Novel insights on the role of Endoplasmic Reticulum calcium in the sensitivity and reversibility of UPR activation

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Novel insights on the role of Endoplasmic Reticulum calcium in the sensitivity and reversibility of UPR activation

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Abstract

Perturbations to the Endoplasmic Reticulum (ER) homeostasis negatively impact on a very crucial ER function that is protein folding. Accumulation of misfolded proteins in the lumen of the ER creates a condition of organelle stress known as ER stress. In order to restore normal proteostasis, cells have developed a series of mechanisms that aim to increase protein folding capacity and relieve misfolded protein burden of the ER. This phenomenon is known as Unfolded Protein Response (UPR) and consists of a network of signaling pathways activated by ER transmembrane sensors that promote cell adaptation or surrender to conditions of ER stress.

ER luminal protein folding ability is performed also by a series of calcium (Ca^{2+}) -binding proteins that also participate to maintain the high luminal Ca^{2+} concentration of the ER, a characteristic that makes this organelle fundamental for cellular Ca^{2+} signaling. For this reason, perturbations at the ER Ca^{2+} concentration leads to increase in ER stress and activation of UPR.

Partial ER Ca^{2+} depletion and activation of UPR are features observed in several human disease but the relationship between these two phenomena and the sensitivity of UPR to Ca^{2+} decrease have not been explored.

Results presented in this thesis show how the UPR sensors' activation tightly reports the variation of ER Ca^{2+} levels. Development of mathematical model allowed the prediction of PERK and IRE1 UPR sensors deactivation upon ER Ca^{2+} refilling. This prediction was validated by experimental results that revealed how in these conditions sensor deactivation occurred in a very rapid manner. These results demonstrate how cell rapidly adapt stress response signaling pathways to variations in ER homeostasis.

Ca^{2+} signaling alterations is a features also of cell infection by intracellular bacterial pathogens. Many of them have also been shown to modulate UPR activation in order to favor host cell colonization. However, for its role in promoting immune host defense, the role of UPR during bacterial infections is controversial.

Several bacterial species such as the ones belonging to *Shigella,* the causative agent of bacillary dysentery, are able to subvert a variety of cellular pathways through the secretion of bacterial effectors into the host cell. However, the role of UPR during infection of epithelial cells by *Shigella* has not been explored yet.

This works presents results demonstrating that *Shigella flexneri* is able to induce activation of UPR by secreting effectors into the host cell and negatively impacting of ER Ca^{2+} concentration. Moreover, *S. flexneri* is able to counteract UPR activation by promoting sensors' degradation, a feature that still remains poorly understood. These results suggest how, by dysregulating cellular pathways, pathogens could reveal unknown intracellular mechanisms.

Altogether this work contributes to clarify some aspects of the complexity of UPR activation and UPR regulation, helping to better dissect its role in many pathological conditions.

Résumé

Les perturbations de l'homéostasie du réticulum endoplasmique (RE) ont un impact négatif sur une des fonctions cruciales du RE : le repliement des protéines. L'accumulation de protéines mal repliées dans la lumière du RE crée une condition de stress connue sous le nom de stress du RE. Afin de restaurer une protéostase normale, les cellules ont développé une série de mécanismes visant à augmenter la capacité de repliement des protéines et diminuer la charge des protéines mal repliée dans le RE. Ce phénomène est connu sous le nom de « réponse aux protéines non répliées » (Unfolded Protein Response-UPR) et consiste en un réseau de voies de signalisation activées par des récepteur transmembranaires du RE qui favorisent l'adaptation cellulaire ou la mort des cellules. Trois protéines transmembranaires du RE initient cette réponse : PERK, IRE1 et ATF6. L'activation de ces voies de signalisation vise à réduire la charge protéique du RE en induisant la dégradation de l'ARNm, en diminuant la traduction de l'ARNm et en favorisant la dégradation associée au RE (ERAD). L'UPR favorise également une réponse transcriptionnelle qui conduit à une production accrue d'enzymes de repliement, à la synthèse lipidique, à l'expansion du RE et à une sécrétion accrue de protéines.

Le repliement des protéines de la lumière du RE est également assuré par une série de protéines liant le calcium (Ca^{2+}). Ces protéines participent également au maintien de la concentration luminale élevée en Ca^{2+} du RE, une caractéristique qui rend cet organite fondamental pour la signalisation cellulaire du Ca^{2+}. Pour cette raison, les perturbations de la concentration de Ca^{2+} dans le RE ($[Ca^{2+}]re$) entraînent une augmentation du stress du RE et l'activation de l'UPR.

La réduction partielle de $[Ca^{2+}]re$ et l'activation de l'UPR sont des caractéristiques observées dans plusieurs maladies humaines, mais la relation entre ces deux phénomènes et la sensibilité de l'UPR à la diminution du Ca^{2+} n'a pas été explorée. Expérimentalement, le stress du RE est généralement induit par un traitement avec des doses élevées de thapsigargine, un inhibiteur de la pompe SERCA. Cela entraîne un blocage de l'importation de Ca^{2+} dans le RE et une diminution massive de $[Ca^{2+}]re$. Cette condition ne récapitule pas ce qui pourrait arriver dans un contexte plus physiologique ou pathologique. Pour cette raison, il est nécessaire de mieux caractériser l'activation de l'UPR dans des conditions de diminutions intermédiaires de $[Ca^{2+}]re$.

Les résultats présentés dans cette thèse montrent comment l'activation des récepteurs UPR rapporte fidèlement la variation des niveaux de $[Ca^{2+}]re$. L'UPR s'active également dans des conditions de diminution partielle du $[Ca^{2+}]re$ et ne nécessite pas une libération totale du Ca^{2+}. De plus, dans ces conditions, PERK, IRE1 et ATF6 montrent des sensibilités et suivent des cinétiques d'activations

différentes. IRE1 est le plus sensible aux petites diminutions des niveaux de [Ca^{2+}]re. De plus, contrairement à PERK et ATF6, IRE1 suit une cinétique d'activation non linéaire.

Un modèle mathématique, nous a permis de prédire l'inactivation des récepteurs PERK et IRE1 lors du remplissage de Ca^{2+} dans le RE. Cette prédiction a été validée par des résultats expérimentaux. Cependant, expérimentalement, la désactivation des récepteurs est plus rapide par rapport aux simulations mathématiques, montrant une inactivation complète des récepteurs en 15 minutes environ. Le modèle rend compte de cet événement de déphosphorylation rapide, quand nous y introduisons une inactivation de IRE1 et de PERK par une phosphatase sensible au Ca^{2+}. Nos résultats ouvrent ainsi de nouvelles perspectives sur l'impact de la signalisation Ca^{2+} sur la réversibilité de l'activation de l'UPR. Ils mettent en évidence la relation directe entre les variations de Ca^{2+} du RE et l'induction et l'inactivation de l'UPR, démontrant ainsi comment les cellules s'adaptent rapidement à un stress du RE.

Du fait de la complexité de la réponse UPR, des modélisations mathématiques de l'activation, de l'effet ou de l'implication de l'UPR dans un contexte pathologique spécifique ont été proposées. Au cours de mon travail de thèse, j'ai fait une synthèse de tous les travaux de modélisation existants à ce jour, relatifs à l'UPR. J'ai ainsi pu réaliser que le modèle développé et présenté dans la section résultats de ce travail constitue la première approche mathématique prenant en compte les modifications du [Ca^{2+}]re lors de l'induction de l'UPR.

Afin d'étudier les interactions entre ces deux phénomènes dans un contexte plus spécifique, cette thèse s'intéresse à une pathologie particulière qu'est l'interaction hôte-pathogène et plus particulièrement l'infection des cellules par des bactéries pathogènes. Cette interaction induit des altérations de la signalisation Ca^{2+} et il a également été démontré que de nombreuses bactéries pathogènes provoquent l'activation de l'UPR afin de favoriser la colonisation des cellules hôtes. Cependant, le rôle de l'UPR lors d'infections bactériennes est controversé. L'UPR participe à favoriser la survie bactérienne et la réplication intracellulaire, mais il a été aussi montré que l'UPR peut favoriser la défense immunitaire de l'hôte, la signalisation pro-inflammatoire et l'apoptose des cellules infectées.

Plusieurs espèces bactériennes telles que celles appartenant à *Shigella*, l'agent causal de la dysenterie bacillaire, sont capables de perturber diverses voies cellulaires grâce à la sécrétion d'effecteurs bactériens dans la cellule hôte. Ainsi *Shigella* modifie la signalisation Ca^{2+}, favorisant l'induction d'augmentations locales durables du Ca^{2+} cytoplasmique et l'atténuation de la

signalisation globale du Ca^{2+}. Ceci est le résultat d'une diminution de la production d'IP_3 due à l'effecteur spécifique ipgD.

Les résultats présentés dans cette thèse montrent que l'infection des cellules épithéliales par *Shigella flexneri* entraîne une déplétion partielle de $[Ca^{2+}]$re, soulignant une possible induction des voies de l'UPR. L'activation et le rôle de l'UPR lors de l'infection des cellules épithéliales par *Shigella* n'ont pas encore été explorés de manière approfondie.

Ce travail présente des résultats démontrant que *Shigella flexneri* est capable d'induire une activation transitoire d'IRE1 et PERK en sécrétant des effecteurs dans la cellule hôte. De plus, *S. flexneri* est capable de contrecarrer l'activation de l'UPR en favorisant la dégradation de ces protéines 2 heures après l'infection via un processus qui reste mal compris. L'inhibition de la dégradation dépendante du protéasome entraîne la récupération des niveaux de protéines totales d'IRE1 et PERK mais ne bloque pas la disparition des protéines phosphorylées suggérant l'existence d'un mécanisme supplémentaire induit par la bactérie pour contrecarrer l'activation de l'UPR.

Nos résultats montrent ainsi que l'effecteur ipgD pourrait participer à ces mécanismes. L'infection des cellules épithéliales par une souche mutante de *S. flexneri* dépourvue d'ipgD entraîne un retard de l'activation de PERK et une diminution de l'activation de IRE1. Les résultats montrent aussi que l'infection par la souche mutante ipgD présente une plus faible déplétion de $[Ca^{2+}]$re par rapport à l'infection par la souche sauvage.

Concernant la dégradation d'IRE1 et de PERK, ipgD intervient dans la dégradation de PERK mais pas d'IRE1. La dégradation de PERK est médiée par la production de PI5P induite par l'ipgD, ce qui suggère une implication possible de l'E3-ubiquitine ligase induite par la PI5P. Concernant la dégradation de IRE1, les résultats préliminaires présentés dans ce travail montrent que la sécrétion de l'effecteur bactérien ipgB2 pourrait être responsable de cet événement. Cet effecteur est impliqué dans la modulation de la contraction de l'actomyosine lors de l'entrée bactérienne dans les cellules hôtes. Ces résultats indiquent que si l'activation des récepteurs UPR dans ce contexte semble être modulée par un mécanisme commun, le processus de dégradation est plus spécifique et diffère selon la protéine impliquée.

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List of abbreviations

σ1R = Sigma1 Receptor
ATF4 = Activating Transcription Factor 4
ATF6 = Activating Transcription Factor 6
BCV = Bacterial Containing Vacuole
BiP = Binding immunoglobulin Protein
CHOP = C/EBP Homologous Protein
CICR = Calcium-Induced Calcium Release
CNX = Calnexin
CRAC =Calcium Release-Activated Channel
CRT = Calreticulin
DAG = Diacylglycerol
DR5 = Death Receptor 5
eIF2α = eukaryotic Initiation Factor α
ER = Endoplasmic Reticulum
ERdj = Endoplasmic Reticulum DnaJ (protein)
ERES = Endoplasmic Reticulum Exit Site
ERO1α = Endoplasmic Reticulum Oxidoreductase α
GADD34 = Growth Arrest and DNA Damage 34
GPCR = G Protein-Coupled Receptor
GRP78 = Glucose Regulated Protein 78
ILs = Interleukins
IP_3 = Inositol Triphosphate
IP_3R = Inositol Triphosphate Receptor
IRE1 = Inositol-REquiring protein 1
JNK = c-Jun N-terminal kinase
LPS = Lipopolysaccharide
MAM = Mitochondria-Associated Membranes
MCS = Membrane Contact Site
MCU = Mitochondrial Calcium Uniporter
Mfn = Mitofusin
mPTP = mitochondrial Permeability Transition Pore
NAADP = Nicotinic Acid Adenine Dinucleotide Phosphate
NBD = Nucleotide Binding Domain
NCX = Na^+/Ca^{2+} exchanger
NMMHC II = Non-Muscle Myosin Heavy Chain II
Nrf2 = NF-E2-related factor-2

ORF = Oper Reading Frame
PDI = Protein Disulfide Isomerase
PERK = PKR-like Endoplasmic Reticulum kinase
PI(4,5)P_2 = Phosphatidylinositol 4,5-bisphosphate
PI(5)P = Phosphatidylinositol-5-phosphate
PMCA = Plasma Membrane Calcium ATPase
PM =Plasma Membrane
PMN = Polymorphonuclear leukocytes
RyR = Ryanodine Receptor
RIDD = Regulated IRE1-Dependent Decay
ROS = Reactive Oxygen Species
S1P/S2P = Site-1 Protease/ Site-2 Protease
SBD = Substrate Binding Domain
SERCA = Sarco-Endoplasmic Reticulum Calcium ATPase
SOCE = Store-Operated Calcium Entry
SR = Sarcoplasmic Reticulum
T3SS = Type III Secretion System
TCA = tricarboxylic acid
TLR = Toll-like Receptor
TRAF2 = TNF Receptor-Associated Factor
TRPC = Transient receptor potential (channel)
TUDCA = Tauroursodeoxycholic acid
UPR = Unfolded Protein Response
VDAC = Voltage-Dependent Anion Channels
XBP1 = X-box binding protein 1

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INTRODUCTION

1 The Endoplasmic Reticulum

The Endoplasmic Reticulum (ER) is the largest membrane-bound organelle which forms a continuum with the nucleus. Its structure is very complex and highly dynamic, exploring the cytoplasmic volume very rapidly. The main functions of this organelle include protein synthesis, protein folding and quality control, lipid synthesis and calcium (Ca^{2+}) storage and release (Schwarz and Blower 2016).

It is made of tubules and sheet-like structures (cisternae) that are connected by junctions in order to form an intricated and complex network. This structure is maintained by a series of proteins that assure the correct ER curvature, the optimal distance to cytoskeleton, junction stabilization and tubules fusion. Indeed, the ER is a very dynamic organelle, constantly undergoing rearrangements. Interaction of ER with cytoskeleton is fundamental for the control of its movement and the general distribution in the cell volume via ER peripheral extension or retrograde retraction (Obara, Moore, and Lippincott-Schwartz 2023).

The ER sheets are ER domains made of nearly flat membranes with constant luminal spacing which localize mainly (but not only) around the nucleus, while the tubules radiate from the nuclear envelope and from cisternae and can also form dense matrices. The reticular structure enables ER to reach the extremity of the cell but providing space for other organelles to traffic around it (Perkins and Allan 2021) (Figure 1).

The high curvature of tubules compared to sheets confers them a broader surface, meaning that tubules have higher surface-to-volume ratio than sheets, making them better suited for surface-dependent functions. On the other hand, sheets would be a good location for luminal processes.

It has been noted that the intricated morphology of the ER is tightly linked to its function. Indeed, the ratio between sheets and tubules varies depending on cell type and reflects the cells' requirement for processes that occur in these two types of structures (Westrate et al. 2015).

The sheets are richer in ribosomes than tubules, although ribosome-bound tubules have been observed. This suggests cisternae are privileged site for protein synthesis while tubules might be preferred for accumulation of integral membrane proteins and for processes involved in lipid synthesis. At ultra-structural level the ER is classified into two parts: the presence of many ribosomes on part of the ER confers it a rough aspect (rough ER) and distinguish it from the smooth ER.

The most known function of the ER is its involvement in proteins synthesis via protein translocation through the translocon complex and subsequent folding and post-translational modification. The ER is the primary site of production of integral membrane and secretory proteins that are later transported to Golgi via ER exit sites (ERES). Specialized secretory cells present a higher proportion of cisternae compared to other cells such as neurons and these sheets have been shown to be enriched in ribosomes. On the contrary, ER tubules are enriched in lipid synthesis enzymes and lipid metabolizing machinery (English and Voeltz 2013b). Tubules have also been shown to be more enriched in contact sites with other organelles such as plasma membrane, mitochondria and endosomes compared to cisternae. Indeed, lipid droplet formation occurs in the ER especially in sites where the ER comes into contact with other organelles suggesting that these sites are specialized in lipid metabolism as well as lipid exchange (English and Voeltz 2013a).

In addition, the ER plays a fundamental role as an intracellular Ca^{2+} store. In some specific type of cells such as muscle cells this ER function is of particular importance for regulating an additional function such as muscle contraction: in these cells the ER is commonly referred to as Sarcoplasmic Reticulum. The importance and regulation of ER Ca^{2+} storage are described in the next sections.

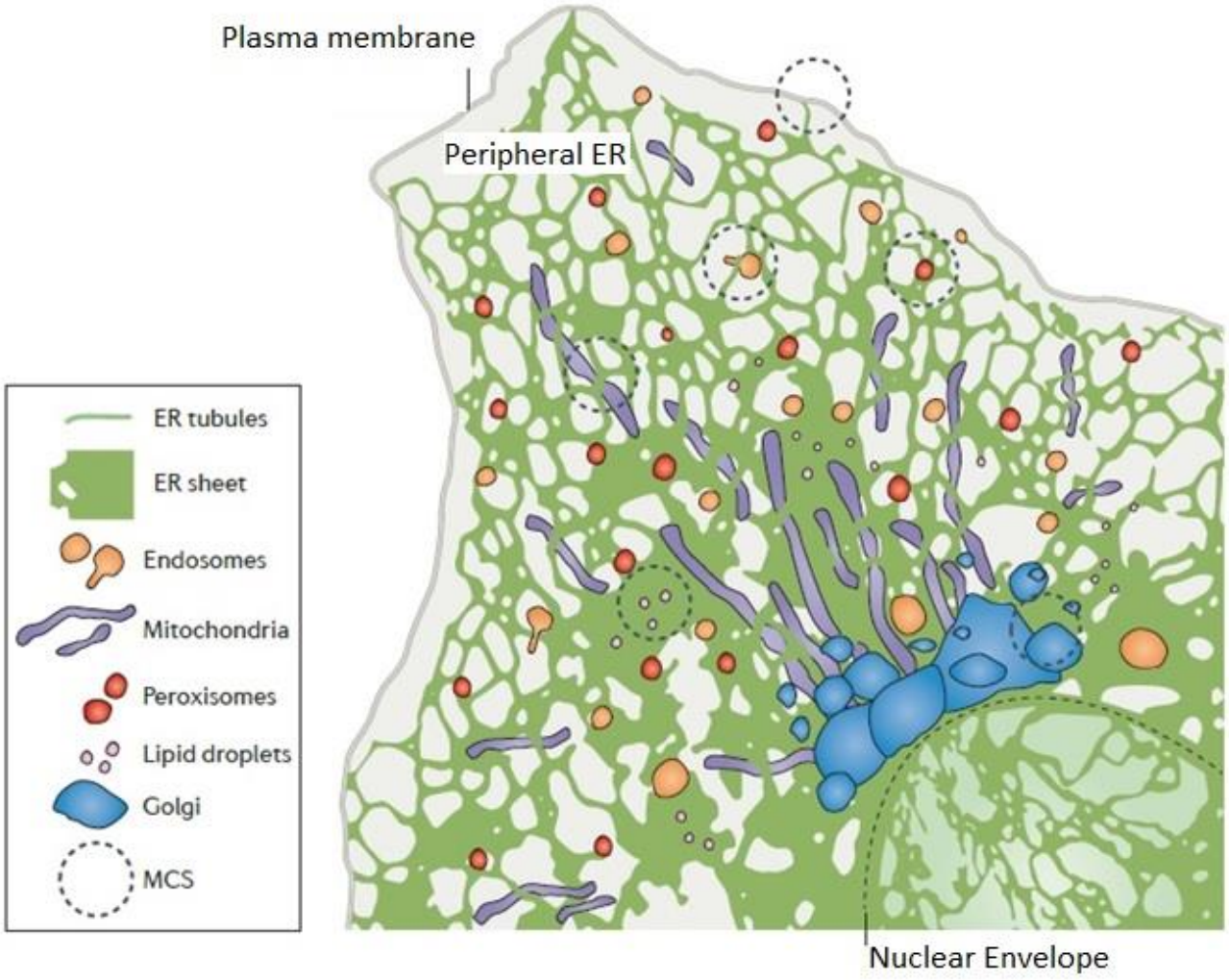

Figure 1. Structure of the Endoplasmic Reticulum. The ER is formed by the nuclear envelope (dashed line) and the peripheral ER, that consists in a network of sheets and tubules that spread in the cytosol. The ER forms contact sites (MCS) with the plasma membrane, mitochondria, endosomes, peroxisomes, lipid droplets and the Golgi Apparatus. Modified from Phillips and Voeltz 2016.

2 Ca^{2+} homeostasis regulation in the ER

Ca^{2+} is an ubiquitous and versatile ion that cells use as second messenger to regulate several physiological functions, such as muscle contraction, metabolism, cell motility, fertilization, cell division and neural activity (M J Berridge, Lipp, and Bootman 2000; Michael J Berridge, Bootman, and Roderick 2003). The ability of the cell to regulate such a plethora of cellular events by using this ion derives from the fact that the cell is able to decode different Ca^{2+} signals that vary according to space and time. The efficiency of these mechanisms is obtained also by establishing a steep Ca^{2+} gradient between the cytoplasm (~100nM) and not only the extracellular space (~2mM) but also intracellular compartments. Among them the ER is the most active to participate in calcium signaling. A high concentration of this versatile molecule in this organelle participates to the regulation of several processes not only in terms of Ca^{2+} release in the cytoplasm but also for the correct functioning of the ER itself. For this reason, several mechanisms contribute to the maintenance of a high Ca^{2+} concentration gradient inside the ER : these involve the action of Ca^{2+} buffering proteins, releasing mechanisms and importing processes that maintain a steady state concentration (Figure 2) (Michael J Berridge, Bootman, and Roderick 2003; Carreras-Sureda, Pihan, and Hetz 2018; Krebs, Agellon, and Michalak 2015). Alteration of ER Ca^{2+} homeostasis has been shown to participate to several diseases such as diabetes, cancer and neurodegenerative diseases (Mekahli et al. 2011), underlying the essential role of ER Ca^{2+} in cell physiology.

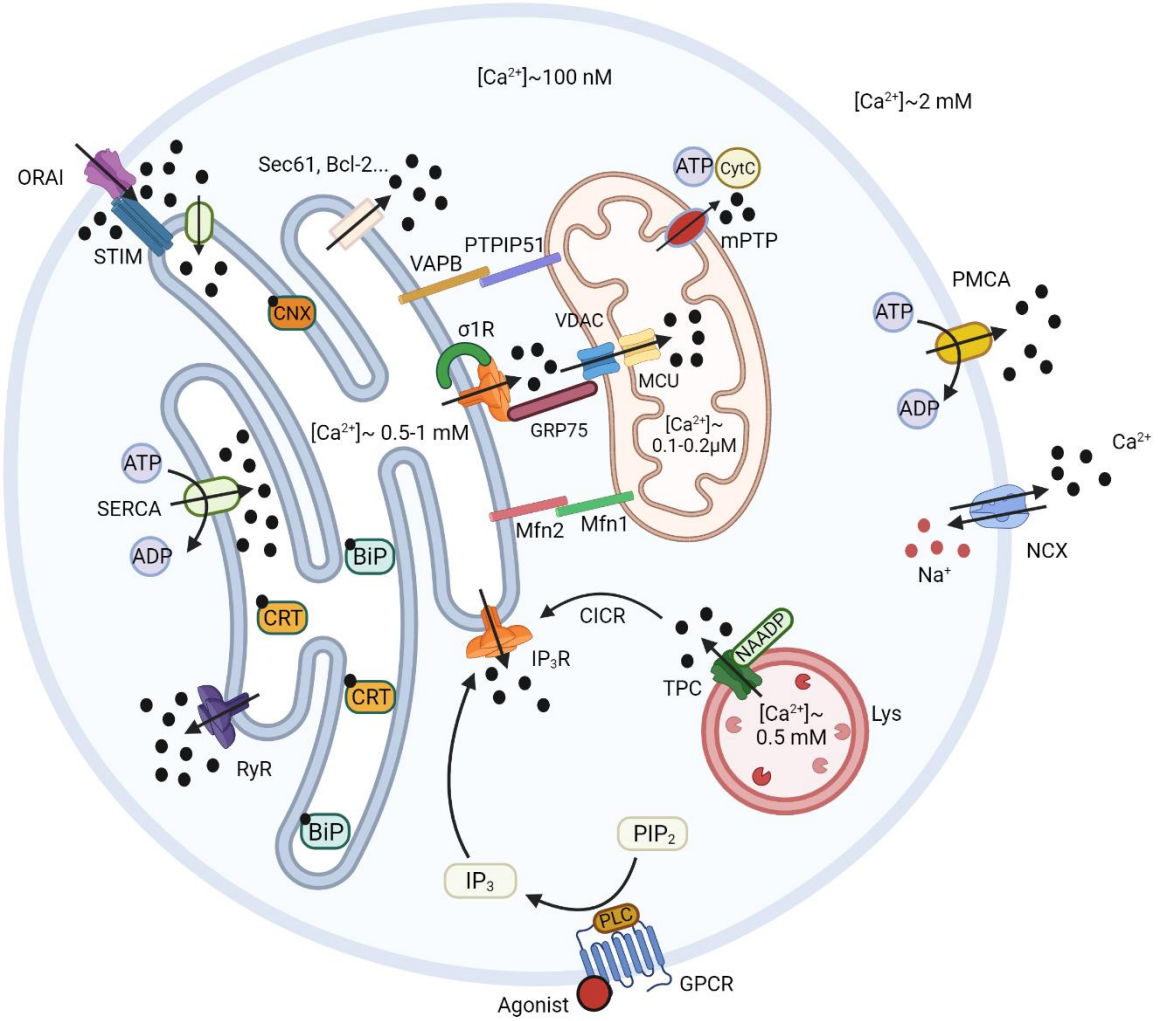

Figure 2 Ca^{2+} homeostasis in the Endoplasmic Reticulum. Ca^{2+} uptake into the ER from the cytosol is driven by SERCA. Meanwhile, Ca^{2+} release from the lumen of the ER into the cytosol is mediated mainly by IP_3R and RyR. IP_3R is activated by binding of IP_3 that is produced from PI (4,5)P_2 through G protein-coupled receptor (GPCR)-mediated activation of PLC. Constant Ca^{2+} leakage from the ER is mediated by series of proteins such as Sec61 or Bcl-2. Once Ca^{2+} is depleted in the ER lumen, dissociation of Ca^{2+} from the STIM proteins leads to STIM oligomerization and interaction with the plasma membrane Ca^{2+} channel ORAI to increase Ca^{2+} influx from the extracellular space. SERCA colocalizes in ER-PM Ca^{2+} microdomains to allow ER Ca^{2+} store refilling. A series of Ca^{2+} binding proteins such as BiP, Calnexin (CNX) and Calreticulin (CRT) buffer Ca^{2+} concentration in the ER allowing it to reach concentrations up to 1 mM. ER Ca^{2+} homeostasis is regulated also at the levels of contact sites with other organelles. Very well characterized is the close proximity with mitochondria tethered by ER-mitochondrial protein association involving among others Mitofusin 1 and 2 (Mfn), VAPB and PTPIP51. This site is enriched with IP_3R, whose Ca^{2+} release is followed by mitochondrial uptake by VDAC and MCU channels. This release is modulated by many proteins such as Sigma1 Receptor (σ1R) or GRP75. Extreme mitochondrial Ca^{2+} uptake can lead to mPTP opening and release of mitochondrial content in the cytoplasm. Here Ca^{2+} concentration is kept very low by extrusion in the extracellular milieu by Plasma Membrane Ca^{2+} ATPase (PMCA) and Na^+/Ca^{2+} exchanger (NCX). ER Ca^{2+} release is also modulated by the action of NAADP-responsive Two-Pore Channels (TPC) on endolysosomal vesicles. Ca^{2+} release from these vesicles induces a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism from IP_3R but also RyR.

2.1 Ca^{2+} releasing mechanisms

Ca^{2+}-releasing mechanisms involve the activation of channels responsive to inositol-1,4,5-triphosphate (IP_3R) and to ryanodine (RyR) which are located on the ER membrane.

The IP_3R is activated by IP_3 which is produced together with diacylglycerol (DAG) by action of phospholipase C (PLC) on Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), following cell stimulation by extracellular agonists, hormones, growth factors or neurotransmitters. The IP_3-induced Ca^{2+}release can spread across the entire cell or be localized in microdomains. These signals are modulated in a complex spatio-temporal manner in order to give rise to a plethora of responses that participate to the regulation of many cellular processes such as modulation of autophagy, apoptosis, proliferation and differentiation (Decuypere et al. 2011; Ivanova et al. 2014).

The IP_3R is ubiquitously expressed and is localized on the ER membrane. Cells have three different isoforms of IP_3R (IP_3R1, IP_3R2 and IP_3R3) which form large tetrameric channels (Taylor and Tovey 2010) and are characterized by a different affinity for IP_3 with IP_3R2 being the most sensitive, while IP_3R3 the least (Tu et al. 2005). IP_3R activity is modulated by cytoplasmic Ca^{2+} itself: at low concentrations, below 300nM, channel activity is increased, while higher Ca^{2+} concentrations have an inhibitory effect (Bezprozvanny, Watras, and Ehrlich 1991; Finch, Turner, and Goldin 1991). Indeed, after a fast increase in surrounding cytoplasmic Ca^{2+} due to Ca^{2+}-induced Ca^{2+} release mechanisms (CICR), the channel closes. The combination of the regulation of IP_3-dependent Ca^{2+} release and restoration of ER Ca^{2+} levels participate to the regulation of oscillatory Ca^{2+} signals that cells decode differently to mount several cellular responses (Dupont and Combettes 2016; Wacquier et al. 2019).

Also, ER luminal Ca^{2+} concentration has been shown to play a role in the regulation of IP_3R response to IP_3. It has been observed that IP_3-responsive stores spontaneously release Ca^{2+} when containing high concentration of this ion through a mechanism requiring a threshold level of IP_3 and sensitizing the receptor to this ligand (Missiaen, Taylor, and Berridge 1991). Moreover, other studies report that the decrease of luminal Ca^{2+} concentration in IP_3-responsive stores slows down the further release of Ca^{2+} (Missiaen et al. 1992) due to reduced sensitivity to IP_3 (Nunn and Taylor 1992). The regulation of IP_3R activity is also modulated by other factors such as the ER environment (pH, redox state, ATP and Mg^{2+} concentrations), its phosphorylation status and the action of several regulatory proteins (Parys and De Smedt 2012; Parys and Vervliet 2020; Vanderheyden et al. 2009).

The RyRs are located in the SR membrane and are the largest known ion channels. They are less expressed than IP_3Rs (or not expressed at all) in most cellular types. However, their expression level is high in skeletal and cardiac muscle and brain (Lanner 2012). There are three different isoforms of RyRs (RyR1-3); RyR1 is the prevalent form in skeletal muscle (Takeshima et al. 1989), while RyR2 is abundantly expressed in cardiac tissue (Nakai et al. 1990). On the contrary, RyR3 is enriched in cortical and hippocampal regions of the brain (Hertle and Yeckel 2007). These channels are able to induce very rapid transient increases in cytosolic Ca^{2+} concentration (J. S. Smith, Coronado, and Meissner 1985). Indeed, action of RyR is key in the excitation-contraction coupling in both cardiac and skeletal muscle: in cardiomyocytes plasma membrane (PM) depolarization induces an extracellular Ca^{2+} influx that triggers Ca^{2+} release from intracellular stores via CICR mechanism. In skeletal muscles activation of RyR is triggered by conformational change of voltage-dependent Ca^{2+} channel DHPR in T-tubules. Similarly to IP_3R, RyR activity can be regulated by cytoplasmic and ER Ca^{2+} levels, as well as other small molecules such as ATP, Cyclic-ADP ribose and caffeine and proteins (Kobayashi et al. 2021).

In addition to IP_3 and Ryanodine-dependent Ca^{2+} release, the ER membrane is characterized by an inherent Ca^{2+} leakage. It has not been fully elucidated which is the mechanisms responsible for this constant leakage but several proteins have been considered as candidates such as Bcl-2 (P Pinton and Rizzuto 2006), Bax (Oakes et al. 2005), members of the Transmembrane BAX inhibitor motif-containing (TMBIM) family (Y. Chang et al. 2014; Lisak et al. 2015) or the Sec61 translocon complex in a ribosome-free condition or in a condition in which it is still bound to ribosome but not occupied by polypeptide chain (Van Coppenolle et al. 2004; Lomax et al. 2002; Hwei L Ong et al. 2007). Indeed, it has been shown that knockdown of Sec61α complex results in reduced ER Ca^{2+} leakage (Lang et al. 2011). Passage of Ca^{2+} ions can be negatively regulated by direct binding of Ca^{2+}-bound calmodulin, which mediates channel closure (Erdmann et al. 2011) or by BiP-dependent gating through luminal BiP-Sec61α protein binding (Schäuble et al. 2012). Several other proteins have been proposed to mediate Ca^{2+} leak even though for many of them the role in this context has not been fully elucidated yet (Lemos, Bultynck, and Parys 2021).

2.2 Ca^{2+} influx mechanisms

These releasing mechanisms are counteracted by a series of importing mechanisms that aim to maintain and restore Ca^{2+} homeostasis inside the reticular lumen. The most important of these

mechanisms is the ATP-dependent action of the Sarco/Endoplasmic- reticulum Ca^{2+} ATPase (SERCA) proteins which transport cytoplasmic Ca^{2+} in the ER lumen against a concentration gradient. SERCA pumps are encoded by three different genes (*ATP2A1-3*), but several splice variants increase the number of the possible proteins. The housekeeping SERCA2 protein has two splice variants that are the most expressed in vertebrates: the ubiquitous SERCA2b and the more specialized SERCA2a which is highly expressed in skeletal and cardiac muscles and at a lower level in smooth muscle and neurons (Vandecaetsbeek et al. 2009, 2011). Here it pumps Ca^{2+} back in the SR, leaving it available for its release in the next contraction and therefore playing a fundamental role in determining the force and speed of cardiac contraction (Periasamy and Huke 2001). SERCA pumps are subjected to tight regulation. The best studied modulators of these ATPases in muscle cells are phospholamban and sarcolipin proteins, which interact with SERCA pumps to reduce the affinity for cytoplasmic Ca^{2+} (A. G. Lee 2003). In addition, other studies have shown that another antiapoptotic protein, Bcl-2, is able to affect SERCA2 function by destabilizing it, affecting its localization or its expression levels (Dremina et al. 2004; Dremina, Sharov, and Schöneich 2006; Kuo et al. 1998). Moreover, a series of ER-resident Ca^{2+}-binding proteins have been shown to bind to SERCA2 pumps such as ER chaperones calreticulin and calnexin (John, Lechleiter, and Camacho 1998; Roderick, Lechleiter, and Camacho 2000), calumenin (Sahoo et al. 2009) and histidine-rich Ca^{2+}-binding protein (HRC) (Arvanitis et al. 2007).

Following depletion of ER Ca^{2+} content, store refilling is mediated by a specific mechanisms termed Store-Operated Calcium Entry (SOCE) (Jousset, Frieden, and Demaurex 2007). This process is triggered by a depletion of luminal Ca^{2+} concentration and is mediated mainly by the action of Ca^{2+}-release activated channels (CRAC) which import Ca^{2+} from the extracellular space through the PM. Reduction of the luminal Ca^{2+} concentration levels is sensed by Stromal Interacting Molecule (STIM) proteins that localize on the membrane of the ER. Upon store depletion they oligomerize and redistribute into “puncta” at the ER-PM contact sites where they associate with and activate the ORAI channels located on the PM, to induce the entry of a Ca^{2+} current (reviewed in Prakriya & Lewis, 2015). The ER-PM contact sites are areas where high Ca^{2+} concentration microdomains are established and are enriched in SERCA proteins that participate to ER refilling (Burgoyne, Patel, and Eden 2015).

Vertebrates display two isoforms of STIM proteins, STIM1 and STIM2, which are ubiquitously expressed, although STIM2 is more enriched in the nervous system. Of the two, STIM1 has been shown to be the major activator of SOCE (Liou et al. 2005; Roos et al. 2005). On the other hand,

STIM2 governs the basal calcium levels and has been shown to be more sensitive to smaller ER Ca^{2+} depletions (Brandman et al. 2007). Among the three isoforms of ORAI channels, several studies have demonstrated that the CRAC currents are largely mediated by the ORAI1 isoform (Yeromin et al. 2006). However, a recent study by Yoast and colleagues has shown that ORAI2 and ORAI3, by forming heteromeric complexes with ORAI1, are required to fine-tune SOCE and Ca^{2+} signaling events according to the strength of agonist stimulation and in this way modulate the activation of different NFAT isoforms in order to respond differently to physiological needs (Yoast et al. 2020).

Further studies have revealed that other PM-localized Ca^{2+} channels, namely the transient receptor potential canonical (TRPC) channels are involved in mediating SOCE (reviewed in (Hwei Ling Ong, de Souza, and Ambudkar 2016)). Indeed, STIM1 is able to activate TRPC1 channel (Huang et al. 2006; Zeng et al. 2008) requiring the presence of ORAI1 (Cheng et al. 2011; Hwei Ling Ong et al. 2007).

2.3 Ca^{2+} buffering proteins

The levels of Ca^{2+} inside the ER can reach concentration of 1 mM (Combettes et al. 1996; Meldolesi and Pozzan 1998). This is the result of the high buffering capacity of the ER which is obtained by a series of Ca^{2+}-binding proteins that serve as ER chaperones or folding enzymes, responsible for correctly fold proteins transiting through the ER: these include lectins such as calnexin and calreticulin, heat shock proteins such as glucose-regulated protein/immunoglobulin heavy chain binding protein (GRP78/BiP) and GRP94 and the protein disulfide isomerases (PDIs) (Coe and Michalak 2009; Halperin et al. 2014).

Most of these proteins bind ER Ca^{2+} with low affinity but high capacity. The most abundant Ca^{2+} -binding chaperones are GRP94 and calreticulin. GRP94 is a low-affinity, high-capacity Ca^{2+} binding protein with 15 moderate-affinity sites with low capacity and 11 low-affinity sites with high capacity. In vitro experiments have shown that its interaction with other proteins could be modulated by Ca^{2+} binding (Argon and Simen 1999). GRP94 is able to bind to ER luminal peptides and this binding is increased in the absence of Ca^{2+} (Ying and Flatmark 2006). GRP94 has been shown to counteract apoptosis via stabilization of ER Ca^{2+} homeostasis (Y Bando et al. 2004), suggesting that its antiapoptotic effects are a consequence of its Ca^{2+} buffering rather than its chaperone activity. GRP94 also protects neurons against cell death induced by ischemic injuries (Yoshio Bando et al. 2003).

Calreticulin mediates a great Ca^{2+} buffering action: calreticulin-deficient mouse embryonic fibroblasts have been shown to have half of the ER Ca^{2} storage capacity without any modification on free luminal Ca^{2+} concentration (Nakamura et al. 2001). In addition, calreticulin overexpression results in augmented Ca^{2+} levels in intracellular stores and decreased Store Operated Calcium Entry (SOCE -explained later in the text) (Bastianutto et al. 1995; Mery et al. 1996). Its Ca^{2+} binding role is critical for its functioning and involvement in several cellular processes (Michalak et al. 2009). As for its protein homologue calnexin, a critical function is to bind to nascent glycoproteins via recognition of glycosylated proteins residues. However, cells lacking calreticulin have impaired Ca^{2+} homoeostasis, but only a modest decrease in protein folding (Molinari et al. 2004).

25% of Ca^{2+} binding capacity of the ER is mediated by HSP70-type chaperone BiP also known as GRP78 (Lièvremont et al. 1997). BiP is expressed at a higher level than is calreticulin, therefore its Ca^{2+} binding should be considered low capacity and low affinity (Lièvremont et al. 1997). The Ca^{2+} binding site of BiP is located in the ATPase domain (Preissler et al. 2020). Intriguingly, BiP binding to ADP or ATP alters its affinity for Ca^{2+}, suggesting a regulation between ER Ca^{2+} filling and BiP chaperone activity (Lamb et al. 2006; Preissler et al. 2020).This protein recognizes the exposed hydrophobic regions of client proteins and its function is tightly dependent on Ca^{2+} binding. Indeed, Ca^{2+} levels modulate the association/dissociation dynamics of BiP with client substrates: reduction in ER Ca^{2+} levels decrease binding ability of BiP to substrates (Preissler et al. 2015; C. K. Suzuki et al. 1991). On the other hand, altered Ca^{2+} concentration modify its ATPase activity (Kassenbrock and Kelly 1989; Preissler et al. 2020). Moreover, Ca^{2+} impacts on BiP's equilibrium between a monomeric and oligomeric form (Preissler et al. 2015) as well as on its stability (Preissler et al. 2020). These works highlight the crucial link between Ca^{2+} homeostasis and ER chaperone activity (see below).

The protein disulfide isomerase (PDI) family members are characterized by oxidoreductase activity and plays a key role in disulfide bridge formation and oligomerization of client proteins (Feige and Hendershot 2011). PDI is an ER luminal protein that is capable of isomerizing disulfide bonds on proteins transiting through the ER. It has been shown to bind Ca^{2+} with high capacity (Lebeche, Lucero, and Kaminer 1994; Macer and Koch 1988). Microsomes derived from CHO cells overexpressing PDI displayed increased Ca^{2+} storage (Lucero, Lebeche, and Kaminer 1998). Moreover, it was demonstrated that PDI was catalytically more active in the presence of high Ca^{2+} concentration (Lucero and Kaminer 1999), showing modulation of enzyme activity by ER

Ca^{2+} levels. On the contrary, another work demonstrated that Ca^{2+} does not impact on the catalytical activity of PDI, though it can regulate substrate binding (Y. Li and Camacho 2004).

PDI family protein ERp72, functions as a molecular chaperone (Nigam et al. 1994) participating in isomerization of disulfide bonds (Rupp et al. 1994). Though it does bind Ca^{2+}, the enzymatic activity of this protein is not influenced by Ca^{2+} concentrations (Rupp et al. 1994). In addition overexpression of ERp72 does not increase ER Ca^{2+} stores, suggesting its protein folding activity is more important than its Ca^{2+} binding (Lièvremont et al. 1997).

Notably, multiple works have demonstrated that members of the PDI family participate in the regulation of ER Ca^{2+} signaling. ERp44 was shown to be able to bind to inositol-1,4,5-triphosphate receptor 1 (IP_3R1) leading to an inhibition of ER Ca^{2+} release, therefore serving as a connecting modulator between ER conditions and cytoplasmic Ca^{2+} signaling (Higo et al. 2005). In addition, ERp57/PDIA3 can interact with STIM1 on its luminal side and where it modulates intramolecular disulfide bond formation. This impacts negatively on STIM1 oligomerization and subsequently on ER Ca^{2+} influx via SOCE mechanism (Prins et al. 2011). ERp57/PDIA3 has also been shown to bind to SERCA2b pump in a Ca^{2+} dependent manner and via the action of calreticulin. Catalytical activity of ERp57/PDIA3 on SERCA2b reduces its Ca^{2+} uptake and decreases frequency of Ca^{2+} oscillations in *Xenopus* oocytes (Y. Li and Camacho 2004). Interestingly, this work observed no influence of Ca^{2+} concentration on catalytical activity of ERp57/PDIA3, suggesting that ERp57/PDIA3 Ca^{2+}-dependent effect on SERCA2b involves the regulation of their association.

2.4 Interaction with organelles

Ca^{2+} homeostasis is also influenced by the interaction of the ER with other organelles.

In recent years there has been an increasing interest in the study of membrane contact sites (MCS), which refer to areas where two organelles are in close proximity to one another (less than 30 nm) with the help of tether proteins that ensure the membrane closeness and prevent membrane fusion. MCS are crucial in regulating molecule transfer among organelles and participate in modulating organelles dynamics and biogenesis (Phillips and Voeltz 2016). It is now well established that MCS between the ER and several organelles can contribute to the regulation of inter-organelles Ca^{2+} exchange (Burgoyne, Patel, and Eden 2015).

A key role in this context is played by MCS between the ER and PM. These MCS are particularly important in the physiology of muscle cells where Sarcoplasmic Reticulum is in close contact with invaginations of the PM (T-tubules) and participates to the Ca^{2+}-induced Ca^{2+} release (CICR)

mechanism to initiate muscle contraction. In addition, as previously mentioned, ER-PM contact sites are key in the regulation of ER refilling by SOCE.

The MCS tethering ER with the mitochondria are commonly referred to as Mitochondria-Associated Membranes (MAMs) (Vecellio Reane et al. 2020). It has been shown that Ca^{2+} release via IP_3Rs in these microdomains is taken up by the mitochondria via VDAC channels in the outer mitochondrial membrane and via the Mitochondrial Ca^{2+} Uniporter (MCU) in the inner one (De Stefani et al. 2011). Several studies have underlined the importance of the role that the mitochondria-ER crosstalk plays in the maintenance of Ca^{2+} homeostasis and in the pathogenesis of several human diseases (Paolo Pinton 2018). Mitochondrial Ca^{2+} uptake is very crucial not only for regulating cytosolic Ca^{2+} increase but also for regulating Ca^{2+}- dependent energy supply, since multiple enzymes of the tricarboxylic acid (TCA) cycle require Ca^{2+} for their correct function (Cárdenas et al. 2010; Gherardi et al. 2020). Of note, extreme mitochondrial Ca^{2+} uptake and subsequent overload are involved in the induction of apoptosis via the opening of the mitochondrial Permeability Transition Pore (mPTP), which induces membrane permeabilization and release of the matrix content (de Ridder et al. 2023). For this reason, Ca^{2+} release via IP_3Rs is tightly regulated. Among the many proteins that participate to this mechanisms there is the Sigma1 Receptor, a MAM enriched protein that has been shown to play an important role in cell fate (Pontisso and Combettes 2021).

In addition, ER forms MCS with another important intracellular Ca^{2+} store: the lysosomes. These organelles are estimated to have a high Ca^{2+} concentration and have been shown to mobilize their store in response to nicotinic acid adenine dinucleotide phosphate (NAADP) via the Two-pore channels (TPCs).

ER-lysosome MCS are key for regulation of Ca^{2+} crosstalk between these organelles: indeed it has been shown that lysosomal Ca^{2+} release is able to trigger Ca^{2+} release from the ER (via CICR activation on IP_3R and RyR) and *vice versa* (Morgan et al. 2013).

3 ER Ca^{2+} concentration and chaperones function

As briefly exposed in section 2.3, the ER expresses several proteins that are able to buffer Ca^{2+} with different affinities and capacities (Coe and Michalak 2009). Most of them are also involved in modulating protein folding. Indeed, the ER is the major site involved in protein folding and quality control of newly synthesized proteins. It is estimated that around one third of cellular proteins are

folded in the ER before reaching their final destination (Ellgaard and Helenius 2003). In addition, most ER Ca^{2+}binding chaperones are capable of dynamic variations in response to alterations in ER Ca^{2+} levels, particularly when it comes to conformation. Therefore, it is no surprise that Ca^{2+} buffering capacity of the ER is tightly linked to other cellular processes that occur in this organelle such as ensuring a balanced and functional proteome (proteostasis). This is one of the most important functions of the ER. In mammalian cells, secretory and proteins that reside in the lumen of organelles are initially directed to the ER through a hydrophobic signal sequence, which is subsequently cleaved by signal peptidase within the lumen of the ER (Blobel and Dobberstein 1975; von Heijne 1985; Martoglio and Dobberstein 1998). Similarly, also integral membrane proteins are initially assembled at the ER and inserted in the membrane either co-translationally or post-translationally (Borgese and Fasana 2011; Pool 2022; Shao and Hegde 2011; Wickner and Schekman 2005).

In the ER all these proteins obtain their final topology which involves achievement of ternary and quaternary structures with the help of protein modification such as glycosylation or disulfide bond formation. Several of ER chaperones and foldases assist to each of the folding and maturation steps (Figure 3). The abundant Ca^{2+} binding chaperones calreticulin and calnexin bind unfolded regions of glycosylated polypeptides (Williams 2006) by recognition of N-linked oligosaccharide intermediate (Hammond, Braakman, and Helenius 1994; Kozlov et al. 2010). The binding between this protein chaperone and carbohydrates serves to prevent the aggregation of recently synthesized polypeptides, protect them from degradation, and ensure accurate folding prior to the progression of proteins along the secretory pathway.

Another key aspect of protein maturation in the ER is disulfide bond formation. Calnexin and calreticulin play a role in presenting polypeptides to ERp57/PDIA3 for disulfide bond formation and isomerization. ERp57/PDIA3 function as a folding enzyme, directly binding to calnexin and calreticulin. ERp57/PDIA3 acts as a PDI-like oxidoreductase and primarily facilitates disulfide bond formation in glycoproteins, contributing to the stabilization of their native protein conformations (Coe and Michalak 2010; Leach et al. 2002). Another abundant ER oxidoreductase, PDI, along with its Ca^{2+} storage role, plays a fundamental role in disulfide bridge formation and avoidance of protein aggregation (Ali Khan and Mutus 2014). Although the dependence on ER Ca^{2+} concentration for its activity is not clear, it has been shown that Ca^{2+} depletion in the lumen of the ER results in a decreased PDI mobility: it has been suggested that this phenomenon can be explained by the formation of a Ca^{2+} responsive complex between PDI and calreticulin resulting in

the sequestration of a crucial component of the oxidative homeostasis of the ER (Avezov et al. 2015). The authors suggest that this could partially explain the observed reductive shift of the ER upon Ca^{2+} depletion that was previously shown (Avezov et al. 2013; Enyedi, Várnai, and Geiszt 2010). This indicates that alteration to Ca^{2+} homeostasis clearly impacts on the activity of chaperones involved in disulfide bond formation leading to alteration to normal proteostasis.

Another important chaperone system of the ER involves the recognition of misfolded hydrophobic regions. Among others, BiP chaperone has been shown to bind to aromatic and hydrophobic residues that are normally found at the internal side of proteins (Blond-Elguindi, Cwirla, et al. 1993; Flynn et al. 1991). As all HSP70 proteins, the activity of BiP is regulated by ATP hydrolysis, which governs substrate binding and release. ATP hydrolysis blocks BiP in a conformation that has higher affinity for substrate. ERdj chaperone proteins, belonging to the DnaJ protein family, play an additional role in regulating this cycle by directly interacting with unfolded proteins, transferring them to the ATP-bound form of an Hsp70. Simultaneously, they induce ATP hydrolysis. Nucleotide-exchange factors then release ADP, enabling ATP to rebind and facilitating the release of the client. This process allows for the client to be folded or targeted for degradation (Behnke, Feige, and Hendershot 2015). It has been shown BiP AMPlation blocks BiP in a conformation similar to ATP-bound state that is impaired for its interaction with ERdj cofactors subsequent ATP hydrolysis: this results in incapacity of BiP to obtain a conformation with high affinity for client substrates (Preissler et al. 2017). Researchers have demonstrated that BiP is able to form reversible oligomers that present a reduced client binding capability (Blond-Elguindi, Fourie, et al. 1993; Freiden, Gaut, and Hendershot 1992). This strategy could be a cellular attempt to rapidly buffer the folding capacity of the ER in conditions in which there is a reduced unfolded protein burden in the lumen of this organelle.

BiP oligomerization results from intermolecular association of the substrate binding domain (SBD) and the interdomain linker, which is disfavored by ATP binding (Preissler et al. 2015). Moreover, accumulation of BiP unfolded substrates led to decrease in BiP oligomers, revealing the role of SBD in tethering between clients and oligomers. Notably, Ca^{2+} alteration impacts on this mechanism: it has been demonstrated that Ca^{2+} depletion in thapsigargin treated cells results in oligomer stabilization and reduced ability of the chaperone to bind client substrates (Preissler et al. 2015). In addition, Ca^{2+} plays a role not only in nucleotide exchange phase of BiP ATPase cycle, favoring ATP hydrolysis but also it enhances BiP affinity for ADP. This results in slower kinetics

of BiP-substrate dissociation due to ATP binding, thus decreasing structural flexibility and stabilizing BiP association with client proteins (Preissler et al. 2020).

All these examples are indicative of how Ca^{2+} homeostasis disruption in the ER impacts on the ability of this organelle to obtain a balanced and functional proteostasis, impinging on its the ability to properly cope with cellular demand of protein folding and quality control. This condition leads to accumulation of misfolded proteins in the lumen of the ER and is known as ER stress.

To cope with altered protein folding cells have developed a network of signaling pathways that aims to reduce the unfolded proteins burden in the ER and restore proteostasis. This is commonly referred to as Unfolded Protein Response (UPR).

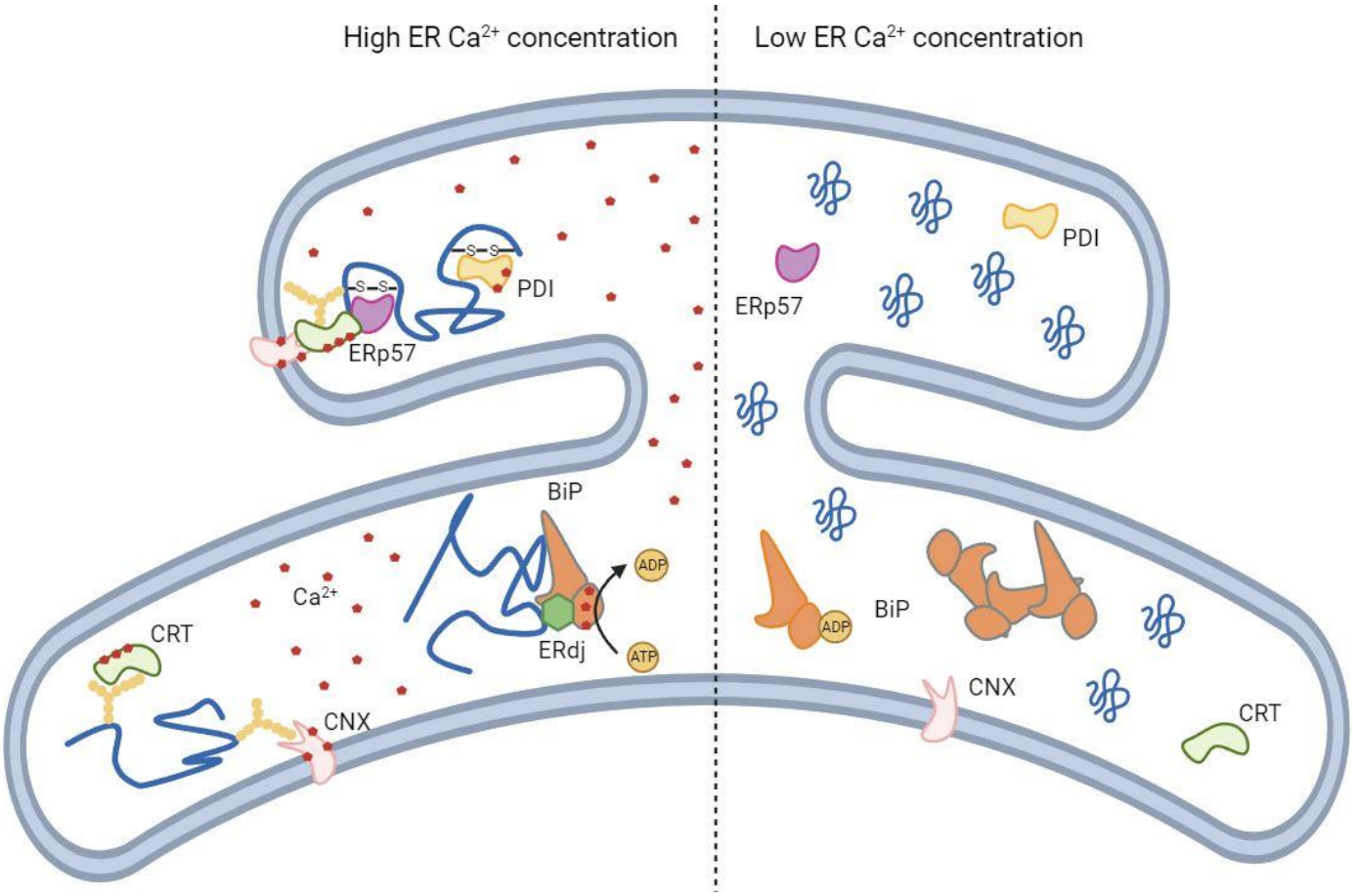

Figure 3. Role of Ca^{2+} on chaperones' activity. The lumen of the ER is a major site for folding and maturation of proteins directed to the secretory pathway and of transmembrane proteins. The correct final conformation is assisted by luminal chaperones many of which require Ca^{2+} for their correct functioning. In homeostatic conditions, Ca^{2+}- binding chaperones calreticulin (CRT) and calnexin (CNX) bind to glycosylated polypeptides and help to direct oxidoreductase ERp57 to client glycosylated proteins in order to favor disulfide bond formation. This reaction is also catalyzed by PDI protein, whose activity is enhanced by high Ca^{2+} concentration. BiP chaperone binds to hydrophobic residues of client substrates with SBD domain. NBD domain is able to hydrolyze ATP to favor its catalytical activity. This is promoted by co-chaperones ERdj proteins that also enhance BiP binding to clients. In conditions of luminal Ca^{2+} depletion chaperones are not able to correctly assist in protein folding and this results in accumulation of misfolded proteins in the ER. In the case of BiP it has also been shown that reduction in ER Ca^{2+} concentration not only impinges on its ability to bind clients by enhancing its affinity for ADP but also promotes stabilization of oligomers, that further reduce its availability in the lumen.

4 The Unfolded Protein Response

Many conditions other than aberrant ER Ca^{2+} regulation are responsible for ER homeostasis disruption and altered proteostasis. Among them we find defective protein modification, impaired ER-to-Golgi trafficking and stressful cellular intrinsic or extrinsic conditions such as hypoxia, lack of nutrients, acidosis but also bacterial and viral infections. In order to restore cellular homeostasis, the UPR firstly activates an adaptive program, which involves decrease in mRNA translation, increased degradation of misfolded luminal proteins via autophagy or ER-associated protein degradation (ERAD), expansion of ER membrane and the increase in the ER folding capacity, by augmented production of components of the folding and quality control machinery (Almanza et al. 2019). In case of persistent or severe ER stress this response may switch from pro-adaptive to pro-apoptotic response, inducing cell death. How the UPR sensors integrate information about the intensity and duration of ER stress in order to determine the cell fate is still an open question. The dynamics of activation and deactivation of UPR sensors that could regulate this aspect have not been fully elucidated yet.

Altered ER proteostasis and abnormal UPR signaling have been implicated in the occurrence of a variety of human diseases, including cancer, neurodegeneration, metabolic diseases and chronic inflammation. Hence, researchers in the last years have increased their effort for developing drugs targeting UPR pathways or UPR pathways modulators (Hetz et al. 2019).

Moreover, it has become clear that UPR plays crucial role in modulating several cellular aspects that go beyond normal proteostasis such as membrane contact sites, cellular bioenergetics, cytoskeletal dynamics, DNA damage response, cell signaling crosstalk and cell non-autonomous proteostasis modulation (all reviewed in Hetz, Zhang, and Kaufman 2020).

The implication of UPR in many cellular functions underlies the importance of dissecting the functioning and regulation of this phenomenon at the activation level but also at the downstream effects.

4.1 Sensing of ER stress by UPR sensors

Three parallel signal transduction pathways are activated by three ER transmembrane proteins, namely PKR-like ER kinase (PERK), inositol-requiring protein 1α (IRE1), and activating transcription factor 6α (ATF6), initiating the UPR.

The precise mechanism by which UPR sensor/transducers detect an accumulation of misfolded proteins in the ER lumen remains a subject of ongoing discussion. It is widely accepted that activation of IRE1 and PERK is mediated by their homo-dimerization and homo-oligomerization (C. Y. Liu, Schroder, and Kaufman 2000), while inhibiting formation of dimers and oligomers prevents UPR signaling (Anne Bertolotti et al. 2000; Credle et al. 2005). The luminal domain of IRE1 and PERK is very similar, therefore it is expected that their ability to sense accumulation of unfolded proteins uses similar mechanisms (Carrara, Prischi, Nowak, and Ali 2015; J. Zhou et al. 2006). IRE1 being the most conserved among the three transducers, most studies focused on studying the activation mechanisms of this sensor (Figure 4a-c). The first model proposed suggests that BiP chaperone maintains the sensor is a non-active monomeric form (Figure 4a). Upon accumulation of misfolded proteins, BiP is titrated away from IRE1, leaving it free to dimerize. This competition model, in which unfolded proteins compete for BiP binding with the sensors has been shown to be similarly involved in PERK activation (Anne Bertolotti et al. 2000; Morris et al. 1997). Further evidence demonstrated that BiP is recruited to IRE1 by ERdj4, which stimulates ATP hydrolysis and promotes the stability of the binding between IRE1 and the substrate binding domain (SBD) of BiP. This counteracts the intrinsic tendency of the sensor to dimerize (Amin-Wetzel et al. 2017, 2019). However, it has been shown that IRE1 lacking BiP binding site is still stress inducible (Kimata et al. 2004; Pincus et al. 2010), suggesting that BiP could play a more critical role in fine tuning stress response rather than solely modulating the activation, which could be dependent on other mechanisms such as direct unfolded protein binding (see below).

Another indirect model proposes an allosteric activation of IRE1 by BiP (Figure 4b). Based on *in vitro* experiments, Carrara and coworkers demonstrated that BiP binds IRE1 and PERK with its nucleotide binding domain (NBD), preventing its dimerization, while the SBD is free to bind to unfolded clients. This binding therefore triggers the dissociation of BiP from the UPR transducer, allowing for subsequent UPR pathway activation (Carrara, Prischi, Nowak, Kopp, et al. 2015; Kopp et al. 2018). However more recently it has been demonstrated that even in absence of unfolded proteins, BiP is capable of regulating the oligomeric state of IRE1, thereby opposing to a key aspect of this model (Amin-Wetzel et al. 2019).

Structural studies revealed that luminal domains of dimeric IRE1 forms a hydrophobic pocket resembling the peptide binding groove of the major histocompatibility complex (MHC), which could direct bind unfolded proteins (Credle et al. 2005; J. Zhou et al. 2006) (Figure 4c). Direct association with unfolded proteins and subsequent oligomer formation was demonstrated later in

time, event that promotes allosteric changes that promote IRE1 dimerization and activation (Credle et al. 2005; Gardner and Walter 2011; Karagöz et al. 2017). This similar way of sensor activation was also characterized for PERK (Carrara, Prischi, Nowak, and Ali 2015; P. Wang et al. 2018; P. Wang, Li, and Sha 2016).

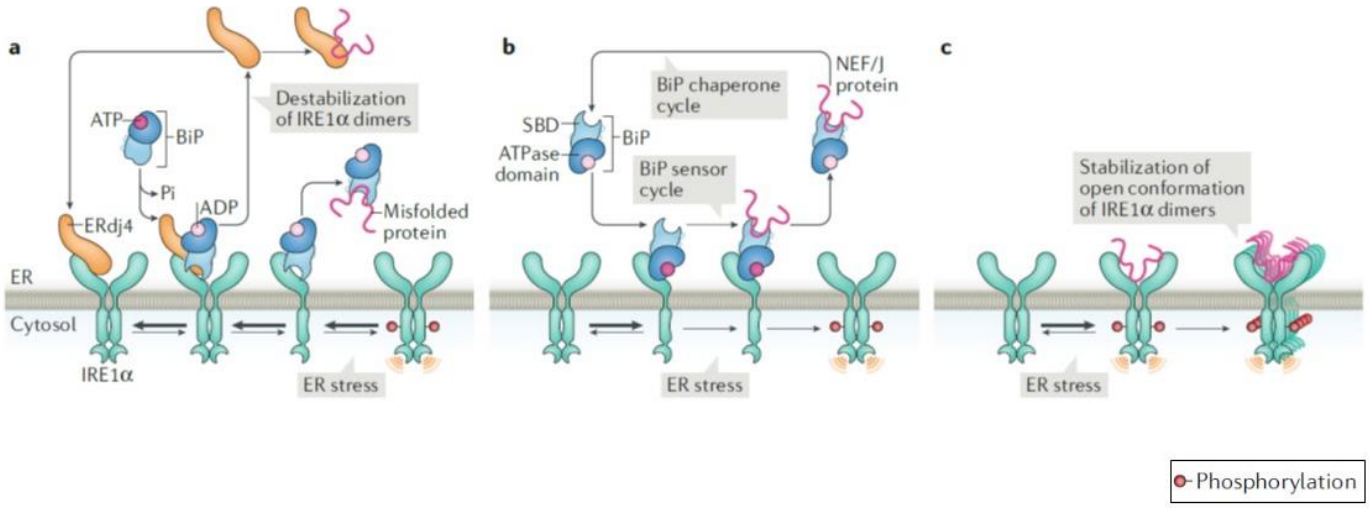

Figure 4. Activation of IRE1 sensor. **a.** Competition model for IRE1 activation. In absence of ER stress, IRE1 is maintained in an inactive state by its association with the ER chaperone BiP through its SBD. Upon accumulation of unfolded proteins, BiP preferentially binds to unfolded protein peptides, thereby releasing the ER stress sensor to allow its spontaneous dimerization and activation. In this model, BiP destabilizes IRE1 dimers and maintains it in an inactive state. This activation model has been proposed also for PERK sensor (not shown for clarity). Moreover, BiP co- chaperone ERdj4 is needed to promote BiP binding to IRE1 and repression of its activation. **b.** Allosteric model for IRE1 activation. It has been proposed that BiP binds misfolded proteins through the SBD and sensors IRE1 and PERK (here only IRE1 shown for clarity) through the ATPase domain. Association of SBD to misfolded proteins mediates the release of the repressive interaction over IRE1 and PERK. **c.** Direct recognition model. This model proposes that unfolded proteins bind directly to the luminal domains of IRE1 and PERK (not shown for clarity), facilitating the assembly of highly ordered oligomers. J protein, J- domain protein; NEF, nucleotide exchange factor. Modified from Hetz, Zhang, and Kaufman 2020.

Interestingly, it has been demonstrated that peptides preferably bound by IRE1 differ from the ones bound by BiP (Gardner and Walter 2011; Karagöz et al. 2017), suggesting that these two proteins could bind different parts of the same polypeptide and the two direct and indirect modes of activation could both participate to favor sensor oligomerization.

Indeed, BiP competition model and a direct binding model are not necessarily mutually exclusive. It has been proposed that clusters of IRE1 resulting from BiP detachment from the sensor are able to bind to unfolded peptides, thus involving a two-step activation (Kimata et al. 2007).

In addition, it has been shown that UPR activation induced by overexpression of immunoglobulin heavy chain correlates with excess of BiP compared to the levels of unfolded protein reached in the lumen: this impinges on BiP availability and hence is the ratio of complexes formation between BiP-client, client-sensor and sensor-BiP the key point that drives the dynamics of activation of UPR sensors. (Bakunts et al. 2017; Vitale et al. 2019).

On the other hand, a recent work has demonstrated by using native PAGE immunoblotting that UPR sensors exist in preformed complexes even in unstressed conditions. Induction of ER stress leads to formation of larger oligomers for PERK but not for IRE1 sensor. Surprisingly, BiP depletion had no effect on the formation of complexes but reduced the activation of the sensors upon ER stress (Sundaram et al., 2018). In line with this, Belyy and coworkers by using single molecule imaging approach also observed the existence of inactive IRE1 dimers at basal conditions that formed larger clusters upon ER stress induction. Interestingly, authors propose a model in which oligomerization promotes trans-autophosphorylation of IRE1 molecules. Active dimers then dissociate and can be brought back to an inactive state by phosphatases (Belyy et al., 2022).

Until now there is no evidence that the third UPR sensor ATF6 could directly bind to unfolded proteins. However, similarly to IRE and PERK, it has been shown that ATF6 is able to bind to BiP and this association is reduced in conditions of ER stress, where the unmasked Golgi localization signals allow its transport the Golgi and subsequent activation (Figure 5) (Shen et al. 2002). ATF6-BiP dissociation is favored by competition of unfolded proteins for BiP (Schindler and Schekman 2009; Shen et al. 2002). ATF6 presents two conserved cysteine residues in the luminal domain which allow the protein to exist in monomer, dimer, and oligomer forms in unstressed conditions. ER stress results in reduction of these disulfide bonds and translocation to Golgi occurs only for the monomeric form (Nadanaka et al. 2007). In line with these findings, more recently it has been demonstrated that ATF6 forms complexes in unstressed conditions and induction of ER stress reduces their size (Sundaram et al. 2018).

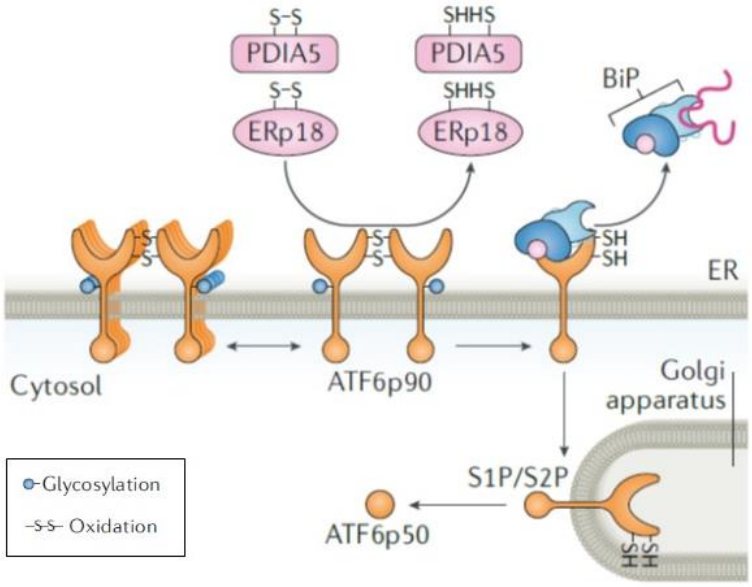

Figure 5. Activation of ATF6. ATF6 activation is modulated by its glycosylation and redox state. Multiple disulfide isomerases such as PDIA5 and ERp18 have been shown to regulate ATF6 redox state. Sensor reduction favors monomer formation and BiP binding. Competition for unfolded protein binding by BiP regulates further the subsequent transport to Golgi apparatus and cleavage of the full-length protein (p90) into smaller portion (p50). ATF6p90, full- length AFT6; S1P, site-1 protease; S2P, site-2 protease. Modified from Hetz, Zhang, and Kaufman 2020.

It has been shown that regulation of redox status of ATF6 plays a role in its dissociation from BiP (Schindler and Schekman 2009) and in its subsequent activation: this could be mediated by disulfide exchange proteins such as ERp18 and PDIA5 (Higa et al. 2014; O. Oka et al. 2019). In contrast to what was previously shown, it has recently been proposed that changes in the redox state of ATF6 occurring after BiP dissociation are crucial in mediating dimerization of the sensor which is the one that is translocated to the nucleus and activated upon both proteotoxic and chemo toxic stresses (O. B. V. Oka et al. 2022).

Interestingly, in recent years it has become more and more evident that the UPR sensor could be activated by a mechanism that does not involve accumulation of unfolded proteins in the lumen of the ER. Alteration to ER membrane composition, commonly known as lipid bilayer stress, can directly activate the sensors via their transmembrane domains (Volmer et al. 2013), inducing a transcriptional and non-transcriptional program specific for lipotoxic conditions (Fun and Thibault 2020). This suggests that the sensor could integrate specific perturbations into very precise program. Among other mechanisms, it has been shown that membrane stiffening, caused also by mis-localized proteins or accumulation of ER membrane proteins, controls the oligomeric state of the UPR transducers (Radanović and Ernst 2021).

In conclusion, the mechanisms that lead to sensors activation are still under debate. Each of them leads to activation of a specific pathway that modulates several cellular outcomes (see below).

4.2 The PERK pathway

PERK is a type I transmembrane protein that presents a cytosolic serine/threonine kinase domain (Harding, Zhang, and Ron 1999). Upon oligomerization the sensors trans-autophosphorylate and activate their kinase domain. This mediates the phosphorylation of target substrates such as the eukaryotic translation initiation factor 2 (eIF2α) on Ser51, preventing the formation of a ternary complex with GTP and $tRNA_{met}$, responsible for translational initiation. Phosphorylation of eIF2α increases the affinity for GDP molecules, blocking a step necessary for translation initiation. In this way PERK activation regulates an immediate adaptive reaction to ER stress by attenuating the rate of global protein synthesis and allowing the release of ribosomes for selective translation of specific UPR response genes (Scheuner et al. 2001).

Upon PERK activation the expression of subset of genes harboring an upstream Open Reading Frame (ORF) in their 5' untranslated regions is enhanced: among them, there is the transcription factor ATF4 (Harding et al. 2000). This promotes transcription of genes involved in redox homeostasis, amino acid metabolism, protein synthesis, apoptosis and autophagy (Wortel et al. 2017) (Figure 6).

In addition, ATF4 induces the expression of GADD34, which, by forming a complex with PP1 phosphatase, is responsible for the dephosphorylation of eI2Fα and restoration of protein synthesis (Novoa et al. 2001). Moreover, ATF4 transcribes for C/EBP homologous protein (CHOP), which is involved in the apoptotic response following prolonged or severe ER stress (Palam, Baird, and Wek 2011). CHOP inhibits transcription of anti-apoptotic proteins such as Bcl-2 (McCullough et al. 2001) and promotes the activation of the pro-apoptotic factors BIM (Puthalakath et al. 2007) and Bax (Szegezdi et al. 2006), but also promotes the expression of Death Receptor 5 (DR5) (H. Yamaguchi and Wang 2004).

Another target of CHOP is the oxidoreductase ERO1α, which plays a role in disulfide bond formation and promotes the creation of an oxidizing environment in the ER lumen, thus favoring cell death (Marciniak et al. 2004).CHOP and ATF4 have short half-lives and CHOP accumulation correlates with cell death. For this reason, prolonged PERK activation is necessary to keep high levels of CHOP and trigger an apoptotic response. Thus, PERK not only is able to promote a pro-survival response following ER stress but also converges the cell fate towards apoptosis according to stress severity and persistence (Rutkowski et al. 2006).

In addition, PERK can directly phosphorylate NF-E2-related factor-2 (Nrf2). In resting conditions, this protein is bound to Keap1. Upon activation of the UPR, PERK-dependent phosphorylation of

Nrf2 dissociates the Keap1/Nrf2 complex. Consequently, Nrf2 is translocated to the nucleus where it activates transcription of proteins with antioxidant activity counteracting ROS levels in cells undergoing ER stress. In this way, the cell triggers an adaptive response to disruption of redox homeostasis (Cullinan and Diehl 2004).

Moreover, PERK-eIF2α signaling, together with ATF4 activation, also favors ATF6 expression, transport to Golgi, cleavage and subsequent activation during ER stress (Teske et al. 2011). In addition, expression of dominant negative version of PERK induces a compensatory mechanisms that helps cells to adapt to ER stress by enhancing ATF6 and *Xbp1* splicing (Y. Yamaguchi et al. 2008).

This implies that the three branches of the UPR are tightly intertwined and PERK plays a key role as a central integrator of UPR signaling.

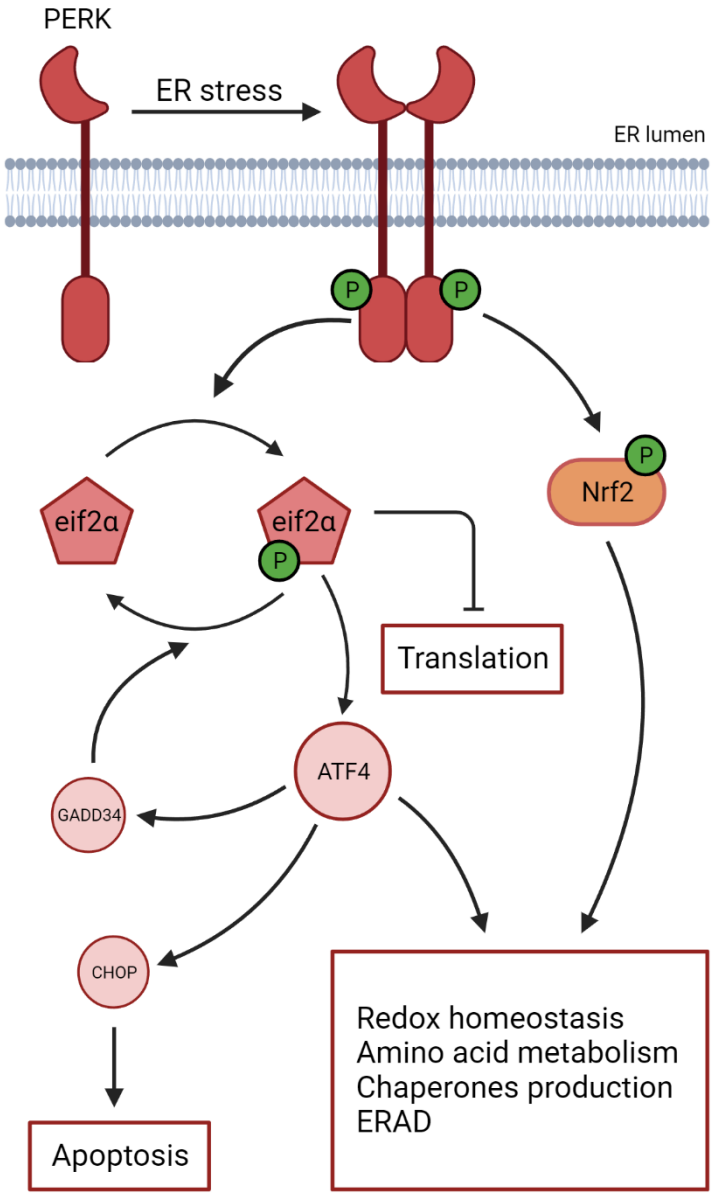

Figure 6. PERK pathway. After dimerization, PERK phosphorylates eIF2α, which in turn blocks global translation and increases the expression of ATF4. This transcription factor induces the expression of target genes, including CHOP and GADD34. The latter participates to a negative feedback loop that leads to eIF2α dephosphorylation. CHOP is involved in promoting cell death by regulating both anti- and pro-apoptotic proteins. In addition, PERK also phosphorylates and activates Nrf2 transcription factor. UPR target genes downstream of PERK activation are involved among other things in enhancing Redox homeostasis and amino acid metabolisms.

4.3 The IRE1 pathway

Similarly to PERK, IRE1 is a type I transmembrane protein that presents a cytosolic serine/threonine kinase domain. IRE1 is the most conserved UPR sensor and has been discovered initially in yeast where it has been shown to be responsible for transducing unfolded protein signal in the cell and promote cell survival upon ER stress (Cox, Shamu, and Walter 1993). Human IRE1 has two isoforms: IRE1α, which has a ubiquitous and constitutive expression, and IRE1β which is only found in cells lining mucosal surfaces such as intestine and lung epithelial cells (A Bertolotti et al. 2001). IRE1 dimerization creates an endoribonuclease domain, which is responsible for the splicing of X-box binding protein 1 (*Xbp1*) mRNA through cleavage of a 26-base intron (Han et al., 2009). Spliced *Xbp1* (*Xbp1s*) contains a translational frameshift, which allows the expression of an active bZIP transcription factor that reaches the nucleus and modulates the expression of numerous target genes. (Shaffer et al. 2004). Activation of this transcription factor upon ER stress enhances cell survival by favoring the protein folding capacity and ERAD response (Park, Kang, and So 2021).In addition, XBP1s can also play a role in the regulation of numerous metabolic pathways such as lipid biosynthesis (Sriburi et al. 2004), glucose metabolism (Y. Zhou et al. 2011), insulin signaling (Akiyama et al. 2013), redox metabolism (Y. Liu et al. 2009) and it influences cell fate including cell survival [123], cell differentiation (Reimold et al. 2001) and development (Reimold et al. 2000; Sha et al. 2009; Sone et al. 2013) (Figure 7).

Unlike PERK signaling, IRE1 pathway is immediately activated upon ER stress but is attenuated when the stress is prolonged (Han Li et al. 2010). Moreover, researchers demonstrated that the unspliced form of *Xbp1* (*Xbp1u*) is able to act as a negative regulator of UPR by promoting degradation of both XBP1s and ATF6 transcription factors (Hiderou Yoshida et al. 2009).

Hyperactivation of IRE1α triggers a cellular response termed regulated IRE1-dependent decay (RIDD). This process implies the cleavage and degradation of microRNAs and mRNAs encoding for membrane and secretory proteins (Hollien and Weissman 2006). The cleavage site is similar to the one of *Xbp1* mRNA, having a consensus sequence within a stem loop structure (Tirasophon et al. 2000). RIDD helps to decrease mRNA abundance and therefore protein folding load in the ER. Interestingly it has been shown that a target of the RNAse domain of IRE1 is IRE1 mRNA itself (Tirasophon et al. 2000). While the quantitative impact of RIDD on ER protein folding homeostasis is not clear, it was found to regulate multiple cellular processes by degrading selected mRNAs in a cell type- dependent and stimulus- dependent manner (Coelho and Domingos 2014; Maurel et al.

2014). Notably, it has been shown that RIDD activity is exerted at a higher degree by IRE1β, while its paralog exhibits a greater level of *Xbp1* mRNA splicing activity (Imagawa et al. 2008).

Furthermore, active IRE1 binds TNF-receptor associated factor 2 (TRAF2), which in turn activates apoptosis-signal regulating kinase 1 (ASK1) and JUN N-terminal kinase (JNK) downstream signaling, thereby promoting programmed cell death (Urano et al., 2000).

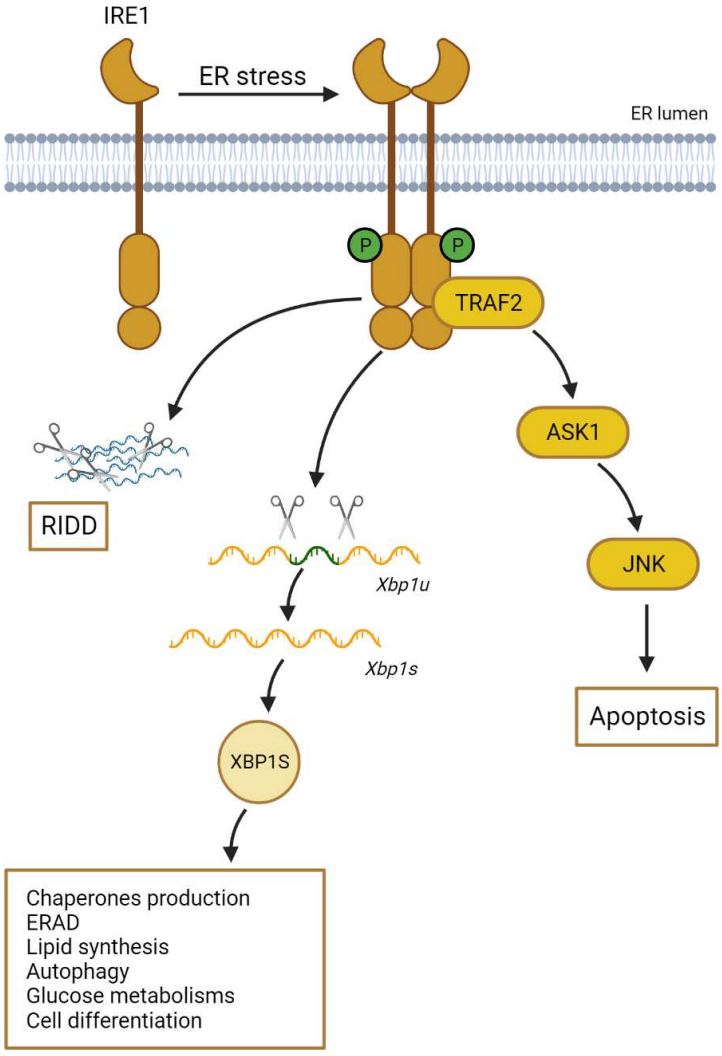

Figure 7. IRE1 pathway. Upon activation, IRE1 induces the alternative splicing of Xbp1 mRNA and participates to general RNA degradation (Regulated IRE1-dependent decay-RIDD). XBP1S enhances transcription of genes involved in protein folding, ER associated degradation and lipid synthesis. It also plays a crucial role in cell differentiation and organismal development. IRE1 also signals through the recruitment of TRAF2 and activation of the ASK1-JNK cascade that induces apoptosis.

4.4 The ATF6 pathway

ATF6 is the third signal transducer that transmits ER stress signal to the nucleus. Mammals express two isoforms of ATF6 (ATF6α and ATF6β) that show high sequence homology.

ATF6 is a type II transmembrane protein that is regulated by intramembrane proteolysis (Figure 8). Upon ER stress ATF6 is translocated to Golgi apparatus via CopII vesicles (Schindler and Schekman 2009). Here it is cleaved by Site-1 and Site-2 proteases (S1P and S2P) (Ye et al. 2000) releasing a ~400 amino acids N-terminal cytosolic fragment: p50-ATF6 or ATF6f (Shen et al., 2002). This is a bZIP transcription factor that induces expression of target genes forming a complex with NF-Y (M. Li et al. 2000; Hiderou Yoshida et al. 2000).

ER stress response genes activated by ATF6 participate to the increase in the ER folding capacity, ERAD and protein secretion (Adachi et al. 2008; Wu et al. 2007). Among the major ATF6 targets there are folding enzymes and chaperones such as GRP94 and GRP78/BiP. In addition, ATF6 transcribes for genes involved in cholesterol and lipid biosynthesis (Maiuolo et al. 2011; Maruyama et al. 2013). Lacking a transcriptional activation domain, ATF6β is a poor transcriptional activator but it has been shown to inhibit the transcriptional activity of ATF6α (Thuerauf, Morrison, and Glembotski 2004).

Interestingly, one of the ATF6 consensus sequence on promoters can be bound also by XBP1s (Yamamoto et al. 2004) and it has been shown that ATF6 and XBP1s transcriptional programs, despite being divergent, partially overlap (Shoulders et al. 2013). Indeed, formation of Xbp1s/ATF6 heterodimers display an increased affinity for DNA binding (Is et al. 2007).

Moreover, ATF6 regulates Xbp1 transcription (Yoshida et al., 2000, 2001) and ATF6 processing collaborates with IRE1α activity to enhance downstream Xbp1transcriptional activity (K. Lee et al. 2002). It has also been shown that ATF6 is able to modulate the expression of CHOP (Yang et al. 2020) thereby showing an intricated regulation among the UPR signaling pathways.

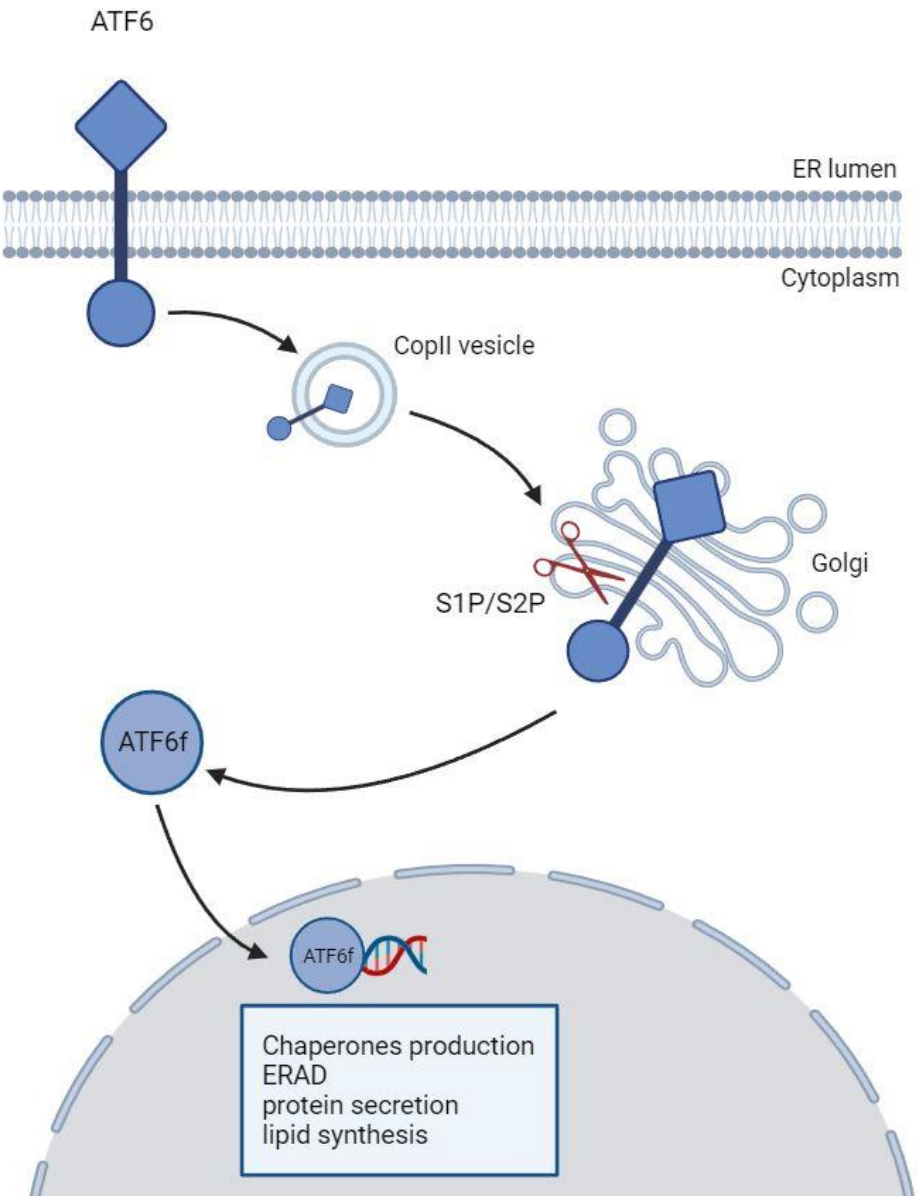

Figure 8 ATF6 pathway. ATF6 is exported from the ER to the Golgi complex via CopII vesicles. In the Golgi ATF6 is cleaved by proteases S1P and S2P, thus releasing its cytoplasmic domain, which is also a transcription factor (ATF6f). ATF6f induces expression of genes involved in protein folding and secretion and ER associated degradation.

5 Modeling the Unfolded Protein Response

UPR pathways and cellular outcomes have been extensively studied in the past two decades. Because of the complexity of the UPR signaling network and the crucial role in regulating many cellular outcomes, many scientist tried to explore several aspects of this phenomenon by using computational approaches. This allowed to better understand some aspects that range from sensor activation to UPR role in specific pathological contexts. Here I present a review entitled “A journey in UPR modeling” that summarizes the work that has been done to highlight UPR aspects through development of mathematical models. This review was published in *Biology of the cell* at the beginning of 2023 together with our collaborators Roberto Ornelas Guevara and Professor Geneviève Dupont from Université Libre de Bruxelles, Belgium.

REVIEW

A journey in UPR modelling

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Abstract

Protein folding and protein maturation largely occur in the controlled environment of the Endoplasmic Reticulum (ER). Perturbation to the correct functioning of this organelle leads to altered proteostasis and accumulation of misfolded proteins in the ER lumen. This condition is commonly known as ER stress and is appearing as an important contributor in the pathogenesis of several human diseases. Monitoring of the quality control processes is mediated by the Unfolded Protein Response (UPR). This response consists in a complex network of signalling pathways that aim to restore protein folding and ER homeostasis. Conditions in which UPR is not able to overcome ER stress lead to a switch of the UPR signalling program from an adaptive to a pro-apoptotic one, revealing a key role of UPR in modulating cell fate decisions. Because of its high complexity and its involvement in the regulation of different cellular outcomes, UPR has been the centre of the development of computational models, which tried to better dissect the role of UPR or of its specific components in several contexts. In this review, we go through the existing mathematical models of UPR. We emphasize how their study contributed to an improved characterization of the role of this intricate response in the modulation of cellular functions.

KEYWORDS
ATF6, ER stress, IRE1, computational model, mathematical model, PERK, signalling, unfolded protein response

INTRODUCTION

The Endoplasmic Reticulum (ER), among other functions, is responsible for the entry of many cellular proteins into the secretory pathway and for this reason it is in this organelle that these proteins need to be folded and obtain their correct conformation. Thus, ER environment must be tightly controlled to allow correct folding, maturation and quality control of protein homeostasis (Hetz et al., 2015).

Defective protein modification, impaired ER-to-Golgi trafficking and stressful cellular intrinsic or extrinsic conditions such as hypoxia, lack of nutrients, acidosis or perturbations in Ca^{2+} homeostasis, are all causes of ER perturbations and homeostasis disruption. This situation results in defective protein folding and accumulation of unfolded or misfolded proteins inside the lumen of the ER, condition that is commonly known as "ER stress." Cells have developed a series of mechanisms that aim at adapting to homeostasis alteration and restoring normal proteostasis. A network of signalling pathways referred to as Unfolded Protein Response (UPR) tries to reduce the protein load inside the ER by impacting on mRNA translation, increasing protein trafficking, inducing protein degradation via autophagy and ER

Abbreviations: ASK1, apoptosis-signal regulating kinase 1; ATF6, activating transcription factor 6; BiP/GRP-78, Binding immunoglobulin protein/78 kDa glucose-regulated protein; CHOP, C/EBP homologous protein; eIF2α, eukaryotic translation initiation factor 2; ER, endoplasmic reticulum; ER^+ breast cancer, estrogen receptor-positive breast cancer; ERAD, endoplasmic reticulum associated degradation; ERAF, endoplasmic reticulum associated folding; GADD34, DNA damage-inducible protein 34; IRE1, inositol requiring protein 1; JNK, JUN N-terminal kinase; ODE, ordinary differential equation; PERK, PKR-like ER kinase; RIDD, regulated IRE1-dependent decay; S1P, Site-1 protease; S2P, Site-2 protease; TA, translational attenuation; TRAF2, TNF-receptor associated factor 2; UP, unfolded proteins; UPR, unfolded protein response.

associated degradation (ERAD) pathways, reprogramming gene transcription and boosting folding capacity (Ron & Walter, 2007).

Depending on the duration and intensity of ER stress, UPR can turn from an adaptive response to a terminal outcome, initiating a cell death program that has been shown to participate to the pathogenesis of several diseases. Moreover, UPR has an impact on several cellular processes and functions such as autophagy, cytoskeleton remodeling, organelle contact sites, DNA damage, Ca^{2+} homeostasis and DNA damage response (Hetz et al., 2020).

The components of the UPR and their interactions have been extensively studied. However, given its high complexity, not only involving feedback loops and crosstalks between the three branches, but also many transcriptional and translational steps, it is difficult to understand it as a whole by using only verbal explanations. To understand how all its components act together, as well as the mechanisms underlying cell fate decisions, it has been necessary to develop computational models. This approach has proven to be very useful for testing hypotheses and exploring a myriad of scenarios, as the flexibility of the models allows to simulate the dynamics of compounds that are not easily measured, or cannot be measured, in the laboratory. Models, therefore, can provide with well-founded and testable predictions. Furthermore, it has become increasingly common to apply advanced mathematical techniques to study biological phenomena, such as bifurcation analysis and control theory.

Virtually all mathematical models of the UPR that have been proposed are deterministic models, based on ordinary differential equations. They differ mainly by the number of UPR sensors they consider, as well as by the UPR stages they study. In this review we provide a state of the art of the work that has been done to develop mathematical models of the UPR and the findings that these models brought in order to contribute to our understanding of the UPR.

THE UNFOLDED PROTEIN RESPONSE

The UPR is coordinated by three distinct ER-resident sensors: PKR-like ER kinase (PERK), inositol-requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6), which coordinate three corresponding and parallel signal transduction pathways (Figure 1). In resting conditions, sensors are maintained inactive via their binding to ER chaperon GRP78/BiP. Upon ER stress proteins, BiP dissociates from the sensors and binds to misfolded proteins, allowing the activation of the signalling pathways (Bertolotti et al., 2000; Shen et al., 2002). On the other hand, studies based on structural observations propose a direct sensing mechanisms for IRE1 and PERK, in which the luminal domains themselves bind to unfolded proteins in the ER (Credle et al., 2005; Karagöz et al., 2017; P. Wang et al., 2018).

Upon activation, PERK homodimerises and transautophosphorylates (Harding et al., 1999; Liu et al., 2000). The activated receptor phosphorylates the eukaryotic translation initiation factor 2 (eIF2α) on Ser51, preventing the formation of a ternary complex with GTP and $tRNA_{met}$, responsible for translational initiation. This results in a transient attenuation of the rate of global protein synthesis and permits the release of ribosomes for selective translation of specific UPR response genes (Scheuner et al., 2001). Among these, transcription factor ATF4 is responsible for the upregulation of DNA damage-inducible protein 34 (GADD34), which regulates the dephosphorylation of eIF2α and restoration of protein synthesis. In addition, ATF4 transcribes for C/EBP homologous protein (CHOP), which is involved in promoting a pro-apoptotic response following prolonged or severe ER stress (McCullough et al., 2001; Puthalakath et al., 2007).

Similarly to PERK, activated IRE1 oligomerizes and autophosphorylates (Tirasophon et al., 1998; Wang, 1998) creating an endoribonuclease domain, which is responsible for the splicing of Xbp1 mRNA through cleavage of a 26-base intron (Calfon et al., 2002; Yoshida et al. 2001). Spliced XBP1 (Xbp1s) contains a translational frameshift that allows the expression of an active transcription factor that modulates the expression of several genes involved in protein folding, lipid biosynthesis, ERAD response and protein secretion (Park et al., 2021). Researchers demonstrated that the unspliced form of Xbp1 (Xbp1u) is able to act as a negative regulator of UPR by promoting degradation of both Xbp1s and ATF6 transcription factors (Yoshida et al., 2009).

Hyper activation of IRE1 triggers a cellular response termed regulated IRE1-dependent decay (RIDD). This process implies the cleavage and degradation of precursors of microRNAs and of mRNAs encoding for membrane and secretory proteins, with the aim to relive the burden of proteins directed to the ER (Han et al., 2009; Hollien & Weissman, 2006; Maurel et al., 2014).

Moreover, active IRE1 binds TNF-receptor associated factor 2 (TRAF2), which in turn promotes programmed cell death by activating apoptosis-signal regulating kinase 1 (ASK1) and JUN N-terminal kinase (JNK) (Urano et al., 2000).

Notably, the IRE1 branch of UPR is the most conserved one. Indeed, it is the only one found in yeast and has been extensively studied in *Saccharomyces cerevisiae*. In this organism *Sc*IRE1 has a similar structure as mammalian IRE1 and is kept inactive by binding to ER chaperon Kar2 (BiP) (Okamura et al. 2000). Upon ER stress, Kar2 dissociates from the ER luminal domain of *Sc*IRE1 and allows the sensor to oligomerize, transautophosphorylate and bind directly to unfolded proteins, a condition that seems to be essential for

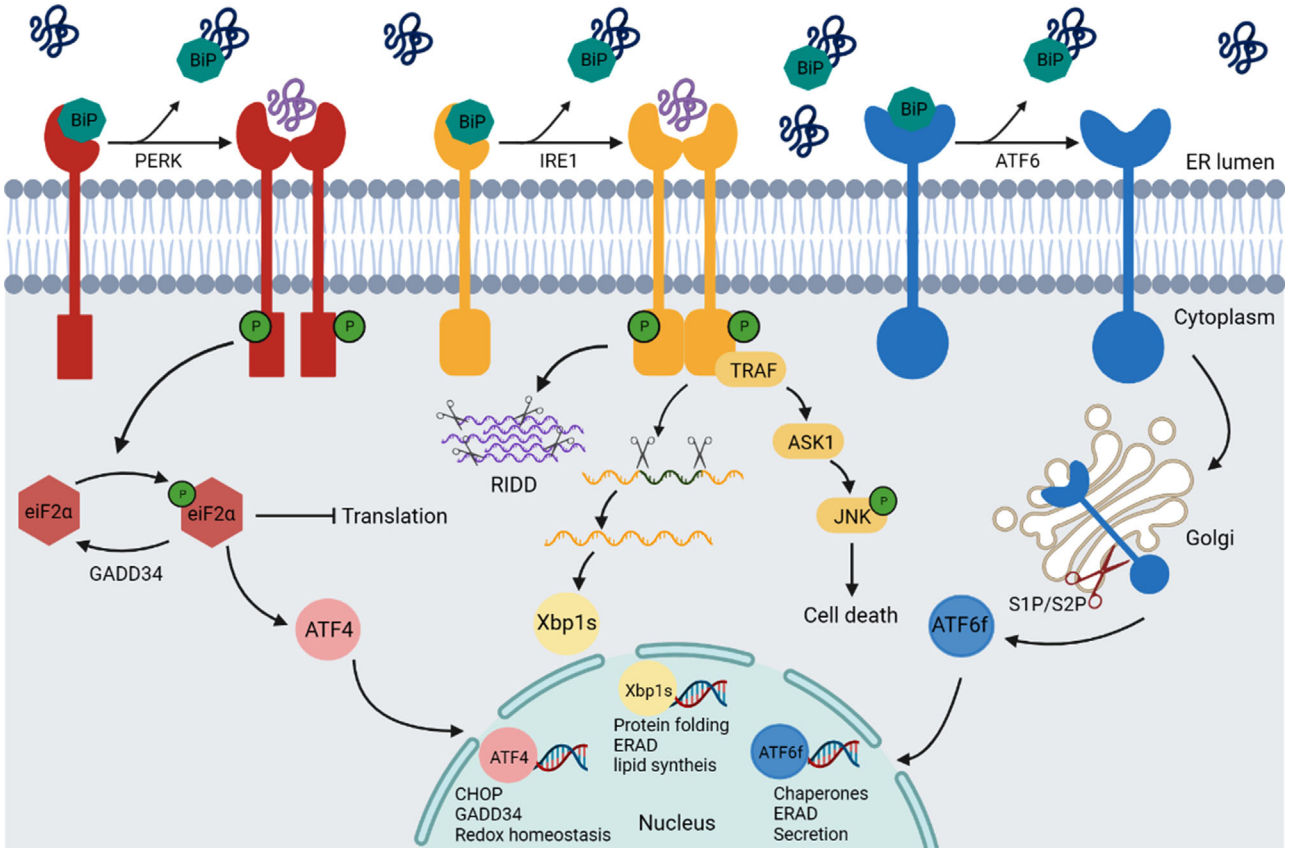

FIGURE 1 PERK, IRE1 and ATF6 activation are the initial steps of the three pathways of the UPR. After dimerization, PERK phosphorylates eIF2α, which in turn blocks global translation and increases the expression of ATF4. This transcription factor induces the expression of target genes, including CHOP and GADD34. The latter participates to a negative feedback loop that leads to eIF2α dephosphorylation. Upon activation, IRE1 induces the alternative splicing of Xbp1 mRNA and participates to general RNA degradation (Regulated IRE1-dependent decay-RIDD). IRE1 also signals through the recruitment of TRAF2 and activation of the ASK1-JNK cascade that induces apoptosis. Finally, ATF6 is exported from the ER to the Golgi complex where it is cleaved by proteases S1P and S2P, thus releasing its cytoplasmic domain, which is also a transcription factor. The UPR sensors' activation is inhibited by their binding to the ER luminal chaperon BiP that prevents their dimerization or translocation. Unfolded proteins compete with the sensors for BiP binding, therefore their accumulation results in dissociation of BIP-sensor complex and UPR activation. Moreover, PERK and IRE1 have been shown to be able to directly bind to unfolded proteins and this binding seems to impact on their activation, although this regulation remains to be validated in living cells. Figure created in Biorender.com

*Sc*IRE1 activation (Gardner & Walter, 2011; Kimata et al., 2007). Active *Sc*IRE1 promotes alternative splicing of Hac1 mRNA (Xbp1) leading to the production of the HAC1 transcription factor and induction of UPR target genes such as chaperones and degrading enzymes (Sidrauski & Walter, 1997).

The activation of the third sensor ATF6 is instead regulated by intramembrane proteolysis. Indeed, ATF6 contains two redundant Golgi localization signals in its luminal domain that are masked by the chaperone GRP78/BiP. Upon accumulation of misfolded proteins, ATF6 translocate to the Golgi apparatus, where it is cleaved by Site-1 and Site-2 proteases (S1P and S2P) resulting in the release of N-terminal cytosolic fragment commonly termed p50-ATF6 or ATF6f (Chen et al., 2002; Haze et al., 1999). The cleaved portion acts as a transcription factor to induce the expression of chaperon proteins such as GRP94 and BiP, as well as genes involved in protein secretion, protein degradation and lipid biosynthesis and ER expansion (Adachi et al., 2008; Maiuolo et al., 2011; Maruyama et al., 2013; Wu et al., 2007). Interestingly, it has been shown that ATF6 and Xbp1 transcriptional programs, despite being divergent, partially overlap. Indeed, simultaneous stress-independent activation of ATF6 and Xbp1s results in a specific transcriptional profile that is upregulated by the cooperation of these two transcription factors on gene activation (Shoulders et al., 2013).

Activation and modulation of the three branches of UPR has different activation kinetics and outcomes dependently on the experimental system, the type of stress, its duration and its intensity. Because of its key role in impacting on cell fate, the UPR needs to be fine-tuned, in order to switch its signalling transduction to an adaptive or pro-apoptotic program (Hetz & Papa, 2018; Jäger et al., 2012).

In addition, many studies uncovered that UPR has a pivotal function in modulating several aspects of cell physiology that go beyond control of ER proteostasis. Indeed, its signalling pathways are involved in regulating not only cellular metabolism, proliferation and differentiation but also cytoskeleton remodelling, DNA damage, organelles contact sites and Ca^{2+} signalling related cellular bioenergetics (Almanza et al., 2019; Carreras-Sureda et al., 2019; González-Quiroz et al., 2020; Hetz et al., 2020; Urra et al., 2018; van Vliet & Agostinis, 2017, 2018). For this reason, an aberrant regulation of the UPR pathways contributes to the pathogenesis of multiple diseases and potentiating or downregulating its pathways is increasingly recognized as potential targets for therapeutic strategies (Doultsinos et al., 2017; Hetz et al., 2013; M. Wang & Kaufman, 2016).

COMPUTATIONAL MODELS DESCRIBING THE UPR

Because the majority of the network components and of their interactions are long known, the building of a mathematical model that describes the complexity of UPR is an efficient tool to improve the analysis of such an intricate signalling network (Table 1). During the past 15 years, researchers have started developing mathematical models of UPR in order to better describe and analyze this complex network and explore its role also in very specific contexts such as in disease progression and drug resistance. While some models focus on a specific aspect of UPR activation, some others try to integrate all UPR signalling pathways and explore not only their mutual impacts but also their crosstalk with other cellular phenomena such as autophagy and cellular proliferation. Notably, because of the relative simpler UPR signalling pathway present in yeast, analysis and modelling of UPR in this organism allowed to better characterize some UPR-related aspects without taking into consideration the complexity of the crosstalks of all the three branches. These include not only a better characterization of UPR adaptation and recovery but also of the mechanisms involved in IRE1 activation.

In addition, because UPR plays a crucial role in the modulation of cell fate and because the regulation of a selective program driving the cell towards an adaptive or pro-apoptotic outcome has not been fully understood, the use of computational models greatly helped to better dissect the action of UPR and its components in this context.

Minimal models of UPR

Early models of UPR typically described the time evolution of a limited number of variables and focussed on some specific aspect of UPR activation. These models are classified below—quite arbitrarily—depending on the main question that was investigated.

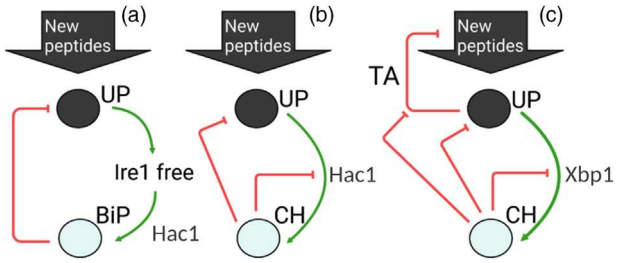

FIGURE 2 Minimal models of the UPR. (a) Model for yeast by Axelsen and Sneppen (2004). (b) Model for yeast and (c) model for mammalian cells by Trusina et al. (2008). Green arrows indicate positive regulation and red arrows negative regulation. The networks describe ER stress as an increase in Unfolded Proteins (UP), followed by activation of Ire1 and subsequent up-regulation of Hac1 or Xbp1 that promotes the expression of BiP or Chaperones (CH), which in turn decreases the amount of UP. The only differences in each network are that in A there is no inhibition of Hac1 by BiP and that in C, Translation Attenuation (TA) is considered.

Translational attenuation

Early models of UPR addressed the role of translational attenuation (TA) in conditions of relatively mild stress, excluding situations of unmitigated stress that overwhelms homeostatic outputs. To this end, Axelsen and Sneppen (2004) built a phenomenological model of 4 ordinary differential equations (ODE) describing IRE1 activation, subsequent *Hac1* splicing (the yeast homologue of Xbp1) and Hac1-promoted BIP expression (Figure 2a). Stress is simulated by an increase in the amount of unfolded proteins (UP), leading to an immediate re-equilibration of BiP between UP and IRE1. This sensor is assumed to activate upon BiP dissociation. The model reproduces the recovery towards nearly basal levels of active IRE1 and spliced Hac1 in the presence of stress due to the increased expression of BiP. The authors found that the key role in providing adaptation to stress is played by the degradation rate of spliced Hac1 mRNA, which needs to exceed the splicing rate and the unspliced degradation rate. This is necessary to maintain a pool of inactive mRNA allowing Hac1 to be highly responsive to changes in IRE1 activity (Axelsen & Sneppen, 2004).

Using a similar framework, Trusina et al. (2008) analyzed the interplay between chaperone synthesis and TA to reduce ER stress. As an additional component with respect to the previous model, these authors also considered additional molecules that increase the protein folding capacity of the ER such as chaperones, oxidoreductases or glycosylating enzymes—all gathered into the unique "Chaperones (CH)" variable (Figure 2b,c), CH being under the control of Xbp1s. Two versions of the model are considered, one for yeast (Figure 2b), as in

TABLE 1 Summary of the models for UPR

Reference model type	Characteristics	Relevance	Validation
Axelsen & Sneppen, 2004 *Minimal*	Simplified model of ODE's Describes IRE1 activation that increases the BiP/Kar2p production	The model shows that translational control is important for fast adaptation	Theoretical study whose predictions were not validated
Chakrabarti et al., 2016 *UPR and cell fate*	ODE model BiP-dependent activation of the three UPR sensors Division in three stages of UPR: adaptation, alarm and apoptosis.	The model proposes that signalling mediated by the three sensors compete for modulation of UPR. The result of this competition determines cells fate The model shows that the activity of the UPR sensors is controlled by BiP dynamics during the response	The model was validated by minimizing the difference between simulations and experimental measurements using multiobjective optimization
Cho et al., 2013 *UPR-related diseases*	Discrete dynamic model. Focus on PERK-eIF2alpha-ATF4 axis in the context of free fatty acids toxicity	The model suggests that palmitate dependent prolonged ATF4 upregulation is affected by the action of three different kinases in a time dependent manner Simulations show that AT4-dependent apoptosis of hepatocytes can be regulated by CREB1-dependent ATF4 upregulation	The model was validated by in vitro experiments
Cook et al., 2014 *UPR-related diseases*	ODE model describing UPR activation, ROS production, autophagy and cell proliferation.	The model uncovered a possible mechanism by which UPR plays a role in the sensitisation of breast cancer cells to fulvestrant treatment	The model was validated by making predictions that were subsequently tested in experiments
Curtu and Diedrichs, 2010 *Detailed model*	Detailed system of ODEs, including the three UPR sensors, network cross-talks and feedback loops.	This is the first model describing the three UPR sensors at a high level of molecular detail	This model was not simulated, nor validated
Dasgupta et al., 2020 *UPR-related diseases*	Model taking into account the three sensors of UPR Application of PID controllers It considers insulin signalling	The model predicts that ER stress reduction impacts positively on insulin sensitivity It validates p-AKT as a good target in high stress conditions, to boost insulin response.	Simulations were validated by in vivo experiments
Diedrichs et al., 2018 *Detailed model*	A detailed ODE model, based on Curtu and Diedrichs (2010), which makes the distinction between mRNA and protein evolutions	Parameters were fitted on observations in MEF treated by thapsigargin The model shows the importance of feedback loops in the regulation of UPR and its influence on cell fate	The study was validated by performing knockout experiments *in silico*
Erguler et al., 2013 *UPR and cell fate*	An ODE model that includes the three UPR sensors Based on bifurcation analysis, the model distinguishes between the phases of adaptation and apoptosis	The model predicts that sufficient elevation of BiP can suppress UPR signalling and prevent elevation of the pro-apoptotic CHOP It predicts oscillations if BiP quantity is too low to cope with unfolded proteins and if CHOP is mildly activated	The authors did not validate the predictions related to the different levels of stress.
Kapuy et al., 2013 *UPR and cell fate*	An ODE model that describes the Bcl2-Beclin1-Casps minimal network	The model predicts stress-controlled switch between autophagy and apoptosis, based on bistability	The model qualitatively reproduces previous experimental observations
Márton et al., 2017 *UPR and cell fate*	ODE model It considers IRE1 and PERK roles in modulating autophagy and apoptotic pathways.	The model sheds light on the influence of PERK and IRE1 in the shift from adaptive to apoptotic phase.	The model was developed based on their experimental results.
Parmar et al., 2013 *UPR-related diseases*	ODE model It considers the three UPR sensors It describes both autophagy and apoptosis	The model quantifies the relative importance of BiP in a crosstalk between UPR, autophagy and apoptosis in the context of resistance to anticancer treatment.	Theoretical study that was used to recapitulate experimental observations

(Continues)

TABLE 1 (Continued)

Reference model type	Characteristics	Relevance	Validation
Pincus et al., 2010 *Minimal*	ODE model Describes only the IRE1 branch	The model aims at testing the IRE1 activation at different stress levels (induced by DTT) It predicts that BiP-mediated IRE1 inhibition is important for the fine tuning and adaptation of UPR	The model was validated by experiments
Rutkowski et al., 2006 *UPR and cell fate*	ODE model that describes the relation of BiP with the ATF4-CHOP-GADD34 axis	The model allows to analyse the effect of the degradation rates of the simulated mRNA's and proteins on cell-fate decisions	The model was developed based on their experimental results
Schnell, 2009 *Minimal*	ODE model based on Michaelis-Menten kinetics to describe the fate of unfolded proteins It is considered that UP can be folded, converted to misfolded proteins or degraded via ERAD	The model shows that increasing the folding capacity of the ER and ERAD simultaneously could be a good strategy to reduce misfolded protein accumulation and impact on pathogenesis of diseases	Theoretical study whose predictions were not validated
Stroberg et al., 2018 *Minimal*	ODE system accounting for the action of unfolded proteins and chaperones on sensors' activation, with specific stress-sensing mechanism. Model development is based on multi-objective optimization	The model shows that chaperone-mediated sensor activation and inactivation is most efficient in modulating UPR	The model was parameterised with values from the literature.
Stroberg et al., 2019 *Minimal*	Model consisting of a deterministic module accounting for chaperone-assisted protein folding, and a stochastic module accounting for the stress-sensing proteins	Simulations show that combination of chaperone- and unfolded protein-mediated stress-sensing mechanisms allows for the fine-tuning of the UPR by stress level	Theoretical study whose predictions were not validated
Trusina et al., 2008 *Minimal*	ODE model in which UPR activation level is described by the amount of chaperones It only considers the adaptive phase The stress is delivered in pulses	The model shows that translation attenuation is crucial only when the number of unfolded proteins to be dealt with is large	Theoretical study whose predictions were not validated
Trusina & Tang, 2010 *Minimal*	Extension of their previous model considering different types of stress pulses	The model shows that the role of translation attenuation (TA) is more important for translational than for chemical stress It predicts that the need for TA is dictated by the pulse duration.	Theoretical study whose predictions were not validated
Wiseman et al., 2007 *Minimal*	Michaelis-Menten based ODE model It describes protein export	The model reveals that export efficiency is strongly influenced by folding and unfolding energetics	Theoretical study whose predictions were not validated
Yang et al., 2020 *UPR and cell fate*	ODE model to explore the kinetics of transcription factors in response to Tunicamycin It is an extension of the models of Trusina et al. including ATF6 branch	The model shows that ATF6 participates in the tuning of CHOP dynamics at early UPR activation, while it is more under the influence of ATF4 later in time.	The model was validated by doing knockout experiments

Axelsen and Sneppen (2004), and one for metazoans that includes the PERK pathway (Figure 2c). In contrast with the previous study of Axelsen and Sneppen (2004), in Trusina et al.'s (2008) model, UPR sensors become active upon binding of UP (and not BiP unbinding). Through simulations, the authors showed that translational attenuation is crucial when the amount of unfolded proteins to be dealt with is large, a condition encountered in secretory cells, whereas if it is small, it is not necessary. In addition, the authors carried out simulations

considering stress pulses, which are observed in insulin-secreting pancreatic β-cells, allowing them to predict that translational attenuation is important to avoid a non-desirable chaperone overload between pulses (Trusina et al., 2008). The model has been later expanded to assess the effects of chemical vs translational stress. Chemical stress is modelled as a direct source of UP, while translational stress is described as an increase in the rate of UP influx. In consequence, attenuation by the PERK pathway acts at different levels for the two types of stress. The model showed that the role of translational attenuation is greater under the conditions of translational stress, where both the amount of unfolded proteins and the amount of chaperones are minimized as compared to chemical stresses. Moreover, it also predicted that the need for translational attenuation is dictated by the pulse duration: when the time between two pulses is shorter than the half-life of the chaperones, translational attenuation only affects the first pulse. This can be explained by the fact that residual chaperones produced in response to the first pulse are still active to deal with subsequent stress pulses (Trusina & Tang, 2010).

Homeostasis of protein folding and export

Together with the reduction in mRNA translation and increase in protein folding ability, UPR alters the cell regulation of protein export. To investigate the interplay between ER assisted folding and the ER associated degradation (ERAD) pathways that are controlled by UPR, Wiseman et al. (2007) developed a computational model describing protein import, folding, degradation and export into the cytoplasm, using the Michaelis-Menten formalism. The model does not account for UPR activation but simply considers the activation of the ERAD pathway at high levels of unfolded proteins. Because the model explored the influence of protein folding on rates of protein degradation and protein export, it provides a useful complement to models of UPR. The model highlighted that export efficiency is strongly influenced by folding and unfolding energetics, which they control by varying kinetic parameters, as well as by cell-specific features such as their protein folding capacity (Wiseman et al., 2007).

A similar point of view was used to describe how UPR activation regulates the amount of misfolded proteins inside the lumen in the case of pancreatic β-cells, where this feature is particularly important because of their high secretory demand (Schnell, 2009). This simple, 2-variable model describes the evolution of unfolded and misfolded proteins, which can enzymatically transform into properly folded proteins or be degraded. The model does not incorporate the activation of the UPR transducers, but it contains Michaelis-Menten terms describing the fate of proteins entering the ER: either their successful folding (ER-associated folding-ERAF) or their conversion to misfolded proteins and degradation via ERAD. The model predicts that increasing the folding capacity of the ER reduces misfolded protein accumulation, explaining the effectiveness of the treatment with chemical chaperones of mouse models of diabetes. Moreover, misfolded proteins accumulation is highly sensitive to their degradation rate via ERAD. This suggests that improving ERAD and ERAF simultaneously could represent a strategy for targeting misfolded proteins diseases such as diabetes (Schnell, 2009).

Role of BiP in IRE1 activation

Among the chaperones that assist protein folding, BiP is of particular importance as it also plays an important role in the activation of the UPR sensors (Bertolotti et al., 2000; Liu et al., 2000). However, its exact contribution in UPR activation is not fully elucidated. At rest, the luminal domain of IRE1 are in complex with BiP, which represses IRE1 autoactivation. Upon conditions of stress, the new partitioning of unfolded proteins between BiP and IRE1 shifts the equilibrium away from IRE1, which allows for activation of this branch of UPR. This classical "BiP unbinding hypothesis" was considered in the model of Axelsen and Sneppen (2004) discussed here above. An alternative hypothesis, considered in the model of Trusina et al. (2008), poses that the main role is played by the binding of unfolded proteins to IRE1's luminal domain, which stabilizes IRE1 dimers and thereby favours downstream signalling. Because of the competition between BiP and IRE1 for binding of unfolded protein, BiP also plays an important role in this scenario. To discriminate between these two mechanisms, Pincus et al. (2010) set out to investigate the role of BiP in IRE1 activity in yeast by carrying out both experiments and computational simulations. The 14-variable model provides a careful description of the interplay between BiP, unfolded proteins and IRE1 dynamics. The model considers that the IRE1 molecules detect stress by being both sequestered by free BiP and activated by free unfolded proteins. It predicted that IRE1 deactivation dynamics, once stress is removed, is delayed if IRE1/BiP interactions are removed from the model. This prediction was confirmed by experiments comparing wild type IRE1 and IRE1 mutant lacking the BiP binding site ($IRE1^{bipless}$). Wild type IRE1 was indeed inactivated after 60 min, while $IRE1^{bipless}$ activity lasted for 120 min. The model allowed to conclude that while BiP release from IRE1 does not play a major role in UPR activation, this chaperon controls IRE1 deactivation once the stress has been relieved. Moreover, it enlightens a buffering role of the IRE1/Bip complexes. Upon small fluctuations of the protein load, these complexes would provide a source of chaperon without triggering IRE1 dimerization that requires a higher level of unfolded proteins (Pincus

et al., 2010). An interesting discussion of this study can be found in Onn and Ron (2010).

In line with this work, a minimal model aiming to determinate the optimal design for ER stress-sensor network demonstrated that sensing stress through modulation of free chaperone concentration rather than through binding of the sensors to unfolded proteins, allowed a better response during acute stress induction and a faster deactivation in case of stress removal. In addition, an hypothesis combining both sensor activation through unfolded proteins binding and chaperone unbinding does not improve the efficiency of the response (Strberg et al., 2018). In order to better understand why this hybrid sensing model evolved for IRE1 and PERK sensors in cells and which one is the best in monitoring unfolded proteins accumulation in the ER, the authors further developed a minimal model containing BiP-titration and stochastic unfolded protein binding. The model showed that while direct unfolded protein binding provides more information in sensing their accumulation in the ER lumen, chaperone-mediated sensing is more precise in measuring available chaperone concentration. However, an activation pathway considering both mechanisms of stress sensing is more informative about ER state and cell ability to respond to stress, allowing a better fine-tuning of UPR (Stroberg et al., 2019).

Detailed models of UPR

Among the first models of the UPR that have been developed, the most detailed one accounting for network crosstalks and feedback loops has been proposed by Curtu and Diedrichs (2010). In their first work the authors focused on the mass action law-based derivation of ODEs describing the signalling pathways of PERK, IRE1 and ATF6 and did not discuss any calibration or validation. Instead, they focused on the establishment of a coherent kinetic scheme of the three UPR branches and of their multiple crosstalks. A more recent work used this model as a starting point and further modified it by taking additional regulations into account and parameterizing it by fitting simulations results to experimental data (Diedrichs et al., 2018). Because the model focusses on the long-term signalling by UPR (up to 3 days), activation of the sensors is described by quasi-equilibrium relations. Time evolutions are described for unfolded proteins, BiP, eIF2α, ATF4, ATF6, CHOP and GADD34. For most of these molecules, the model discriminates between mRNA and protein levels, which allows to distinguish between transcriptional and translational regulations. Optimization of parameters was performed using kinetic data of CHOP mRNA, GADD34 mRNA and BiP mRNA in mouse embryonic fibroblasts treated with 2.5 or 10 nM thapsigargin for 8 h. The model was then validated by performing in silico knockouts of UPR components (PERK, IRE1, ATF6, and ATF4) and confronting them with experimental results. Interestingly, the model suggests that the main role of the eIF2α- mediated translational stimulation of CHOP and GADD34 is to allow for a faster eIF2α dephosphorylation, which reveals the existence of an autoregulatory negative feedback loop. In addition, the model predicts that signalling crosstalks between pro-survival and pro-apoptotic components are key for the balance between adaptive or apoptotic cellular response (Diedrichs et al., 2018).

UPR MODELS THAT STUDY CELL FATE UNDER ER STRESS

Mathematical models of UPR have been particularly useful in exploring ER stress control of cell fate and the decision mechanisms modulating the switch between adaptive and maladaptive responses.

It has been shown that adaptation to ER stress is not a result of selective activation of a specific UPR pathway. On the contrary, this is a result of differential stabilities of mRNA and proteins involved in cell fate decision (Rutkowski et al., 2006). With the help of a mathematical model considering the expression patterns of BiP and the ATF4-CHOP-GADD34 axis, Rutkowski and colleagues showed by modulating their degradation rates that differential instability at mRNA and protein level participates to promote a more adaptive response in cases of mild and transient ER stress. Indeed, in these conditions, expression levels of BiP are maintained longer in time compared to the protein level of the pro-apoptotic ATF4-CHOP-GADD34 axis. The model predicts that if one or more components of the pro-apoptotic axis had the same stability as BiP, cell death would be favoured even at low UPR activation (Rutkowski et al., 2006).

In line with the better characterization of the adaptive phase of UPR, a recent model (Yang et al., 2020) tried to mechanistically understand the complexity of UPR and especially the activity of transcription factors during the adaptive phase. This model, in the form of a set of ODEs, aims at exploring the kinetics of transcription factors in response to Tunicamycin, a drug that induces ER stress by blocking N-linked glycosylation. The model is a modified version of Trusina et al. (2008) in which they included equations describing the ATF6 branch explicitly. In addition, the authors introduced a pharmacokinetic module in the form of a threshold function of the effective intracellular concentration of the drug that can activate UPR. This model was able to show that ATF6 participates in the modulation of CHOP dynamics at early time points, while the latter is more under the control of ATF4 later in time (Yang et al., 2020).

By resorting to a bifurcation analysis, another model explored the UPR signalling responses to different levels and duration of stress (Erguler et al., 2013). Depending on the stress level, three behaviours are identified.

The first one corresponds to a low-activity state, which is characterised by the ability to improve folding capacity by increasing BiP. An interesting prediction is that sufficient elevation of BiP can suppress UPR signalling and prevent the increase of the pro-apoptotic transcription factor CHOP. On the contrary, in the second intermediate state, the amount of BiP is not sufficient to cope with unfolded proteins and CHOP, although activated, does not reach its maximal limit. Notably, the model predicts that several UPR components exhibit oscillations in this intermediate state. The high activity state is characterized by high levels of CHOP and a virtually arrested translation. However, the model has not been entrained on experimental data and the predictions obtained by the model for different levels of stress were not validated (Erguler et al., 2013).

Different conclusions were reached by another model exploring UPR-dependent cell fate decisions. The model describes a BiP concentration-dependent activation of the UPR, as well as the three stages of the UPR: adaptation, alarm and apoptosis. The model predicts that the overall outcome of the UPR then results from the simultaneous triggering of, and competition between the signalling pathways mediated by the three UPR sensors. IRE1 and PERK are more important at the beginning, while the role of ATF6 becomes predominant at a later stage. The model thus concluded that the activity of the UPR sensors appears to be controlled by BiP dynamics during the UPR (Chakrabarti et al., 2016).

Both the IRE1 and the PERK pathway participate in the induction of autophagy during ER stress, a situation that is generally considered as beneficial for cellular survival and that occurs prior to apoptosis induction (Ogata et al., 2006). The autophagy-apoptosis crosstalk plays an important role in driving cell fate decisions during ER stress induction. Computational modelling allowed to understand how autophagy modulates the switch to an apoptotic cell death during stress conditions, by impacting on the threshold for apoptosis activation (Kapuy et al., 2013; Holczer et al., 2015). The crosstalk between these two phenomena is known to be modulated by the IRE1 and the PERK pathways in conditions of ER stress. More recently, the development of an additional computational model helped to reveal the existence of a positive feedback loop between IRE1 and PERK at high stress levels, which leads to irreversible programmed cell death induction. In support of experimental results, the model shows that after acute ER stress induction, the cells can be either in a state of autophagy-dependent survival (more dependent on IRE1) or in a state of apoptotic cell death (more dependent on PERK). This bistable behaviour relies on a double negative feedback. The switch between the two states is regulated by pro-apoptotic inducers that are initially active but that become rapidly downregulated by pro-survival mechanisms. The model confirmed that reduced activity of PERK promoted pro-survival program, increasing the stress threshold that needs to be reached for the cell death induction. The opposite happens for IRE1 inactivation, leading to an apoptosis activation for lower levels of stress (Márton et al., 2017).

MODELS USED TO ANALYSE UPR-RELATED DISEASES

Since the UPR has been shown to be involved in several diseases, the development of mathematical models is important for investigating the contribution of these signalling networks in the context of pathogenesis and drug resistance. In this context, it is well known that breast cancer is treated with anti-oestrogen drugs to which most patients develop resistance. It has been demonstrated that in breast cancer BiP plays a crucial role in the development of drug-resistance. Through mathematical modelling, Parmar et al. (2013) coupled UPR, autophagy and apoptosis, known to play crucial roles in cell fate decision in response to different drugs with the aim of testing whether this is sufficient to reproduce the observations on the role of BiP in anti-oestrogen resistance. The model helped characterise the relative importance of each pathway and demonstrated that the interplay between UPR, autophagy and apoptosis is sufficient to simulate previous experimental findings (Parmar et al., 2013).

In addition, it has been shown that Estrogen Receptor α (ERα) inhibitor fulvestrant (ICI) is able to induce UPR activation and stimulate autophagy. Knockdown of ERα increased reactive oxygen species (ROS) production and cell death induction after treatment with ICI, suggesting a beneficial role for the combination of anti-estrogen therapy with UPR and autophagy inhibitors for the treatment of ER^+ breast cancer patients. A mathematical model, based on the experimental results, combined equations describing UPR activation, ROS production, autophagy and cell proliferation. The model helped to explain how ICI negatively affects cell proliferation in conditions in which the inhibitor's target (ERα) is depleted. ICI helps to sensitize cells to anti-estrogen therapy by reducing the UPR-induced antioxidant response, thereby allowing ROS accumulation. Although, ERα knockdown and ICI are able to stimulate pro-survival autophagy, this response does not seem to be sufficient to overcome the apoptotic response due to ROS accumulation. These data suggest that UPR and autophagy inhibitors could be beneficial for treatment of ER^+ breast cancer patients (Cook et al., 2014). Human obesity and non-alcoholic fatty liver disease (NAFLD) are characterized by chronic activation of UPR, high levels of free fatty acids, inflammation, and insulin resistance (Baiceanu et al., 2016). Researchers hypothesized that high ER stress could contribute to reduce insulin sensitivity in obese models and that this could be induced by the action of functional peptide catestatin

(CST). To test this hypothesis, Dasgupta et al. (2020) developed an extensive model based on state transition equations, integrating the key steps of the activation of the three pathways of UPR and insulin signalling. They applied two Proportional-Integral-Derivative (PID) controllers targeting either UPR activation (in the form of active PERK) or key components of the insulin response (such as insulin receptor activation or insulin receptor substrate levels) and set them to a high or low value. The model predicted that at low levels of active PERK along with increased phosphorylated levels of AKT, this leads to increased insulin receptor activation and modification of its substrate, all signs of enhanced insulin sensitivity. Therefore, inhibition of ER stress by means of CST peptide could result in increased responsiveness to insulin, suggesting that targeting ER stress induction in obese patients could be a strategy to overcome the insulin resistance phenotype observed in these cases (Dasgupta et al., 2020). In this context, Cho et al. (2013) used computational modelling to investigate free fatty acids toxicity. This work focuses only on a small part of the UPR induction pathway, and more precisely on the eIF2α-ATF4 axis. The study aims at elucidating the molecular mechanisms responsible for ATF4-dependent cell death in hepatocytes caused by free fatty acids toxicity. Palmitate treatment of HepG2 cells results in a transient increase in phosphorylated eIF2α and in a prolonged upregulation of ATF4 protein levels. By building a mathematical model consisting of discrete variables, the authors showed that palmitate-dependent prolonged ATF4 upregulation is affected by three different proteins in a time-dependent manner (PKR, PERK and PKA, successively). Moreover, the binding of p-CREB on the *Atf4* promoter, but also that of ATF4 itself, play a significant role in the palmitate-dependent ATF4 upregulation (Cho et al., 2013).

CONCLUSIONS

Computational models provide an excellent tool to explore the regulatory complexity of signalling pathways, as well as their contribution and impact on cellular processes and human pathophysiology. The mathematical models addressed in this review contributed to explore and test in silico many aspects of the UPR and its consequences on cell physiology. This network of signalling pathways can be modulated in several ways and the means that lead to alteration to normal proteostasis can be multiple, from the modification of protein glycosylation to changes in ER oxidizing environment. One of the best known is the alteration of the luminal ER Ca^{2+} concentration, which impacts on the activity of ER protein chaperones and foldases. Surprisingly, existing mathematical models of UPR do not much explore the quantitative relationship between UPR and stress induction, especially when it comes to Ca^{2+} homeostasis alteration. We therefore think there is a need for a better understanding of this aspect and that computational modelling could be beneficial to this aim.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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6 ER stress and bacterial infections

Several reports highlight the role of the UPR in infectious diseases and regulation of the immune system, extensively explored in the case of viral infections (reviewed in S. Li, Kong, & Yu, 2015; Prasad & Greber, 2021). Viral infections elicit ER stress due to the increased demand of protein production and protein folding. UPR induction, in turn, plays an important role in promotion of host immune defense. However, UPR activated by host cells as defense mechanisms may also be exploited by viruses. Many studies have demonstrated how viruses are able to promote or blunt specific UPR pathways in order to promote cell survival and viral life cycle and dampen pro-apoptotic signaling to ensure productive infection.

In contrast, findings exploring relationships between ER stress and bacterial pathogens are more limited. While the ER is expected to serve as a welcoming and nutrient-rich organelle lacking antimicrobial abilities, only few studies reported that bacterial pathogens exploit the ER to survive and multiply within cells. Some bacterial species such as *Legionella, Chlamydia* and *Brucella* can reside and multiply intracellularly within vacuoles. These bacteria-containing vacuoles are in close interaction with the ER, allowing for a permissive proliferation environment that involves impairment of vesicle trafficking and secretory pathways. These conditions could promote ER stress induction and elicit UPR response.

The fact that only a few species exploit the ER for their infection cycle raises the question whether the ER is actually a replication permissive organelle for many bacterial species. This suggests that during infection, bacteria must confront consequences of ER alteration leading to UPR induction (Celli and Tsolis 2015). Indeed, as in the case of viruses, UPR activation during bacterial infection triggers an immune response and inflammation.

It is nowadays clear that UPR is crucial for highly secretory cells such as immune cells to cope with high protein folding demand elicited during inflammation. Moreover, it has been demonstrated that UPR components are fundamental for immune cells differentiation and signaling. In this context, it has been shown that IRE1 and XBP1 play a role in the maturation of B cells and plasma cells maturation (Reimold et al. 2001; Zhang et al. 2005). In addition, XBP1 signaling plays a role in maturation of CD8+ T cell (Kamimura and Bevan 2008), dendritic cell function (Osorio et al. 2014) and pro-inflammatory signaling in macrophages (Martinon et al. 2010). In macrophages, modulation of PERK pathways is crucial to promote cell survival during immune response (Woo et al. 2009).

ER stress is involved in several chronic pathological conditions that involve inflammation such as inflammatory bowel disease, diabetes or cancer (Grootjans et al. 2016). Researchers have shown the existence of a reciprocal regulation between ER stress and inflammation: pro-inflammatory stimuli such as ROS, TLR ligands and cytokines can trigger ER stress, while ER stress can induce inflammatory pathways. Indeed, all three branches of UPR are involved in potentiating NF-κB signaling by maintaining basal IKK activity and downregulating its degradation or via modulation of the Akt pathway (Tam et al. 2012; Yamazaki et al. 2009).

Also, cytokine production can be regulated by UPR branches (Meares et al. 2014). As previously mentioned, activated IRE1 can form a complex with TRAF2, activating JNK upstream of the AP-1 transcription factor, which induces expression of proinflammatory cytokines (Khalaf et al., 2010; Urano et al., 2000). In addition, XBP1 is responsible for transcription of several cytokines such as IL-6 and IFN-β (Martinon et al. 2010).

Promotion of inflammation by UPR pathways provides a positive feedback loop for the intensification of pathological conditions, revealing a negative role of UPR in exacerbating various diseases (Grootjans et al. 2016). Inflammatory conditions and apoptosis induction during host infection could contribute to further infected tissue destruction and disease progression.

Due to its role in host cell survival and the inflammatory response, ER stress is a common feature of many bacterial infections (Alshareef et al. 2021). ER stress can arise from the action of virulence factors that bacteria secrete into the host cell which could directly interfere with the ER and its secretory processes. Alternatively, ER stress can be indirectly induced when essential nutrients and cofactors necessary for proper ER function become depleted. An example is given by intracellular bacterial pathogens that have adapted to survive within a eukaryotic host and depend on the host cell nutriment supply, consequently leading to the activation of ER stress (Shames 2023). Bacteria have evolved strategies to regulate ER stress and UPR activation in order to ensure their survival, persistence and proliferation. On the other hand, UPR activation and subsequent apoptosis induction or promotion of inflammatory response counteracts pathogenesis of bacterial infections, suggesting that this particular signaling network plays a dual role in this context (Figure 9) (Alshareef, Hartland, and McCaffrey 2021).

6.1 UPR promotes bacterial infections

Many works indicate that UPR could be beneficial for intracellular pathogens during infection of host cells. UPR has been shown to promote life cycle of bacteria belonging to *Brucella* spp. These bacteria infect phagocytic cells, where they are included in a vacuole (BCV) that modify ER structure and induce UPR activation (J. A. Smith et al. 2013). ER stress induction during *Brucella* infection has been shown to be dependent on bacterial effectors injected into the host cells that act on the secretory pathway (Myeni et al. 2013). Enhancement of ER stress by tunicamycin promoted bacterial intracellular replication while its inhibition by chemical chaperone Tauroursodeoxycholic acid (TUDCA) resulted in a marked reduction of bacterial replication (J. A. Smith et al. 2013). *Brucella* replicates less efficiently in IRE1 but not in PERK and ATF6 knockdown cells (Qin et al. 2008). This could be due to the impairment of IRE1-dependent autophagic vacuole biogenesis (Ogata et al. 2006), which is a required step for *Brucella* infectious cycle. Indeed, inactivation of host autophagy pathways dramatically impaired *Brucella* replication (Pandey et al. 2018). In addition, IRE1 via RIDD process participates in dysregulation of endosomal trafficking and lysosomal fusion, thereby increasing susceptibility to *Brucella* infections (Wells et al. 2022).

On the same line, ER stress and UPR activation are also induced during infection of alveolar macrophages by *Mycobacterium tuberculosis*. Researchers demonstrated that *M. tuberculosis* dampens eIF2α phosphorylation and subsequently CHOP expression in order to downregulate pro-apoptotic signaling and promote cell survival for productive cellular infection (Lim et al. 2011). Induction of the three UPR pathways is required by *Chlamydia* spp. to boost glucose uptake, energy production, lipid synthesis and nutrient production during host cell infection. Moreover, inhibition of IRE1 and PERK resulted in decreased infection ability of epithelial cells and reduction in *Chlamydia* intracellular replication (George et al. 2017). *Chlamydia* activates UPR sensors by secretion of effector proteins that bind non-muscle myosin heavy chain II (NMMHC-II) (George et al. 2019).

UPR promotes intracellular replication also in the case of *Salmonella* via activation of XBP1 and ATF6. Induction of ER stress and activation of UPR leads to phospholipid synthesis. Phospholipid remodeling seems to be crucial to promote *Salmonella* intracellular replication (Antoniou et al. 2019). ER stress is caused either by accumulation of misfolded Human Leukocyte Antigen B27 (Antoniou et al. 2019) or by bacterial-induced degradation of BiP co-chaperone Erdj3 (Bernal-Bayard et al. 2010).

In the case of *Shigella dysenteriae*, it has been demonstrated that Shiga-toxin1 activates PERK and IRE1 pathways and induces a pro-apoptotic program in macrophages. However, unlike in undifferentiated monocytic cells, Shiga-toxin1 also upregulates the anti-apoptotic factor Bcl-2, leading to delayed apoptosis and longer cell survival for intracellular replication (M.-S. Lee et al. 2009).

During *Helicobacter pylori* infection, activation of the PERK-ATF4-CHOP signalling pathway induces autophagy, thereby reducing apoptosis of infected cells (Halder et al. 2015).

These works suggest that UPR activation could play a role in promoting successful bacterial infection of host cells.

6.2 UPR is protective against bacterial infections

Many bacterial toxins assemble on the host cell membranes and form pores that permeabilize cells to metabolites and proteins therefore activating stress responses and UPR (Alshareef, Hartland, and McCaffrey 2021). *In vivo*, the activation of the UPR provides protection against pore-forming toxins, as demonstrated by increased sensitivity to toxins in animals lacking either the IRE1-XBP1 or the ATF6 branches of the UPR (Bischof et al. 2008). In addition, the *Listeria monocytogenes* listeriolysin O toxin induces UPR (possibly via intracellular Ca^{2+} stores depletion), cellular apoptosis and limits bacterial infection: indeed, stressed cells are more resistant to bacterial infection than control (Gekara et al., 2008; Pillich et al., 2012). Subtilase cytotoxin (SubAB) is a toxin produced by Shiga toxigenic *Escherichia coli* (STEC) whose catalytic A-subunit has been shown to cleave BiP chaperone and induce all the three branches of UPR as well as activation of a pro-inflammatory response and cytotoxicity (Paton et al. 2006; Wolfson et al. 2008; Zhao et al. 2011). Shiga-toxin1 production by *Shigella dysenteriae* induces strong UPR activation in monocytic cells and rapid apoptotic cell death by increasing CHOP expression, DR5-TRAIL signaling and decreasing Bcl-2 expression. This response is different from the one induced in more mature macrophages, suggesting that Shiga-toxin1- dependent response and apoptosis regulation differs according to cell maturation (S.-Y. Lee et al. 2008).

Activation of pro-inflammatory responses by UPR pathways plays a crucial role to counteract bacterial infections. UPR-dependent inflammation is induced during *Brucella* infection of mice and contribute to mount host response to infection. This response is triggered by NOD1 and NOD2-mediated IRE1 activation and subsequent induction of NF-κB and IL-6 production. These events

are particularly involved in induction of acute placentitis and decreased pup viability in pregnant mouse model (Keestra-Gounder et al. 2016). Similarly, ER stress induction following *M. tuberculosis* infection results in activation of pro-apoptotic program that limits bacterial spreading in tissues (Cui et al. 2016). Mechanistically, this happens through increase in cytosolic Ca^{2+} concentration and ROS production (H.-H. Choi et al. 2010; J.-A. Choi et al. 2013). However, more recent research proposed that induction of pro-apoptotic program in granulomas at later stages of infection could be a strategy developed by *M. tuberculosis* bacteria to favor their dissemination in the infected tissue (Grover et al. 2018).

The UPR-dependent up-regulation of inflammatory response also limits the bacterial burden of mice infected with *Chlamydia*. Animals treated with the chemical chaperone TUDCA to inhibit ER stress show higher bacterial infection. This occurs by UPR-dependent regulation of the NOD1 and NOD2- induced proinflammatory pathways (Pham et al. 2020). Similarly, it has been shown that IRE1 activation downstream of TL-R4 signaling is required for interferon-β transcription following PKR activation, which might be due to modified host RNA species following IRE1 RNAse activation (Webster et al. 2016).

UPR signaling is in some cases blocked by bacterial invasive species to avoid enhanced pro-inflammatory cytokine signaling and promote bacterial intracellular survival. Effector-dependent inhibition of *Xbp1* splicing and XBP1 transcription factor production occurs during *Legionella* infection of macrophages with a process that requires translation inhibition (Hempstead and Isberg 2015; Treacy-Abarca and Mukherjee 2015). Interestingly, cells infected with heat-killed *Legionella* induce *Xbp1* splicing, but this does not occur with living bacteria (Hempstead and Isberg 2015). In addition, it has been proposed that *Legionella*-induced translation inhibition is responsible for suppression of BiP and CHOP translation despite their transcriptional up-regulation during infection downstream of ATF6 signaling (Treacy-Abarca and Mukherjee 2015). The intracellular chlamydial pathogen *Simkania negevensis* induces the UPR response but down-regulates it at later stages of infection to promote massive intracellular proliferation and block UPR-induced apoptosis (Mehlitz et al. 2014). In addition, a more recent study reported a decreased transcriptional and protein levels of CHOP during infection of epithelial colonic cells by *Enteropathogenic E. coli* (EPEC). Mechanistically, the secreted EPEC effector NleE interacts with ZPR1 protein and affects its action on transcriptional regulation. The authors speculate that suppression UPR during EPEC infection could be a strategy for the bacteria to escape from host defense, as production of IL-8 is upregulated by CHOP and downregulated by NleE (Ouyang et al. 2023).

Figure 9 summarizes the works regarding the dual role of UPR during bacterial infections. Our knowledge about the crosstalk between ER, intracellular bacteria and pathogenic conditions is far from being complete and needs further investigation.

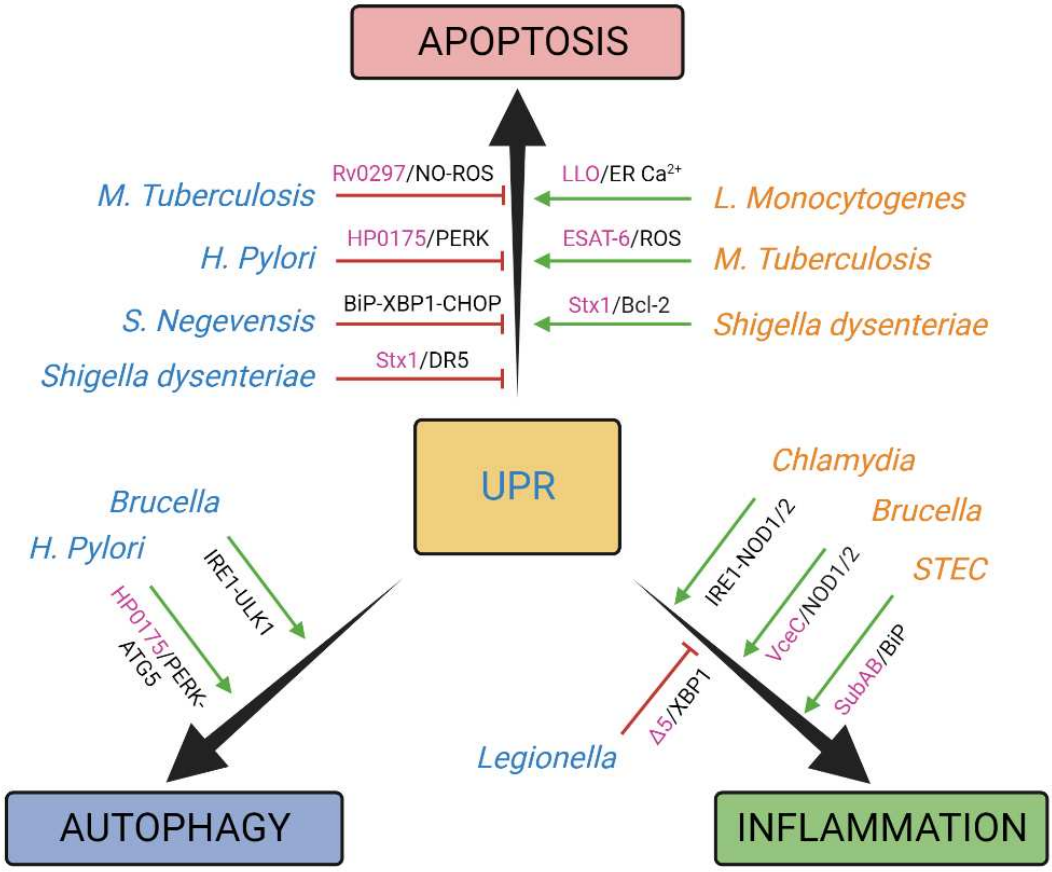

Figure 9. Co-opting of UPR by bacterial species. UPR has been shown to play a dual role in the control of bacterial infections. It may promote bacterial infection or restrict it via the induction of inflammatory program and activation of immune system. UPR also induces pro-autophagic and pro-apoptotic programs regulating infection. Induction or inhibition of a specific UPR-induced cellular response can be either beneficial (blue) or detrimental (orange) for bacterial infection cycle and disease progression. In some cases, the effects on UPR response can be opposite depending on the stage of infection or the cell type. These effects are induced by identified bacterial effectors (purple) that elicit specific cellular signaling pathways (black). STEC: Shiga toxigenic *E coli*; Stx1: Shiga toxin 1; LLO: Lysteriolysin O; Δ5: mutant for Lgt1–3, SidI, Sid; ROS: Reactive Oxygen Species; NO: Nitric Oxide; SubAB: subtilase cytotoxin.

7 *Shigella* pathogens

7.1 Shigellosis

Shigella species are intracellular pathogens and etiological agents of bacillary dysentery (shigellosis), a disease caused by an infection of the colon that causes watery diarrhea containing blood and mucus. Other clinical symptoms include fever and abdominal pain. *Shigella* was the second leading cause of diarrheal mortality in 2016 accounting for more than 200 thousand deaths worldwide (Khalil et al. 2018). However, these estimates are substantially reduced compared to numbers registered in previous decades due, among other things, to reduction in malnutrition and improvement in therapies (Karen L Kotloff et al. 2018). The great majority of mortality and morbidity occurs in children under the age of 5. While adults often experience swift recovery, elderly individuals may face the possibility of developing severe illness (Raso et al. 2023). According to World Health Organization, it is estimated that *Shigella* causes about 270 million diarrheal episodes annually among all ages (WHO 2023).

Shigella bacteria invade and proliferate within the colonic mucosa, leading to a vigorous inflammatory response. This process ultimately culminates in the destruction of infected tissues, however rarely followed by a systemic infection.

Shigellosis mostly occurs in low socioeconomic countries, especially in south Asia and Africa with a strong correlation with poverty, malnutrition, poor water supply and sanitation (Nisa et al. 2020) (Figure 10). Incident rates in higher income countries are very low: the Annual Epidemiological Report for 2020 stated a notification rate of 0.7 per 100 thousand population in countries participating to European Economical Agreement (ECDC 2023).

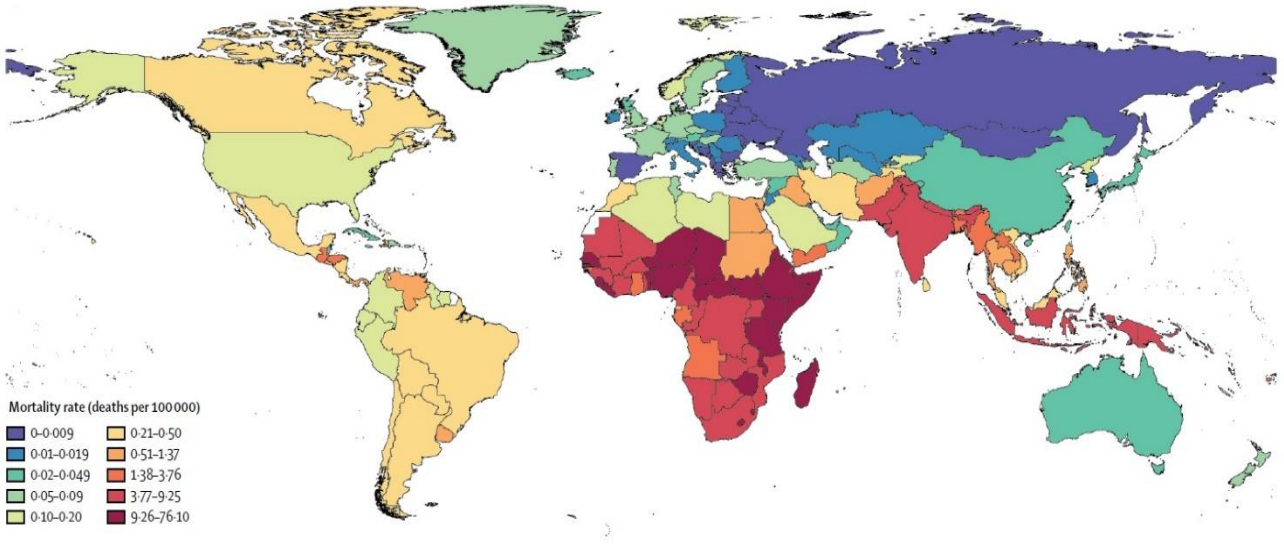

Figure 10. *Shigella* mortality rate. *Shigella* is one of the main causes of diarrheal mortality worldwide, especially in developing countries. Map reports mortality rate per 100 000 people in 2016 for all ages. Modified from Khalil et al., 2018

Shigella has only humans as natural hosts. It has been shown that a low infection dose (10-100 bacteria) is sufficient to cause a symptomatic infection (DuPont et al. 1989). *Shigella* transmission is largely favored through contaminated food or water, but houseflies have also been reported as bacterial vectors (Farag et al. 2013). In addition, it was estimated that almost 10% of traveler's diarrheal cases could be attributed to shigellosis (Shah, DuPont, and Ramsey 2009) and in fact it is responsible for the majority of cases reported in Europe (ECDC 2023). Also, *Shigella* outbreaks have been reported in past decades among men who have sex with men (Karen L Kotloff et al. 2018).

In developing countries, treatment for *Shigella* infection involves rest and rehydration with low osmolarity solutions. The administration of antimotility drugs is not recommended because it has been shown that they could result in symptoms prolongation and promotion of bacterial dissemination. The use of antibiotics for shigellosis treatment, systematic in industrialized countries, is beneficial to shorten fever and diarrhea and limit bacterial transmission. The most recommended antibiotics are ciprofloxacin and azithromycin (Karen L Kotloff et al. 2018). However, resistance of *Shigella* spp. to broad spectrum of antibiotics has been reported and it has been estimated that about half of *Shigella* strains present resistance to multiple drugs (Raso et al. 2023). This greatly impacts on successful treatment of *Shigella* infection. *Shigella* overcomes

antibiotics via several mechanisms including extrusion of drugs by active efflux pumps, reduction in cellular permeability, and overexpression of drug-modifying and -inactivating enzymes (Ranjbar and Farahani 2019).

Regarding shigellosis prevention, in addition to improvement of hygiene practices and segregation of ill people, research is focusing on development of vaccines. Unfortunately, nowadays there is no vaccine worldwide available. Only two vaccines are now commercialized but they are restricted to Russia and China (Raso et al. 2023). The difficulty of vaccine development for shigellosis prevention is due to the need for a multivalent vaccine to protect against multiple *Shigella* spp. but also to the lack of adequate animal models to mirror human infection. However, there are currently several vaccines under development both in pre-clinical and clinical trials with some candidates approaching phase 3, leaving hope for the development of licensed product in the incoming years (Raso et al. 2023).

7.2 *Shigella* species

Shigella bacteria are rod-shaped Gram-negative, facultative anaerobic and non-motile facultative intracellular pathogens. The name *Shigella* derives from the Japanese microbiologist Kiyoshi Shiga who first isolated the bacteria during an epidemic in Japan at the end of 19th century (Shiga 1898).

Shigella includes four species that are subdivided into serotypes based on the saccharide units that form the O-antigen, a portion of lipopolysaccharide (LPS). The Shiga bacillus is now known as serotype 1 of *Shigella dysenteriae* that has in total 14 serotypes. The other species are *Shigella flexneri* (15 serotypes), *Shigella boydii* (19 serotypes) and *Shigella sonnei* (1 serotype) (Karen L Kotloff et al. 2018).

Shigella spp. belong to the family of *Enterobacteriaceae* and are very related to *E. coli*. Indeed, *Shigella* was originally considered a separate genus because of its epidemiology, clinical disease, and specific features (non-flagellated, lack of lactose fermentation). It is now clear that *Shigella* spp. are in fact different clones of *E. coli* (Lan et al. 2004; Rolland et al. 1998).

S. flexneri and *S. sonnei* are the most clinically relevant species. The first one is responsible for the majority of bacillary dysentery cases in developing countries (Connor et al. 2015), while *S. sonnei* induces milder symptoms and is predominant in high income countries (Figure 11) (K L Kotloff et al. 1999). *S. sonnei* is also responsible for the majority of cases of traveler's diarrhea in Europe (ECDC 2023). Indeed, this latter species emerged from Europe at the end of the 17th century and

disseminated worldwide via travelers into 4 different lineages especially during the last decades (Holt et al. 2012).

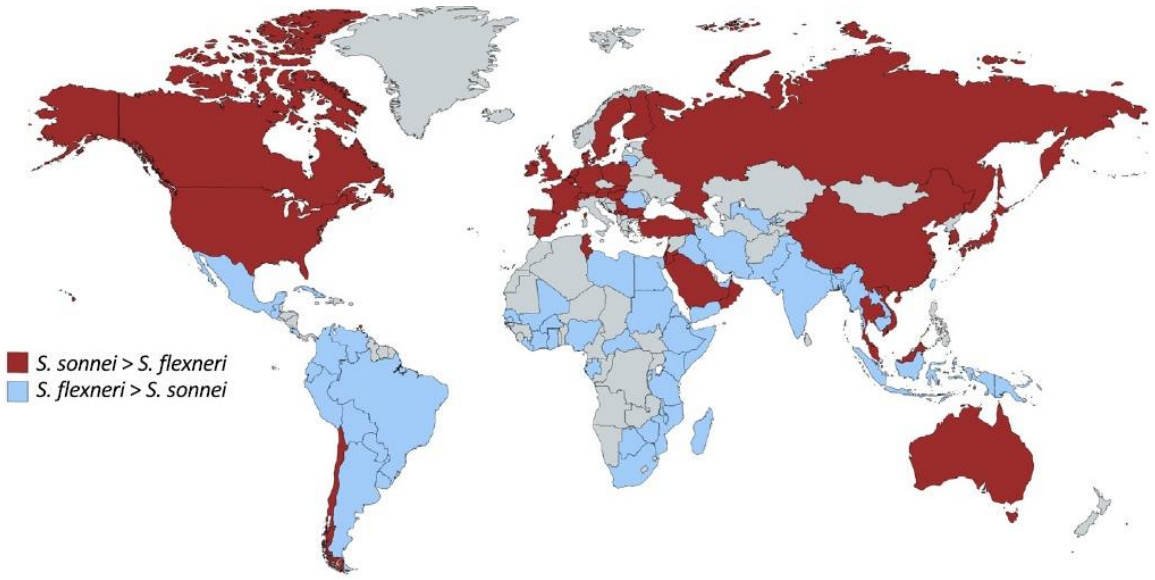

Figure 11. Cases of *S. sonnei* versus *S. flexneri.* Map reporting ratios between cases of *S sonnei* and *S. flexneri*. Red: countries where *S. sonnei* is the dominant cause of shigellosis when compared with *S. flexneri*. Blue: countries where a higher proportion of *S. flexneri* is still being reported (although *S. sonnei* cases may be rapidly increasing). Picture modified from Torraca et al., 2020

However, with the recent rapid economic development of some Asian, Latin America and Middle East countries, we are assisting to a shift from *S. flexneri* towards a *S. sonnei* prevalence (Sousa et al. 2013; Thompson, Duy, and Baker 2015; Vinh et al. 2009). Among different reasons responsible for this phenomenon, it has been proposed that this could be due to the greater ability of *S. sonnei* in assimilating antimicrobial resistance from mobile genetic elements of other bacteria, thereby conferring a selective advantage for survival (Thompson, Duy, and Baker 2015). On the other hand, *S. dysenteriae* serotype 1 is known to be responsible for explosive pandemics characterized by high mortality in populations undergoing disruptive events (Karen L Kotloff et al. 2018).

7.3 *Shigella* infection cycle

The main site of *Shigella* infection are terminal ileum, colon and rectum that are reached by the bacteria after surviving stomach acid environment (Karen L Kotloff et al. 2018). Interestingly, *Shigella* does not penetrate further into deeper tissues but mainly disseminates in the colonic mucosa (Carayol and Tran Van Nhieu 2013a). It has been observed that *Shigella* traverse the intestinal epithelium through M cells and infects macrophages present in the intestinal tissue (Figure 12). After promoting inflammation-induced cell death (pyroptosis) of macrophages, the bacteria are released in the basolateral side of colonic epithelium and are able to colonize epithelial cells. Moreover, the release of large amount of pro-inflammatory cytokines such as IL-1β and IL-18 causes an intense inflammatory response that contributes to tissue damage and disease pathogenesis by promoting further infection and dissemination of luminal bacteria. Also, infection of epithelial cells results in initial activation of pro-inflammatory signaling and cytokine production by epithelial cells (especially IL-8). This attracts polymorphonuclear leukocytes such as neutrophils that initially exacerbate the infection by destabilizing the epithelial barrier and facilitating the entry to the basolateral space of more bacteria (Schnupf and Sansonetti 2019).

Microscopy techniques on colonic samples of guinea pig models presented an enrichment of *Shigella* at the mouth of colonic crypts at early stages of infection and showed an entry of bacterial from an apical side of colonic cells at this site, demonstrating an alternative route for *Shigella* colonization of the tissue (Arena et al. 2015).

Although *Shigella* lacks classical adhesion proteins, the bacteria are able to interact with epithelial cells and induce its uptake by micropinocytosis using a Type III Secretion System (T3SS), (described later in the text) used by the bacteria to translocate effector proteins into the host cell (Killackey, Sorbara, and Girardin 2016). Secretion of bacterial effectors through this system mediates host cytoskeleton alteration and the creation of membrane ruffles rich in actin (Figure 13), which surround the intruding bacteria and enclose them within a vacuole. Adhesion and cytoskeletal remodeling are also favored by the IcsA protein, whose adhesin function depends on T3SS activation (Brotcke Zumsteg et al. 2014). Later, bacteria escape the BCV and are released into the cytosol where they replicate and disseminate in the cell using actin-based motility to avoid destruction by host cell via autophagy and to colonize neighboring cells. During cell to cell spread, bacteria form protrusions that push against adjacent cells. Lysis of the donor and recipient cell membranes occurs and a new infectious cycle begins in adjacent cells (Carayol and Tran Van Nhieu 2013a; C. M. Valencia-Gallardo, Carayol, and Tran Van Nhieu 2015).

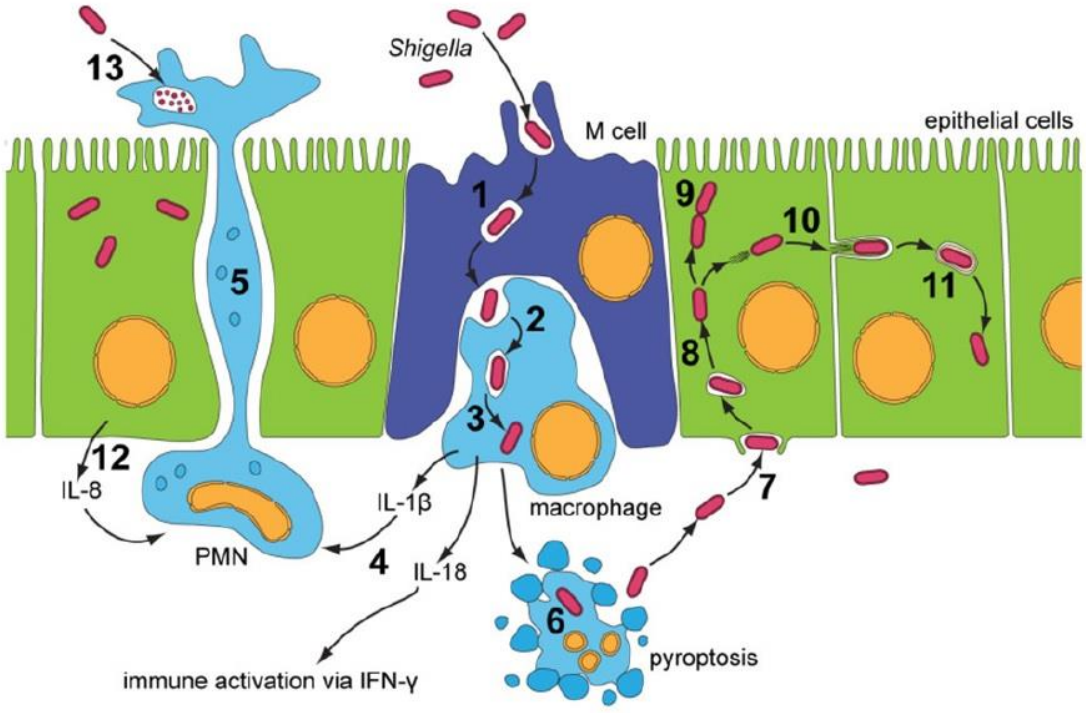

Figure 12. **Infectious cycle of *Shigella*** Entry into the colonic epithelium is mediated by M cells and epithelial barrier destabilization. *Shigella* enters M-cells (1) and is then transported to the other side of the epithelium where it is endocytosed by resident macrophages (2). *Shigella* escapes from the phagocytic vacuole (3) and triggers release of a large quantity of pro-inflammatory cytokines (4). These together with chemotaxic cytokines (IL-8) produced by infected epithelial cells mediate epithelial barrier destruction. Indeed, recruitment of polymorphonuclear leukocytes (PMN) (12), which travel the apical side of colonic epithelium, destabilizes the junctions between the epithelial cells and allows further invasion of *Shigella* (5). After colonizing macrophages and inducing their cell death (6), *Shigella* is internalized by epithelial cells via a macropinocytic-like process at the basolateral membrane (7). Lysis of the vacuole allows *Shigella* to gain access to the cytoplasm, where it rapidly replicates and subverts host cell behavior to escape autophagy and promote cell survival (8 and 9). Exploitation of the actin cytoskeleton allows *Shigella* to move both intra- and intercellularly (10). Protrusions mediated by bacteria are endocytosed by the neighboring cells and engulfed in a double membrane vacuole (11). PMN leukocytes eventually eliminate *Shigella* infection from the colonic epithelium (13). From Mattock and Blocker, 2017

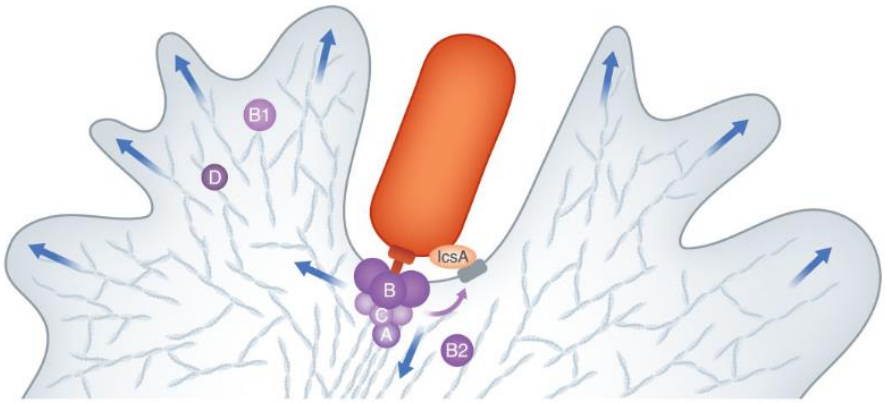

Figure 13. Membrane ruffles formation during *Shigella* entry. Upon contact with the host cell and insertion of type III secretion apparatus in the plasma membrane, the bacterium secretes effector proteins in the host cell thereby inducing cytoskeletal remodeling and subsequently pulling forces (blue arrows). This leads to formation of membrane ruffles that surround the invading bacteria and favors its internalization. Adhesion of *Shigella* to the host cells and promotion of actin remodeling are favored by IcsA-mediated adhesion following type III secretion system activation (purple arrow). Effectors playing a role in this context are described in the next sections: A (IpaA), B (IpaB), C (IpaC), B1 (IpgB1), B2 (IpgB2), D (IpgD). Modified from Valencia-Gallardo et al. 2015.

Cytosolic replication of *Shigella* is facilitated by the injection through T3SS of additional effector proteins into the host cell that suppress the host's inflammatory reaction, enhance the survival of host cells, and counteract antimicrobial mechanisms. This strategy ensures the preservation of its cytosolic environment, creating optimal conditions for replication and sustained spreading (Schnupf and Sansonetti 2019).

Over time, the initial inflammatory reaction that initially facilitates effective infection ultimately results in the clearance of *Shigella*. Eventually, immune cells such as Natural Killer cells and neutrophils eradicate the infection in 5-7 days in individuals who are in good health (Mattock and Blocker 2017).

7.4 The Type III secretion system (T3SS) or injectisome

The virulence of *Shigella* is largely mediated by the presence of 220 kb virulence plasmid that has been shown to be necessary for invasive phenotype: introduction of the plasmid in non-invasive *E.coli* K-12 strain is sufficient to confer ability to bacteria to enter HeLa cells (Sansonetti et al. 1983).

The majority of crucial virulent factors are encoded in a 30 kb region, named “entry region”. Within this specific area lies the *mxi-spa* locus, encoding the T3SS, along with the *ipa* genes. These genes play a key role in invasion of epithelial cells and in the *Shigella* infection process. In addition, some virulence factors are encoded on the bacterial chromosome in regions known as “pathogenicity islands” (PAI) (Mattock and Blocker 2017). Expression of these genes is under the control of a regulatory network that responds to environmental stimuli encountered during transit through the host: the principal trigger is a temperature shift to 37°C which allows expression of VirF transcription activator and downstream of it of VirB, which mediates transcription of *mxi* and *spa* genes (Bajunaid et al. 2020). This allows the assembly of the Type III Secretion System Apparatus or “injectisome” that is a needle-like structure that spans the bacterial inner and outer membranes (Muthuramalingam et al. 2021). VirB is also responsible for transcription of many bacterial effector proteins that mediate the first steps of *Shigella* invasion (Bajunaid et al. 2020).

The injectisome is related to the bacterial flagella and contains three parts: the basal body, the needle and the tip complex (Figure 14). The needle is constituted by MxiH monomers disposed in helical manner that form a channel of about 2.5 nm diameter (Cordes et al. 2003). At the top of the needle, there is a tip complex formed by IpaD and IpaB proteins (Olive et al. 2007). These proteins keep the T3SS in an inactive state. Indeed, it has been shown that deletion of either IpaD or IpaB renders the T3SS constitutively active (Ménard, Sansonetti, and Parsot 1993).

MxiD, MxiJ and MxiG proteins form T3SS ring structures in the outer and inner membrane of the bacteria, constituting the basal body of the structure (Muthuramalingam et al. 2021). At the bottom of this, in bacterial cytoplasm, the sorting platform recognizes type III substrates (Lara-Tejero et al. 2011) via the ATPase Spa47, which promotes their unfolding and engagement in the T3S apparatus (Akeda and Galán 2005) .

Activation of T3SS and translocation of unfolded effectors through the needle and into the host cell are induced upon direct contact between *Shigella* and host cell: contact of T3SS tip complex with phospholipids, cholesterol or unidentified host receptors induces a conformational change of tip complex proteins and subsequently mediates recruitment to the needle tip of IpaC, that together with IpaB forms a translocon pore into host cell plasma membrane (Epler et al. 2009). These steps are then followed by injection of type III effectors stored in the bacterial cytoplasm (Parsot 2009).

Successful invasion and host colonization are mediated by a set of ~30 effector proteins that are involved in subverting a plethora of host cell processes (Killackey, Sorbara, and Girardin 2016).

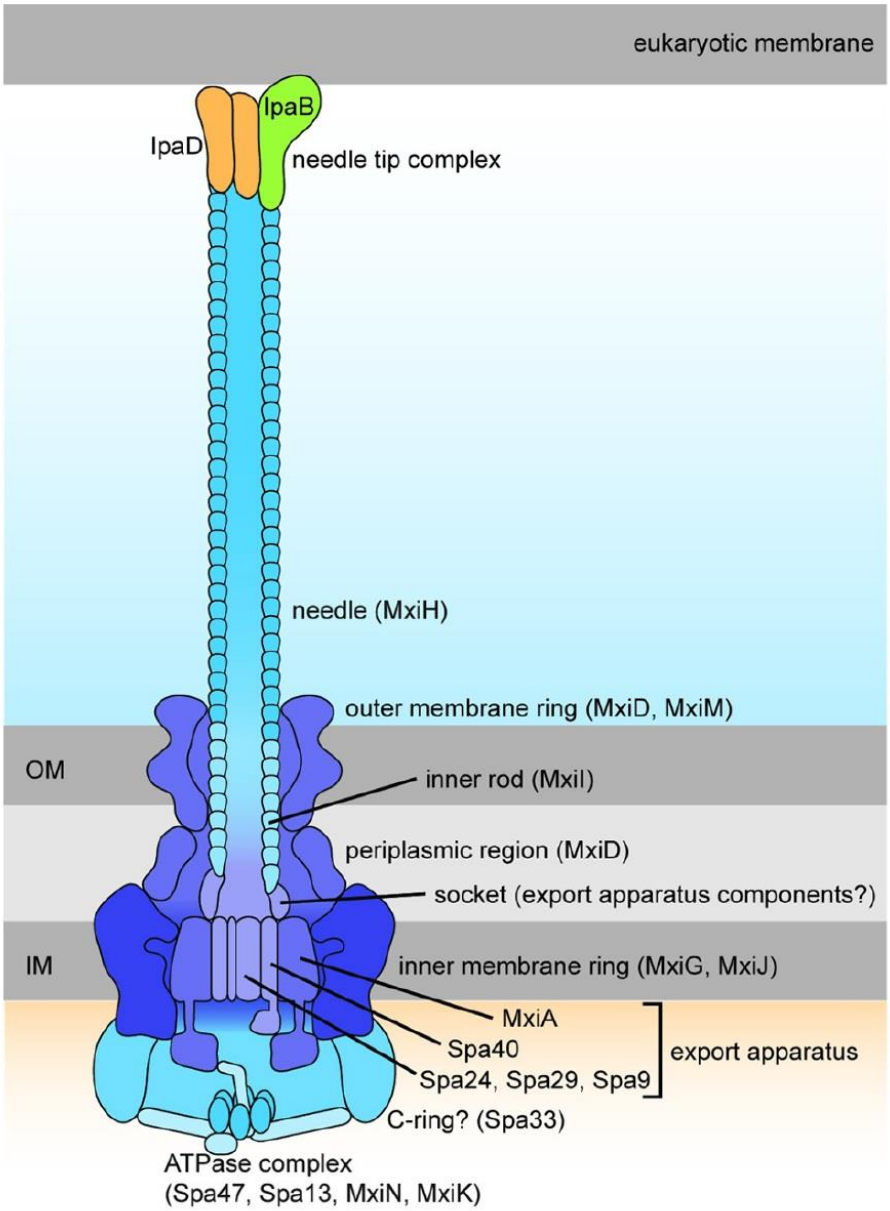

Figure 14. Schematic drawing of the *Shigella* Type III Secretion System. The T3SS is a needle-like structure that protrudes from the bacteria. The genes that encode for components of the T3SS are encoded in a virulence plasmid essential for its pathogenicity. *Shigella* uses the T3SS to inject in the host cells several protein effectors in order to promote infection. From Mattock and Blocker, 2017

7.5 Bacterial effectors

Shigella by secreting several protein effectors in the host through the injectosome subverts several cellular processes in order to favor host colonization and productive infection (Figure 15). These effectors are divided into two waves. First wave effectors are constitutively expressed together with components of the injectisome. Upon cell contact, these first wave effectors rapidly secreted following insertion of translocon components in host cell membranes (Table1) (Parsot 2009). These effectors are involved, among other functions, in promoting bacterial internalization favoring bacterial anchoring, cytoskeletal remodeling and vacuolar escape (Carayol and Tran Van Nhieu 2013b).

Effectors belonging to the "second wave" are up-regulated following induction of secretion and play a role during bacterial intracellular growth (Table 3). Their genes are under the control of transcription activator MxiE and co-activator IpgC. MxiE is normally kept inactive by binding to type III OspD1 anti-activator and its chaperone Spa15, while IpgC acts as a chaperone for IpaB and IpaC proteins. Secretion of IpaB, IpaC and OspD1 allows MxiE to associate with IpgC and to promote transcription of the second wave effectors (Parsot et al. 2005). MxiE also up-regulates some first wave effectors termed middle effectors (Table 2) (Bajunaid et al. 2020). Second wave effectors modulate several host signaling pathways to dampen host immune response (Mattock and Blocker 2017).

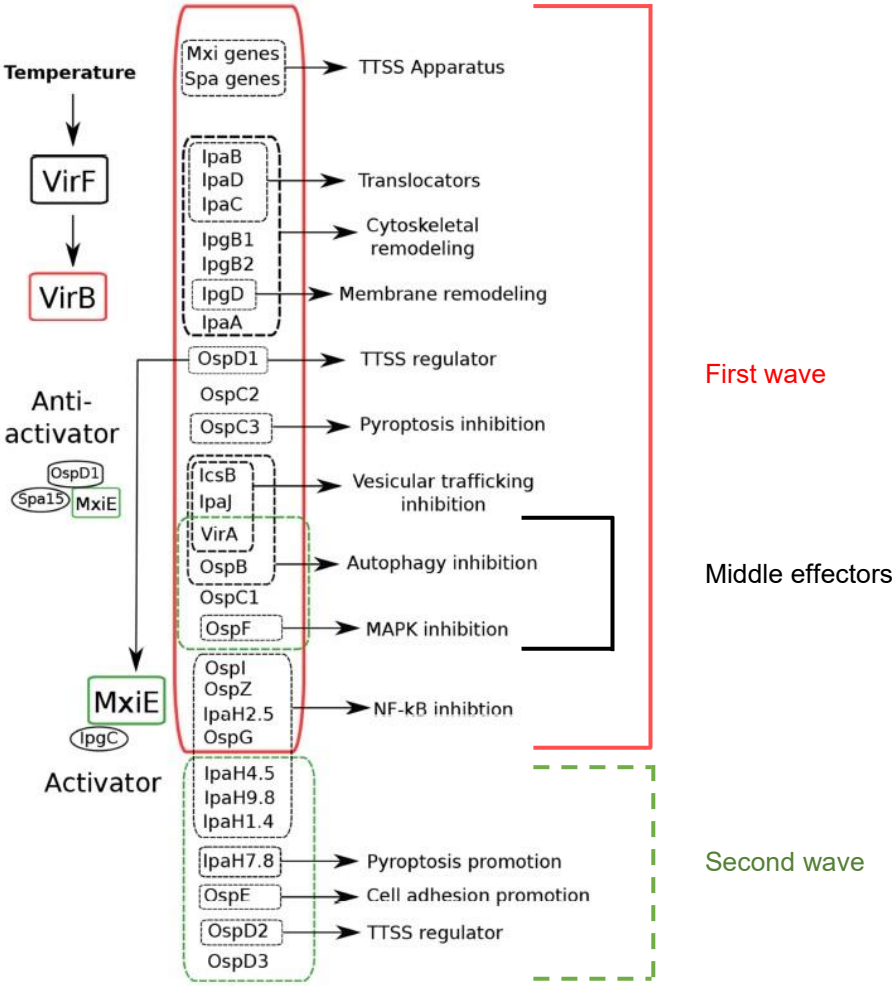

Figure 15 *Shigella* T3SS and effectors regulation. Expression of the T3SS apparatus and its effectors is regulated by environmental factors such as temperature that, through VirF, controls the expression of the transcription factor VirB, leading to their constitutive expression in the host. Upon activation of the T3SS apparatus, first wave of effectors are rapidly injected in host cells, MxiE inhibition by OspD1 is relieved and this event allows the transcription of the second wave effectors. Some effectors (middle effectors) are under the control of VirB promoter but undergo an increase in their expression after MxiE activation. Modified from Schnupf and Sansonetti, 2019.

Table 1. *Shigella* T3SS-secreted first wave effectors and their functions

Effector	Function	Effect	Reference
IpgB1	GEF for Rac1 and CDC42	Induces membrane ruffles via actin remodeling to favor bacterial uptake	(Hachani et al. 2008; Handa et al. 2007; Ohya et al. 2005)
IpgB2	GEF for Rho	Promotes actin remodeling to facilitate invasion	(Hachani et al. 2008; Klink et al. 2010)
IpgD	$PI(4,5)P_2$-4-phosphatase to generate PI(5)P and alter phosphoinositide composition	Stimulation of actin polymerization at bacterial entry site Promotion of cell survival via activation of PI3K/Akt Promotion of efficient vacuolar disruption Stimulation of endocytosis and degradation of ICAM1 and impairment of neutrophil recruitment Blocking of endosomal maturation and lysosomal degradation of EGFR Alteration of intracellular Ca^{2+} signaling Inhibition T cell migration Limitation of ATP release through connexins and reduction of inflammation	(Boal et al. 2016; Konradt et al. 2011; Mellouk et al. 2014; Niebuhr et al. 2002; Pendaries et al. 2006; Puhar et al. 2013; Ramel et al. 2011; C. H. Sun et al. 2017)
IpaA	Binds to vinculin Binds to talin Activates Rho	Promotes formation of focal adhesions and cytoskeletal anchorage Stabilization of filopodial adhesions and stimulation of bacterial capture Promotes actin remodeling to facilitate invasion	(Demali, Jue, and Burridge 2006; Tran Van Nhieu et al. 1997; C. Valencia-Gallardo et al. 2019)
IpaB	Inserts into membranes and binds cholesterol	Translocon pore formation Vacuolar escape Golgi fragmentation and inhibition of cellular trafficking and secretion	(High et al. 1992; Lafont et al. 2002; Mounier et al. 2012; Veenendaal et al. 2007)
IpaC	Inserts into membranes Activates Src Kinase and Cdc42	Translocon pore formation Actin polymerization	(Mounier et al. 2009; G Tran Van Nhieu et al. 1999; Veenendaal et al. 2007)
IpaJ	Blocks STING exit from the ER Removal of the myristoyl group from ARF1	Inhibition of Interferon induction and immune activation Golgi fragmentation	(Burnaevskiy et al. 2013; Dobbs et al. 2015)
IpaH2.5	Ubiquitin-E3-ligase	Inhibition of NF-κB activation	(de Jong et al. 2016)
IcsB	Inhibits LC3 recruitment to bacterial vacuole Involved in protrusion lysis	Inhibition of autophagy Mediates escape from autophagic vacuole Promotion of cell-to-cell spreading	(Allaoui et al. 1992; Campbell-Valois et al. 2015)
OspC3	Inhibits caspase 4/11 activation Binds to calmodulin	Downregulation of inflammatory cell death of epithelial cells Inhibition of Interferon-mediated immune response	(Alphonse et al. 2022; Taira Kobayashi et al. 2013; Z. Li et al. 2021)
OspD1	Binds to MxiE	Regulates T3SS activity	(Parsot et al. 2005)
OspI	Deamidates of UBC13	Inhibition of TRAF6- NF-κB signaling pathway	(Sanada et al. 2012)
OspZ	Prevents IκB degradation	Suppression of NF-κB activation Reduced PMN transepithelial migration	(Newton et al. 2010; Zurawski et al. 2008)
OspG	Prevents p-IκBα degradation	Inhibition of NF-κB activation	(D. W. Kim et al. 2005)

Table 2. *Shigella* T3SS-secreted middle effectors and their functions

Effector	Function	Effect	Reference
VirA	GAP for Rab proteins Activates calpain	Inhibits autophagosome formation and endosomal trafficking Escape from entry vacuole p53 degradation and inhibition of apoptotic cell death Necrosis induction at later stages of infection	(Bergounioux et al. 2012; Campbell-Valois et al. 2015; Dong et al. 2012)
OspB	Activates MAPK signaling pathway Activates mTORC1	Promotion of PMN recruitment and transepithelial migration Induces cell proliferation	(Ambrosi et al. 2015; Lu et al. 2015)
OspC1	Activates of ERK pathway Binds to Calmodulin Prevents caspase-8 activation	Promotion of PMN recruitment and transepithelial migration Inhibition of Interferon-mediated immune response Inhibition of apoptosis	(Alphonse et al. 2022; Ashida, Sasakawa, and Suzuki 2020; Zurawski et al. 2006)
OspF	Inactivates p38 and ERK kinases Activates JNK and NF-κB responses	Modulation of inflammatory signaling	(Hongtao Li et al. 2007; Reiterer et al. 2011)

Table 3. *Shigella* T3SS-secreted second wave effectors and their functions

Effector	Function	Effect	Reference
OspD2	Limits VirA translocation into host cell	Timing control of infection-induced cell necrosis	(Mou et al. 2018)
OspD3	Degrades RIPK1 and RIPK3	Prevents necroptosis	(Ashida, et al. 2020)
OspE1/2	Binds integrin-linked kinase	Stabilization of focal adhesions Inhibition of infected cells' detachment	(M. Kim et al. 2009)
IpaH1.4	Ubiquitin-E3-ligase	Inhibition of NF-κB activation	(de Jong et al. 2016)
IpaH7.8	E3 ubiquitin ligase for glomulin	Inflammasome activation and macrophagic pyroptosis induction	(S. Suzuki et al. 2014)
IpaH9.8	E3 ubiquitin ligase for U2AF35 E3 ubiquitin ligase for NEMO/IKKγ	Downregulation of immune response Inhibition of NF-κB signaling	(Ashida et al. 2010; Okuda et al. 2005)
IpaH4.5	E3 ubiquitin ligase for p65 E3 ubiquitin ligase for TBK1	Inhibition of NF-κB signaling Dampening of inflammatory response	(F. Wang et al. 2013; Zheng et al. 2016)

7.6 The *Shigella* effector IpgD

IpgD effector has been characterized as a PI(4,5)P_2-4-phosphatase to produce PI(5)P but a recent work showed that it could have also a phosphotransferase activity producing PI(3,4)P_2 similarly to its *Salmonella* ortholog SopB (Walpole et al. 2022).

This effector is crucial for *Shigella* to alter multiple cellular processes through its alteration of host cell phosphoinositides composition. PI(5)P production by IpgD promotes cell survival and cell proliferation by activation of PI3K and Akt kinases (Pendaries et al. 2006). Due to the recent discovered phosphotransferase activity, it is possible that Akt activation is mediated also via the production of PI(3,4)P_2. Increased PI(5)P levels produced by IpgD are responsible for the internalization and subsequent lysosomal degradation of ICAM-1 from the cell surface (Boal et al. 2016). ICAM-1 has been shown to bind to integrins of leukocytes and mediate recruitment of neutrophils at the site of infection. Indeed, internalization of this protein promoted by PI(5)P results in reduced neutrophil adhesion and decreased recruitment at intestinal epithelial surface in a *in vivo* model (Boal et al. 2016). IpgD-produced PI(5)P triggers the activation of EGFR, which in turn sustains Akt signaling. Notably, upon PI(5)P treatment EGFR is internalized in early endosomes and its degradation impaired, resulting in prolonged pro-survival cues through endosome-specific signaling of activated EGFR (Ramel et al. 2011).

IpgD also promotes the closure of connexins hemichannels and so inhibits ATP release. Decrease in ATP concentration in extracellular medium results in a decrease in danger signal release and reduction of the inflammation of the intestinal mucosa (Puhar et al. 2013).

In addition, IpgD is involved in promotion of actin polymerization at bacterial entry site (Niebuhr et al. 2002). PI(5)P activates Rac1 GEF Tiam1 and mediates their recruitment at early endosomes next to cell surface, enhancing cellular actin and membrane dynamics that favor pathogen invasion (Viaud et al. 2014).

This effector seems to be involved also in actin regulation after bacterial entry in host cell by modulating the actin cage that surrounds the BCV and by sustaining cytoskeletal remodeling at protrusions that form between two adjacent cells at the moment of cell-to-cell spreading (Tran Van Nhieu et al. 2022).

Although internalization kinetics of IpgD mutant bacteria are similar to WT *Shigella*, vacuolar rupture is delayed. IpgD effector is important for recruitment of Rab11 positive vesicles to the BCV and rupture of the vacuole is impaired, leaving bacteria trapped in actin cages (Mellouk et al. 2014).

The alteration of cell phosphoinositides composition by IpgD has repercussions on cellular Ca^{2+} homeostasis, a topic that will be addressed in the next section.

7.7 Ca^{2+} signaling alteration during infection of *Shigella*

Many bacterial pathogens cause modification to intracellular Ca^{2+} concentration either by impacting on intracellular Ca^{2+} stores or by triggering Ca^{2+} influx from extracellular medium. This latter mechanism is largely mediated by the action of pore-forming toxins that are inserted into the host plasma membrane. Increase in cellular Ca^{2+} concentration leads to perturbation of several host cell processes such as cell adhesion, inflammation and cell death (Tran Van Nhieu et al. 2018). In the case of *Shigella* infection, bacteria subvert host Ca^{2+} signaling and differently regulate multiple endogenous processes (Bonnet et al. 2016).

Shigella infection of epithelial cells induces transient increases in global cytoplasmic Ca^{2+} that are mediated by a functional T3SS. These Ca^{2+} signals are IP_3-dependent and their frequency is increased by the presence of functional Connexin 26 that mediates ATP release in extracellular medium and increases Ca^{2+} signaling in neighboring cells. This results in increase bacterial capture and internalization as well as augmented bacterial spreading among neighboring cells (Tran Van Nhieu et al. 2003). Moreover, during host cell invasion, *Shigella* induces atypical IP_3-dependent cytoplasmic Ca^{2+} increases that localize at the entry site and can last for many seconds. These are caused by an accumulation of IP_3 whose diffusion, as well as Ca^{2+} diffusion, is restricted by the formation of a dense actin meshwork induced by cytoskeletal remodeling events triggered by bacterial invasion (Tran Van Nhieu et al. 2013). This response is regulated by the IpgD type III effector, whose action on $PI(4,5)P_2$ reduces also IP_3 pool in infected cells, thus shaping cellular Ca^{2+} signaling and impacting on calpain-mediated focal adhesions destruction and cell detachment (C. H. Sun et al. 2017). Infection of epithelial cells with mutant lacking IpgD results in reduced local Ca^{2+} responses but presented increase in global Ca^{2+} signals consistent with increased IP_3 levels and diffusion. Cells infected with an *ipgD* mutant are more responsive to IP_3-dependent Ca^{2+} signals, suggesting that IpgD effector negatively regulates IP_3-dependent ER Ca^{2+} release (C. H. Sun et al. 2017). Modulation of Ca^{2+} signals by *Shigella* has been shown to downregulate host inflammatory response. By activation of calpain by cytosolic Ca^{2+} increase *Shigella* promotes cleavage of pro-apoptotic protein BID. This induces SMAC/Diablo release for mitochondria and inhibition of XIAP-mediated induction of NF-κB (Andree et al. 2014). Moreover calpain activation, together with the action of VirA effector, promotes p53 degradation and inhibits p53-mediated induction of

apoptosis to promote cell survival. However, in later stages of infection calpain activation triggers necrotic cell death (Bergounioux et al. 2012).

Ca^{2+} signaling is involved in orchestrating a plethora of cellular events and alteration of this regulation has an impact on several processes. Understanding the differential regulation of this versatile ion during the steps of *Shigella* infection could help us highlight how these bacteria integrate several signaling pathways and cellular processes to promote host cell infection.

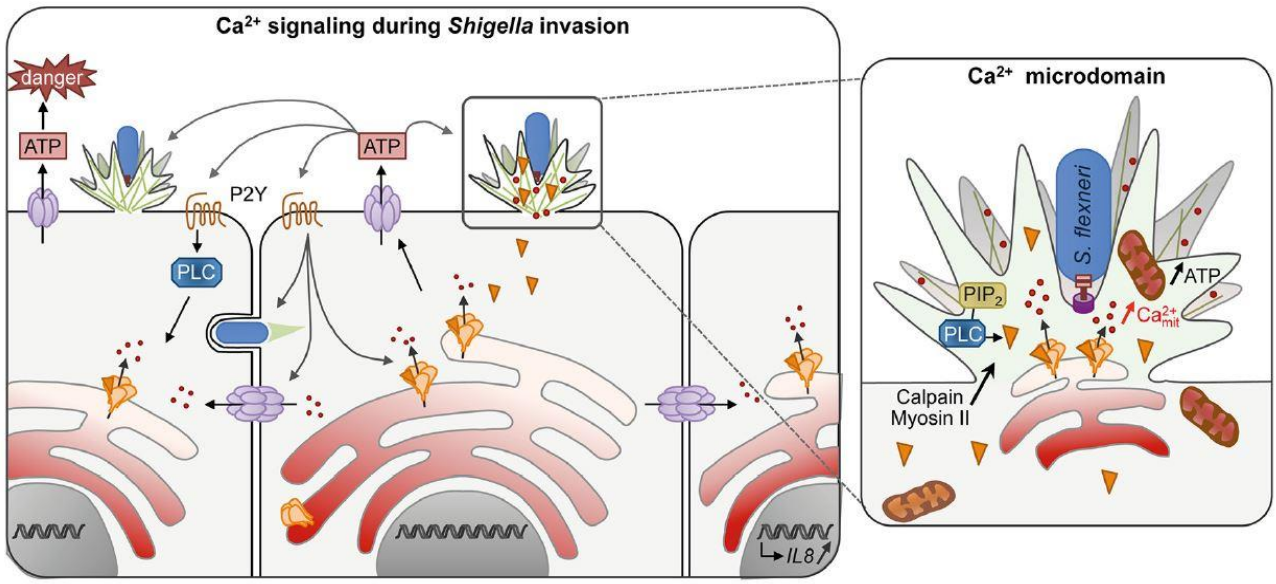

Figure 16. Ca^{2+} signaling during *Shigella* invasion. In the process of invading epithelial cells, *Shigella* triggers Ca^{2+} signaling by recruiting PLC and IP3R at the entry sites. This creates Ca^{2+} microdomains at this level favored by presence of a dense actin network. Increase in mitochondria Ca^{2+} levels of organelles trapped at this location increase the production of ATP necessary for cytoskeletal remodeling and foci formation. Elevation of Ca^{2+} levels at the entry site are required for activation of calpain and myosin II. *Shigella* infection produces also global Ca^{2+} responses that mediated the opening of connexins hemichannels and release of ATP in extracellular medium. This induces activation of ATP receptors (P2Y) and further increases global Ca^{2+} signal. ATP release also mediates recognition of danger signals by hot immune defense as well as favoring invasion of epithelial cells by more bacteria. Ca^{2+} signals spread to neighboring cells via gap junctions. From (Bonnet et al. 2016)

Aim of the thesis

Storage of high concentration of Ca^{2+} is a key function of the ER and plays a crucial role for cellular physiology. Alteration of ER Ca^{2+} levels strongly impacts on ER protein folding capacity and activated the UPR. Many human diseases are characterized by dysregulation of ER Ca^{2+} concentration and activated UPR. In order to induce UPR activation, scientists use high doses of SERCA pump inhibitors, leading to release of total ER Ca^{2+} content within few minutes. These conditions do not resemble ER Ca^{2+} homeostasis dysregulation that could occur in physiological or pathological context.

For this reason the aim of my thesis is to better characterize the activation of UPR in conditions of gradual ER Ca^{2+} release. In order to dissect the UPR sensor activation kinetics and sensitivities to ER Ca^{2+} release and because of the complexity of the UPR response I collaborated with a team of modelers to combine experimental and mathematical modeling approaches.

Furthermore, to frame this into a specific context, part of my work aimed to explore the role of UPR activation in condition of infection of epithelial cells by *Shigella flexneri*, an intracellular pathogen that has been shown to cause Ca^{2+} homeostasis dysregulation. The role of UPR in bacterial infections has not been largely explored, especially in conditions of *Shigella* colonization of epithelial cells. This work aims to highlight some unknown aspects of host-pathogen interaction in order to better understand all the mechanisms involved in bacterial infections.

RESULTS

Article 1

This work entitled "*Gradual ER Calcium depletion induces a progressive and reversible UPR signaling*" focuses on the relationship between gradual Ca^{2+} depletion and induction of UPR. We combined experimental and modeling approaches to investigate sensitivity and kinetics of UPR activation in conditions of moderate decrease in ER Ca^{2+} levels.

Gradual ER Calcium depletion induces a progressive and reversible UPR signaling

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Abstract

The unfolded protein response (UPR) is a widespread signal transduction pathway triggered by endoplasmic reticulum (ER) stress. Because calcium (Ca^{2+}) is a key factor in the maintenance of ER homeostasis, massive Ca^{2+} depletion of the ER is a potent inducer of ER stress. Although moderate changes in ER Ca^{2+} drive the ubiquitous Ca^{2+} signaling pathways, a possible incremental relationship between UPR activation and Ca^{2+} changes has yet to be described. Here, we determine the sensitivity and time-dependency of activation of the three ER stress sensors, IRE1α, PERK and ATF6α in response to controlled changes in the concentration of ER Ca^{2+} in human cultured cells. Combining Ca^{2+} imaging, FRAP experiments, biochemical analyses and mathematical modeling, we uncover a non-linear rate of activation of the IRE1α branch of UPR, as compared to the PERK and ATF6α branches that become activated gradually with time and are sensitive to more important ER Ca2+ depletions. However, the three arms are all activated within a 1h timescale. The model predicted the deactivation of PERK and IRE1α upon refilling the ER with Ca2+. Accordingly, we showed that ER Ca2+ replenishment leads to the complete reversion of IRE1α and PERK phosphorylation in less than 15 min, thus revealing the highly plastic character of the activation of the upstream UPR sensors. In conclusion, our results reveal a dynamic and dose-sensitive Ca^{2+}-dependent activation/deactivation cycle of UPR induction, which could tightly control cell fate upon acute and/or chronic stress.

Significance Statement

Partial ER Ca^{2+} depletion and activation of UPR are features observed in several human disease but the relationship between these two phenomena and the sensitivity of UPR to Ca^{2+} decrease have not been extensively explored. In this work we demonstrate how the UPR sensors show high plasticity in sensing physiological alterations by closely reporting the variations in ER Ca^{2+} levels.

Using a mathematical model, we predicted the deactivation of PERK and IRE1α upon refilling the ER with Ca^{2+}. This prediction was confirmed experimentally revealing that ER Ca^{2+} replenishment led to the complete dephosphorylation of IRE1α and PERK in a few minutes. These results demonstrate how cell rapidly adapt to stress response signaling pathways to variations in ER homeostasis.

Introduction

Many physiological processes are modulated by the variation in intracellular concentration of calcium ions (Ca^{2+}) (1). Ca^{2+} is accumulated in the major intracellular store, the Endoplasmic reticulum (ER) where it helps to maintain ER functions. Alteration of ER Ca^{2+} homeostasis leads to the accumulation of improperly folded proteins in the ER lumen, leading to a situation known as ER stress. To cope with this, cells have evolved an adaptive signaling pathway aiming at restoring ER homeostasis. This mechanism is collectively named the Unfolded Protein Response (UPR) and is operated by the activation of three ER transmembrane proteins: the Protein Kinase R-like ER kinase (PERK), the inositol-requiring protein 1 alpha (IRE1α, referred to IRE1 hereafter) and the activating transcription factor 6 alpha (ATF6α, referred to ATF6 hereafter), which activate three parallel signal transduction pathways (2). In non-stressed conditions, sensors are maintained inactive through at least their binding to ER chaperone BiP. Upon accumulation of improperly folded proteins in the ER, BiP dissociates from the UPR sensors allowing for their activation and that of the downstream signaling pathways (3, 4). In addition, it has also been shown that the luminal domains of both IRE1 and PERK are able to bind to unfolded peptides, suggesting the existence of a direct sensing mechanism (5–7). To study UPR signaling, one of the most used ER stress inducers is linked to the depletion of luminal ER Ca^{2+}, generally obtained using inhibitors of the SERCA pumps (e.g. thapsigargin). Although these experiments have allowed significant advances in the understanding of the mechanisms underlying the activation of the UPR sensors and their downstream signals, they failed so far in exploring the tight relationships between ER Ca^{2+} concentrations and UPR activation especially when it comes to the sensitivity and early activation of the UPR by moderate and more physiological luminal Ca^{2+} stores modulation (8, 9). In this work, by combining experimental and mathematical modelling approaches, we have explored how UPR sensor activation closely reports the ER Ca^{2+} depletion in a gradual and reversible manner.

Results

Partial calcium depletion in the Endoplasmic Reticulum incrementally and reversibly activates the Unfolded Protein Response sensors

To assess the impact of gradual ER Ca^{2+} store depletion on UPR activation, we used a reversible SERCA2B inhibitor at various concentrations. 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) has a relatively low potency as compared to the more commonly used thapsigargin, with an IC_{50} in

the range of 1 to 2 μM (10, 11). We monitored the kinetics of $[Ca^{2+}]_{ER}$ using GEM-CEPIA1er, the ratiometric version of CEPIA1er (12). This genetically encoded indicator has a K_D of 558 μM and a good dynamic range (R_{max}/R_{min} = 21.7). When expressed in HeLa cells, GEM-CEPIA1er correctly localized to the ER (Figure 1a) and accurately reported changes in $[Ca^{2+}]_{ER}$ either upon treatment of the cells with histamine (Figure 1b) or in response to SERCA2B pump inhibition by supra-maximal dose of tBuBHQ (Figure 1c). At lower concentrations, a dose-dependent plateau in $[Ca^{2+}]_{ER}$ was reached in less than 1 h. As compared to the $[Ca^{2+}]_{ER}$ decrease induced upon treatment with 30 μM tBuBHQ, $[Ca^{2+}]_{ER}$ was stabilized at 50%, 60% and 85% of this value upon treatments with 3, 5 and 10 μM tBuBHQ, respectively.

As shown in Figure 2a, b, the evolution of $[Ca^{2+}]_{ER}$ upon treatment with tBuBHQ was consistent with kinetic simulations (see the Material and Methods section for a description of the model), taking into account the 2 μM IC50 of tBuBHQ binding to SERCA pumps (10, 11). As such, we next investigated if the graded decreases in $[Ca^{2+}]_{ER}$ impacted on different levels of ER stress.

High ER Ca^{2+} concentration plays a key role in the activity of many ER luminal chaperones and foldases (13), that, if not properly functioning, lead to ER protein homeostasis imbalance and UPR activation (14). BiP is one of the most abundant ER chaperones, which can bind a variety of substrates/clients. Given that measurement of global levels of misfolded proteins is technically very challenging, we followed BiP's mobility after treatment with tBuBHQ as a read-out of ER crowding. Fluorescence recovery after photobleaching (FRAP) experiments indeed reveal that ER stress induced by Tunicamycin or DTT treatment reduces BiP's mobility in FRAP, suggesting an increased ability of BiP to bind to larger proteins and aggregates (Lai et al. 2010). To evaluate the changes in BiP mobility that could result from the ER Ca^{2+} reduction induced upon treatment with tBuBHQ, we used HeLa cells expressing BiP-mGFP (Lai et al. 2010) and evaluated the mobility of this protein using FRAP. Expression of BiP-mGFP *per se* neither induced ER stress nor UPR activation (Figure S1). Under basal conditions, the half-time of BiP-mGFP recovery in the photobleached areas was of 3.33 ± 0.17s (Figure 2c). This value increased upon treatment with 30 μM tBuBHQ ($t_{1/2}$ = 4.83 ± 0.33 s after 30 min, $t_{1/2}$ = 6.16 ± 0.40 s after 1 h and $t_{1/2}$ = 6.53 ± 0.95 s after 2 h). The effects of tBuBHQ on BiP-mGFP mobility were also observed in Hela cells expressing BiP-mCherry, suggesting that the effect on the mobility of the protein of interest is not impacted by the fluorescent reporter used (Figure S2). Because longer times of tBuBHQ treatment were associated with larger depletion of ER Ca^{2+}, we concluded that BiP mobility, hence the accumulation of improperly folded proteins are sensitive to $[Ca^{2+}]_{ER}$. To quantify the rate of

accumulation of improperly folded proteins (UP), we generated a data-driven mathematical model of the $t_{1/2}$ of recovery after photobleaching. The diffusion coefficients were inferred from the half-times, either by explicit simulations of the FRAP experiments or by using an approximate equation that relates the two quantities. Assuming that BiP diffuses more slowly when bound to improperly folded proteins and that this association increases with the amount of improperly folded proteins present in the ER lumen, this allowed us to determine a relative concentration of improperly folded proteins (Figure 2d). These values were in agreement with the temporal evolution of [UP] predicted by a simple model assuming that ER Ca^{2+} is necessary for correct protein folding and taking into account the observed evolution of $[Ca^{2+}]_{ER}$ (Figure 2d, inset and equations (5)-(8) in the Material and Methods section). The model also revealed the existence of a delay between the decrease in $[Ca^{2+}]_{ER}$ and UP accumulation. The correspondence between the model and the independent computational analysis of BiP diffusion $t_{1/2}$ pointed to an adequate quantification of the concentrations of UP or, at least, of their relative values. In conclusion, treatment of HeLa cells with appropriate concentrations of tBuBHQ allowed for a fine-tuned control of the rates and levels of depletion of ER Ca^{2+} depletion and subsequent accumulation of improperly folded proteins, thereby providing a tunable system to explore ER Ca^{2+} impact on activation of the UPR.

Moderate ER Ca^{2+} depletion triggers activation of the three UPR pathways with different sensitivities and kinetics

Since we focus on the early activation of UPR, we monitored early markers of UPR induction that precede transcriptional and translational responses. Given that activation of both IRE1 and PERK depends on their autophosphorylation following oligomerization, we monitored their phosphorylation status using Phos-tag™ SDS PAGE and Western blot with anti-IRE1 and anti-PERK antibodies (16). Figure 3a shows that the phosphorylation patterns of IRE1 and PERK were distinct: IRE1 displayed a single slow migrating band upon induction while PERK activation resulted in several bands likely to reflect multiple phosphorylation. By quantifying the amount of phosphorylated sensor over total protein quantity, we determined the percentage of sensor activation after treatment with different concentrations of tBuBHQ. Under basal conditions, HeLa cells displayed a phosphorylation status of about 15% for both IRE1 and PERK (Figure 3b). For these two sensors, tBuBHQ induced an increase in phosphorylation in a dose dependent manner suggesting that the pattern of sensor activation correlates with the extent of ER Ca^{2+} depletion. Notably, IRE1 phosphorylation reached its maximal level at 1h post treatment for all doses tested

and then plateaued at a level proportional to the extent of ER depletion (Figure 2b). In comparison, PERK phosphorylation was slower and kept increasing gradually over the duration of the experiment (2h). Moreover, the extent of phosphorylation after 2h was significantly higher for 30 µM tBuBHQ than for lower doses. PERK activation thus appeared to be slower and less sensitive to Ca^{2+} depletion than IRE1 in our experimental setup.

To monitor ATF6 activation, HeLa cells expressing pEGFP-ATF6 were live-imaged upon ER stress induction with tBuBHQ. Immunofluorescence experiments for colocalization with giantin (Golgi complex marker) revealed an increased fluorescence in the Golgi complex upon ER stress (Figure 3c). Quantification of Golgi/ER intensity ratios revealed a dose dependent ATF6 activation, as for the other sensors (Figure 3d). Kinetics were similar to those obtained for PERK, while dose sensitivity appeared bimodal with similar patterns for the two lowest and the two highest tBuBHQ concentrations. These results reveal that ER luminal Ca^{2+} concentration decrease is detected by the three UPR sensors thus impacting on the three branches of the UPR in an hour time. Moreover, IRE1, PERK and ATF6 are activated at different rates and sensitivities, suggesting different characteristics of activation by the increase in [UP] triggered by the decrease in $[Ca^{2+}]_{ER}$, a mechanism that might be cell-type specific.

IRE1 and PERK activation is reversible upon repletion of ER Ca^{2+}

To gain insight into the possible mechanisms controlling the initial activation kinetics and sensitivities of the three branches of the UPR, we developed minimal computational models describing IRE1, PERK and ATF6 activation. These models, schematized in Figure 4a-b, assume the existence of pre-formed oligomers of IRE1 and PERK and describe activation of the sensors upon dissociation of BiP. Bip-free IRE1 and PERK can autophosphorylate. For ATF6, the free sensor can translocate to the Golgi apparatus. For each branch, the evolution of BiP concentration is imposed by the changes in ER Ca^{2+} concentration shown in Figure 2b. In the absence of available information about the kinetic constants, we used the genetic algorithm for optimization of the parameters provided in the COPASI software (17). It predicts that IRE1 has a lower affinity for BiP and autophosphorylates in larger oligomers than PERK. Computational simulations for the activation of each sensor is reported in Figure 4c. Because the molecular steps involved in the model for IRE1 and PERK are all reversible, it is implicitly considered in the models that the inductions of the branches should be reversible if $[Ca^{2+}]_{ER}$ is brought back to its initial value. To assess the validity of this assumption, we used the model to predict the evolution of the levels of

phosphorylated sensors upon washing of the reversible SERCA inhibitor tBuBHQ. As shown in Figure 4d, in the presence of external Ca^{2+}, $[Ca^{2+}]_{ER}$ is predicted to increase rather rapidly, reaching about 80% of its full content in ~30 min, with a slower rate of replenishment afterwards. Concomitantly, the levels of phosphorylation of both IRE1 and PERK both decrease and tend to recover their basal levels (Figure 4e).

To validate this prediction, we imaged $[Ca^{2+}]_{ER}$ changes as described above in response to a 90 min treatment with 30 μM tBuBHQ, followed by washout. The steep decrease in $[Ca^{2+}]_{ER}$ was followed by store replenishment displaying biphasic kinetics (Figure 5a). In another set of experiments, we monitored IRE1 and PERK phosphorylation status in cells subjected to the tBuBHQ treatments (Figure 5b). As predicted by the model, drug washout resulted in a decrease in the amount of the phosphorylated sensors, which both reach their basal level in about 1h, thus mirroring the evolution of $[Ca^{2+}]_{ER}$ recovery (Figure 5b, c). However, de-activation of IRE1 and PERK was surprisingly faster in the experiments than in the model, with half-times of restoration equal to 15.4 min and 14 min for IRE1 and PERK, respectively, instead of 74 min and 20 min in the simulations. Moreover, the return to pre-treatment activation/phosphorylation levels was faster for IRE1 than for PERK, in contrast to the model's predictions. In the model, the faster return of PERK was likely due to its larger affinity for BiP, which is in agreement with the observed faster activation of the IRE1 branch. Such discrepancies in the recovery times cannot be alleviated by changing the ratios between the values of the rate constants of phosphorylation and dephosphorylation but point to the existence of an additional mechanism that regulates IRE1 and PERK recovery. Accordingly, when considering the activation of a phosphatase activity by Ca^{2+}, simulations are able to account for the observed fast recovery (Figure 5d).

In summary, observations and modeling indicate that the three branches of UPR are initiated with different kinetics that can be ascribed to differences in the early steps following BiP unbinding. Hence, the activation status of the IRE1 and PERK UPR sensors rapidly mirrors the variations in $[Ca^{2+}]_{ER}$ upon both depletion or restoration.

Discussion

In this work, we show that gradual ER luminal Ca^{2+} depletion results in increased production and accumulation of improperly folded proteins and that UPR induction is triggered even in conditions in which the ER Ca^{2+} content is only partially reduced. This approach differs form the classical studies on UPR activation in which massive depletion of Ca^{2+} is maintained over a long period

using the irreversible SERCA inhibitor thapsigargin. Previous studies explored the relationship between moderate depletion of ER Ca^{2+} and induction of the UPR (8, 9). In contrast with our work, these studies did neither specifically focus on the immediate activation of the sensors nor on the mechanism that allows the UPR tranducers to sense luminal Ca^{2+} alteration. Moreover, our experimental investigation was accompanied by mathematical modelling. Similar approaches already allowed characterizing the kinetics and cross-regulations between the three UPR branches (18), but until now no model has focused on the first steps of UPR induction in response to moderate changes in ER Ca^{2+} concentration.

Herein, the application of different doses of tBuBHQ led to intermediate ER Ca^{2+} steady states that more closely recapitulate possible Ca^{2+} alterations likely relevant to both physiological and pathological contexts. UPR sensors readily respond to Ca^{2+} variations with different kinetics and sensitivities. The model considers BiP titration-dependent sensors' activation and predicts a lower affinity of IRE1 for BiP compared to PERK. This could explain the faster activation kinetics of IRE1 in response to Ca^{2+} depletion compared to PERK and suggests that IRE1 more rapidly responds to BiP dissociation than PERK. Indeed reduction in ER Ca^{2+} concentration negatively influences BiP's ability to bind to client proteins and therefore also to sensors (19, 20). The model also suggests the existence of larger oligomers of IRE1 than PERK, which relates to a more progressive activation of the latter sensor. Previous studies have shown that ER stress sensors could exist in preformed complexes at the ER membrane and upon ER stress would form even larger structures (21, 22). Our model suggests that dimerization/oligomerization step does not play a key part in the activation of IRE1 and PERK sensor when it comes to a BiP titration-dependent activation. Decreases in BiP mobility were considered as a proxy for the accumulation of improperly folded proteins in the ER. While this is in line with previous studies (Lai et al., 2010), it has also been demonstrated that BiP forms oligomers which can participate to the regulation of BiP active monomers. Ca^{2+} depletion favors oligomer formation and detachment from substrates (20) thereby allowing the cell to respond more readily to Ca^{2+} fluctuations by regulating BiP accessibility. We can therefore not exclude that Ca^{2+} dependent reduction in BiP mobility that we observed in our FRAP experiments is partially due to formation of BiP oligomers.

To our knowledge, this work is the first to closely explore the reversion of UPR sensor activation in conditions of ER Ca^{2+} replenishment. Ca^{2+} dependent induction of ER stress is generally obtained by treatment with thapsigargin, which inhibits SERCA pumps in an irreversible manner and therefore is not suited to explore reversion phenotypes. Previous studies have shown that washout

of cyclopiazonic acid (CPA), another reversible SERCA2B inhibitor, results in ER stress resolution, and restoration of PERK sensor activation but without having a close look at the kinetics (23–25). In the present study, we found that SERCA2B inhibitor washout reversed IRE1 and PERK phosphorylation in less than 1h, mirroring very closely the kinetics of Ca^{2+} store replenishment and suggesting that these proteins sense luminal Ca^{2+} levels very well. Such a rapid reversion indicated that there could be an induction of a Ca^{2+} sensitive phosphatase involved in the dephosphorylation of the sensors. A well-documented Ca^{2+} sensitive cytoplasmic phosphatase is calcineurin. It has been shown that calcineurin can associate with active PERK in the presence of high Ca^{2+}, so presumably soon after ER stress induction by SERCA pump inhibition. This interaction increases PERK phosphorylation and plays a role in PERK downstream signaling (26). At this stage it is difficult to rule out that another phosphatase could be responsible for the deactivation of the sensors. Of note, our knowledge regarding phosphatases that counteract the activation of UPR sensors IRE1 and PERK is very limited, although some phosphatases have been shown to dephosphorylate IRE1 in yeast and mammals (27–30). In addition, a ER localized tyrosine phosphatase, PTP-1B, has been shown to regulate IRE1 signaling and *Xbp1* mRNA splicing via dephosphorylation of RtcB ligase (31, 32) as well as the PERK pathway (33, 34). Importantly, it has been shown that PTP-1B is negatively regulated by Ca^{2+} influx through TRPV1 and via store-operated Ca^{2+} entry (SOCE) mehanisms (35, 36).

Inactivation of the UPR sensors still remains largely unexplored and current research mainly focuses on the most conserved sensor IRE1. Indeed, IRE1 is negatively regulated by a complex formation with anti-apoptotic protein BAX inhibitor-1 (BI-1) (37) and by direct binding with PDIA6 which acts on possible disulfide formation to stabilize oligomers, a mechanism also found for PERK (38). In addition, some other studies have suggested that reversion of sensors' activation could be finely tuned by modulation of their oligomeric status and its association with BiP via Sec61 and the Sec63 translocon proteins. (39, 40). It might be possible that restoration of ER luminal Ca^{2+} impacts and accelerates these processes. Along this line, we showed here that the observed fast reversion of IRE1 and PERK activation is best described by the model when taking into account an activation of the phosphatase activity by Ca^{2+}. This activation would take place at the phosphorylation sites of the sensor located on the cytoplasmic side of the ER membrane, where significant fluxes are supposed to occur upon store replenishment. The high SOCE activity is indeed at the origin of microdomains in which Ca^{2+} concentrations can become larger than in the rest of the cytoplasm (41). Our work did not explore reversion of activation occurring at the level of

ATF6 sensor. Our experiment monitors translocation of ATF6 to the Golgi complex while the models assume that the pool of translocated sensor is immediately cleaved by Golgi proteases to reach the nucleus. However, we cannot exclude that restoration of ER Ca^{2+} levels could impact on the downregulation of ATF6 signaling via modulation of retrograde transport, the inhibitory binding to BiP or redox dependent ATF6 dimerization (42) or through the fact that that ATF6 is a natively unstable protein in the ER (43, 44).

In conclusion, our work documents the tight relationships existing between ER Ca^{2+} concentrations and the activation of the three UPR transducers. The combined use of experimental approaches and mathematical modeling has allowed us to describe for the first time a rapid mechanism of IRE1 and PERK deactivation upon Ca^{2+} replenishment in the ER lumen which could explain the very high plasticity of the UPR upon physiological management of ER stress.

Materials and Methods

Cell culture and treatments - Hela cells were cultured in RPMI medium (Gibco™-Thermo Fischer Scientific) supplemented with 10% FBS (Gibco™ 10270 -Thermo Fischer Scientific) at 37°C in a 5% CO2 incubator. tBuBHQ was purchased from Sigma Aldrich (112976) and dissolved in DMSO. Cells were treated with the corresponding concentrations of tBuBHQ for the indicated times.

Transfection and plasmids - Plasmid transfections were carried out 24h after cell plating (except when otherwise indicated) using FuGENE® HD Transfection Reagent (Promega), following manufacturer's instructions. After 6h, cells were washed with PBS for two times and fresh culture medium was added. pCIS GEM-CEPIA1er (#58217), peGFP-ATF6 (#32955), BiP-mGFP (#62231) and BiP-mCherry (#62233) were purchased from Addgene.

Live imaging for calcium measurements and ATF6 translocation - HeLa cells seeded on 25 mm coverslips and transfected with pCIS GEM-CEPIA1er or peGFP-ATF6 24 and 48h after plating respectively. Cells were placed in an observation chamber in imaging medium containing 115 mM NaCl, 5.6 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM $NaHPO_4$, 10 mM $NaHCO_3$, 20 mM HEPES (pH = 7.4) and 1 mg/ml glucose. Samples were analyzed at room temperature on an

inverted Nikon eclipse TE200 fluorescence microscope, using a 60x objective. Illuminating sources used were ex. 380 nm; em. 480nm/535nm for GEM-CEPIA1er and ex. 485 nm; em. 535 nm for GFP-ATF6 driven by Simple 32 Software from Compix Incorporated. Images were captured using a CMOS camera (Hamamatsu). Images were analyzed with Simple 32 software. Changes in the ratio of GEM-CEPIA1er were transformed into ER Ca^{2+} percentage by taking the mean intensity value at resting conditions as 100% and lower value as 0%. Fold change of GFP-ATF6 translocation was calculated by dividing Golgi intensity values by ER intensity values for each time point.

Cell manipulations and Western Blot analyses - To obtain total cell extracts cells were lysed in sample buffer 1x (62.5 mM Tris pH=8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 5% β-mercaptoethanol), sonicated and boiled at 95°C for 5 minutes. Proteins from total lysates were separated by SDS PAGE or Phos-tag ™ SDS PAGE as reported in (16) and transferred to nitrocellulose membrane (0.45µM Amersham™ Protran™). Western Blot analysis were performed according to standard procedure using the following primary antibodies: PERK (Cell Signaling Technologies C33E10), IRE1α (Cell Signaling Technologies 14C10), HSP90 (Santa Cruz Biotechnologies sc-13119). HRPO conjugated anti-mouse (Cytiva) and anti-rabbit (Sigma) were used as secondary antibodies.

Immunofluorescence analyses - After fixation with PFA, cells were permeabilized with 0.1% Triton X-100 for 5 min. Samples were washed three times with PBS and blocked with PBS containing 3% FBS for 30 min. Cells were incubated for 2h with primary mouse antibody against giantin (1:200) provided by E. Chevet. Cells were incubated with the corresponding secondary anti-mouse IgG antibodies for 1h. Samples were mounted in Dako mounting medium (DAKO) and analyzed using a Nikon Ti2 confocal microscope with a 60× objective, controlled by Nikon software.

FRAP experiments - Cells were cultured on 25-mm-diameter coverslips and transfected with BiP-mGFP or BiP-mCherry 24h before performing the experiment. Cells were mounted in an observation chamber in imaging medium (see above). Experiments were performed using an inverted spinning disk confocal microscopy (Gataca system) and analyzed with a 100x objective, using an sCMOS camera (Photometrics) equipped with a FRAP module and driven by the

Metamorph software (Roper Scientific Instruments), allowing to the acquisition of images during the bleaching phase. Samples were treated with 30µM tBuBHQ for the indicated times or left untreated. For each sample, bleaching and image acquisition were performed using identical conditions. Bleaching was performed for 50ms using 50%of maximal laser power. Fluorescence curves were corrected for background levels and for fluorescence bleaching. Normalized recovery curves were fitted using easyFRAP for MATLAB (45)

Statistical Analyses - Data are expressed as mean ± SEM of three or more independent experiments. Differences were analyzed by Anova test using Prism 7 (GraphPad). P-values < 0.05 were considered significant.

Model description

Calcium dynamics - Ca^{2+} is pumped from the cytosol into the endoplasmic reticulum through the SERCA pump. The latter is inhibited by tBuBHQ (tBu), with a K_D value equal to 2 □M (10). In the opposite way, Ca^{2+} leaks from the ER into the cytosol. Ca^{2+} exchanges between the cytoplasm and the extracellular medium are mediated by the PMCA that extrudes Ca^{2+} out of the cell and by Store-Operated-Ca^{2+}-entry (SOCE), a STIM/ORAI mediated mechanism that is sensitive to the Ca^{2+} concentration inside the ER. The resulting changes in ER (C_{ER}) and cytosolic (C_c) Ca^{2+} concentrations are given by the following equations (Dupont et al. 2016) :

$$\frac{dC_{ER}}{dt} = \alpha\left(V_e \frac{C_c^2}{C_c^2+K_e^2} \cdot \frac{K_I^n}{tBu^n+K_I^n} - k_{out}[C_{ER} - C_c]\right) \quad (1)$$

$$\frac{dC_c}{dt} = k_{out}[C_{ER} - C_c] - V_e \frac{C_c^2}{C_c^2+K_e^2} \cdot \frac{K_I^n}{tBu^n+K_I^n} - V_p \frac{C_c^2}{C_c^2+K_p^2} + V_S \frac{K_S^4}{C_{ER}^4+K_S^4} \quad (2)$$

Definitions and default values of the parameters are listed in **Table S1**.

Estimation of the effective diffusion coefficients of BiP and of the related concentrations of unfolded proteins - FRAP experiments were performed after different times of treatment of HeLa cells with tBu 30 □M and revealed distinctive half-times of recovery of BiP-associated fluorescence (Figure 2c). The observed step-up in the half-times reflects the increasing binding of unfolded proteins to

BiP. To infer the value of the diffusion coefficient of BiP –representing a weighted average of free and UP-bound forms– from the measured values of half-times, we performed 3D simulations of diffusion of BiP molecules at a concentration of 100 µM (47), surrounding an empty hole with the same radius as the photobleached region. We looked for the values of D_{BIP} allowing to obtain half-times of recovery corresponding t observations. For each experimental point (corresponding to a given time after tBu treatment, see Figure 2c), best agreement was always close to the expected value with the measured half-times, i.e. with

$$D_{BIP} = 0.224 \cdot \frac{r_n^2}{t_{1/2}} \quad (3)$$

where r_n is the radius of the uniform bleach laser and $t_{1/2}$ the measured half-time (48).

From these effective diffusion coefficients, the corresponding concentrations of UP can be evaluated by

$$[UP] = \frac{D_{BIP}^0 - D_{BIP}}{D_{BIP} - D_{UP}} \left(\frac{(K+[BiP])^2}{K} \right). \quad (4)$$

This equation is analogous to that used to quantify the decrease in the effective diffusion coefficient of Ca^{2+} (the "equivalent" of BiP) in the presence of Ca^{2+} buffers (the "equivalent" of UP). In Equation (4), D_{BIP}^0 stands for the diffusion coefficient of free BiP, D_{UP} for the diffusion coefficient of an average UP in the ER and K, for the dissociation constant of UP from BiP. The value of D_{BIP}^0 was estimated from half-time at time 0 (Figure 2c) and D_{UP} was calculated using the Einstein-Stokes relation and was found to be equal to 0.009086727 $\mu m^2 s^{-1}$, considering a hydrodynamic radius equal to 0.035µm (49). [BiP] was taken equal to 100 µM (47) and K, to 8.7 µM (50).

Activation of the UPR sensors - Activation of the three branches of the UPR is triggered independently by BiP unbinding from the corresponding sensors. In conditions of a full ER and in the absence of stress inducer corresponding to a low concentration of unfolded proteins (UP) in the ER, IRE1, PERK and ATF6 sensors are bound to BiP. The relation between ER Ca^{2+} changes and BiP dynamics is schematized in Figure 1D. It is assumed that unfolded protein (UP) production in the ER is inhibited by ER Ca^{2+}, as a simplified way of representing the Ca^{2+} dependence of protein chaperons and foldases. UP bind to BiP with high affinity. This initiates the folding process, after

which free BiP is recovered. The model also allows for the possibility of incomplete protein folding, in which case BiP is freed together with the modified UP. Corresponding evolution equations are:

$$\frac{dUP}{dt} = v_{prod} \cdot P_{UP} - k_{BU} \cdot BiP \cdot UP + k_2 \cdot BiP\text{–}UP - k_d \cdot UP \quad (5)$$

$$\frac{dBiP}{dt} = (k_1 + k_2) \cdot BiP\text{–}UP - k_{BU} \cdot BiP \cdot UP \quad (6)$$

$$\frac{dBiP\text{–}UP}{dt} = k_{BU} \cdot BiP \cdot UP - (k_1 + k_2) \cdot BiP\text{–}UP \quad (7)$$

$$\frac{dP_{UP}}{dt} = \frac{1}{\tau_{UP}}\left(\frac{K_{ER}}{C_{ER}+K_{ER}} - P_{UP}\right) \quad (8)$$

where UP, BiP and BiP-UP stand for the concentrations of unfolded proteins, free BiP and UP-bound BiP, respectively. P_{UP} is a function that modulates the production of UP, depending on the concentration of ER Ca^{2+}. Parameter τ_{UP} accounts for a possible delay between ER Ca^{2+} decrease and accumulation of UP. Eq. (5)-(7) correspond to the mass action law description of the mechanism schematized in Figure 2d. Definitions and default values of the parameters are listed in Table S1.

We assume that the activation of the IRE1 and PERK sensors obey the same mechanisms (Figure 4a) but differ in their kinetics, i.e. involve different rate constants. Thus, in the following description, the term "sensor" stands for IRE1 or for PERK. Preformed oligomers of the sensor are supposed to be present in the ER membrane (21, 22). S represents the concentration of BiP-unbound, unphosphorylated oligomers. These oligomers can bind BiP, with a stoichiometry that corresponds to the size of the oligomer. As the sensor can be free (S), bound to Bip (BiP-S) or phosphorylated (S_p), the conservation relation for the sensor reads:

$$S_{tot} = S + BiP\text{–}S + S_p \quad (9)$$

We assume rapid equilibrium on the reactions of BiP binding and unbinding to and from the sensors, i.e.

$$n_{SB} \cdot k_{SB}^{+} \cdot (S_{tot} - S_P - BiP\text{–}S) \cdot BiP^n = n_{SB} \cdot k_{SB}^{-} \cdot BiP\text{–}S. \quad (10)$$

The rate of change of the concentration of sensor oligomers due to phosphorylation and dephosphorylation is given by:

$$\frac{dS_P}{dt} = k_{sk}S - k_{sp}S_p \quad (11)$$

where k_{Sk} and k_{Sp} stand for the rate constants of the kinase and the phosphatase, respectively.
Changes in the concentration of phosphorylated sensor can be described by a single differential equation:

$$\frac{dS_P}{dt} = \frac{1}{\tau_{SP}}\left(S_{tot} \cdot K_{sp} \cdot \frac{K_{SB}^{ns}}{BiP^{ns} + K_{SB}^{ns}} - S_P\right) \quad (12)$$

In eq. (12), τ_{SP} is the inverse of the rate constant of dephosphorylation, i.e. $1/k_{sp}$). S_{tot} stands for the total concentration of oligomers of the sensor, K_{Sp} for the ratio between the first order kinetic constants of phosphorylation (k_{Sk}) and dephosphorylation (k_{sp}), and K_{SB} for the ratio between the unbinding (k_{SB}^{-}) and binding (k_{SB}^{+}) rate constants of BiP from and to the sensor. The Hill-type factor follows the assumption of rapid equilibrium for BiP binding and unbinding to and from the sensor, with ns being the number of monomers that form an oligomer. It is also assumed that the concentration of phosphorylated sensor is much smaller than the total concentration of sensor ($S_P << S_{tot}$) given that we model early and moderate activation of UPR. Table S1 lists the values of the parameters, with the "S" being replaced by "I" and "P" for the IRE1 and PERK pathways, respectively.

Similarly, activation of the ATF6 branch of UPR follows the unbinding of BiP from this sensor. This triggers the cleavage of ATF6 and its translocation to the Golgi, as schematized in Figure 4b.

Thus, the evolutions of BiP-bound, free and translocated ATF6 are given by the following equations:

$$\frac{dA\text{–}BiP}{dt} = k_{AB}^{+} \cdot A \cdot BiP - k_{AB}^{-} \cdot A\text{–}BiP \quad (13)$$

$$\frac{dA}{dt} = k_{AB}^{-} \cdot A\text{–}BiP \; - \; k_{AB}^{+} \cdot A \cdot BiP - k_{AC} \cdot A \quad (14)$$

$$\frac{dA_c}{dt} = k_{AC} \cdot A - k_{dAC} \cdot A_c \quad (15)$$

Definitions and values of the parameters are listed in Table S1.

Model extension to account for the observed fast kinetics of reversion - Although the model qualitatively predicted reversion of IRE1 and PERK activation, the simulated dephosphorylation of the two sensors was slower than in the experiments (compare Figure 4e and Figure 5c). We therefore extended the model to take into account the possibility that a Ca^{2+} sensitive phosphatase participates in the inactivation of the sensors. Such phosphatase activity has progressevily accumulated near the sensors during their activation. The rate constant of the phosphatase k_{sp} (eq. 9) now reads:

$$k_{sp} = \overline{k_{sp}} \left(1 + \alpha_{sr} \frac{C_{ER}}{K_{sr} + C_{ER}}\right) \quad (16)$$

The Ca^{2+} concentration in the ER is taken as a proxy for the Ca^{2+} concentration on the cytoplasmic side of the ER membrane where the phosphorylation sites of the sensors are located. Definitions and default values of the parameters are listed in Table S1.

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Data availability

Detailed experimental and modeling data are available upon request.

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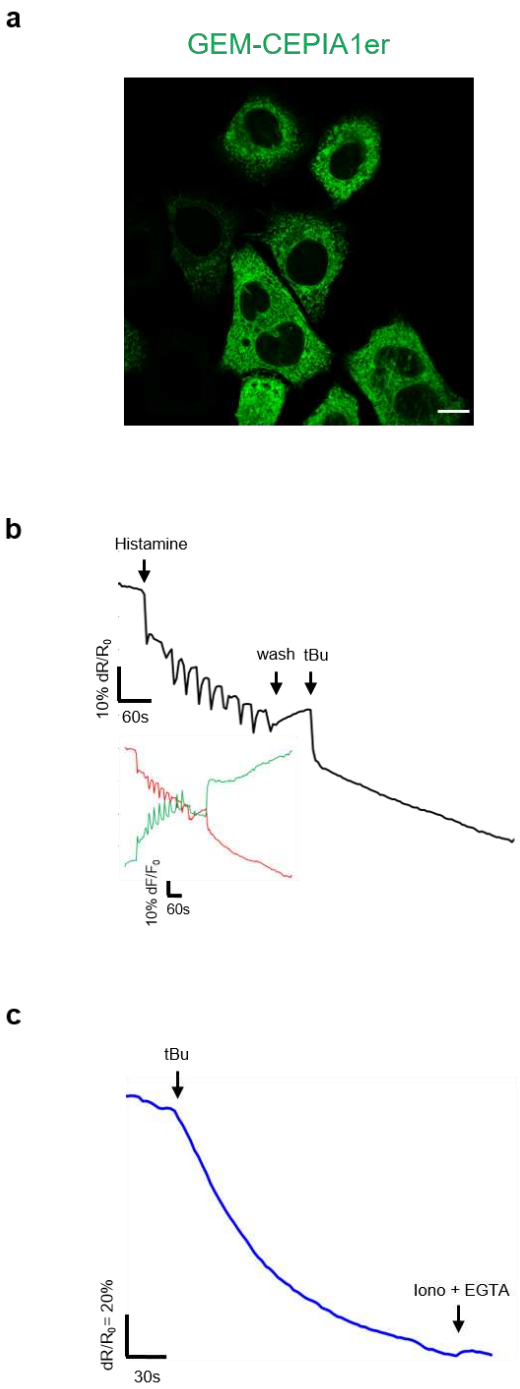

Figure 1. tBuBHQ-mediated Calcium depletion in the ER. **a.** HeLa cells expressing GEM-CEPIA1er as analysed using confocal microscopy. Scale bar = 10µm**. b.** Representative Ca^{2+} traces obtained in HeLa cells expressing GEM-CEPIA1er Ca^{2+} indicator treated successively with histamine 50µM and 30µM tBuBHQ. Black trace - ratio values between emission wavelengths F_{480}/F_{535}, red and green traces report fluorescence variation at 480nm and 535nm, respectively. **c.** Representative Ca^{2+} traces obtained in HeLa cells expressing GEM-CEPIA1er and treated with 30µM tBuBHQ. After plateau was reached, cells were treated with Ionomycin (Iono) 2µM and EGTA 20mM as controls.

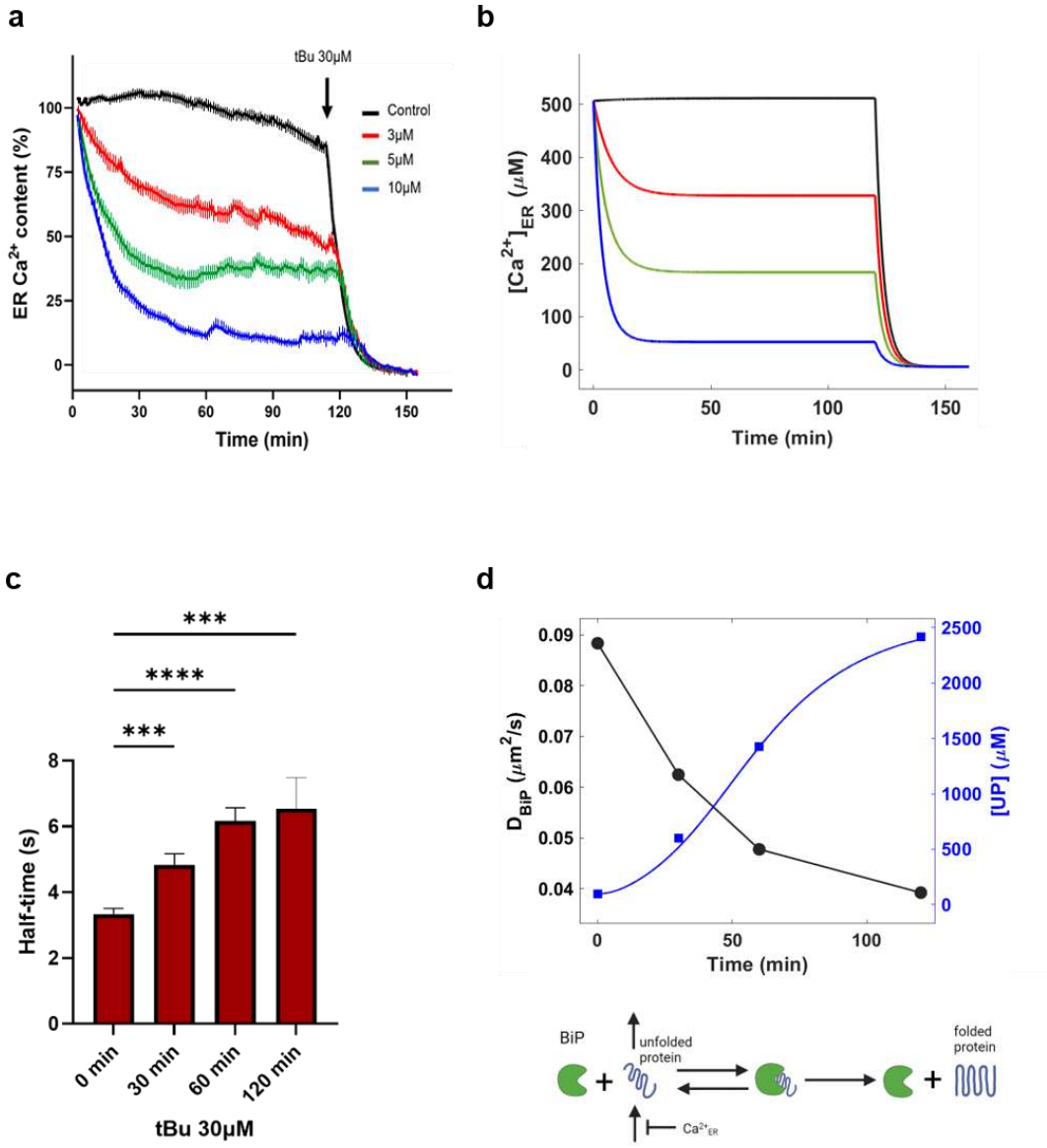

Figure 2. ER Calcium concentration-dependent BiP diffusion in the ER. a. HeLa cells expressing GEM-CEPIA1er were treated with indicated concentration of tBuBHQ (tBu). Percentage of ER Ca2+ was calculated from ratio of emission wavelengths values (F480/F535). Data display mean ± SEM of n=117 (control), n=66 (3µM), n=32 (5µM), n=36 (10 µM). b. Computational simulation of the time evolution of ER Ca2+ concentration after the addition of the tBu inhibitor for 2h at different concentrations (3 µM, (red); 5 µM, (green); 10 µM, (blue)) as in a). At 2h, addition of 30 µM tBu is simulated for all conditions. c. Half-time values resulting from FRAP analyses performed in HeLa cells expressing BiP-mGFP and treated with 30µM tBu for the indicated times. Data display mean ± SEM of n=49, n=32, n=36, n=24. d. Theoretical evaluation of BiP's effective diffusion coefficients and the corresponding concentrations of unfolded proteins on the basis of the half-times of recovery of fluorescence after photobleaching (panel c). The blue curve represents the simulated evolution of UP for 30µM tBu, using equations (5)-(8) described in the Material and Methods section.

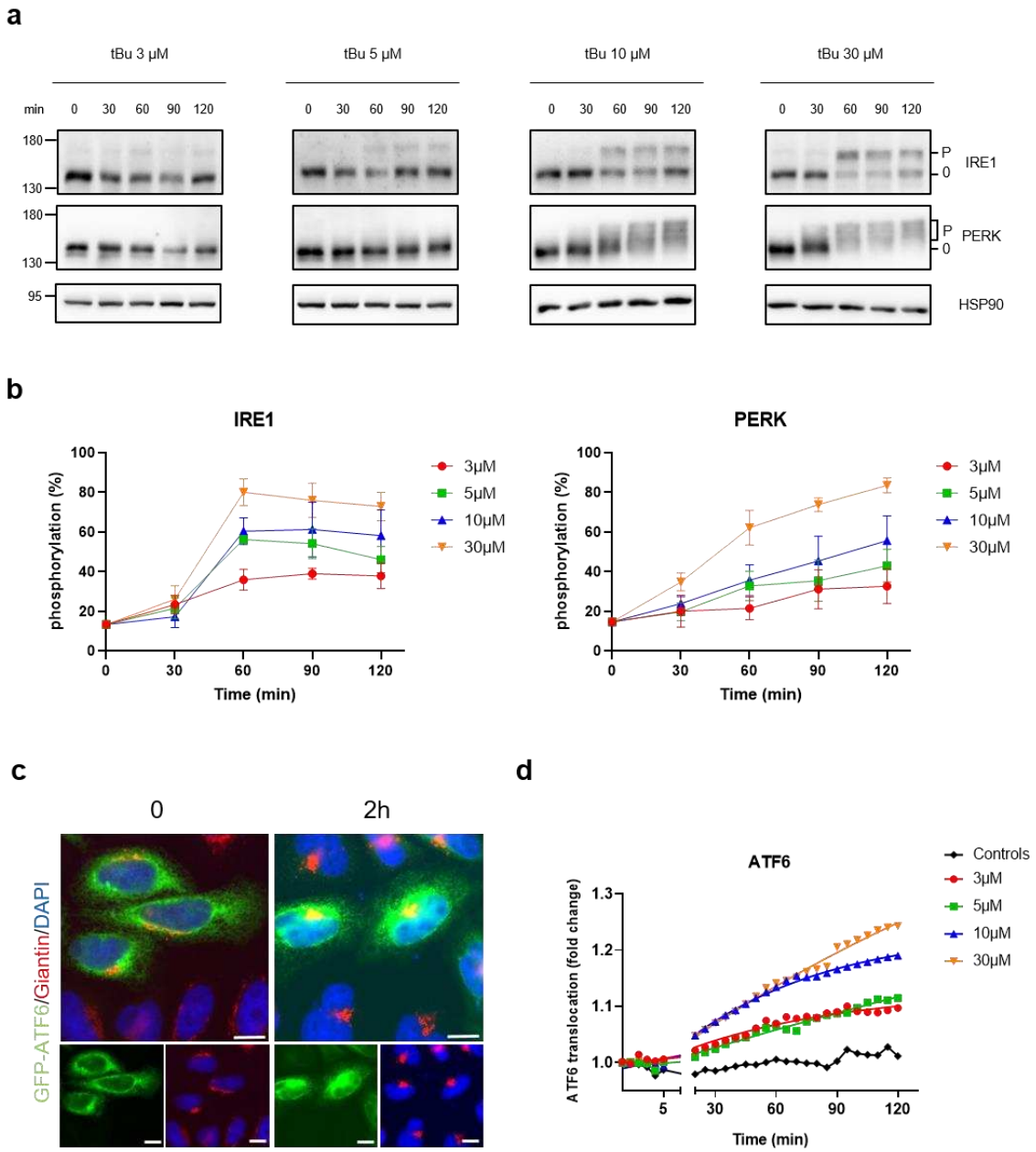

Figure 3. Incremental activation of the UPR sensors upon Calcium depletion in the ER. a. HeLa cells were treated with the indicated concentrations of tBu. PERK and IRE1 phosphorylation status was monitored using Phos-tag™ SDS-PAGE and Western blot. b. Quantification of results presented in (a) reported in percentage of phosphorylated protein over the total PERK/ IRE1 protein quantity (mean ±SEM of n=3). c. ATF6 subcellular colocalization with Giantin (Golgi) and DAPI (nucleus). Scale bar =10µM. d. ATF6 translocation to the Golgi following treatment with the indicated doses of tBu. Values of Golgi/reticular intensity ratio were normalised to basal values observed before the addition of tBu. Mean of n=45 (control), n= 43 (3µM), n=39 (5 µM), n=22 (10 µM), n=51 (30 µM). SEM was omitted for the sake of clarity (data are provided in Figure S3).

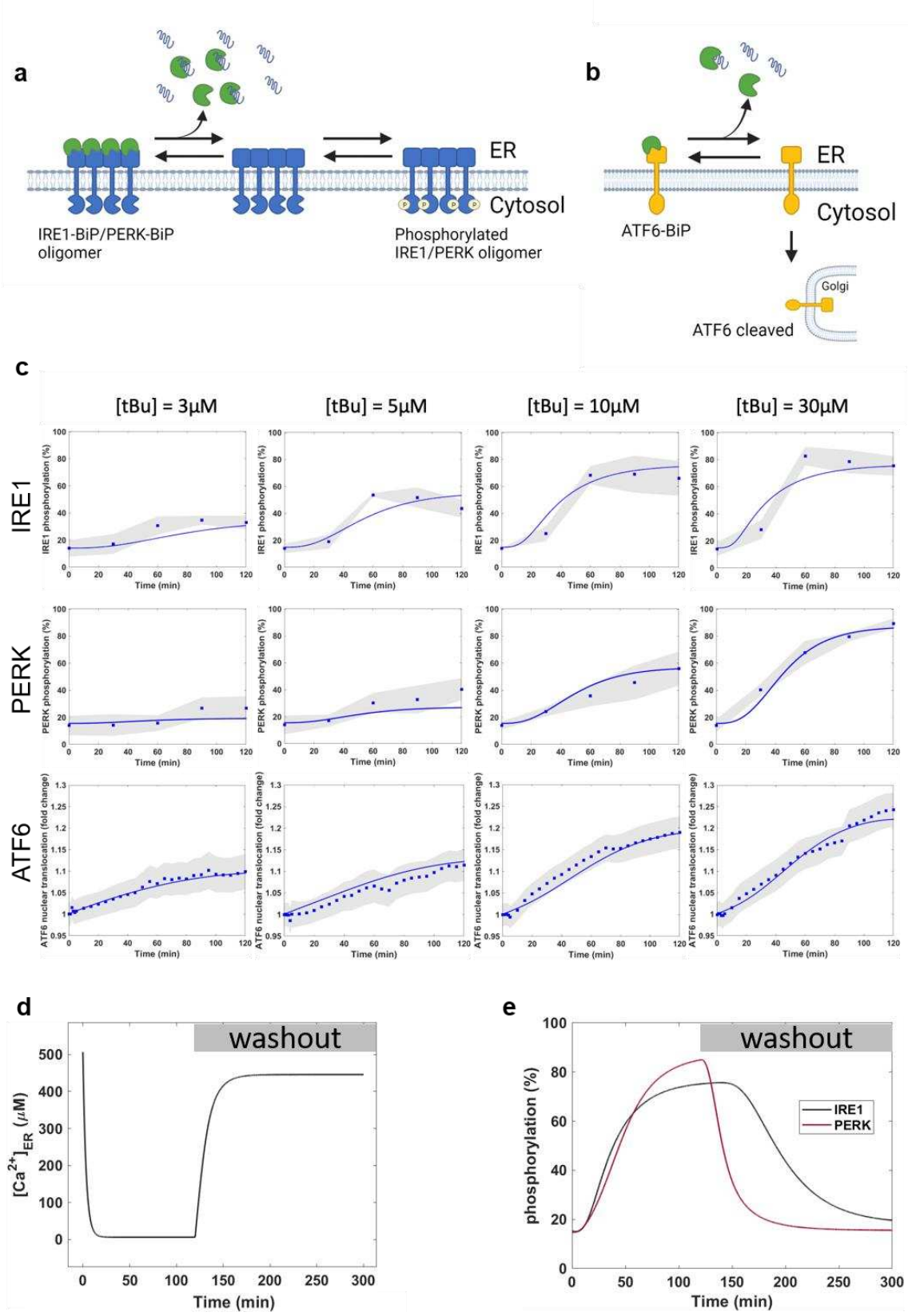
a
ER
Cytosol
IRE1-BiP/PERK-BiP
oligomer
Phosphorylated
IRE1/PERK oligomer
b
ER
Cytosol
ATF6-BiP
Golgi
ATF6 cleaved
c
[tBu] = 3μM
[tBu] = 5μM
[tBu] = 10μM
[tBu] = 30μM
IRE1
PERK
ATF6
IRE1 phosphorylation (%)
PERK phosphorylation (%)
ATF6 nuclear translocation (fold change)
Time (min)
d
washout
$[Ca^{2+}]_{ER}$ (μM)
Time (min)
e
washout
phosphorylation (%)
IRE1
PERK
Time (min)

Figure 4. Data-driven computational simulation of IRE1, PERK and ATF6 activation. a. Schematic representation of the model of activation of IRE1 and PERK based on the dissociation of BiP from the sensors upon accumulation of UP. The modes of activation are assumed to be similar for IRE1and PERK, although the two differ by the values of the kinetic constants. The model assumes the existence of pre-formed oligomers of IRE1 and PERK. Equations of the model are given in the Material and Methods section. b. Schematic representation of the model of activation of ATF6 based on the dissociation of BiP upon accumulation of UP. When freed from BiP, ATF6 translocates to the Golgi complex where it is cleaved by S1P and S2P. Equations of the model are given in the Materials and Methods section. c. Computational simulations of the activation of the 3 branches of the UPR after treatment with different concentrations of tBu. Blue lines correspond to the results of the simulations of the models schematized in a and b, with the equations (1), (2), (5)-(15) and the parameter values listed in Table S1. Blue squares represent experimental data and the grey shaded region, the curve interpolated between those data ± SEM. d. Model prediction of the evolution of ER Ca2+ concentration after washout of tBu 30 µM. In the model equations (1), (2), tBu concentration is multiplied by 0.05 at time 120 min. e. Model prediction of the reversion of IRE1 and PERK activation after washout of tBu 30 µM, corresponding to the evolution of [Ca2+]ER shown in panel d. Results were obtained by integration of Equations (1), (2), (5)-(12).

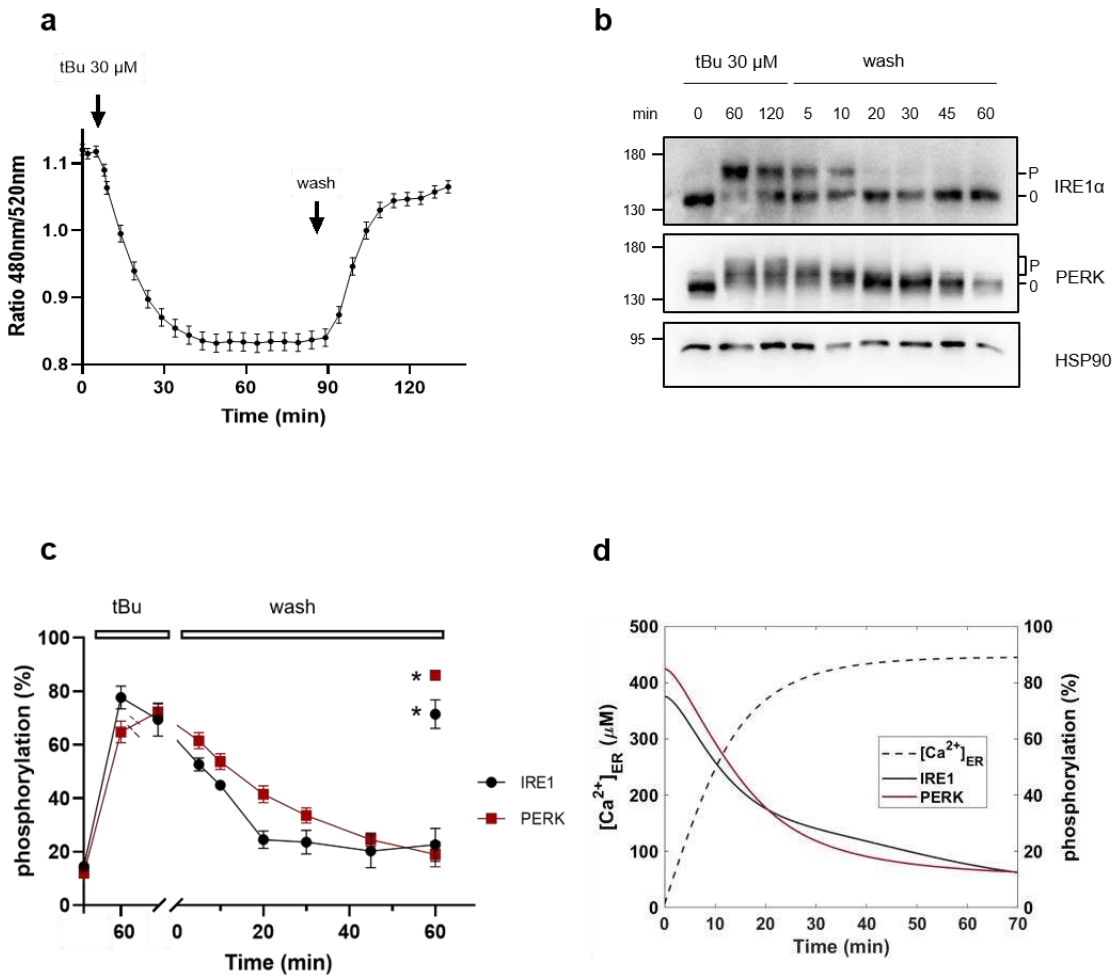

Figure 5. Reversible activation of the UPR sensors upon ER Calcium restoration. a. HeLa cells expressing GEM-Cepia1er Ca2+ were treated with 30 µM tBu for 90 min then washed twice to remove all traces of SERCA inhibitor. The graph shows ratio values between emission wavelengths F480/F535 (mean ± SEM of n=46). b. HeLa cells were treated with 30 µM tBu for 2h and washed twice before being incubated in normal culture medium. PERK and IRE1 phosphorylation status was monitored using Phos-tag™ SDS-PAGE/Western blot. c. Quantification of results presented in (b) as percentage of phosphorylated protein over the total PERK/ IRE1 protein quantity (mean ± SEM of n=4). Asterisks (*) at 60 min indicate IRE1 and PERK values when tBu treatment was maintained for the entire duration of the experiment and no washing was performed. d. Model simulation of the faster reversion of IRE1 and PERK activation after washout of tBu 30 µM when taking into account a possible Ca2+ activation of the IRE1 and PERK phosphatases. Results have been obtained with Equations (1), (2), (5)-(11), (16) with the evolution of [Ca2+]ER shown in Figure 4d.

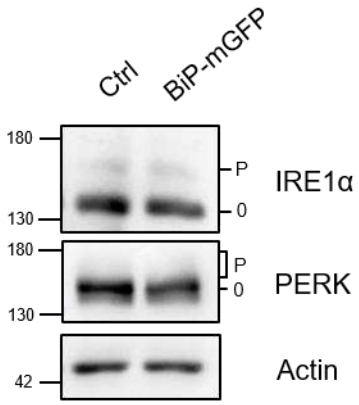

Fig. S1. HeLa cells were transfected with Bip-mGFP 48h after plating. The next day lysates were prepared and PERK and IRE1 phosphorylation status was monitored by Western blot after normal and Phos-tag™ SDS-PAGE respectively.

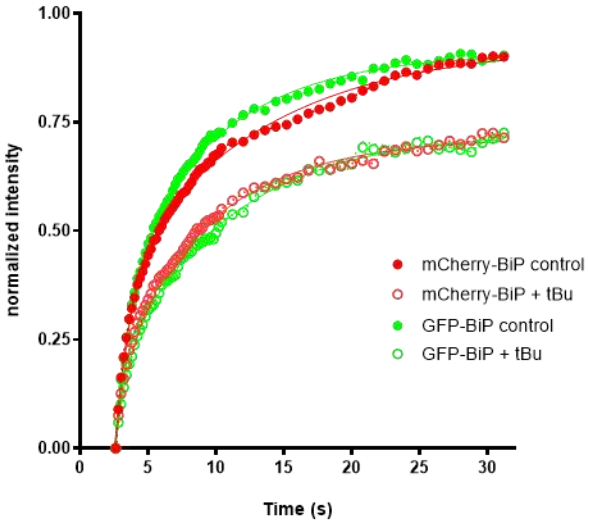

Fig. S2. Curves and non-linear fitting resulting from FRAP experiments performed on HeLa cells transfected 48h after plating with BiP-mGFP or BiP-mCherry for 24h. Cells were treated with 30µM tBu for 1h. Mean curves of (BiP-mCherry Ctrl n=40, BiP-mCherry tBu n=22, BiP-mGFP Ctrl n=30, GFP tBu =22). SEM has been omitted for clarity.

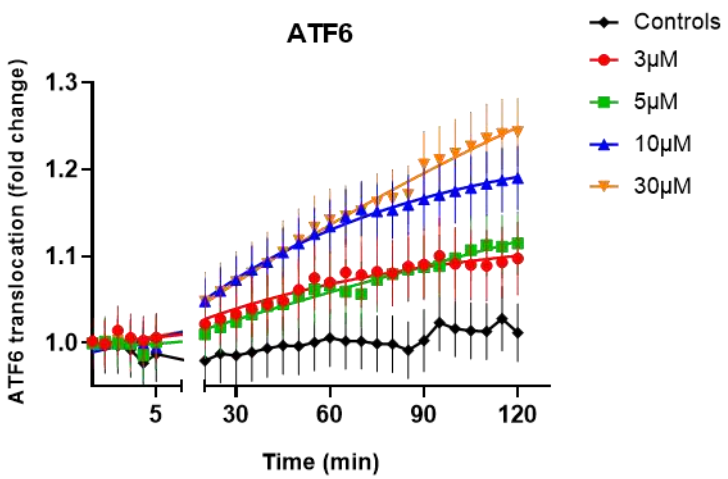

Fig. S3. Fig. 3d with the addition of SEM. Mean±SEM of n=45 (control), n= 43 (3μM), n=39 (5 μM), n=22 (10 μM), n=51 (30 μM).

Table S1. List of the values of the parameters used in simulations. To reach best agreement with observations, values have been optimized using the genetic algorithm provided in the COPASI software (1).

Param.	*Definition*	*Value*	*Reference*
	Ca^{2+} dynamics (Equations (1)-(2))		
K_e	SERCA Ca^{2+} affinity	$0.125\mu M$	(2)
K_I	IC50 of tBuBHQ binding to SERCA	$2\mu M$	(3)
k_{out}	Ca^{2+} leak rate from ER	$5(10)^{-4}s^{-}$	This work
K_P	PMCA Ca^{2+} affinity	1.5µM	(4)
K_s	STIM2 ER Ca^{2+} affinity	$400\mu M$	(5)
V_e	Maximal rate of Ca^{2+} pumping through SERCA	$1.82\mu M /s$	(2)
V_P	Maximal rate of Ca^{2+} pumping through PMCA	5µM/s	(4)
V_s	Maximal Ca^{2+} flux through SOCE	$0.02\mu M /s$	This work
n	Hill coefficient of tBuBHQ binding with SERCA	2	This work
α	Volumic ratio between the cytosol and the ER	10	(6)
	BiP-UP interactions (Equations (3)-(8))		
k_1	Rate constant of protein folding	$15s^{-1}$	This work
k_2	Rate constant of BiP unbinding from unfolded proteins	$0.7s^{-1}$	(7)
k_{BU}	Rate constant of BiP binding to unfolded proteins	$0.0804 \mu M^{-1}s^{-1}$	(7)
k_d	Unfolded protein degradation rate	$0.087s^{-1}$	This work
K_{er}	$K_{1/2}$ for the Ca^{2+} inhibition of unfolded proteins generation	$243\mu M$	This work
V_{prod}	Maximal rate of production of unfolded proteins	$1649\mu M /s$	This work
τ_{UP}	Unfolded protein generation time constant	$1772s$	This work
BiP	Total concentration of BiP	$100\mu M$	(8)

K	Dissociation constant of BiP binding to unfolded proteins (k_2/k_{BU})	$8.7\mu M$	(7)
UPR activation (Equations (9)-(16))			
I_{total}	Total concentration of preformed IRE1 oligomers	$0.249\mu M$	This work
K_{Ip}	Ratio between IRE1 phophorylation and dephosphorylation rate constants	0.7631	This work
K_{IB}	Dissociation constant of BiP from IRE1 (k_{IB}^-/k_{IB}^+)	$51\mu M$	This work
n_{IB}	IRE1 oligomerisation degree	5.38	This work
τ_{IB}	IRE1 phosphorylation timescale	$1579s$	This work
P_{total}	Total concentration of preformed PERK oligomers	$0.249\mu M$	This work
K_{Pp}	Ratio between PERK phophorylation and dephosphorylation rate constants	1.2213	This work
K_{PB}	Dissociation constant of BiP from PERK (k_{PB}^-/k_{PB}^+)	$14.3581\mu M$	This work
n_{PB}	PERK oligomerisation degree	1.3	This work
τ_{PB}	PERK phosphorylation timescale	$185.6s$	This work
k_{AB}^+	Rate constant of BiP binding to ATF6	$1.3\mu M^{-1}s^{-1}$	This work
k_{AB}^-	Rate constant of BiP unbinding from ATF6	$4.54s^{-1}$	This work
k_{Ac}	Rate constante of ATF6 cleavage	$5.08 (10)^{-4}s^{-1}$	This work
k_{dAc}	Transformation rate of cleaved ATF6	$1.71 (10)^{-5}s^{-1}$	This work
α_{IT}	Factor of stimulation by Ca^{2+} of IRE1 dephosphorylation	2.7	This work
K_{IT}	$K_{1/2}$ for the Ca^{2+} activation of IRE1 dephosphorylation	$150\mu M$	This work
α_{PT}	Factor of stimulation by Ca^{2+} of PERK dephosphorylation	0.7	This work
K_{PT}	$K_{1/2}$ for the Ca^{2+} activation of PERK dephosphorylation	$420\mu M$	This work

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Article 2

In order to study the relationship between Ca^{2+} and UPR in a more specific context I focused my attention on the role of UPR during infection of epithelial cells by *Shigella flexneri*. Infection of this pathogen lead to alteration of Ca^{2+} signaling but its impact on the UPR is not known. This project here entitled *The role of UPR during infection of epithelial cells by Shigella flexneri* is still ongoing. Here I present the results that have obtained so far.

The Role of UPR during infection of epithelial cells by *Shigella flexneri*

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Abstract

The Unfolded Protein Response (UPR) is a cell response to the alteration of ER protein folding and quality control (ER stress) that elicits an adaptive signaling pathway, restoring ER homeostasis and promoting cell survival. Due to its role in host inflammatory response and immune cells development and activation, the UPR is activated by various pathogens and regulates bacterial infection. Depending on the infection model, the UPR may orchestrate host responses to restrict infection, or conversely, may be diverted to promote intracellular pathogen survival and replication. *Shigella* is an invasive pathogen that is able to colonize the colonic mucosa, triggering severe inflammation and tissue destruction leading to dysentery. The role of UPR in *Shigella* pathogenesis is poorly understood. It has been shown that Shiga toxin, a virulence factor produced by the enteric pathogens *Shigella dysenteriae* serotype 1, triggers ER stress sensors activation and induces a pro-apoptotic signaling in monocytic and macrophage-like cells. In this study, we explore the role of UPR during epithelial cell infection with *Shigella flexneri* that does not express the Shiga toxin. In preliminary results, upon infection of HeLa cells with *S. flexneri*, we observe that this pathogen induces UPR activation suggesting a role during the colonization of epithelial cells by this pathogen. Interestingly, initial UPR activation is followed by sensor degradation 120 minutes post-infection, an effect that is promoted by specific bacterial effectors secreted by *Shigella* into the host cell. Degradation of UPR sensors mediated by this pathogen shed light on a possible role of sensors' turnover as means of modulating UPR.

Introduction

The endoplasmic reticulum (ER) is the major site of protein folding and quality control. The Unfolded Protein Response (UPR) is a cellular stress response mechanism activated when there is an accumulation of misfolded or unfolded proteins within the ER, commonly termed ER stress (Hetz et al. 2020). The UPR is a complex and highly regulated network of signaling pathways that aims to restore normal cellular proteostasis by increasing ER folding capacity and removing misfolded proteins. Depending on the extent and persistence of ER stress, the UPR can shift from being an adaptive mechanism to becoming a triggering factor for cellular death. This transition may activate an apoptotic program, which has been implicated in contributing to the development of various diseases (M. Wang and Kaufman 2016). The UPR is initiated by the activation of three ER transmembrane proteins: PKR-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α, hereby referred to as IRE1) and activating transcription factor 6 (ATF6), which activate three distinct signal transduction pathways (Almanza et al. 2019). Upon activation, PERK and IRE1 oligomerize and autophosphorylate(Harding, Zhang, and Ron 1999; Liu, Schroder, and Kaufman 2000; Tirasophon, Welihinda, and Kaufman 1998; X. Z. Wang et al. 1998). Activated PERK phosphorylates the eukaryotic translation initiation factor 2 (eIF2α) on Ser51. This results in a transient attenuation of global protein translation and promotes translation of the transcription factor ATF4 and downstream transcriptional response. Activated IRE1presents an RNAse domain that degrades precursors of microRNAs and mRNAs, a process termed regulated IRE1-dependent decay (RIDD). IRE1 also triggers the splicing of Xbp1 mRNA and the expression of an active transcription factor that modulates the expression of several genes (D. Han et al. 2009; Hollien and Weissman 2006; Calfon et al. 2002; Yoshida et al. 2001; Park, Kang, and So 2021). ATF6 activation is regulated by the Site-1 and Site-2 protease- dependent cleavage in the Golgi apparatus. The ATF6 released fragments acts as a transcription factor inducing the expression of UPR responsive genes (Chen, Shen, and Prywes 2002; Haze et al. 1999; Wu et al. 2007; Maiuolo et al. 2011; Adachi et al. 2008).

UPR has also been shown to be implicated in modulation of immune responses by playing a crucial role in immune cell maturation and in regulation of pro-inflammatory response. However, UPR pathways that promote inflammation often amplify the progression of pathological conditions (Hotamisligil 2010). This highlights the detrimental role of UPR in exacerbating a wide range of diseases such as inflammatory bowel disease or cancer (Grootjans et al. 2016). The UPR has been

shown to be activated during infection by several pathogens (Smith et al. 2013; George et al. 2017; Antoniou et al. 2019; Halder et al. 2015). Depending on the infection, the UPR can play a different role: it may coordinate host responses to limit infection, or conversely, may be co-opted to support the survival and replication of intracellular pathogens (Alshareef et al. 2021).

Shigella is a gram-negative invasive pathogen causing bacillary dysentery, a diarrheal disease primarily affecting children below the age of five in developing countries. Upon ingestion by human hosts, *Shigella* invades the colonic mucosa, initiating an intense inflammatory reaction leading to its destruction. After invading the host cell, *Shigella* bacteria escape form the phagocytic vacuole to replicate inside the cell. *Shigella* utilizes actin polymerization at one end of the bacterium to create actin comet tails. These comet tails enable the formation of protrusions on the cell's plasma membrane, which contain bacteria and invade neighboring cells. Once the membranes of both the donor and recipient cells are ruptured, the bacteria resume intracellular replication, facilitating their spread into the epithelial tissue.

The virulence of *Shigella* requires a type III secretion system (T3SS) that injects bacterial effectors into host cells. This process requires IpaB and IpaC proteins, that upon direct cell contact between the bacteria and the host cell form a translocon that allows the insertion of effector proteins. These type III effectors subvert many host cellular processes, including the remodeling of the actin cytoskeleton and downregulation of pro-inflammatory responses to favor tissue colonization. In the initial phases of bacterial intracellular replication, T3SS effectors play a crucial role by inhibiting autophagy, reducing inflammatory reactions, enhancing cell survival, and strengthening cell adhesion. These actions are aimed at maintaining the integrity of epithelial cells, facilitating the spread of bacteria. In the later stages of intracellular replication, the rise in cytosolic calcium levels and mitochondrial impairment trigger necrotic cell death, causing the release of proinflammatory signals (Carneiro et al. 2009).

Among *Shigella*-secreted effectors, IpgD is a phosphatidylinositol (4,5) biphosphate ($PI4,5P_2$)-4-phosphatase or phosphotransferase that modulates several host cell processes (Tran Van Nhieu et al. 2022). IpgD alters phosphoinositides' levels, by decreasing $PI4,5P_2$ and increasing of PI5P, $PI3,4P_2$, $PI3,4,5P_3$ levels. IpgD, via $PI4,5P_2$ depletion, also indirectly decreases $InsP_3$ levels and InsP3-mediated signaling. Through $PI4,5P_2$ hydrolysis, IpgD promotes cortical actin disassembly and actin polymerization at bacterial entry sites to favor bacterial internalization or inhibit T-cell migration to infected sites (Boal et al. 2016; Konradt et al. 2011). Via PI5P production, IpgD also promotes host cell survival by activating the PI3K/Akt pathway (Pendaries et al. 2006). Moreover,

IpgD plays a role during rupture of *Shigella*-containing vacuole formed after bacterial invasion (Mellouk et al. 2014).

Among cellular mechanisms that are modulated by *Shigella* bacteria the role and regulation of UPR during *Shigella* infection remains poorly understood. It has been shown that Shiga toxin-producing *S. dysenteriae* serotype I induces UPR activation in monocytes and macrophages, leading to a different modulation of UPR-mediated apoptotic response according to the maturation state of the cell. While on undifferentiated monocytic cells UPR activation leads to rapid apoptotic cell death, in more mature macrophages UPR counteracts programmed cell death in order to favor cell survival (S.-Y. Lee et al. 2008; M.-S. Lee et al. 2009).

The UPR activation and its consequences in case of *Shigella* infection of epithelial cells have not been explored so far.

In this work we investigated the UPR modulation in epithelial cells infected with *S. flexneri*, a *Shigella* species that does not produce Shiga toxin and is responsible for the majority of *Shigella* infections in the developing countries. Here we demonstrate that *S. flexneri* induces a transient activation of IRE1 and PERK sensor and promotes degradation of these proteins 120 minutes post infection. This events seems to be dependent on two different bacterial effectors that subvert host cellular pathways to reach similar outcome for both sensors. Our result demonstrate that PERK degradation is mediated by PI5P production by IpgD effector. Moreover we show that this protein plays a role in mediating a decrease in ER Ca^{2+} concentration which could contribute to induce sensors' activation. UPR sensors turnover has not been extensively addressed. Our results suggest that degradation of UPR sensors could be a way to modulate UPR response.

Results

Infection of epithelial cells by *S. flexneri* induces a transient activation of UPR sensors

The role of UPR in the modulation of bacterial infections is still controversial and many aspects have not been explored yet. UPR modulation and its consequences triggered by infection of epithelial cells by *Shigella* and not dependent on the action of toxins have not been so far investigated. To highlight this aspects we decided to work with a *Shigella* flexneri, a *Shigella* strain that is very diffused in developing countries and lacks production of Shiga toxin.

In order to understand whether infection of epithelial cells by *S. flexneri* induced the activation of UPR we infected HeLa cells with *S. flexneri* M90T WT strain or the non-invasive MxiD mutant that lacks a functional T3SS. Both strains express afaE adhesin to favor adhesion to host cells. Infection of epithelial cells with WT bacteria but not MxiD mutant induces activation of PERK sensor and downstream phosphorylation of eIF2α (Figure 1a) that peak at 60 minutes post infection and then drop dramatically after 120 minutes. Similarly, in the case of infection with WT bacteria we observed increased in phosphorylation levels of IRE1 sensor increasing very rapidly up to 30 mins post infection, reaching basal levels after 120 minutes (Figure 1b). These results suggest that invasion of epithelial cells by *S. flexneri* results in a transient activation of PERK and IRE1 sensors with different kinetics.

S. flexneri promotes proteasome-dependent degradation of PERK and IRE1

Interestingly, we observed that 120 minutes post infection epithelial cells infection with WT but not MxiD strain results in a dramatic decrease in the total protein levels of both PERK and IRE1 sensors (Figure 2a) suggesting that invasion by *S. flexneri* promotes rapid sensor degradation between 60 and 120 minutes. Although not extensively explored, it has been shown that UPR sensors turnover is mediated by proteasomal degradation (Hong et al. 2004; S. Sun et al. 2015; Papaioannou et al. 2018; Namba et al. 2015; Qiu et al. 2013). To test if proteasome inhibition could decrease the degradation of PERK and IRE1 in our experimental conditions, HeLa cells were treated with MG132 and infected with WT bacteria. MG132 treatment caused the stabilization of PERK and IRE1 total protein levels (Figure 2b). Surprisingly, proteasome inhibition did not result in stabilization of phosphorylation status of UPR sensors (Figure 2c), supporting the previous observation of their transient activation upon *S. flexneri* infection. This is supported also by the decrease in phosphorylation status of eIF2α. These data indicated that bacteria induce proteasomal-

dependent degradation of PERK and IRE1, while mediating sensor-deactivation by a different mechanism.

IpgD effector impacts on sensor activation and mediates degradation of PERK

The observed effects of sensor activation and degradation did not occur in case of infection of epithelial cells with non-invasive strain MxiD which does not present a functional T3SS. This led us to hypothesize that the observed phenomenon could be mediated by one of the ~ 30 bacterial effector that *S. flexneri* secretes through the T3SS into host cell in order to favor cell infection. Among them there are some effectors which have been shown to act as E3-ubiquitin ligases and mediate degradation of host cellular proteins in order to downregulate pro-inflammatory signaling (de Jong et al. 2016; S. Suzuki et al. 2014; Okuda et al. 2005; Ashida et al. 2010; F. Wang et al. 2013; Zheng et al. 2016). These belong to the so-called "second wave" due to their secretion in later stages of the *Shigella* infection cycle and are under the control of MxiE transcriptional activator. We sought to investigate if one of these effector was responsible for UPR sensor degradation. Surprisingly, infection of HeLa cells with M90T strain lacking MxiE activator did not revert the effect observed with WT bacteria (Supplementary Figure 1), suggesting that sensor degradation is mediated by an effector belonging to the "first wave". We therefore tested the effect "first wave" effectors on the degradation of UPR sensors. Among the first effectors to be secreted during bacterial entry we tested cytoskeletal remodeling effectors IpgB1 and IpgB2 and PI4,5P_2-4-phosphatase IpgD. Strains lacking cytoskeletal remodeling effectors IpgB1 and IpgB2 presented a delayed PERK activation but still sensor degradation 120 minutes post-infection. On the contrary in the case of IRE1 only IpgB2 mutant showed reduced sensor activation and degradation (Supplementary Figure 2). Interestingly, infection of HeLa cells with strain lacking IpgD effector displayed delayed activation of PERK and reduced activation of IRE1 (Figure 3a). Moreover it resulted in reduced degradation of PERK but not of IRE1 (Figure 3b), suggesting that this effector is responsible for PERK degradation but not IRE1's. To corroborate the effect of IpgD effector on PERK degradation we transiently overexpressed GFP-IpgD protein or the catalytically mutant version C438S. Overexpression of the WT but not the mutated protein resulted in degradation of PERK sensor (Figure 4a), suggesting that this results from a catalytical activity of the effector. IpgD is not only a PI4,5P_2-4-phosphatase but has been shown to be also a PI4,5P_2 phosphotransferase producing PI3,4P_2 (Walpole et al. 2022). To verify which activity is involved in mediating PERK

degradation we treated cells with PI5P for 120 minutes. The treatment resulted in reduction of PERK protein levels (Figure 4b), suggesting that PI4,5P_2-4-phosphatase activity and subsequent PI5P production is responsible for mediating PERK degradation. These results suggest that many effectors contribute to induce UPR activation by regulating a common mechanism that activates both PERK and IRE1. On the contrary, sensors' degradation seems to be mediated by different mechanisms induced by different sensors.

S. flexneri induces partial ER Ca^{2+} depletion

Shigella has been shown to modify Ca^{2+} signaling in order to promote host infection by favoring bacterial invasion, dissemination, host cell survival and dampen inflammation (Bonnet et al. 2016). Alteration of Ca^{2+} signaling by depletion of ER Ca^{2+} content negatively impacts on ER protein folding and therefore is a common source of ER stress and UPR activation. We hypothesized that by altering Ca^{2+} signaling mechanisms *Shigella* could impact on ER Ca^{2+} content thereby leading to UPR activation. To address this point we transfected cells with genetically encoded ratiometric Ca^{2+} indicator GEM-CEPIA1er (Suzuki et al. 2014) to monitor Ca^{2+} content in the ER. In order to verify if *S. flexneri* results in a decrease of ER Ca^{2+} levels, we infected cells with bacteria for 1h before monitoring GEM-CEPIA1er intensity values. By obtaining minimal and maximal ratio values by EGTA treatment followed by Ca^{2+} wash we could estimate the Ca^{2+} concentration in the ER as previously shown (Suzuki et al. 2014) (Figure 5a). Figure 5b reports mean ER Ca^{2+} concentration data of cells prior to EGTA treatment. Infection of cells with WT *S. flexneri* results in a decrease of total ER Ca^{2+} content (0.61 ± 0.03 mM) compared to control (1.04 ± 0.08 mM) or the non-invasive mutant MxiD (1.10 ± 0.07). This results demonstrate that infection of epithelial cells by *S. flexneri* for 1h results in a 40% depletion of ER Ca^{2+} which could be at least partially responsible for UPR activation.

IpgD effector has been shown to modulate several cellular processes such as pro-inflammatory signaling, actin polymerization but also Ca^{2+} signaling. Our group demonstrated that IpgD reduces PI4,5P_2 pool available for IP_3 production by PLC and thereby affects IP_3-mediated signaling (Sun et al. 2017). Since IpgD mutant resulted in a delayed activation of PERK and IRE1 sensors we asked whether this correlated with a minor ER Ca^{2+} depletion. Results in Figure 5b show that indeed infection of cell with IpgD mutant does not induce a significant decrease in ER Ca^{2+} levels compared to control (0.89 ± 0.07 mM). This suggests that IpgD effector subverts Ca^{2+} signaling by impacting on a mechanisms that participates in regulating ER Ca^{2+} content. Altogether, these results

indicate that *S. flexneri* infection alters ER Ca^{2+} concentration by the action of bacterial effectors such IpgD, thereby inducing UPR activation.

Discussion

In this work we explored the role of UPR during the infection of epithelial cells by *S. flexneri*. We observed the transient activation of PERK and IRE1 sensors that follows different kinetics. Indeed, IRE1 activation is more rapid and increases as fast as 15 minutes post infection. This suggests that a very rapid mechanisms possibly induced by the initial steps of *Shigella* infection such as cytoskeletal remodeling. It has been demonstrated that IRE1 activation and signaling is affected by cell contractility. He and coworkers have shown that IRE1 associates to non-muscle myosin heavy chain IIB (NMHCIIB), a subunit of non-muscle myosin IIB (NMIIB) in an ER stress-dependent manner (He et al. 2012). Most importantly, this association increases activation of IRE1 and downstream signaling and required a NMIIB activation and motor activity. It is therefore possible that actomyosin contractility induced by *Shigella* entry into the host cells contributes to IRE1 activation. Indeed, mutant lacking IpgB2 effector responsible for mimicking RhoA activation and thereby inducing stress fiber formation and cytoskeleton contractility displays a delayed IRE1 activation. Recently it has also been demonstrated that IRE1 itself is able to modulate cytoskeletal remodeling (Urra et al. 2018). IRE1 dimers/oligomers serve as scaffold for association with filamin A, a protein involved in crosslinking cortical actin filaments, and promotion of its and phosphorylation. This events are required for actin cytoskeleton remodeling and cell migration. Indeed, IRE1 KO cells present reduced filopodia and lamellipodia formation as well as reduced Rac1 activity, that controls actin polymerization (Urra et al. 2018). It is possible that bacterial entry promotes IRE1 dimerization and filamin A activation in order to increase filopodial formation and filopodial-dependent capture of *Shigella* bacteria (Valencia-Gallardo et al. 2015).

Association with filamin A has also been demonstrated with PERK sensor (van Vliet et al. 2017). Van Vliet and colleagues revealed that PERK-filamin A association drives actin remodeling at the cell surface in a kinase independent manner. PERK KO cells display an increase in cortical actin distribution. PERK dimerization increases PERK-filamin A association and actin remodeling, suggesting a role for PERK in the modulation of cytoskeletal rearrangements required for efficient bacterial entry.

Activation of IRE1 and PERK could serve as a mechanism to increase host immune defense: indeed, activation of UPR pathways has been show to contribute to boost inflammation by

regulating several pro-inflammatory pathways such as NF-κB and JAK/STAT (Kitamura 2011; Meares et al. 2014).

It has also been shown in monocytic cells that IRE1 with its RNAse activity contributes to the assembly of NLRP3 inflammasome and subsequently to pro-IL-1β processing and IL-1β secretion (Talty et al. 2019). The signaling of IL-1β seems to have a primary role in orchestrating inflammation within the intestine and participates together with IL-18 in promoting *Shigella* infection of the colonic epithelium (Schnupf and Sansonetti 2019). Both these cytokines are processed by NLRP3 inflammasome, whose formation is triggered by several PAMP signals and is crucial for intestine immune homeostasis and defense against intestinal pathogens (Lei-Leston, Murphy, and Maloy 2017). In addition PERK activation, together with Atf6 activation, has been demonstrated to increase TLR signaling after PAMP recognition and cytokine production and secretion by pulmonary epithelial cells via MAPK pathway (Mijošek et al. 2016). PERK activation has been shown to participate to NLRP3 inflammasome activation in hepatocytes via CHOP-mediated NLRP3 induction (C. Y. Han et al. 2018). Activation of IRE1 and PERK following invasion of epithelial cells by *Shigella* bacteria could represent a mechanism for promoting faster NLRP3 inflammasome assembly and boos host cell defense.

Infection of epithelial cells by *S. flexneri* results in degradation of PERK and IRE1 sensor between 60- and 120-minutes post-infection. Degradation of these sensors has not been extensively explored. It has been shown that ER E3-ubiquitin ligase Synoviolin/HRD1 mediates ubiquitination and proteasomal degradation of IRE1 (Gao et al. 2008) and this process is strongly regulated by p53 (Namba et al. 2015). Further research demonstrated that IRE1 but not PERK is a target of the ERAD complex Sel1L-Hrd1, that mediates its proteasomal degradation. ER stress induction stabilized IRE1 sensor and attenuates Sel1L-HRD1-mediated IRE1 ubiquitination and degradation (S. Sun et al. 2015). Intriguingly, IRE1-Sel1L interaction is favored by cytomegalovirus M50 protein, resulting in dampening of UPR signaling and promotion of viral protein production in order to favor progeny formation (Hinte, Müller, and Brune 2021). Similar to what we observe with *S. flexneri* infection cytomegalovirus transiently induces IRE1 signaling and mediates degradation of the sensor at later stages of infection (Stahl et al. 2013). In the case of cytomegalovirus infection transient activation of IRE1 and subsequent splicing of *Xbp1* mRNA serve to decrease *Xbp1u* negative regulation on viral promoter (Hinte et al. 2020). Further research is required if a transient activation of IRE1 and PERK signaling could be beneficial for *S. flexneri* infection or it is a mere result of the downregulation of anti-infection properties of UPR activation such as promotion of

inflammation or apoptosis induction. Moreover it could be interesting to explore if a *Shigella* secreted bacterial factor is mediating IRE1 degradation by positively regulating Sel1L-HRD1-mediated protein ubiquitination and degradation.

Information about PERK degradation or PERK turnover are not currently known. We have demonstrated that IpgD mediated PI5P production mediates PERK degradation but not IRE1, suggesting that degradation of the two sensors is triggered by different mechanisms.

Degradation of the sensors is not mediated by a bacterial-injected E3-ubiquitin ligase, as infection with MxiE mutant bacteria does not revert WT-induced events. It is therefore plausible that PI5P mediates activation of a host E3-ubiquitin ligase. It has been reported that PI5P is responsible for activation of nuclear ubiquitin ligase Cullin3-SPOP (Bunce et al. 2008) but knockdown of Cullin3 during *S. flexneri* infection had no effect on PERK degradation (Supplementary Figure 3). Further research is required to identify E3-ubiquitin ligase proteins responsible for PERK degradation. In addition, we cannot exclude that this process could be mediated by another bacterial effector.

Missing IpgD activity resulted also in a delayed activation of the sensors. This suggested that alteration of phosphoinositide composition could play a role in activation of UPR sensors. Although phosphoinositide are not abundant in the ER membrane and PI5P is the less abundant and known phosphatidylinositol variant (McCrea and De Camilli 2009), it is not excluded that could mediate UPR-inducing lipid bilayer stress. Modification of ER membrane lipid composition affects its structural dynamics and can be sensed by the UPR sensors to elicit downstream responses (Radanović and Ernst 2021). In the case of pathogenic intracellular infections researchers have reported changes in host lipids and alteration of lipid droplets. These modifications may serve to maintain an intact and functional bacteria containing vacuole as well as to provide energy sources and membrane material for the pathogens themselves (Allen and Martinez 2020). *Shigella* was shown to recruit cholesterol molecules at the entry site and this represents a key step to allow bacterial invasion of the host cell (Lafont et al. 2002). This leads to a disruption of Golgi lipid composition that results in Golgi fragmentation and impairment of intracellular trafficking (Mounier et al. 2012). Although information about alteration of ER membrane during *Shigella* infection are still missing, it could not be excluded that a lipid bilayer stress could participate to UPR induction in this context.

UPR is often induced by a sustained depletion of Ca^{2+} in ER store that impacts on protein folding ability of the ER. In this work we demonstrate that infection of epithelial cells by *S. flexneri* results in partial ER Ca^{2+} depletion. Interestingly, infection with IpgD mutant bacteria reduced the ER Ca^{2+}

depletion at 60 minutes post-infection correlating with a delayed activation of PERK and reduced activation of IRE1. This results suggests that an IpgD-mediated mechanisms contributes to regulate ER Ca^{2+} homeostasis. In our previous work we demonstrated that infection of epithelial cells by *S. flexneri* IpgD mutant increased the IP_3 levels and recruitment of IP_3R at the bacterial entry site. Moreover, IpgD mutant induced fast oscillating but less sustained local Ca^{2+} responses and more global Ca^{2+} responses than WT *Shigella* (C. H. Sun et al. 2017). Based on these data we cannot assume that *S. flexneri*-induced ER Ca^{2+} depletion is due to massive IP_3-dependent ER Ca^{2+} release. It is possible that IpgD action interferes with other mechanisms involved in ER Ca^{2+} homeostasis such as SERCA pump activity, ER Ca^{2+} leakage or Store Operated Ca^{2+} Entry (SOCE). Interestingly, it has been proposed that cytosolic Ca^{2+}-induced PERK dimerization and association with filamin A favors formation of ER-plasma membrane contact sites and promotes STIM1 localization at the contact sites to regulate SOCE mechanism (van Vliet and Agostinis 2017). It is possible that IpgD effector by impacting on both cytoskeletal remodeling and reducing prolonged local Ca^{2+} accumulations alters PERK dimerization and its filamin A association thereby negatively impacting on ER-plasma membrane contact site and SOCE activation.

In addition, it would be of great importance to understand if there is an increase recruitment of PERK and IRE1 sensors at the bacterial entry site in order to better understand if sensors' activation and their degradation are consequences of a local event induced at the invasion site or if it mostly involves a more global phenomenon.

This work analyzes *S. flexneri* impact on PERK and IRE1 sensor of the UPR (Figure 6). It would be interesting to understand what is the implication of the third branch of UPR, ATF6 pathway, during infection of epithelial cells by *Shigella* and if the bacteria mediate degradation of this sensor as well. Better highlighting this aspects could improve our understanding of cellular mechanisms that are implicated in the modulation of the UPR and on the sensor's turnover, demonstrating how research focusing on host-pathogen interaction could increase our knowledge on yet unknown cellular events.

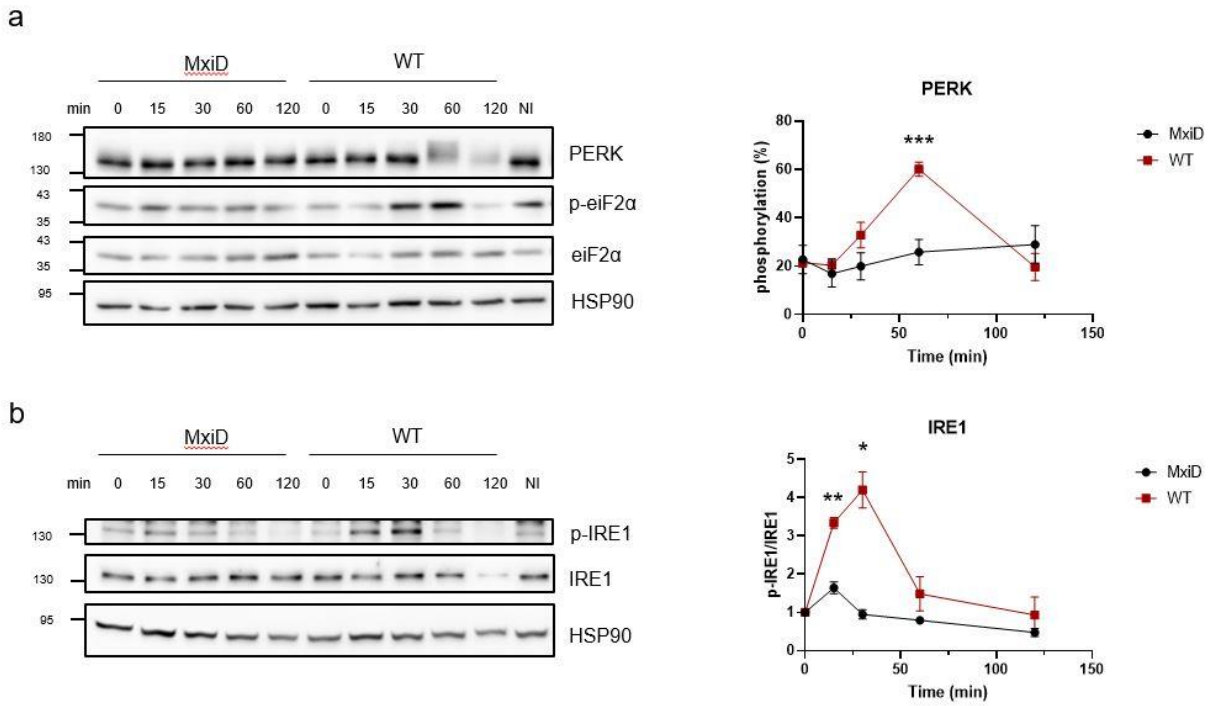
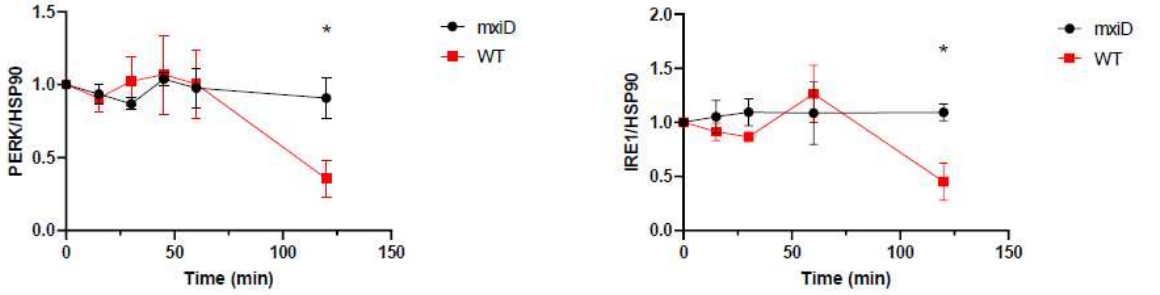
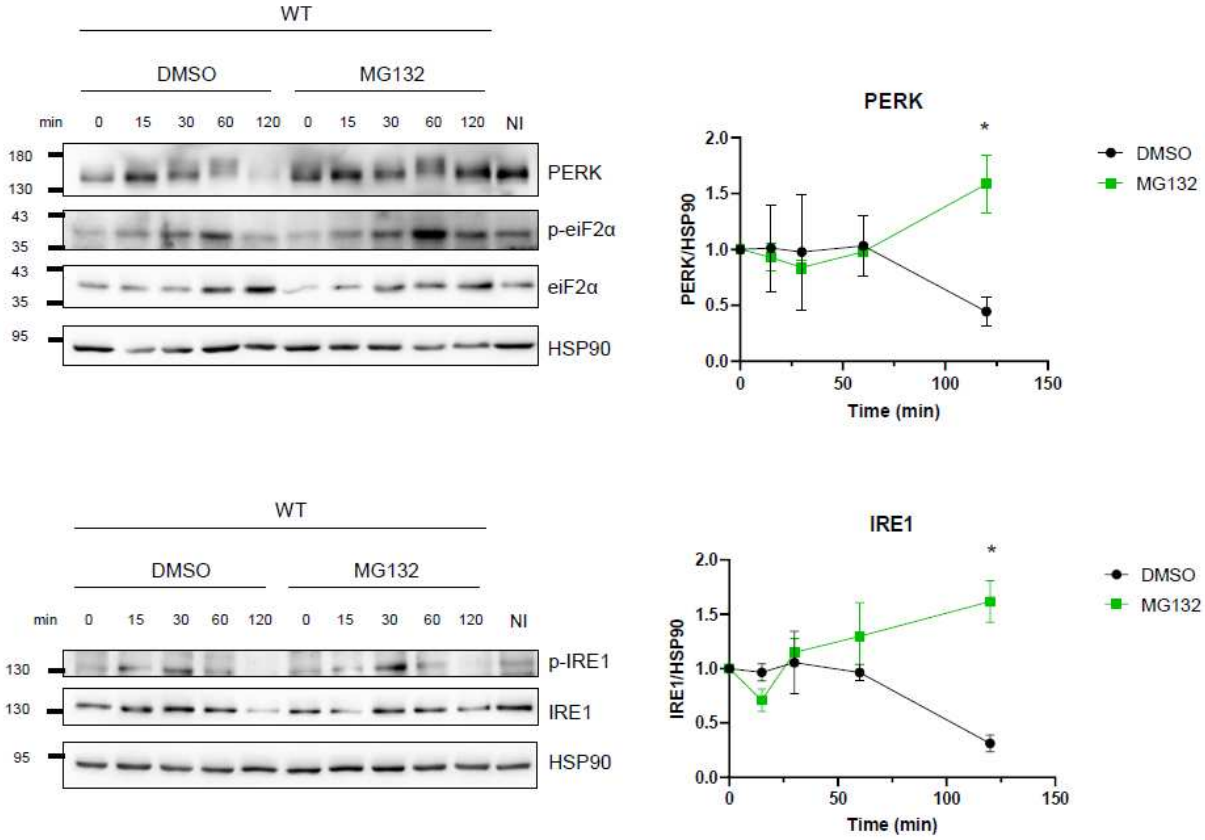


Figure 17. Transient activation of PERK and IRE1 upon infection of epithelial cells by *Shigella* flexneri. Western blot results of HeLa cells challenged with *S. flexneri* for the indicated times and relative quantification of phosphorylation of PERK (a) and IRE1 (b). Mean ±SEM of N=3.

a


PERK/HSP90
Time (min)
mxiD
WT
IRE1/HSP90
Time (min)
mxiD
WT

b


WT
DMSO
MG132
min 0 15 30 60 120 0 15 30 60 120 NI
180
130
43
35
43
35
95
PERK
p-eiF2α
eiF2α
HSP90
PERK
PERK/HSP90
Time (min)
DMSO
MG132
WT
DMSO
MG132
min 0 15 30 60 120 0 15 30 60 120 NI
130
130
95
p-IRE1
IRE1
HSP90
IRE1
IRE1/HSP90
Time (min)
DMSO
MG132

c

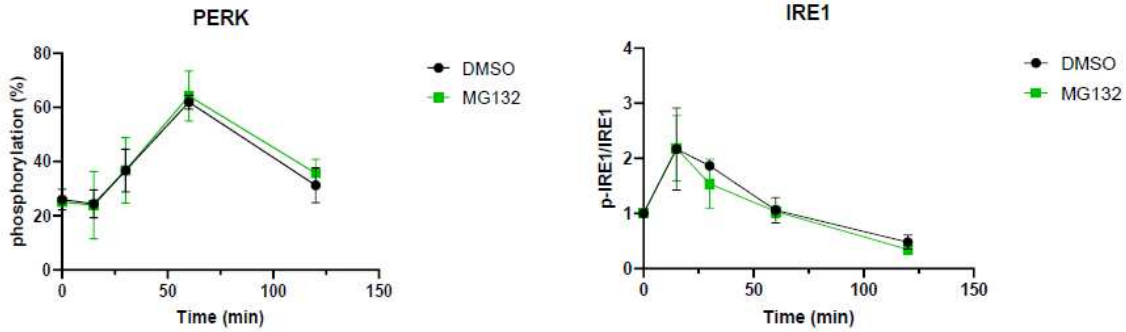

PERK
phosphorylation (%)
Time (min)
DMSO
MG132
IRE1
p-IRE1/IRE1
Time (min)
DMSO
MG132

Figure 18. *S. flexneri* promotes proteasomal-dependent degradation of PERK and IRE1. a) quantification results of total protein levels of PERK and IRE1. Mean ±SEM of N=3. b) Left panel: western blot results of HeLa cells infected with WT *S. flexneri* for the indicated times in presence or absence of MG132. Right panel: quantification of PERK and IRE1 total protein levels. Mean ±SEM of N=3. c) quantification of phosphorylation status of PERK and IRE1 in presence or absence of proteasomal inhibition. Mean ±SEM of N=3.

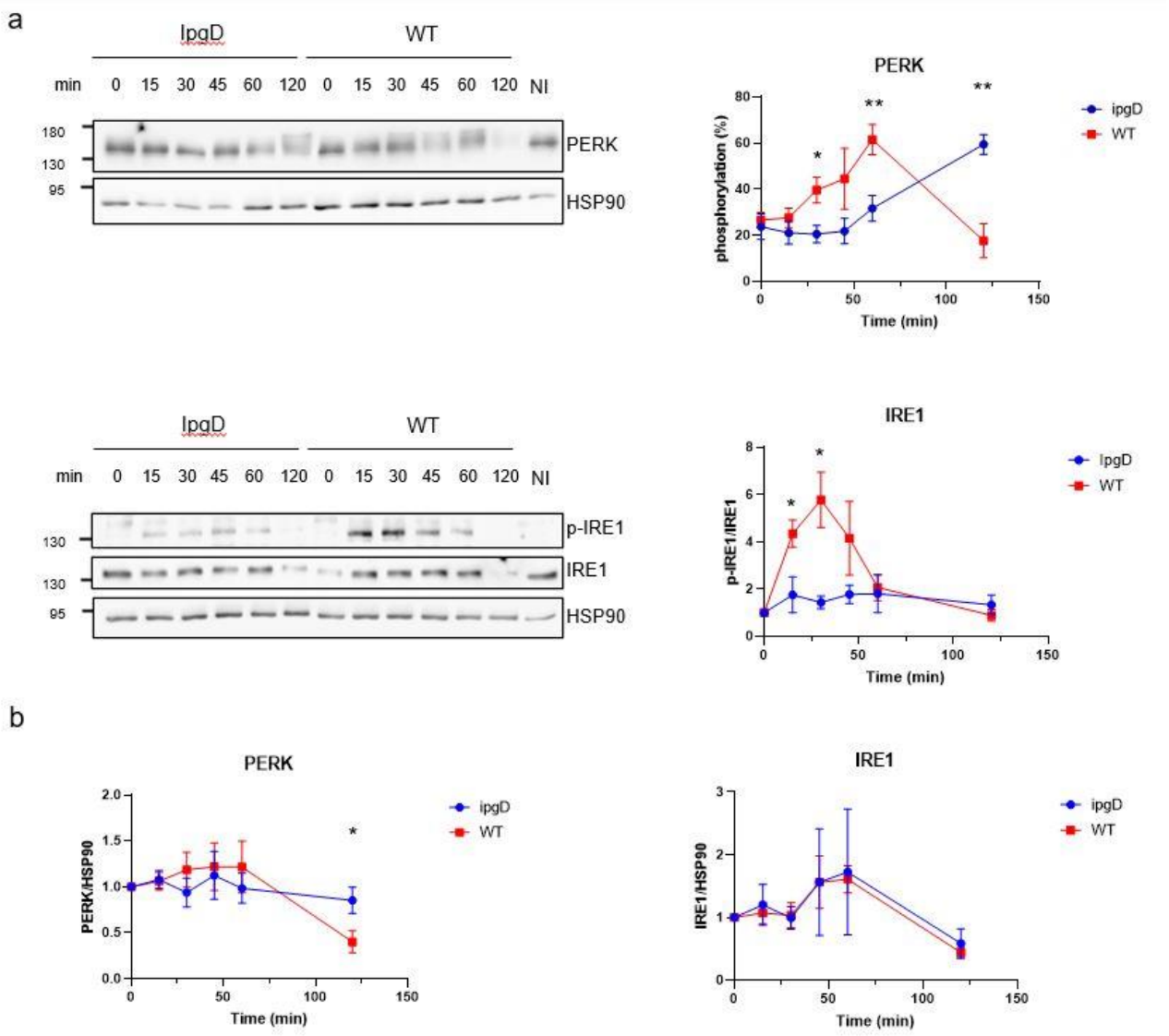


Figure 19. IpgD effector impacts on sensors' activation. a) Left panel: western blot results of HeLa cells infected with WT *S. flexneri* or mutant lacking IpgD for the indicated times. Right panel: quantification of phosphorylation status of PERK and IRE1. Mean ±SEM of N=3. b) quantification of PERK and IRE1 total protein levels. Mean ±SEM of N=3.

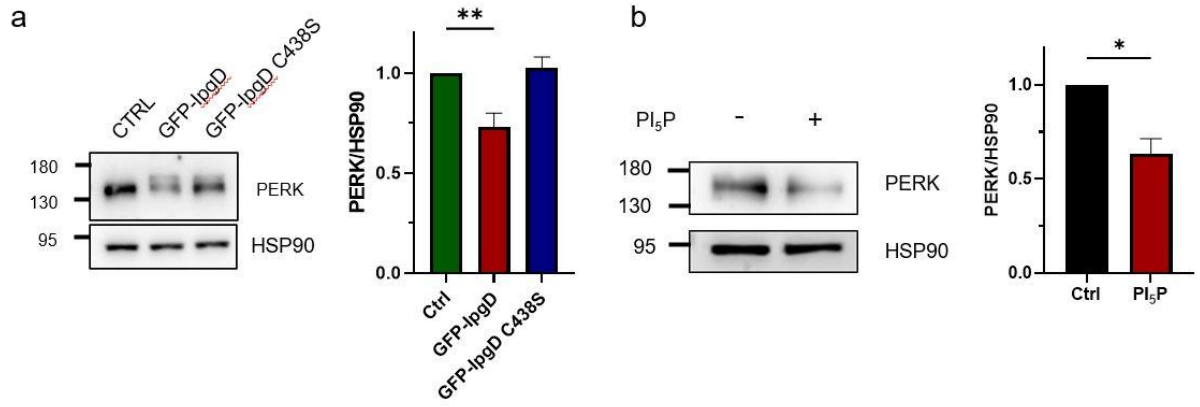


Figure 20. IpgD mediates degradation of PERK sensor. a) Western blot results and relative quantification of HeLa cells overexpressing GFP-IpgD or GFP-IpgD C438S mutant for 8h. Mean ±SEM of N=4. b) Western blot results and relative quantification of HeLa cells treated with 15µM PI5P for 2h. Mean ±SEM of N=3.

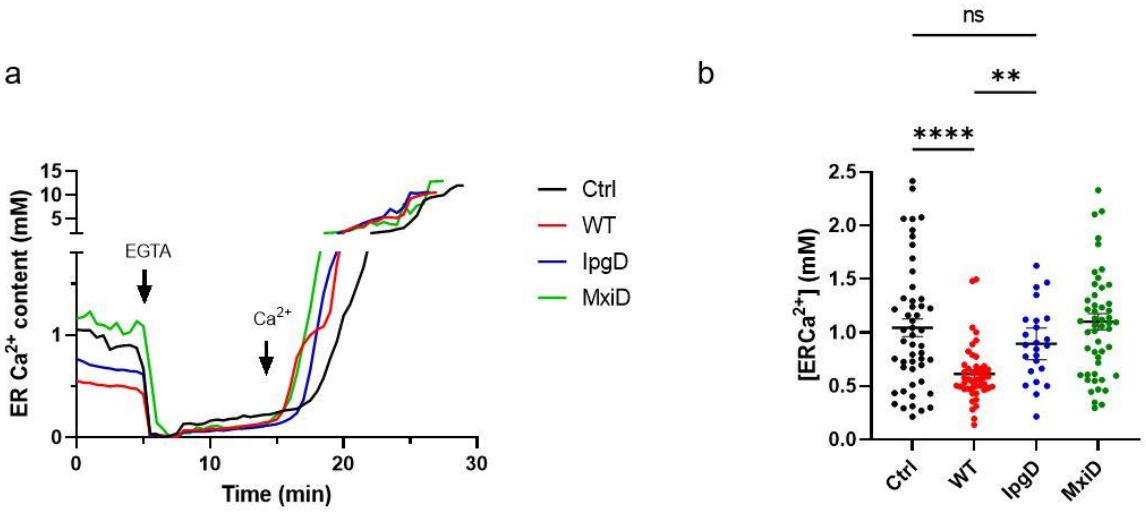


Figure 21. *S. flexneri* induces partial ER Ca^{2+} depletion. a) representative ER Ca^{2+} traces of HeLa cells transfected with pCIS GEM-CEPIA1er challenged with *Shigella* WT or mutant strains for 1h. After initial measurements cells were treated with 5Mm EGTA followed by 10mM Ca^{2+} in RPMI containing no phenol red in presence of 2µM ionomycin. b)ER Ca^{2+} content was calculated using GEM-CEPIA1er ratio values as shown in (J. Suzuki et al. 2014). Mean ±SEM of N=50 (Ctrl) N=47 (WT) N=24 (IpgD) N=51 (MxiD). Statistical analysis was performed using non parametric multiple comparisons test (Kruskal-Wallis).

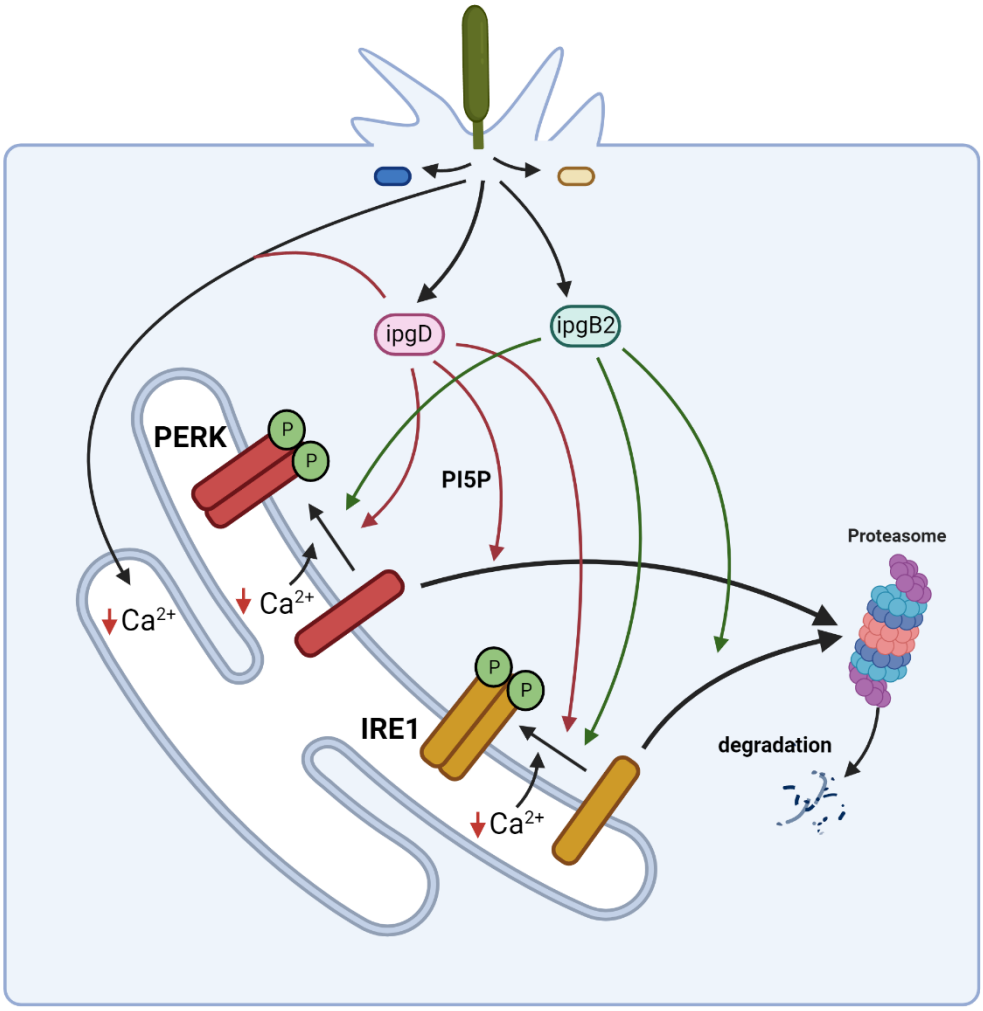
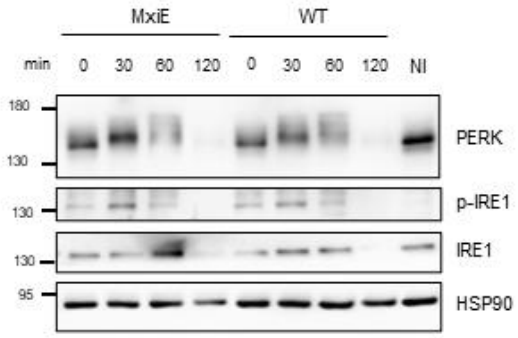
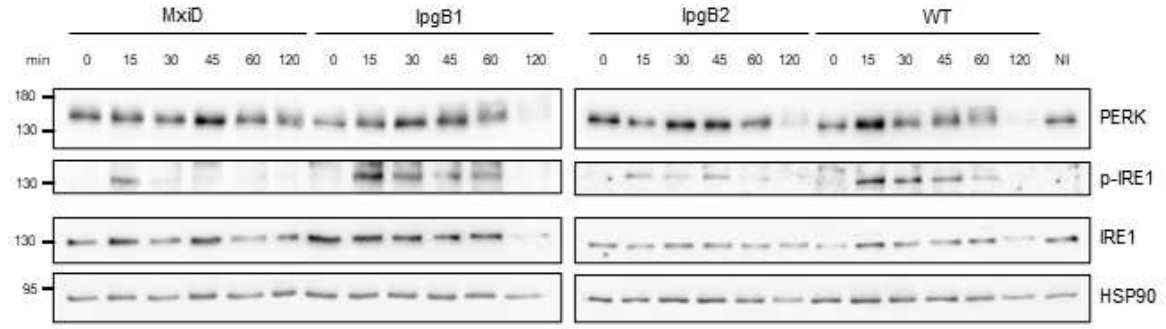


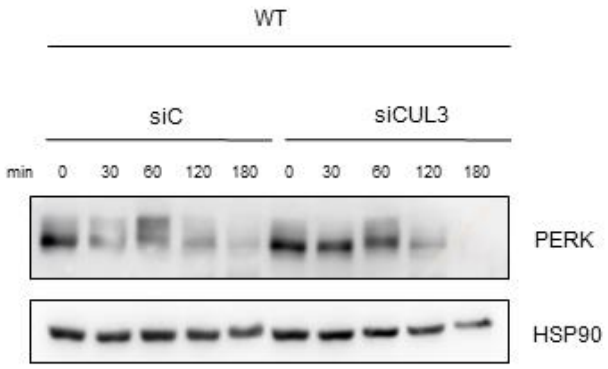
Figure *6. S. flexneri* modulates IRE1 and PERK sensors activation and degradation. Infection of HeLa cells by *S. flexneri* induces IRE1 and PERK activation that is mediated by secreted bacterial effectors such as ipgB2 and ipgD. On the contrary, degradation of the sensors observed 120 minutes post-infection is regulated more specifically by effectors. IpgD participates to degradation of PERK through the production of PI5P while IpgB2 is responsible for promotion of IRE1 turnover. In addition, invasion of cells by *S. flexneri* results in depletion of ER Ca^{2+} which could contribute to induction of ER stress and UPR signaling.



Supplementary Figure 1. Western blot results of HeLa cells challenged for the indicated time with WT *S. flexneri* or mutant lacking MxiE.



Supplementary Figure 2. Effect of IpgB1 and IpgB2 effectors on PERK and IRE1 activation and degradation. Western blot results of HeLa cells challenged for the indicated time with WT *S. flexneri* or mutant lacking MxiD, IpgB1 or IpgB2 effectors.



Supplementary Figure 3. PERK degradation is not mediated by E3-Ubiquitin ligase Cullin 3. Preliminary results of a Western blot of HeLa cells infected with WT *Shigella* for the indicated times after transfection of Cullin3 siRNA or control siRNA.

Materials and Methods

Cell culture and bacterial strains - Hela cells were cultured in RPMI medium (Gibco^{TM}-Thermo Fischer Scientific) supplemented with 10% FBS (Gibco^{TM} 10270 -Thermo Fischer Scientific) at 37°C in a 5% CO_2 incubator. Wild Type *Shigella* serotype V M90T and its derivatives mxiD, IpgD, IpgB1, IpgB2 and MxiE were described in (Ménard and Sansonetti 1994) . Bacterial strains were transformed with the pILL1168 plasmid or pBR322-based p1018 encoding the E. coli AfaE adhesin and an ampicillin and spectinomycin resistance marker respectively to allow for cell adhesion and synchronization of the invasive process. Bacteria were grown in trypticase Soy (TCS) broth containing spectinomycin or ampicillin at a final concentration of 50 µg/ml and 100 µg/ml respectively at 37 °C in a shaking incubator.

***Shigella* infection of Hela cells-** HeLa cells were plated the day before infection in 6-well plates at a density of 2.5×10^5 cells/well. Bacterial strains grown to exponential phase were resuspended in RPMI medium for challenge of HeLa cells. Cells were challenged with bacteria at an $OD_{600} = 0.1$. After 10 mins RT incubation to allow bacteria to deposit on cells, samples were placed at 37 °C in a 10% CO_2 incubator. At the indicated time points, cells were washed three times with PBS and processed for total protein extraction. For proteasomal-inhibition experiment, 10µM MG132 (Merck 474790) or DMSO were added on cells together with bacteria.

Transfection and plasmids - pCIS GEM-CEPIA1er plasmid transfection was performed 24h after cell plating using FuGENE® HD Transfection Reagent (Promega), following manufacturer's instructions. After 6h, cells were washed with PBS for two times and fresh culture medium was added. pCIS GEM-CEPIA1er (#58217) was purchased from Addgene. GFP-IpgD and GFP-IpgD C438S were transfected for 8h and then cells were collected and processed for total protein extraction. GFP-ipgD and GFP-IpgD C438S plasmids were described in (C. H. Sun et al. 2017).

PI5P treatment- Cells were treated 24h after plating with 15µM PI5P diC4 (Tebubio P-5004) in RPMI medium in absence of FBS for 2h. Cells were washed two times with PBS and processed for total protein extraction.

Cell manipulations and Western Blot analyses - To obtain total cell extracts cells were lysed in sample buffer 1x (62.5 mM Tris pH=8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 5% β-

mercaptoethanol), sonicated and boiled at 95°C for 5 minutes. Proteins from total lysates were separated by SDS PAGE and transferred to nitrocellulose membrane (0.45µM Amersham^{TM} Protran^{TM}). Western Blot analysis were performed according to standard procedure using the following primary antibodies: PERK (Cell Signaling Technologies C33E10), IRE1 (Cell Signaling Technologies 14C10), p-IRE1 (Abcam ab124945), HSP90 (Santa Cruz Biotechnologies sc-13119) p-eIF2α (Cell Signaling Technologies 119A11), eIF2α (Cell Signaling Technologies L57A5). HRPO conjugated anti-mouse (Cytiva) and anti-rabbit (Sigma) were used as secondary antibodies.

Calcium measurements - HeLa cells were seeded on 25 mm coverslips and transfected with pCIS GEM-CEPIA1er 24h after plating. Cells were placed in an observation chamber in RPMI medium non containing phenol red and supplemented with 4Mm Glutamine and 25mM Hepes. Cells were incubated at 37 °C in presence or absence of bacteria at a OD_{600} = 0.1 for 1h before analysis. Cells were imaged for 3 minutes before treatment with 5mM EGTA and 2µM Ionomycin and then washed with 10mM Ca^{2+} and 2µM Ionomycin to obtain minimal and maximal ratio values. Samples were analyzed at room temperature on an inverted Nikon eclipse TE200 fluorescence microscope, using a 60x objective. Illuminating sources used were ex. 380 nm; em. 480nm/535nm driven by Simple 32 Software from Compix Incorporated. Images were captured using a CMOS camera (Hamamatsu) and analyzed with Simple 32 software. ER Ca^{2+} concentration before EGTA treatment was estimated for each cell using ratio values of GEM-CEPIA1er as previously shown in (J. Suzuki et al. 2014).

Statistical Analyses - Data are expressed as mean ± SEM of three or more independent experiments. Differences were analyzed by unpaired Student's t-test with unequal variance unless otherwise stated using Prism 7 (GraphPad). P-values < 0.05 were considered significant.

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GENERAL DISCUSSION AND PERSPECTIVES

Ca^{2+} impact on the sensitivity of UPR

This thesis explores the sensitivity of the three UPR sensors to a moderate depletion of ER Ca^{2+} content. In experimental settings induction of ER stress is generally induced by impairing protein folding ability through alteration of protein glycosylation with Tunicamycin, disulfide bond formation with reducing agent DTT or impairment of Ca^{2+}-binding chaperones activity with SERCA pump inhibitors. Among these the most used is the potent and irreversible inhibitor thapsigargin that is able to induce complete ER Ca^{2+} depletion within minutes. In this work, the application of different doses of reversible SERCA inhibitor tBuBHQ allowed us to obtain intermediate ER Ca^{2+} steady states that could more closely recapitulate a possible Ca^{2+} alteration that could happen in a physiological context as well as in pathological one. Indeed, luminal Ca^{2+} depletion has been shown to contribute to the pathophysiology of many diseases, very often coupled with ER stress induction and UPR activation. Indeed, both these phenomena participate to the regulation of cell fate and to induction of cell death (Mekahli et al. 2011). These characteristics are implicated in altered insulin secretion and β-cell survival in diabetes as well as normal neuron functioning and insurgence of neurological disorders (Arruda et al. 2015; Schrank et al. 2020).
It is therefore relevant to better understand the activation of UPR pathways in conditions that better resemble the ER Ca^{2+} alterations that are more likely to occur in a cellular context. Previous studies sought to investigate the connection between moderate decrease of ER Ca^{2+} and the triggering of the UPR. Partial depletion of ER Ca^{2+} concentration in *xenopus* oocytes for 24h induced events downstream of sensor activation such as increase in eIF2α phosphorylation and accumulation of normally secreted proteins (Paredes et al. 2013). More recently, another work demonstrated that total luminal Ca^{2+} depletion is required to observe UPR induction when it comes to downstream effectors proteins and UPR outcomes such as autophagy and apoptosis (Szalai et al. 2018). Contrary to our research, these works however did not specifically focus on the immediate activation of the sensors and explore the close link that allows the UPR transducers to sense luminal Ca^{2+} alteration.

The development of a mathematical model highlighted the relevance of the BiP-competition model on the activation of the three UPR sensors and its impact on the sensitivity of the different proteins. Since the exact mechanisms of activation of IRE1, PERK and ATF6 is still under debate, the use of computational modeling could allow us to shed some light on this topic. Other works sought to investigate with the use of mathematical modeling the importance of the different activation models proposed on sensors' activation (Pincus et al. 2010; Stroberg et al. 2018; Stroberg et al. 2019). It

would be therefore intriguing to explore the significance on sensors' activation by the direct binding to unfolded proteins, especially in the context of ER Ca^{2+} depletion, where the stressor directly impacts on both BiP function and unfolded protein accumulation (see our model). Computational models exploring UPR pathways and consequences generally do not focus on a specific ER stressor (Pontisso et al. 2023). Our interest in Ca^{2+} signaling brought us to investigate the link between UPR induction and alteration to ER Ca^{2+} but further research could assess the sensitivity and activation kinetics of the three UPR branches to other types of stress and a computational approach could help us better dissect the eventual differences and their relevance.

ER stress and proteins involved in the UPR are implicated in the modulation of ER Ca^{2+} signaling (Carreras-Sureda et al. 2018). Higo and colleagues demonstrated that IP_3R1 associates with BiP in neuronal cells and BiP activity is required for IP_3R1 tetramer assembly and channel function. Interestingly, ER stress impairs this association and results in altered IP_3R1-dependent Ca^{2+} release (Higo et al. 2010). Another work suggested that in neurons IRE1 downregulates Ca^{2+} release from IP_3R thereby impacting on neuronal cell death (Son et al. 2014). In addition, PERK by impacting on actin cytoskeleton organization can modulate STIM1 puncta formation and regulate SOCE (van Vliet et al. 2017). PERK is also enriched in MAMs where it has a tethering function assuring the correct Ca^{2+} transfer among organelles (Verfaillie et al. 2012). This process is also regulated by IRE1 (Carreras-Sureda et al. 2019). Given the intricated connection among ER stress, UPR, Ca^{2+} signaling and cell fate it would be interesting further explore the interplay between the activation of UPR sensors and ER Ca^{2+} content by addressing the implication of sensors' activation on ER Ca^{2+}.

Fluctuations at the ER Ca^{2+} content are regularly happening in our cells. Research has always focused the attention on the Ca^{2+}-mediated UPR activation in condition of total ion depletion. Further studies are needed to investigate whether Ca^{2+} oscillations have an impact on UPR sensor activation. Indeed it has been shown that increase in cytosolic Ca^{2+} could mediate activation of PERK (T. Li et al. 2023; van Vliet and Agostinis 2017) Similarly, it is not known whether ER Ca^{2+} release localized in only a part of the organelle causes the activation of localized UPR sensors.
These aspects would be relevant to understand the amount of Ca^{2+}-dependent induction of IRE1 and PERK during infection with *S. flexneri*. 60 minutes incubation of HeLa cells with bacteria results in 40% reduction in ER Ca^{2+} concentration. Such a depletion, when induced by tBuBHQ (3µM) does not induce similar activation of PERK (taken as a reference because the technique of activation

monitoring is similar). This could be explained by induction of another type of stress (such as lipid bilayer stress as discussed previously) or by possible accumulation of the sensor at the invasion site, which could respond to IP_3-dependent Ca^{2+} fluctuations or local major Ca^{2+} depletions induced by bacterial invasion. Further experiments and modeling are needed to address this point.

Reversibility of UPR activation and role of Ca^{2+}

Another important aspect dissected in this work is the reversibility of UPR sensors' activation. The use of tBuBHQ as a SERCA inhibitor, rather than the more commonly used thapsigargin, allowed us to tackle this point. Physiologically cells could be exposed to acute stress induction that when resolved results in sensors' deactivation and UPR downregulation. Surprisingly, research on this topic is less abundant especially when it comes to reversible Ca^{2+} alteration, due to the irreversibility of thapsigargin. Previous works have shown that washout of cyclopiazonic acid (CPA), another reversible SERCA pump inhibitor, results in ER stress resolution, monitored in terms of UPR induced transcripts and relative protein production and activation of Sec62-dependent degradation of ER resident proteins via autolysosomal pathway (Fumagalli et al. 2016). In addition, transient ER stress obtained by CPA treatment and subsequent washout on MEF cells show that complete restoring of PERK phosphorylation levels to resting conditions is obtained between 4 and 8h (Guan et al. 2017). Moreover, in a more recent work, CPA removal resulted in total inactivation of PERK sensor in about 2h and a restoration of lost β-cell functionality. Interestingly, repeated cycles of ER stress negatively impact on β-cell plasticity and ability to restore normal conditions, suggesting that UPR sensors deactivation kinetics could also be impacted in this case (Chen et al. 2022). However, the kinetics and mechanisms of sensors' inactivation upon resolution of ER stress have largely been unexplored.

This thesis presents evidence of a fast deactivation of PERK and IRE1 upon refilling of luminal Ca^{2+} content. The development of computational model help us to test the possibility that this fast inactivation could be mediated by the catalytical activity of a phosphatase. Indeed, when the model considers a Ca^{2+}-sensitive phosphatase, it could more closely recapitulate the experimental results. Further work should explore the action of possible phosphatases that participate to PERK and IRE1 inactivation, specifically in the context of Ca^{2+} store refilling. Phosphatases that act on IRE1 have already been reported (T.-K. Chang et al. 2018; Guo and Polymenis 2006; Y. Qiu et al. 2010; Welihinda et al. 1998), as well as other downregulation mechanisms (Eletto et al. 2014; X. Li et al.

2020; Lisbona et al. 2009; Sundaram et al. 2017). It might be possible that restoration of Ca^{2+} luminal concentration impact and accelerates these processes.

In a more specific context, this works demonstrates that during *Shigella* infection, when proteasomal degradation of IRE1 and PERK is impaired the activation of the sensors still occurs. Disappearance of the phosphorylated species could be due to proteasomal-independent degradation pathway or to bacterial-activated host cell serine-threonine phosphatases as well as bacteria-secreted ones. In *Shigella* the bacterial effector OspF has been shown to remove phosphate from p38 and pERK kinases in the nucleus. This effector is already present in the bacteria at the moment of cell invasion but its production is increased following activation of MxiE transcriptional activator. Although, MxiE mutant retained phosphorylated sensor disappearance further investigation on this hypothesis is required.

Relevance of UPR sensors' turnover

Our work examines some aspects related to UPR sensors turnover. Surprisingly, this topic has not been extensively explored. As previously mentioned, some works investigated on mechanisms of IRE1 and ATF6 degradation (Gao et al. 2008; Hong et al. 2004; Namba et al. 2015; Papaioannou et al. 2018; Q. Qiu et al. 2013; S. Sun et al. 2015). Further investigation is required to explore new pathways and mechanisms that are responsible for the degradation of IRE1, PERK and ATF6 as well as potential ubiquitin-ligases that participate in proteasomal degradation. In the context of *Shigella* infection, a better characterization of the mechanisms responsible for PERK and IRE1 degradation as well as the downstream effects of UPR activation in these conditions could shed light on the possible reason for bacterial induced sensors' destruction.

One possibility is that *Shigella* through degradation of PERK sensor aims to restore general protein synthesis in order to promote host cell production of proteins required for its successful infection cycle. Indeed, our results report that phosphorylation of eIF2α is increased in cells infected with WT bacteria. Upon degradation of PERK, levels of p-eIF2α drop drastically, suggesting that phosphorylation of this factor was mainly mediated by PERK and not by other kinases involved in the Integrated Stress Response. Another hypothesis is related to host immune response inhibition induced by *Shigella*. It has been extensively shown that *Shigella* represses pro-inflammatory program upon infection of host (Ashida et al. 2015). UPR activation is involved in the activation of immune responses and promotion of inflammation e.g. by inducing activation of NF-κB (Tam et al.

2012; Yamazaki et al. 2009) or inducing transcription of cytokines (Martinon et al. 2010). It needs to be clarified if the downregulation of IRE1 and PERK mediated by *S. flexneri* results in a decreased cytokine production. In that case, the observed degradation of the sensors induced by infection of host cell by *Shigella* could be an additional mechanisms that the bacteria regulate in order to restrain host immune defense. Further research is needed to investigate if any other bacteria effector is additionally involved in PERK degradation, as well which secreted factor participates in the induction of IRE1 reduced protein levels. Our work does not investigate the fate of the third UPR sensor ATF6 in these conditions. Analyzing the fate of this third protein will allow to have a complete perspective on the modulation of UPR by *Shigella* and bring us more knowledge about the role of the UPR during infection of host cells. Moreover, due to limited knowledge of reported mechanisms mediating degradation of IRE1, PERK and ATF6, investigation of *Shigella* induced processes could contribute to increase our understanding of existing cellular pathways regulating this processes.

Conclusion

Given importance of UPR in modulating several aspects of cellular physiology and its implication in a plethora of human diseases, the study of mechanisms regulating this cellular response is of great importance. Among the multiple aspects affecting UPR and specifically its activation, three factors involving particularly the three UPR sensors have still many facets to clarify: their sensitivity, their inactivation and their turnover. We have tried to explore new insights related to these points specifically focusing on the role of Ca^{2+} in mediating the induction and reversion of sensors' activation. The investigation of these aspects in a more specific context such as infection of epithelial cells by *S. flexneri* raised the additional still largely unexplored aspect of sensors' degradation pointing out the usefulness of host-bacteria interaction studies to unravel yet undiscovered host cellular processes.

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université PARIS-SACLAY
ÉCOLE DOCTORALE
Signalisations et réseaux intégratifs en biologie (BIOSIGNE)

Titre : Nouvelles connaissances sur le rôle du calcium du Reticulum Endoplasmique dans la sensibilité et la réversibilité de l'activation de l'UPR

Mots clés : Réponse Protéique non repliée, Signalisation calcique, Modélisation mathématique, Infections bactériennes, Shigella

Résumé: Le Réticulum Endoplasmique (RE) est un organite cellulaire qui participe au stockage du Ca^{2+} et au repliement des protéines. La diminution de la concentration de Ca^{2+} du RE conduit à une accumulation de protéines mal repliées et à l'activation de la réponse dite "UPR" (Unfolded Protein Response). La diminution partielle du Ca^{2+} du RE et l'activation de l'UPR sont des caractéristiques observées dans plusieurs maladies humaines, mais la sensibilité de l'UPR à la diminution du Ca^{2+} du RE n'a pas été explorée. Les résultats présentés dans cette thèse montrent, à l'aide d'un modèle mathématique, comment l'activation de l'UPR suit de façon précise le niveau de Ca^{2+} du RE. Ce travail analyse aussi le rôle de l'UPR dans une situation physiopathologique, en l'occurrence lors de l'infection des cellules épithéliales par la bactérie *Shigella flexneri* et démontre que ces bactéries sont capables de reverser l'activation de l'UPR, pour favoriser l'infection des cellules hôtes. Ce travail contribue à mieux comprendre le rôle de l'UPR dans de nombreuses conditions pathologiques.

Le Réticulum Endoplasmique (RE) est un organite cellulaire qui participe au stockage du Ca^{2+} et au repliement des protéines. La diminution de la concentration de Ca^{2+} du RE conduit à une accumulation de protéines mal repliées et à l'activation de la réponse dite "UPR" (Unfolded Protein Response). La diminution partielle du Ca^{2+} du RE et l'activation de l'UPR sont des caractéristiques observées dans plusieurs maladies humaines, mais la sensibilité de l'UPR à la diminution du Ca^{2+} du RE n'a pas été explorée. Les résultats présentés dans cette thèse montrent, à l'aide d'un modèle mathématique, comment l'activation de l'UPR suit de façon précise le niveau de Ca^{2+} du RE. Ce travail analyse aussi le rôle de l'UPR dans une situation physiopathologique, en l'occurrence lors de l'infection des cellules épithéliales par la bactérie *Shigella flexneri* et démontre que ces bactéries sont capables de reverser l'activation de l'UPR, pour favoriser l'infection des cellules hôtes. Ce travail contribue à mieux comprendre le rôle de l'UPR dans de nombreuses conditions pathologiques.

Title : Novel insights on the role of Endoplasmic Reticulum calcium in the sensitivity and reversibility of UPR activation

Keywords : Unfolded Protein Response, Calcium signaling, Mathematical modeling, Bacterial infections, Shigella

Abstract: The Endoplasmic Reticulum (ER) is a cellular organelle that participates to Ca^{2+} storage and to protein folding. Decrease in ER Ca^{2+} concentration leads to accumulation of misfolded proteins and to activation of Unfolded Protein Response (UPR). Partial ER Ca^{2+} depletion and activation of UPR are features observed in several human disease but the sensitivity of UPR to Ca^{2+} decrease has not been explored. Results presented in this thesis show, with the help of a mathematical model, how the UPR activation tightly reports the state of ER Ca^{2+} levels in conditions of Ca^{2+} depletion but also of store refilling. Moreover, this work analyzes the role of UPR during infection of epithelial cells by *Shigella flexneri* pathogens and demonstrates that these bacteria are able to subvert the UPR activation, possibly to promote host cell infection. This work contributes to clarify some aspects of the complexity of UPR activation and regulation, helping to better dissect its role in many pathological conditions.

The Endoplasmic Reticulum (ER) is a cellular organelle that participates to Ca^{2+} storage and to protein folding. Decrease in ER Ca^{2+} concentration leads to accumulation of misfolded proteins and to activation of Unfolded Protein Response (UPR). Partial ER Ca^{2+} depletion and activation of UPR are features observed in several human disease but the sensitivity of UPR to Ca^{2+} decrease has not been explored. Results presented in this thesis show, with the help of a mathematical model, how the UPR activation tightly reports the state of ER Ca^{2+} levels in conditions of Ca^{2+} depletion but also of store refilling. Moreover, this work analyzes the role of UPR during infection of epithelial cells by *Shigella flexneri* pathogens and demonstrates that these bacteria are able to subvert the UPR activation, possibly to promote host cell infection. This work contributes to clarify some aspects of the complexity of UPR activation and regulation, helping to better dissect its role in many pathological conditions.