–71]. The second pathway includes the ubiquitin-like system, which plays a role in the conjugation to phosphatidylethanolamine (PE) lipid and glycine residue of the yeast ATG8 (LC3 in the mammal), and is processed by the cysteine protease ATG4 and then ATG8 is conjugated to PE by E1-like enzyme ATG7 and E2-like enzyme ATG3. Based on that , the ATG4 can act as delipidation or deconjugation enzyme which is involved in the recycling of membrane bound LC3-II on the external layer to the internal layer of the autophagosome [50, 67, 72]. Accordingly, the lipidated form of LC3-II is a stable marker protein associated with the biochemical and microscopic detection of cellular autophagy [73]. In mammals, six orthologs of ATG8 and four of ATG4 exist, among which the LC3, GATE-16 (Golgi-associated ATPase enhancer of 16 kDa), and GABARAP (G-amino butyric acid type A receptor-associated protein) have been the most studied [74]. The lipidation of ATG8/LC3 expedites the interaction with the autophagosome membrane, which leads to the autophagosome maturation steps, such as the extension and shrinkage of the membranes and cargo induction to autophagosome [75]. Once the autophagosome has surrounded the substrate of autophagy, it may merge with the late lysosome or endosome to create the autolysosome [76]. The cellular and molecular machinery that important for the fusion is activated by the small GTPase, RAB7A/RAB7 member of RAS oncogene family, which is necessary for autophagosome maturation [77]; and the RAB7 effector pleckstrin homology and RUN domain containing M1 (PLEKHM1) [78]; other soluble

N-ethylmaleimide-sensitive factor proteins trigger set of SNARE protein including syntaxin-17 (STX17), SNAP29,and VAMP8 [79, 80]; the PI3P-binding protein tectonin beta-propeller repeat containing 1 (TECPR1) [81]; inositol polyphosphate-5-phosphatase-E (INPP5E) [82]; ectopic P-granules autophagy protein 5 homolog (EPG5) [83]; as well as the homotypic fusion and vacuole protein sorting (HOPS) complexes ATG14 [78]; LAMP2B (but not LAMP2A) as well as the phosphorylated and lipidated LC3 which are also involved in the formation of autolysosomes [84]; finally, the autophagosomal-sequestered cargo undergoes degradation upon the acidification of the lysosomal lumen (by the activity of an ATP-dependent proton pump known as the V-type ATPase) [85].

7. ER stress and autophagy

Several studies have demonstrated that the ER stress and autophagy are mechanistically interconnected, in which the UPR, the key ER stress pathway, stimulates the autophagy. The three canonical divisions of the UPR intervened by the three ER membrane-associated proteins, IRE1α (inositol-requiring enzyme 1), PERK (PKR-like eIF2α also known as EIF2AK3), and ATF6α (activating transcription factor-6), regulate the autophagy in distinctive manners during the ER stress. The relationship between autophagosome and the ER stress was first described in 2006 [86, 87].

IRE1α-mediated MAPK8 (mitogen-activated protein kinases 8) phosphorylation is the major regulatory step in this pathway [88]. MAPK8 is considered stress-associated protein kinase," which is involved in numerous manners in stress-induced autophagy and apoptosis, which depend on MAPK8 activation [89]. In particular, the activation of IRE1α leads to MAPK8 phosphorylation, which induces autophagy. JNK (c-Jun N-terminal kinase) interacts with the MAPK8 family, which triggers the downstream mediators of autophagy, both directly and indirectly [90]. Directly, JNK can stimulate cell apoptosis in cancer cells by inducing Atg5 and p53. Indirectly, JNK inhibits the association of Bcl-2 with Beclin-1 and upregulates Beclin-1 expression by c-Jun phosphorylation. Beclin-1 is the autophagy-related gene and is the downstream regulator of MAPK8 and is activated by the direct phosphorylation of Bcl-2, which then obstructs the interaction between Beclin-1 and Bcl-2 and activation of the phosphoinositide-3-kinase (PI3K) complex and induces autophagy in the cancer cell (**Figure 2**) [90, 91]. Additionally, SP600125, a pharmacological inhibitor of JNK, also blocks the Beclin-1 expression and autophagy [92]. Wei Y et al [91] elucidated the starvation-induced autophagy by JNK1, via phosphorylation of ER-specific Bcl-2, at multiresidues T69, S70, and S87A, followed by Beclin-1 disruption from ER-localized Bcl-2 and the induction of autophagy [91]. Similarly, Beclin-1 expression is regulated by the JNK1 pathway, which plays a crucial role at the transcription level, following the ceramide-induced autophagy in mammalian CNE2 and Hep3B cancer cell lines [92]. SP600125 inhibited the autophagosome formation and ceramide-induced upregulation of Beclin-1, and similar phenomenon was observed using the small interfering RNA targeting JNK mRNA. Moreover, immunoprecipitation of chromatin and luciferase reporter analysis demonstrated that c-Jun, a target of JNK1, was activated and directly interacted with the Beclin-1 promoter in ceramide-treated cancer cells. In this respect, the IRE1α/JNK1/c-Jun pathway is the key mechanism for the induction of autophagy. The IRE1α/JNK1-induced autophagy pathways interact with the ATG protein and Beclin-1, which play a key role in vesicle nucleation [93, 94].

In addition, the IRE1α-XBP1s axis has been involved in the induction of autophagy [95]. Initially, the spliced XBP1 indirectly regulates the Bcl-2 expression to induce autophagy (**Figure 2**) [66, 96]. Along with this, the autophagy induction

is also observed in endothelial cells that overexpress XBP1s, which enhances the transformation of LC3-I to LC3-II and increases the Beclin-1 expression [95]. Furthermore, XBP1s binds directly to the −537 and −755 region of the Beclin-1 gene promoter in the nucleus and enhances an autophagy induction via the transcriptional upregulated expression of Beclin-1 gene [97]. The deficiency in XBP1s leads to increased expression of Forkhead box O1, a transcriptional factor that elevates the induction of autophagy in neurons [98].

The major events in autophagy, such as the induction of phagophore and maturation, are coordinated by the LC3-II and the ATG12-ATG5 conjugate [99]. To maintain the autophagy flux, the upregulation of the transcription of the congruent autophagy genes is important [100]. Under the ER stress conditions, the PERK branch of UPR aids in the regulation of the autophagy-related genes. The association of PERK in ER stress-mediated induction of autophagy was first reported by Kouroku et al. [101]. In particular, they demonstrated that the aggregated polyglutamine (72Q) protein in the cytosol decreases the activity of proteasomes and leads to autophagy induction through the activation of the PERK branch of the UPR [102]. Under the hypoxic response, PERK mediates the transcriptional activation of LC3 and Atg5 proteins, through the action of the transcription factors ATF4, CHOP, and DDIT3 induction (**Figure 2**) [101, 103]. PERK may also reduce IkBα translation, as well as NF-kB activation, which promotes the induction of

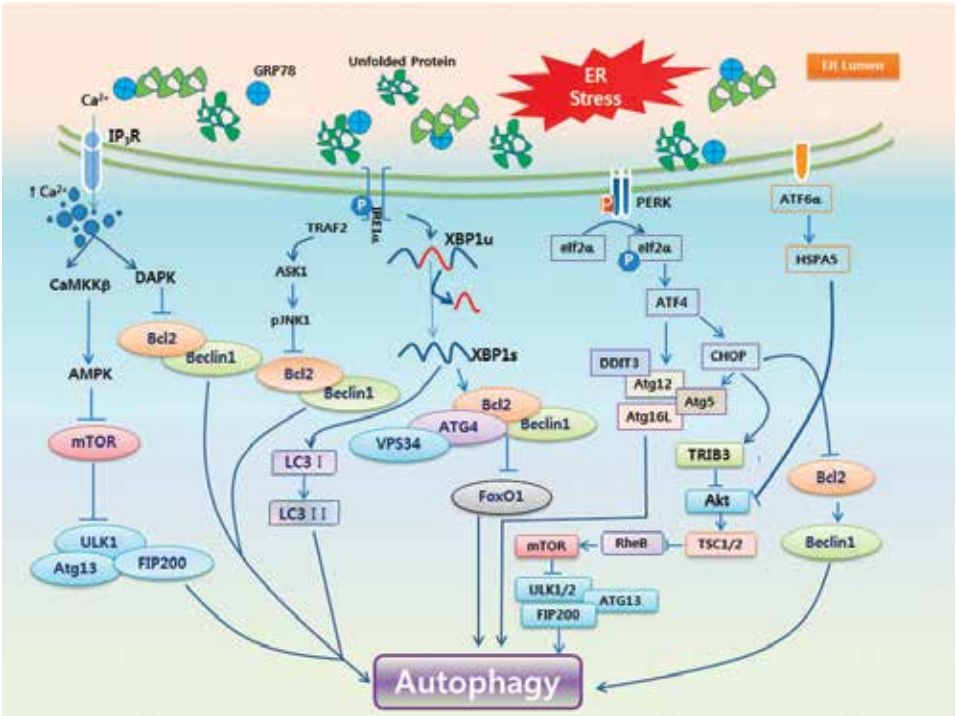

Figure 2.
Overview of the mechanism of UPR-mediated autophagy. The ER stress can activate autophagy through Ca^{2+}, IRE1α, PERK, and the ATF6α signaling pathway. Ca^{2+} from ER lumen can be released through the IP3R channel, which phosphorylates CaMKKβ and activates AMPK, which in turn inactivates ULK1 complex through the inhibition of mTOR; Ca^{2+} activates DAPK which phosphorylates Beclin1 and Bcl2 lead to autophagy induction. The IRE1α arm of UPR activation of JNK1 mediates phosphorylation of Bcl2, which causes Beclin-1 dissociation and induction of autophagy. In addition, spliced XBP1 also enhances the formation of LC3-I and LC3-II, which triggers the Beclin-1 via decrease of FoxO1 activity. Another arm of UPR activated PERK induce autophagy via expression of ATG12, DDIT3, ATG12, ATG16L by ATF4 transcription factor similarly CHOP activate TRIB3 which suppress the activity of Akt/mTOR pathway induced autophagy. ATF6α arm of UPR can also induce autophagy by inhibiting phosphorylation at Akt and mTOR pathway.

autophagy [104]. PERK phosphorylates the downstream regulator eukaryotic initiation factor 2a (eIF2α), at the residue serine 51, and also increases the ATG12 mRNA and protein levels [105]. PERK-mediated ATF4 activation is required for expression of the autophagy genes, including MAP1LC3B, BECN1, ATG3, ATG12, and ATG16L1, while interaction of ATF4 and DDIT3 causes the upregulation of the transcription of SQSTM1/p62, BR1, and ATG7 [100]. In addition, ATF4 directly binds to cyclic AMP response component binding site of the promoter of microtubule-associated protein 1 light chain 3β (LC3β), a vital component of autophagosomal membranes, which alleviates the induction of autophagy. In addition, DDIT3 can activate the formation of autophagosome through downregulation of Bcl-2 expression [106].

CHOP is another potent transcription factor, which is involved in the induction of autophagy [107, 108]. It has been elucidated that the expression levels of ATG5 and BH3 domain proteins are elevated by upregulation of the CHOP expression. Besides, the Bcl-2 expression level is downregulated, which assists in the release of Beclin-1 from Bcl-2. Moreover, the PERK-CHOP pathway instigates tribbles-related protein 3 (TRIB3), which inhibits the activation of the protein kinase B (Akt) [103, 109]. TRIB3-mediated inhibition of Akt regulates the phosphorylation of TSC2 (tuberous sclerosis complex 2) by the serine/threonine kinase, Ras homolog enriched in brain (Rheb), and the inhibition of mTORC1, which dephosphorylates ATG13 and the ULK1/2 complex and results in the induction of autophagosome formation [110].

The ATF6α branch of the UPR is the least understood branch in relation to ER stress and autophagy. Nonetheless, the ATF6α transcription regulator is involved in the initiation of autophagy by the elevated expression of HSPA5 (heat shock70kDa protein 5) (**Figure 2**) and followed by the inhibition of expression and activation of protein kinase B of AKT1/AKT [111]. In addition, the ATF6α interacts with CEBPB (CCAAT/enhancer binding protein) to form a transcriptional heterodimer complex and binds to the CRE/ATF components of DAPK1 (death-associated protein kinase 1) to induce DAPK1 expression. ATF6α knockdown with specific shRNA and ATF6α$^{-/-}$ cells leads to reduced expression of DAPK1, followed by the inhibition of formation of autophagosomes [112]. Beclin-1 phosphorylation leads to decreased Bcl-2 expression and initiates the formation of a complex between the autophagosome initiator Beclin-1 and PIK3C3. Simultaneously, the ATF6α-mediated upregulation of CHOP, XBP1, and GRP78 expression is also initiated, resulting in the induction of autophagy [113].

8. ER stress induces autophagy via the PI3K/AKT/mTORC pathway

The serine/threonine kinase of mTORC is the main regulator of ER stress [114]. It forms two complexes, the mTORC1 and mTORC2, both of which are triggered by extracellular and intracellular stimuli, under favorable conditions for growth [114, 115]. Accordingly, mTORC1 is a critical regulator of the UPR-mediated autophagy and nutrient signaling [116]. mTORC1 is involved in the regulation of the major signaling pathway. Interaction of growth factors with insulin triggers the PI3K complex, which accelerates the plasma membrane adaptation of the lipid phosphatidylinositol-3-phosphate (PtdIns(3)P) to generate PtdIns(3,4,5)P2 and PtdIns(3,4,5)P3. These increase the membrane recruitment of pleckstrin homology domain proteins such as the serine/threonine kinase PDK1 (phosphoinositide-dependent kinase 1) and its substrate Akt protein kinase B to activate Akt in the plasma membrane [117]. The PI3K is elicited as a vesicular protein trafficking mediator, which binds to PtdIns(3)P, resulting in its translocation to intracellular membranes such as endosomal and lysosomal membranes. PI3K is a member of Vps34 family, which plays an important role in the formation of autophagosomes,

by directly interacting with Beclin-1 [118]. Similarly, PtdIns(3)P and PtdIns(3,4,5)P3 initiate autophagy by phosphorylation of the phosphatidylinositol to activate PtdIns(3,4,5)P3 and contributes to the autophagic vacuole sequestration [119].

Akt is a serine/threonine kinase, which is an upstream regulator of mTORC. Several hormone growth factors and the phosphorylation of the oncogene PI3K-Akt-mTORC can stimulate mTORC and the ribosomal protein S6 kinase (RPS6KB1) and inhibit the expression and phosphorylation of TSC1 (tuberous sclerosis 1) and TSC2, which under ER stress conditions inhibits mTORC [90]. Similarly, the inhibition of TSC triggers mTORC activity, which suppresses the initiation of ER stress-mediated autophagy. Furthermore, the knockdown of TSC1/2 can regulate the activation of mTORC, which is elevated under ER stress conditions. This indicates that TSC is essential for the canonical ER stress feedback [120, 121]. Thus, TSC1/2 is a crucial coordinator of several signals, including mTORC and the well-known PI3K-Akt pathway, for the induction of autophagy.

The opposite branch of this pathway is downregulated by mTORC release, and ULK1 initiates the autophagosome formation [122]. Accordingly, ER stress can inhibit the expression of Akt and suppress the mTORC regulation, which can induce autophagy. ATF6α increases the expression of ER chaperone HSPA5 (heat shock 70 kDa protein 5), which can block the phosphorylation of Akt activity, in turn activating the induction of autophagy in placental choriocarcinoma cell [90].

TRIB3 (tribbles homolog 3) is an ER stress-associated protein, which can interact with Akt and downregulate the expression of Akt-mTORC [123, 124]. The defective ATF4-DDIT3 complex in malignant gliomas can activate TRIB3 under ER stress condition, which indicates that TRIB3 activation is ATF4-DDIT3 dependent. Δ9-Tetrahydrocannabinol (THC), the main active compound of marijuana, triggers the TRIB3-dependent autophagy pathway of ER stress, by the suppression of the Akt/mTORC1 pathway. The overactivation of TRIB3 can reduce the transcriptional activity of ATF4 and DDIT3. This indicates that the ER stress-mediated induction of autophagy via the PI3K/AKT/mTOR pathway plays a key role in cell survival [123].

9. ER stress induces autophagy via the AMPK/TSC/mTORC1 pathway

The AMP-activated kinase (AMPK) is a key cellular energy sensor that regulates the transcription of the autophagy genes through the regulation of many downstream kinases [125]. AMPK is a cellular energy sensor that detects increased level of intracellular ATP/AMP concentration ratio [126]. Under several metabolic stress conditions, AMPK is phosphorylated by a serine/threonine kinase and activates genes including liver kinase B1 (LKB1, which is activated upon energy depletion), calcium/calmodulin kinase (CaMKKβ, which is activated by cytosolic Ca^{2+}), and TGFβ-activated kinase-1 (TAK-1, which is involved in IKK activation) [126]. AMPK induces autophagy through the inactivation of mTORC1 via the phosphorylation of the tuberous sclerosis complex 2 (TSC2) and the regulation of the associated protein RAPTOR, after the dissociation and activation of ULK1 [127]. In addition, AMPK-induced autophagy not only inhibits mTORC1 but also directly phosphorylates ULK1 and Beclin-1. AMPK has a major role in preventing the ER stress-induced autophagy-mediated cytotoxicity. In addition, albumin-treated cellular toxicity leads to the activation of AMPK. Similarly, silenced RPS6KA3 (ribosomal S6 kinase 90 kDa polypeptide 3) decreased expression of AMPK induce autophagy which aggregates ER stress mammalian breast cancer model [128, 129]. Involvement of PERK-AMPK mediated and inactivation mTORC initiate autophagy has also demonstrated detachment of extracellular matrix in human epithelial cell. Moreover, AMPK inhibits synthesis protein by inactivation of mTORC and

phosphorylating EIF4EBP1/4E-BP1 and RPS6KB/p70S6K [130]. Moreover, the phosphorylation of eIF2α [101] and the activation of IKK [131] are indispensable for induction of autophagy by starvation.

10. Ca^{2+} in ER stress regulates autophagy

The ER plays a major role in maintaining the intracellular Ca^{2+} store that can compile Ca^{2+} concentrations of 10–100 mM, while in the cytoplasm and remaining cell concentration, the range is 100–300 nm [132]. The multifunctional organelle ER maintains Ca^{2+} homeostasis, which is necessary for proper functioning including protein folding, lipid and protein biosynthesis, and posttranslational modification and regulation of gene expression [133]. The majority of ER-associated proteins participate in maintaining ER Ca^{2+} homeostasis. For maintaining ER Ca^{2+} homeostasis, most of the ER-associated proteins, such as calreticulin, GRP94 or GRP78, histidine-rich Ca^{2+}-binding protein, and protein disulfide isomerase (PDI), uphold to Ca^{2+} buffer in the lumen of ER [134]. Ca^{2+}-binding protein mainly GRP78 is involved in sensing unfolded protein accumulation in the ER and interacts with three other UPRs of ER transmembrane proteins, ATF6α, IRE1α, and PERK [135]. As noted, loss of Ca^{2+} homeostasis in the ER followed to initiate ER stress [136]. In addition, ER lumenal Ca^{2+} can reduce because of ER stress. Upon incitement of plasma membrane ER influx and discharge formation of Ca^{2+} signal, whereas ER reservoir influx and release depend on replenishment of Ca^{2+}. Activity of Ca^{2+} across the membrane of ER is expedited by three kinds of protein receptor: Ca^{2+} release channels—RYR (ryanodine receptor) and ITPR/IP3R (inositol 1, 4, 5-trisphosphate receptor); in the ER, cytosolic Ca^{2+} enters through a Ca^{2+} pump called ATP2A/SERCA (sarco/endoplasmic reticulum Ca^{2+}) [137].

There is multitudinous Ca^{2+} movement through the membrane of ER that assures appropriate functioning of numerous kinases and proteases. It is already well established that cytosolic Ca^{2+} signal regulates protein intricate in several stages of autophagosome formation [138]. In addition, a number of Ca^{2+} dependent pathways involved in autophagy induction have been studied. Indeed, cytosolic Ca^{2+} initiation of autophagy it is ambiguous in many conditions. The numerous Ca^{2+} origin has already involved merely various downstream effectors containing protein kinase C, Ca^{2+}/calmodulin-dependent kinase β (CaMKKβ or CaMKK2), ERK, and Vps34 (a calmodulin protein) [139, 140]. It is already proven that CaMKKβ or CaMKK2 has perceived the majority experimental support, whereas Ca^{2+} refinement of Vps34 and ERK is unsupportable. Activation of Vps34 by Ca^{2+} or calmodulin is insinuated although the activity of Vps34 in cellulo was not affected by cytosolic Ca^{2+} or calmodulin antagonist [139]. CaMKKβ is an inrease the activity of AMPK, thereby inhibition of mTORC1 leads to activate autophagy [141]. Høyer-Hansen et al. demonstrated that in MCF-7 breast cancer cells the mobilize of cytosolic Ca^{2+} from ER by stimulate IP3R generating agonist, such as thasigargin, ionomycin and vitamin D analogue activate CaMKKβ which is initiate autophagy by downregulating of mTORC1 and activation AMPK dependent pathway [142]. In addition, deficient autophagy in T lymphocyte has an extension of ER compartment due to more Ca^{2+} in the ER. Depletion of Ca^{2+} in the ER leads to extension of Ca^{2+} reservoir, which could be the purpose behind unfit to store diminished. This invasion of Ca^{2+} can be recovered by SERCA/ATPase pump blocking with thapsigargin, which means autophagy can maintain Ca^{2+} mobilization across the ER [143]. In total, the connection between autophagy and Ca^{2+} mobilization intimates that they can have impact on each other. Moreover, the elevation of cytosolic Ca^{2+} endogenously induction of autophagy by precipitation of Ca^{2+} phosphate without modifying the condition of ER. In consequence, ER Ca^{2+} plays a key role for induced

autophagy by the UPR, while other sources of Ca^{2+} can induce autophagy but not interaction with the UPR [144, 145].

IP3R receptor is another important cellular pathways which is involved in regulating Ca^{2+} and induced autophagy. This pathway is mTORC-dependent autophagy and ER stress through upon activation of UPR [146]. IP3R is a second messenger which is known for regulating cell survival signaling although its negative role initiating autophagy is also emerging from several experimental studies that suggest the pharmacological and genetic inhibition of IP3R induction of autophagy-independent Ca^{2+} flux [147]. The role of ER Ca^{2+} depletion (SERCA/ATPase antagonist thapsigargin) and luminal ER Ca^{2+}-stimulating compound IP3R antagonist xetospongin B, both of contradictory role, can activate autophagy. Inversely, inhibition of IP3Rs can activate autophagy signal that might be mechanically different from ER stress-attenuated autophagy. Apart from IP3Rs, RYRs have also induced autophagy. In hippocampal neuronal stem cells treated of insulin lead to increase expression of RYR3 isoform which instigate cell death through elevate induction of autophagy [148]. Accordingly, endogenous expression of RYRs in skeletal muscle cells and HEK cells segregates rat hippocampal neurons inhibit the autophagy flux particularly at the autophagosome-lysosome fusion. Inhibition of RYRs increased autophagy flux by mTORC independent pathway [149].

Under ER stress condition, Ca^{2+}-mediated autophagy is induced by known tumor inhibitor DAPK1. Activated DAPK1 mediated direct phosphorylation on BH3 domain of Beclin-1 elevated from Bcl2L1, which promotes autophagy [113]. Accordingly, under hypoxic condition, decrease synthesis of protein through PERK-eIF2α-ATF4 and AMPK-mTORC1 pathway. Similarly, autophagy can be induced upon hypoxic condition, whereas Ca^{2+} influx by initiation of hypoxia and triggered CaMKKβ or CaMKK2 promotes WIPI1 and autophagosome formation [150, 151].

Many evidences suggest that cytosolic Ca^{2+} can initiate autophagy although many reports demonstrate that chelating Ca^{2+} suppresses autophagy. BAPTA-AM (1,2-bis (O-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid tetra (acetoxymethyl) ester), a cell permeable Ca^{2+} buffering agent, can also suppress autophagy initiation following ER stress induced by inhibition of proteasome [152]. In many studies, stimulation of exogenous cytosolic Ca^{2+} signal and the BAPTA-AM effect on autophagy can be rational inhibition activate the influx of Ca^{2+}. In addition, BAPTA-AM effect on cell did not alter the production of IP3Rs by Vps34 but mutated the aggregation of the IP3Rs protein receptor WIPI-1 to the formation of phagophore. Likewise, BAPTA-AM was observed to suppress lysosome fusion [153]. Furthermore, BAPTA-AM inhibits initiating autophagy by experimentally increasing influx Ca^{2+} signal but blocks formation of autophagosome. In the meantime, autophagy inhibition by BAPTA-AM continuously remarks that there are some consequences using Ca^{2+} chelating agents which also defect lysosomal function followed by inhibiting degradation of autophagosome [154]. In addition, hydrolysis of the acetoxymethyl ester modification of Ca^{2+}-dependent intracellular signaling process directly involved autophagy [154]. Nevertheless, BAPTA is Ca^{2+} chelator and limitaion is when Ca^{2+} enters the cell and it can be replete by the influx of Ca^{2+}. In a similar way, mobilization of intracellular Ca^{2+} led to defects in plasma membrane, resulting in the expanded interplay between lysosome and SNAREs, which are more important for membrane fusion, and thereby increase of Ca^{2+} could alleviate autophagosome-lysosome fusion, which induces autophagy [155].

Alternatively, many compounds that inhibit Ca^{2+} signaling led to an ascent of cytosolic Ca^{2+} that blocks initiation of autophagy. Particularly, voltage-operated Ca^{2+} channel antagonist and the IP3R signal can induce autophagy by suppressing activity of Ca^{2+}-sensitive protease called as calpain [156]. Calpain is activated by elevation of cytosolic Ca^{2+}. Inhibition of calpain by pharmacological calpestatin

and calpeptin or knockdown of calpain enhances autophagy flux without turbulence mTORC1 [156]. In addition, in neuronal disease cells, abnormal Ca^{2+} signal obstructs the clearance accumulation of nascent protein through inhibition of autophagy induction. Nonetheless, these studies demonstrate that calpain can suppress autophagy induction although other experimental studies suggest that the activation of calpain is essential for autophagy induction [156]. Cytosolic Ca^{2+} can activate mTORC1, which led to inhibition of autophagy induction. For instance, knockdown of TRPML1 (transient receptor potential cation channel, mucolipin subfamily, member 1) lysosomal Ca^{2+} channel inhibits mTORC1 activity. However, knockdown of TRPML1 channel reversed by thapsigargin, lead to downstream cytosolic Ca^{2+} signal activated by mTORC1 [157].

11. ER stress mediates autophagy in pathological condition

The UPR pathway is not always a reason for autophagy induction. When ER stress is divergent in some contagious situation, defective regulation of autophagy occurs. Notably, in some pathological conditions such as neurodegenerative, cardiovascular, and liver diseases, ER stress negatively regulates autophagy. Alzheimer disease (AD) is one of the most common neurodegenerative diseases, which is mainly caused by the accumulation of extracellular amyloid-β (Aβ), senile plaques, and neurofibrillary tangles protein. Aβ is originating from the cleavage of the amyloid precursor protein (APP) by two aspartic enzymes β-secretase (BACE1) and γ-secretase. This γ-secretase is a membrane-associated complex consisting of a presenilin-1/2 (PS1/PS2) in the ER [158]. UPR and autophagy play a key role in maintaining normal neuron against aggregation of Aβ and PS1 mutation that affect the form of AD. Many reports suggest that mutation in PS1 and accumulation of intracellular Aβ activate ER stress in neurons [159]. However, mutation of Aβ leads to upregulation of the HSPA5 (heat shock 70 kDa protein 5) expression in the neuron, which is the main neuroprotective role despite the ER stress-associated cell death and sustaining Ca^{2+} stability [160]. Interestingly, mutation of ps1 and Aβ suppresses the main arms of UPR, including IRE1α, PERK, and ATF6α [161]. Activation of ER stress is an early sequence of the AD, which initiates autophagy by phosphorylation of PERK-positive neuron via accumulation of MAP1LC3B induced autophagy in cardinal direction for abasement of Aβ and APP [162]. Defective regulation of autophagic function leads to AD progression; Pickford et al. report that downregulation of Beclin-1 was observed in the middle frontal lobe in the brain cortex of AD patients similar to the observation in the mouse model of AD [163]. Similarly, in Parkinson disease model, synaptic protein α-synuclein (α-syn) decreases accumulation of the expression of Beclin-1 gene that suppresses the induction of autophagy [164]. In addition, Huntington's diseases (HD) is also neuropathological disease condition, whereas ER stress impaired the regulation of autophagy. Knockdown of IRE1α-XBP1 increases autophagy in HD model which initiates pathological condition [165, 166]. Similarly, in HD-upregulated expression, USP14 is the deubiquitinating enzyme with His and Cys domains that increase autophagic discharge of mutant HTT protein (huntingtin protein) through non-phosphorylated IRE1α. Phosphorylated IRE1α has not much affinity to interact with USP14, thus increasing accumulation of mutant HTT by suppressing autophagy regulation [167]. Therefore, activation of UPR will not be regulated properly as a result of negative induction of autophagy, which fails to eradicate the accumulation of contagious protein and then consequently leads to neurodegenerative diseases.

UPR and autophagy are also interconnected for inflammation of bowel in the epithelial cell. In cultured intestinal epithelial cell initiate PERK-eIF2α dependent

pathway autophagy because of loss IRE1α activity which intimate that UPR signal maintaining normal mechanism also conserve balance need to possible rebuttal mechanism [168]. In addition, XBP1 conditional knock in intestinal epithelial cell lead to induced autophagy in small intestinal paneth cell, essential for the formation of antimicrobial agents followed by inflammation in small intestine, which is more exacerbated when codeletion of ATG gene like ATG7 or ATG16L1. Double knockout mice XBP1$^{-/-}$, ATG7$^{-/-}$ and XBP1$^{-/-}$, ATG16L$^{-/-}$ demonstrate that Crohn diseases stimulate nuclear factor kappa B (NF-kB) in IRE1α-dependent manner. Moreover, In ATG16L conditional knockout mice enhance GRP78 expression along with phosphorylation of eIF2a and activation of JNK, terminating the expression of IRE1a and increased the XBP1 spicing in intestinal glands, these circumstances increase the inflammation state, which changes the interaction between ER stress and autophagy that increases cell death, which is negative retroaction of ER stress-induced autophagy [168]. Notably, inactivation of XBP1 can induce autophagy but this UPR also can downregulate the induction of autophagy. Nevertheless, defective regulation of XBP1 integrates FoxO1 (Forkhead box O1), a transcription factor that sequentially provokes expression of many genes that positively induce autophagy [98]. The unspliced XBP1 (uXBP1) under glutamine starvation condition regulated FoxO1 depravation by interacting FoxO1 for the 20s proteasome. Similarly, this interaction between uXBP1 and FoxO1 based on phosphorylation of uXBP1 by the extracellular signal-regulated kinase 1/2 (ERK1/2), as well as spliced XBP1 (XBP1s) in overexpression which also interacted, evolved degradation of FoxO1 [169]. Accordingly, recently, the FoxO1 and XBP1 interaction in auditory cells regulates autophagy [170]. Prominently, the consistent mechanism has been proved under severe ER stress in which the UPR loses its activity, whereas it can be considered that another regulatory mechanism FoxO1 maintains the autophagy induction.

12. Conclusion

During the last decade, research has been conducted to determine the mechanism by which ER stress and autophagy maintain intracellular homeostasis. Here, we described the UPR and autophagy in detail with respect to their molecular mechanism and interaction between ER stress and autophagy. However, the detailed mechanism of ER stress and autophagy is yet to be fully understood. In the last few years, research has shown that the ER stress response can not only initiate autophagy but can also negatively regulate autophagy to maintain cell survival. Elucidation of the interactions between the UPR and autophagy will help in the development of novel treatments for several diseases.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Author details

Mohammad Fazlul Kabir[1], Hyung-Ryong Kim[2] and Han-Jung Chae[1*]

1 Department of Pharmacology, School of Medicine, Institute of New Drug Development, Chonbuk National University, Jeonju, South Korea

2 Graduate School, Daegu Gyeongbuk Institute of Science and Technology, Daegu, South Korea

*Address all correspondence to: hjchae@jbnu.ac.kr

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Chapter 5

Endoplasmic Reticulum Stress during Mammalian Follicular Atresia

Nayeli Torres-Ramírez, Rosario Ortiz-Hernández, M. Luisa Escobar-Sánchez, Olga M. Echeverría-Martínez and Gerardo H. Vázquez-Nin

Abstract

Follicles are ovarian structures that contain a single germ cell. During the mammalian reproductive lifetime, ovarian follicles mature through the process of follicular development, with the aim of selecting oocytes for ovulation. As part of this process, several follicles are eliminated by means of follicular atresia, a mechanism that mainly involves apoptosis. Nevertheless, it has been shown that there are other routes of programmed cell death in the ovary including autophagy, paraptosis, and necroptosis. Surprisingly, the endoplasmic reticulum is involved in these different programmed cell death pathways. Moreover, there are several evidences for the pathways triggered by intra- and extracellular signals in endoplasmic reticulum-induced cell death. Thus, it is important to analyze the participation of endoplasmic reticulum in follicular atresia.

Keywords: ovary, follicular atresia, endoplasmic reticulum, apoptosis, autophagy, paraptosis, necroptosis

1. Introduction

The endoplasmic reticulum plays several important roles in normal cellular physiology. Some functions include protein synthesis, folding, and distribution to the Golgi apparatus. Alterations in protein synthesis inside the endoplasmic reticulum have been related to the trigger of different programmed cell death routes such as necroptosis, apoptosis, autophagy, and paraptosis, with apoptosis being the most studied process.

The mammalian ovary is an excellent model to study the mechanisms of programmed cell death because 99% of the follicles, the functional units of the ovary, undergo degeneration through follicular atresia, which maintains intraovarian homeostasis. Follicular atresia involves the physiological elimination of most germinal cells (oocytes) before they are ovulated, both in fetal and reproductive lives.

The presence of different programmed cell death pathways in follicular atresia have recently been shown, and these can be directly related to endoplasmic reticulum signaling. In this chapter we describe evidences of the linkage between endoplasmic reticulum alterations and programmed cell death, with special emphasis on follicular atresia.

2. Follicular development and atresia

The mammalian ovary is a paired organ that is responsible for generating competent oocytes for successful fertilization and early embryonic development. To do this, these germinal cells need to mature within transient functional complexes called follicles. Follicles form for an oocyte surrounded by somatic cells. During reproductive life, follicles are continuously recruited into the pool of growing follicles and change their size, morphology, and physiology, leading to different stage classifications including primordial, primary, secondary, and antral (**Figure 1**).

At birth, the ovaries contain a fixed number of nongrowing primordial follicles, characterized by an oocyte enclosed by flattened pre-granulosa cells. In primary follicles, the oocyte is surrounded by a monolayer of cubical granulosa cells. Secondary follicles are formed by two or more layers of granulosa cells. Antral follicles accumulate fluid and develop an antral cavity. The accumulation of fluid is useful for transporting nutrients and waste products.

Follicular growth is a continuous process that is under strict control by hormones, growth factors, cytokines, and environmental factors. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), insulin-like growth factor (IGF)-I, and estradiol are the principal regulators of follicular growth. FSH, a gonadotropin secreted by the pituitary gland, together with estradiol and IGF-I, is responsible for stimulating follicular growth and maturation. Moreover, FSH, LH, and estradiol enhance IGF-I secretion [1]. Additionally, FSH stimulates granulosa cells to develop LH receptor sites. The main function of LH is stimulating ovulation.

Several follicles grow and undergo ovulation, releasing an oocyte that is available for fertilization, but the principal destiny of ovarian follicles is follicular atresia, which is a physiological process that eliminates more than 99% of the follicles. Follicular atresia can occur in all stages of follicular development and ensures that only healthy follicles that contain optimal quality oocytes will be ovulated. Follicular degeneration occurs by programmed cell death (PCD). Apoptosis is the main route of follicular atresia, but may not be the only process involved (**Figure 2**). Other forms of PCD such as autophagy and paraptosis may also participate in this process [2–4].

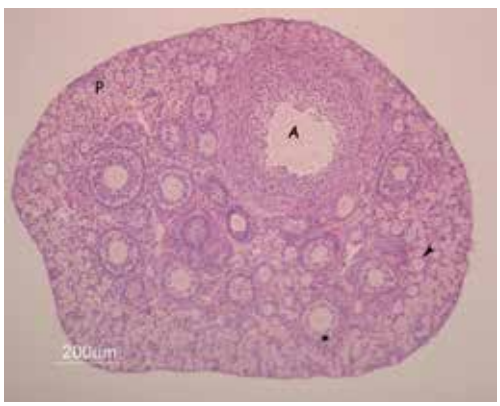

Figure 1.
Ovary of mouse. Follicles are in different stages of growth. Primordial (P), primary (head arrow), secondary (asterisk), and antral (A) follicles.

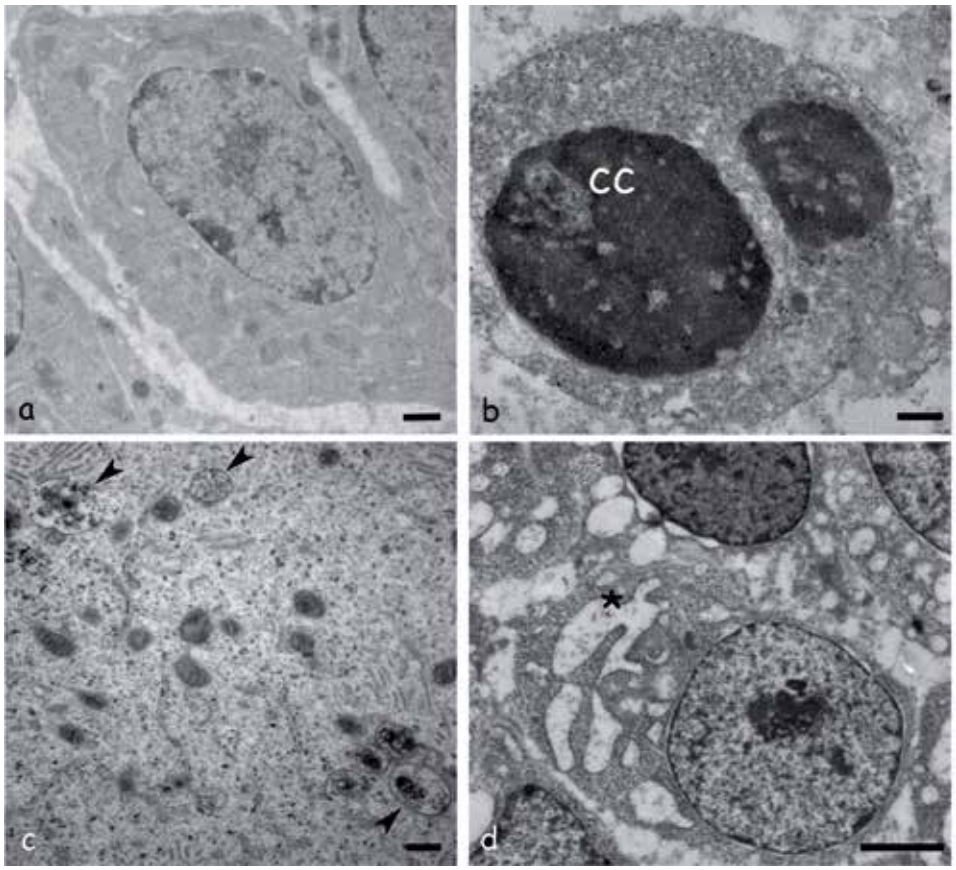

Figure 2.
Transmission electron microscope images of granulosa cells in different programmed cell death pathways. (a) healthy granulosa cell, (b) apoptotic body with highly condensed chromatin (cc), (c) autophagic cell with autophagic vesicles (head arrow), and (d) paraptotic granulosa cell with endoplasmic reticulum swelling (asterisk). Bars (a–c) 500 nm, and (d) 2 μm.

3. The endoplasmic reticulum and cell death

The endoplasmic reticulum (ER) is the organelle that is responsible for the folding and maturation of both transmembrane proteins and proteins that follow the route of secretion. Protein folding is facilitated by chaperones and oxidoreductases including binding immunoglobulin protein/glucose-regulated protein 78-kDa (BiP/GRP78), calnexin, calreticulin, and protein disulfide isomerase (PDI). An increase of cellular translational activity is possible under both normal and altered conditions, causing an overload of accumulating misfolding or unfolded proteins inside the ER. During ER stress, damaged proteins need to be degraded, but there is a limited number of proteases in the ER, and thus misfolded proteins are ejected from the ER and returned to the cytoplasm to be ubiquitinated and degraded by the 26S proteasome. These events are collectively referred to as ER-associated degradation (ERAD) [5]. Also, ER stress triggers the unfolded protein response (UPR), which is orchestrated by three ER-resident UPR sensors, inositol-requiring kinase 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) [6, 7].

The UPR establishes an adaptive program aimed at re-establishing ER homeostasis by increasing the folding capacity of the cell, reducing protein synthesis, and enhancing the clearance of abnormally folded proteins and damaged organelles.

The proteins PERK and IRE1α and β are important players during UPR because they undergo oligomerization and autophosphorylation due to their interactions with peptides and unfolded proteins [8, 9]. Additionally, IRE1 promotes the unconventional splicing of X-box binding protein 1 (XBP-1) mRNA and an unspecific decrease of mRNAs better known as regulated IRE1-dependent decay (RIDD) [10, 11]. Afterward, the protein XBP-1 is translocated to the nucleus to activate the transcription of chaperones and ERAD factors [12]. RIDD suppresses protein inflow by degrading the mRNA of proteins with signal peptides or proteins with transmembrane domains, and in this manner RIDD permits proteins that are incorrectly folded inside the ER to be folded correctly [10]. PERK phosphorylates eukaryotic translation initiation factor (eIF2a), which then accumulates on the cytosolic side and leads to the downregulation of translation and enhances the translation of Grp78 and the transcription factor ATF4 [13, 14]. It has been shown that during early mouse embryonic development, Grp78 suppresses ER stress and pro-apoptotic pathways via ER signaling [15]. ATF6 is regulated by proteolysis in the Golgi apparatus, allowing the N-terminal fragments to be translocated into the nucleus where they function as a transcription factor [16, 17]. The processing of both ATF6- and IRE1α-mediated splicing of XBP1 mRNA is required for the full activation of the UPR [18].

UPR works like a protection mechanism. For example, in pancreatic beta cell line INS-1E, glucosamine and high glucose induce UPR activation and generate a feedback loop at the level of insulin transcription [19]. However, chronic or irreversible UPR can trigger cell death pathways, mainly apoptosis, but ER stress can induce other programmed cell death mechanisms including autophagy, necroptosis, and paraptosis.

3.1 The ER and follicular atresia

Morphological ER disturbances during follicular atresia have been observed for a long time. Henderson et al. [20] observed a higher surface area of endoplasmic reticulum in granulosa cells cultured from atretic follicles. Moreover, researchers have used electron microscopy to observe the dilation and disintegration of RER cisterns and the swelling of mitochondria [21].

These morphological disturbances in ovaries are associated with ER stress and UPR activation under both physiological and pathological conditions [22]. UPR is present during follicular growth and maturation and follicular atresia and in the corpus luteum. ER stress during follicular growth and maturation has been evidenced by means of the expression of XPB1 and heat shock 70 kDa protein 5 (HSPA5) accompanied by the activation of IRE1 and PERK [23]. The ER stress level and cellular response depend on the signal and its intensity. It has been shown that a lipid-rich intrafollicular environment induces ER stress and impaired oocyte nuclear maturation [24]. Likewise, in the ovary a moderate activation of ER stress depends upon PERK and p38 signaling [25], evidencing a UPR response in the cells of this organ.

4. Apoptosis

Apoptosis, the term proposed by Kerr et al. [26], describes an intrinsic suicide mechanism that involves cell shrinkage and the loss of cell contacts, chromatin condensation, and cleavage [27]. This process is better known as programmed cell death type 1 (PCD type 1). The biochemical activation of apoptosis can be directed through extrinsic and intrinsic pathways. The extrinsic pathway is initiated by the activation of cell surface death receptors to their ligands, like the Fas Ligand and TNF. After binding, apoptotic signals are transmitted through dead effector domains

and caspase recruitment domains. The intrinsic pathway is governed by a variety of cellular stresses including DNA damage, endoplasmic reticulum stress, and nutrient deprivation, which culminates in mitochondrial outer membrane permeabilization (MOMP), resulting in the release of mitochondrial proteins including cytochrome c and Smac/DIABLO. Apoptosis pathways converge on a common machinery of cell destruction that is activated by caspases, a family of cysteine proteases that cleave after an aspartate residue [28, 29]. The caspases implicated in apoptosis are divided into initiators and executioners, where initiator caspases (caspase-8 and caspase-9) activate the executor caspases (caspase-3, caspase-6, and caspase-7).

The Bcl-2 family, which are central regulators of MOMP, are a large class of both pro- and anti-apoptotic proteins. The Bcl-2 family is divided into three subfamilies: multidomain anti-apoptotic such as BCL-2, BCL-XL (BCL2L1), MCL-1, BCL-W (BCL2L2), and A1 (BCL2A1), multidomain pro-apoptotic such as BAX and BAK, and pro-apoptotic BH3-only molecules that include BID, BIM, PUMA (p53 upregulated modulator of apoptosis), and NOXA [30]. BH3-only proteins antagonize anti-apoptotic BCL-2 proteins to release and activate Bak/Bax [31]. Bax and Bak induce external membrane mitochondrial permeabilization and cytochrome c release [32]. Nevertheless, some death stimuli can trigger caspase-independent cell death pathways where other organelles such as the endoplasmic reticulum and the mitochondria have an important function in the release and activation of death factors [33].

In atretic follicles, this PCD was thoroughly described by Tilly et al. [34] and can be conducted through the intrinsic or the extrinsic pathway [35]. In ovaries, apoptosis can be triggered by deprivation of various signal molecules, survival factors, growth factors (IGF and EGF), and gonadotropins (FSH and LH). Apoptosis can occur in both oocytes and somatic cells. Cell elimination has been observed in follicles in different stages of development, from fetal to adult organisms [3, 36–38]. Although different routes of PCD can occur during follicular atresia, apoptosis plays a major role (**Figure 2b**).

4.1 The role of the ER in apoptosis

Apoptosis is triggered by chronic or irreversible ER stress and UPR and occurs through either the extrinsic or intrinsic pathway. Further, apoptosis can be carried out by two pathways, a classical Bax-/Bak-dependent apoptotic response that can be inhibited by ERK1/2 signaling and an alternative ERK1-/2- and Bax-/Bak-independent pathway [39]. No single component is entirely necessary, but the interaction of many different mechanisms results in apoptosis during ER stress [40]. Under ER stress Bax and Bak interact with the cytosolic region of IRE1α, which is required for the modulation of IRE1α signaling [41].

The activity of the BH3-only protein Bim is induced through different pathways. The first one involves protein phosphatase 2A-mediated dephosphorylation, which prevents its ubiquitination and the proteasomal degradation of Bim. A second pathway is direct transcriptional induction that is C/EBP homologous protein (CHOP)-C/EBPalpha-mediated, and a third comprises a repression of miRNAs led by PERK [42, 43]. On the other hand, PUMA, p53, and NOXA contribute to ER stress-induced apoptosis [44].

It has been reported that CHOP (a transcription factor of pro-apoptotic proteins such as Bim) increases during ER stress [45]. ATF4 and CHOP increase a generalized protein synthesis, provoking ATP depletion, oxidative stress, and cell death [46]. Also, IRE1α degrades the miRNA that represses caspase-2 mRNA translation, which causes an increase in the protein levels of this initiator protease of the mitochondrial apoptotic pathway [47].

4.2 The role of the ER in apoptosis during follicular atresia

ER stress and UPR during follicular atresia are not fully understood; however, there are several evidences of these processes in the ovary. For example, cisplatin, a widely used chemotherapeutic agent, can induce ER stress, which promotes apoptosis and autophagy in granulosa cells, causing excessive follicle loss and endocrine disorders [48].

In goat ovaries, ER stress is involved in follicular atresia through ATF6 and PERK/eIF2α/ATF4 signaling. Furthermore CHOP, caspase-12, and Grp78 proteins are upregulated in apoptotic granulosa cells during follicular atresia [49, 50]. ATF6 is a protein that is extensively distributed in the granulosa cells of ovarian follicles and oocytes in adult mice, and the amount of ATF6 increases in the presence of FSH and LH. ATF6 regulates apoptosis, the cell cycle, steroid hormone synthesis, and other modulators of folliculogenesis in granulosa cells, which may impact the development, ovulation, and atresia of ovarian follicles [51].

The presence of apoptosis-inducing factor (AIF) has been identified in granulosa cells. This protein mediates caspase-independent apoptosis and causes chromatin condensation and DNA fragmentation. AIF expression increases during follicular atresia, and AIF depletion protects ER stress-mediated goat granulosa cell apoptosis [52].

Reactive oxygen species (ROS) generation and oxidative stress can be upstream or downstream UPR targets. That is, UPR is interconnected with different enzymatic mechanisms of ROS generation, and they may depend on Ca^{2+} levels, ROS themselves, and PDI, which associates with NADPH oxidase and regulates its function [53]. ROS are pro-apoptotic factors in antral follicles. During oxidative stress, JNK activates FoxO1, which increases PUMA and induces apoptosis in granulosa cells [54]. Furthermore, pentosidine, a biomarker for advanced glycation end products, is accumulated in apoptotic human oocytes and increases with age [55].

UPR and ER stresses also have important roles in the regulation of corpus luteum (CL) regression. The overexpression of p-JNK, CHOP, caspase-12, and active caspase-3 during CL regression points to ER stress-dependent apoptosis [56, 57].

5. Autophagy

Autophagy is a catabolic pathway of cell constituents that contributes to cell survival in response to stress. Autophagy does not cause a loss of cell chemical components because the cell reutilizes them. There are three major types of autophagy, microautophagy, chaperon-mediated autophagy, and macroautophagy.

In microautophagy, vesicles bud into the lysosomal lumen by direct invagination of the boundary membrane, resulting in degradation of both cytoplasmic components and the lysosomal membrane by lysosomal hydrolases. This process involves sequential stages of vacuole invagination and vesicle scission [58].

Chaperon-mediated autophagy is the selective transport of proteins into lysosomes. The first step is protein recognition and lysosomal targeting. Protein recognition takes place in the cytosol through the binding of hsc70 to a KFERQ-like motif present in all chaperon-mediated autophagy substrates [59]. In the second step, proteins bind to receptors at the lysosomal membrane, Lamp2A, or a similar protein receptor for subsequent translocation and lysosomal degradation [60]. Receptors are subcompartmentalized in lipid microdomains to engage the processes of degradation, multimerization, and membrane retrieval [61].

Macroautophagy, also referred to as autophagy, involves the engulfment of cytoplasmic portions in a nonselective manner, as well as the degradation of specific proteins, organelles, and invading bacteria by a selective autophagy. Autophagy begins with the formation of an isolation membrane, the phagophore, which is a disk-like structure where the Atg machinery assembles. An isolation membrane grows to generate a double-membrane autophagosome, followed by elongation to form a mature autophagosome that captures cytosolic cargo. The fusion of mature autophagosomes with endosomes or lysosomes results in a single-membrane autolysosome where cargo is degraded by acid hydrolases [62].

Autophagy (Atg)-related proteins are the core machinery for autophagosome biogenesis and consist of several functional units: the ULK1-Atg13-FIP200-Atg101 protein kinase complex; the PI3K class III complex containing the core proteins VPS34, VPS15, and beclin 1; the PI3P-binding WIPI/Atg18-Atg2 complex; Atg9A; and the ubiquitin-like Atg5/Atg12 and Atg8/LC3 conjugation systems [63].

Autophagosome maturation involves the clearance of PI3P by Ymr1, a PI3P phosphatase, triggering the dissociation of the Atg machinery. Mature autophagosomes are transported to lysosomes through the microtubule cytoskeleton. The FYVE and coiled-coil domain containing 1 (FYCO1) protein binds to LC3, PI3P, and the small GTPase Rab7 and acts as an adaptor between autophagosomes and microtubules [64, 65]. Finally, the autolysosome is generated by autophagosome and lysosome fusion, where sequestered cargos are digested.

5.1 The role of the ER in autophagy

Autophagy and ER stress can be physiological processes in organisms. For example, they regulate endometrial function by modulating the mTOR pathway [66]. Also, autophagy contributes to the recovery of cell homeostasis after ER stress. During ER stress, damaged proteins are degraded by ERAD. However, some misfolded proteins are resistant, so autophagy is a final cell protection strategy deployed against ER-accumulated cytotoxic aggregates that cannot be removed by ERAD [67]. Additionally, ubiquitin is a common signal for both the ubiquitin-proteasome system and autophagy. In the mouse neuroblastoma cell line neuro-2a treated with tunicamycin, an ER stress inductor, the proteins involved in proteasomal degradation were downregulated, while proteins involved in ubiquitination were upregulated. Moreover, tunicamycin triggered autophagy, suggesting that it may serve as a compensatory effect to proteasomal degradation [68]. Also, ER-resident chaperones and enzymes that reduce the overload of misfolded proteins need to be removed by autophagy.

The structure or phagophore assembly site (PAS) localizes proximal to the ER. Autophagosome formation and transport to the vacuole are stimulated in an Atg protein-dependent manner. ER stress can induce an autophagic response because it increases Atg1 kinase activity and reflects both the nutritional status and autophagic state of the cell [69]. ER exit sites are essential for autophagy and are proximal to the PAS. Sec62, a constituent of the translocon complex that regulates protein import into the mammalian ER, intervenes during recovery from ER stress to selectively deliver ER components to the autolysosomal system for clearance and therefore is a critical molecular component in the maintenance and recovery of ER homeostasis [70].

The eIF2α/ATF4 pathway directs an autophagy gene transcriptional program in response to amino acid starvation or ER stress. The eIF2α kinase and the transcriptional factors ATF4 and CHOP are required to increase the transcription of a set of genes implicated in the formation, elongation, and function of the autophagosome,

including Atgs and beclin 1, increasing the capacity to maintain autophagy in stressed cells. These autophagy genes exhibit different dependencies on ATF4 and CHOP, which means that they have a differential transcriptional response according to the stress intensity [71]. In human heart failure, the overexpression of the ER stress markers Grp78, PERK, CHOP, and ATF3 correlates with the expression of autophagy genes [72].

IRE1, a UPR sensor, has two isoforms, IRE1α and IRE1β, which both have RNase and kinase activities. However, in *Arabidopsis thaliana*, RNase activity of IRE1β, but not its protein kinase activity, is required for ER stress-mediated autophagy [73]. In *Dictyostelium*, the response to ER stress involves the combined activation of an IRE1α-dependent gene expression program and the autophagy pathway [74]. In mammalian cells, the spliced form of XBP 1 upregulates Nedd4-2, an E3 ubiquitin ligase involved in targeting proteins for subsequent degradation, in response to ER stress. It is also important for the induction of an appropriate autophagic response [75].

Different cancer cell models have allowed a better understanding of the mechanisms involved in autophagy triggered by ER stress. In cervical tumor cells, ER stress and UPR induced by X-ray exposition led to the activation of the NF-κB signaling pathway, autophagy, and apoptosis [76]. NF-κB is important for the proliferation, invasion, and metastasis of cervical cancer cells. Furthermore, in a model of breast cancer, autophagy and apoptosis were triggered through ER stress, UPR, and a high expression of CHOP and JNK [77].

Moreover, ERK and JNK activation is associated with cross talk between autophagy and another PCD. In L929 fibrosarcoma cells, ERK and JNK can link a signal from caspase-8 inhibition to autophagy, which in turn induce ROS production and PARP activation, leading to ATP depletion and necroptosis [78].

Ca^{2+} exchange between the ER and mitochondria is mediated through domains called mitochondria-associated membranes (MAMs). The interruption of Ca^{2+} flux between these organelles generates metabolic stress where AMPK present in MAMs triggers autophagy via beclin-1 phosphorylation [79, 80]. Autophagy activation might prevent proper interorganelle communication that would maintain mitochondrial function and cellular homeostasis [79].

In ER stress, some miRNAs promote the survival of the cells, while others promote cell death. In HeLa cells under RE stress, miR-346 positively regulates the expression of glycogen synthase kinase 3 beta (GSK3B) which reduces the interaction of beclin-1 and BCL2 to induce autophagy, ROS reduction, and cell death [81].

5.2 The role of the ER in autophagy during follicular atresia

Autophagy is mainly induced in granulosa cells (**Figure 2c**) during folliculogenesis and shows a high correlation with apoptosis, and furthermore, both routes of PCD could play active roles in oocyte depletion [82]. According to Meng et al. [83], antral follicular degeneration is initiated by granulosa cell apoptosis, while preantral follicular atresia occurs mainly via enhanced granulosa cell autophagy. Surprisingly, apoptosis and autophagy can be present in the same cell at the same time, just as cells can show caspase-3 active, DNA fragmentation, and immunodetection of LC3 and Lamp 1 [2, 3].

The signals that establish autophagy or apoptosis as the route of cell death are not fully understood. Consistent with Zhang et al. [84], atresia initiation is associated with a cross talk of different PCDs including apoptosis and autophagy, a dramatic shift of steroidogenic enzymes, deficient glutathione metabolism, and vascular degeneration. In a rat model, FSH, a survival factor, decreased autophagy through LC3-II inhibition and Akt-mTOR pathway activation [85]. Shen et al. [86]

assessed the mechanism involved in autophagy inhibition by the Akt-mTOR pathway in granulosa cells exposed to FSH and oxidative stress because mTOR, a negative regulator of autophagy, inhibits FOXO1, which promotes the expression of several autophagy genes. They found that FSH induced granulosa cell survival via FOXO1 inhibition by the PI3K-Akt-mTOR pathway [86]. Nevertheless, in mouse granulosa cells, FSH was related to follicle development and atresia because FSH induces autophagy signaling via HIF-1α [87].

Despite the studies on the role of the ER in autophagy, its specific participation in follicular atresia is still unknown.

6. Necroptosis

Necroptosis is a subtype of regulated necrosis and shares the same morphological changes, including organelle swelling and membrane rupture. Necroptosis is a caspase-independent cell death, and its execution involves the active disintegration of mitochondrial, lysosomal, and plasma membranes. This PCD is triggered by various stimuli, such as TNF, Fas ligand, and TRAIL and depends on the serine/threonine kinase activity of RIP1. Additionally, a set of 432 genes regulates necroptosis and cellular sensitivity to this PCD by a signaling network that mediates innate immunity [88]. Moreover, Bmf, a BH3-only protein, is required for death receptor-induced necroptosis [88].

Moreover, environmental toxicants like cadmium can activate necroptosis. Intermediate levels of cadmium are associated with lost plasma membrane integrity, a decrease of ATP levels, and mitochondrial membrane potential and cell swelling, which are features associated with necroptotic cell death [89].

The core pathway of necroptosis relies on the assembly of an amyloid-like structure termed the necrosome. The necrosome is a multiprotein complex formed by receptor-interacting protein kinase 3 (RIPK3), RIPK1, and mixed lineage kinase domain-like (MLKL). Oligomerization and intramolecular autophosphorylation of RIPK3 lead to the recruitment and phosphorylation of MLKL. RIPK3 and MLKL continuously shuttle between the nucleus and the cytoplasm, whereas RIPK1 is constitutively present in both compartments [90]. Nuclear RIPK1 becomes ubiquitinated, and then nuclear MLKL becomes phosphorylated and oligomerized [90]. MLKL mediates plasma membrane rupture. MLKL forms cation channels that are preferentially permeable to Mg^{2+} in the presence of Na^{+} and K^{+} [91]. MLKL-induced membrane depolarization and cell death exhibit a positive correlation to channel activity.

6.1 The role of ER in necroptosis

The role of the ER in necroptosis has been evidenced using necrostatin-1, an inhibitor of necroptosis, which has a protective effect on the endoplasmic reticulum and mitochondria and alleviates ER stress after spinal cord injury [92]. Furthermore, Grp78 promotes an inflammatory response through the upregulation of necroptosis and subsequent activation of NF-κB and AP-1 pathways [93]. The depletion of reticulocalbin 1, an ER-resident Ca^{2+}-binding protein, induces Grp78, activates PERK, and phosphorylates eIF2α. Moreover, the activation of CaMKII and the inactivation of Akt are important for necroptosis in response to reticulocalbin 1 depletion [94].

The function of MLKL and RIPK in necroptosis has been widely studied. The signal transducer and activator of transcription 3 (STAT3) was demonstrated to be downstream of calpain and regulates RIPK3 expression and MLKL phosphorylation and induces ER stress and mitochondrial calcium dysregulation [95]. Moreover, in cardiomyocytes upregulated RIPK1 and RIPK3 evoke ER stress, accompanied by an

increase in intracellular Ca^{2+} levels and xanthine oxidase expression, which raised cellular ROS that mediated the mitochondrial permeability transition pore opening and necroptosis [96, 97]. In addition, the activation of JNK1/2 is regulated by RIPK3 [96].

Moreover, there are proteins that can participate in necroptosis and other types of PCD such as AIF and MLKL. Apoptosis-inducing factor (AIF), a protein normally located within the intermembrane space of mitochondria, is linked to apoptosis and necrosis. However, it has been shown that mitochondrial depolarization induced by ER stress promotes AIF release and nuclear condensation, which is consistent with necroptotic cell death [98–100]. MLKL, a member of the necrosome, also participates in chelerythrine (CHE)-promoted apoptosis through nuclear MLKL translocation and a special band of MLKL, which is promoted by a mutual regulation between the MLKL and PERK-eIF2α pathways in response to ROS formation [101].

6.2 The role of the RE in necroptosis during follicular atresia

Necroptosis has been widely researched, but there is still much to investigate, including the mechanism that mediates its execution. Nevertheless, necroptosis studies have been carried out under pathological conditions, and thus it is important to use physiological models like follicular atresia.

Necroptosis contributes to follicular atresia and luteolysis [102]. The factors involved in granulosa cell necroptosis can be regulated by acetylcholinesterase (AChE), cytokines, starvation, and oxidative stress via TNFα [103]. Also, an ovarian AChE variant, the read-through isoform AChE-R, has a nonenzymatic function that stimulates RIPK1-/MLKL-dependent necroptosis [103]. Therefore, although the participation of the ER in necroptosis and the contribution of this PCD in follicular atresia have been shown, the interrelation between ER stress-induced necroptosis and follicular atresia is completely unknown.

7. Paraptosis

Sperandio et al. [104] introduced the term paraptosis to describe a route of caspase-independent PCD that has morphological, biochemical, and transcriptional features that are different from apoptosis [104]. Endoplasmic reticulum swelling, mitochondrial swelling, and resistance to apoptosis inhibitors without nuclear shrinkage or pyknosis characterize paraptosis. Although paraptosis is a caspase-independent cell death, participation of caspase-9 has been shown under experimental conditions [104].

Paraptosis can be triggered by different stimuli including insulin-like growth factor I receptor (IGFIR), JAY/TROY, and ROS. IGF-I is a regulator of multiple cell signaling pathways including PI3K-Akt1-RPS6 and ERK1/2 MAPK that are critical for cell proliferation, migration, and survival [105]. IGFIR-induced paraptosis is mediated by caspase-9, and at least two signal transduction pathways participate in the execution of paraptosis, the MAPK and JNK pathways [104, 106].

TAJ/TROY, a member of the tumor necrosis factor receptor superfamily, induces morphological features of paraptosis accompanied by phosphatidylserine externalization, the loss of the mitochondrial transmembrane potential, and independent caspase activation [105]. Moreover, programmed cell death 5 (PDCD5), an apoptosis-promoting protein, enhances TAJ-/TROY-induced paraptotic cell death [107].

ROS production can trigger paraptosis through PINK and mitophagy activation [108, 109]. Covalent modifications of free sulfhydryl groups on proteins cause protein misfolding and the accumulation of misfolded proteins, leading to ER stress, CHOP activation, and paraptosis [110, 111]. In malignant hepatoma cells with

Bcl-xL-mediated apoptotic defects, the disruption of thiol homeostasis and treatment with doxorubicin and pyrrolidine dithiocarbamate induced paraptotic cell death [112].

The full signal transduction pathway and identification of specific markers for paraptosis are still unclear. Nevertheless, phosphatidylethanolamine-binding protein (PEBP-1), a suppressor of the MAPK pathway, has been identified, and prohibitin, a mitochondrial protein, is a mediator of paraptosis [113]. Furthermore, the redistribution of α- and β-tubulin and tropomyosin has been observed in the early stages of paraptosis. Other characteristics of the paraptotic pathway involve alterations mainly in signal transduction proteins, mitochondrial proteins, and some metabolic proteins [113].

7.1 The role of RE in paraptosis

Cancer cells are the best model to study paraptosis because there can be apoptosis and/or autophagy resistance. In melanoma cells, the sustained activation of the IRE1α and ATF6 pathways driven by the MEK/ERK pathway avoids ER stress-induced apoptosis [114].

Different compounds for cancer treatment have shown paraptosis induction. For example, HeLa, A549, and PC-3 cells treated with celastrol induced vacuoles derived from the dilation of ER, a feature of apoptotic cell death; moreover, this was accompanied by autophagy and apoptosis. Furthermore, the ER swelling triggered by celastrol induced ER stress markers including Grp78, PERK, IRE, and CHOP and alterations to proteasome function that resulted in the accumulation of ubiquitinated protein [115, 116]. Moreover, paraptosis can be accelerated by pre-treatment with the proteasome inhibitor MG132 [117]. On the other hand, cyclosporine A treatment of cervical cancerous SiHa cells showed ER stress and UPR preceded by massive cytoplasmic vacuole formation that culminated in a paraptosis-like cell death [118]. Moreover, murine hepatoma 1c1c7 cells and the human non-small cell lung cancer A549 cell line exposed to a combination of photodamage and benzoporphyrin derivative result in ER swelling and paraptotic cell death [119].

For the pathways involved in paraptosis, ER vacuoles can be dependent on the PI3K/Akt signaling pathway [120]. Moreover, in BC3H1 myoblast cell lines exposed to yessotoxin, paraptosis was accompanied by cytoskeletal alterations and the activation of JNK/SAPK1 [121]. However, in acute lymphoblastic leukemia cells, everolimus, a mTOR inhibitor, showed that JNK signaling was not required for paraptotic cell death [122]. Paraptosis in epithelial ovarian cancer (EOC) cells treated with morusin was characterized by VDAC-mediated Ca^{2+} influx into mitochondria, and subsequent mitochondrial Ca^{2+} overload contributes to mitochondrial swelling and dysfunction, leading to the accumulation of ER stress markers, the generation of ROS, and the loss of mitochondrial membrane potential ($\Delta\psi m$) in EOC cells [123].

7.2 The role of the RE in paraptosis during follicular atresia

Knowledge of the role of paraptosis during follicular atresia is still limited. In *Bombyx mori*, apoptosis, autophagy, and paraptosis occur in the ovarian nurse cell cluster during late vitellogenesis, whereas middle vitellogenesis is exclusively characterized by the presence of paraptosis, preceding both apoptosis and autophagy [124]. In mammals, paraptosis was evidenced by ER swelling (**Figure 2d**) and CHOP immunodetection in granulosa cells during follicular atresia in adult Wistar rats [4].

The mechanisms involved in paraptosis during follicular atresia are still unknown. The paraptotic inductor IGFR might be related because it is implicated in follicular growth and selection [104, 125]. Moreover, IGF2R and the binding protein genes IGFBP5 and IGFBP6 are overexpressed in atretic follicles [126]. However, more studies on paraptosis during follicular atresia are necessary.

8. Conclusions

Endoplasmic reticulum stress is a strong signal that triggers different programmed cell death pathways. Interestingly, programmed cell death via endoplasmic reticulum stress is not exclusive to pathological or experimental conditions but is present in physiological processes like follicular atresia. However, the specific mechanisms and signals for choosing a particular cell death pathway are still unknown. In this way, research on the pathways and mechanisms involved in programmed cell death activated by endoplasmic reticulum stress are fundamental, particularly for follicular atresia, as this process ensures the ovulation of competent oocytes for fertilization.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Nayeli Torres-Ramírez, Rosario Ortiz-Hernández, M. Luisa Escobar-Sánchez, Olga M. Echeverría-Martínez and Gerardo H. Vázquez-Nin*
Laboratory of Electron Microscopy, Department of Cellular Biology, Faculty of Sciences, National Autonomous University of Mexico, Mexico City, Mexico

*Address all correspondence to: vazqueznin@ciencias.unam.mx

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The purpose of this book is to concentrate on recent developments on endoplasmic reticulum. The articles collected in this book are contributions by invited researchers with a long-standing experience in different research areas. We hope that the material presented here is understandable to a broad audience, not only scientists but also people with general background in many different biological sciences. This volume offers you up-to-date, expert reviews of the fast-moving field of endoplasmic reticulum. The book is divided in two sections: 1. Introduction and 2. Endoplasmic Reticulum Properties and Functions.

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