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Ketsia Bakambamba

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Par

Ketsia BAKAMBAMBA

Identification of molecular actors regulated by IRE1 that control protein secretion in Glioblastoma cells

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Identification of molecular actors regulated by IRE1 that control protein secretion in Glioblastoma cells

K. BAKAMBAMBA

*For my mum, my grandparents and my dearest
husband.*

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Abstract

Glioblastoma (GB) is an aggressive and deadly brain tumor with a highly heterogeneous pathophysiology, contributing to its resistance to current therapies. Despite advances in research, the prognosis for GB patients remains poor, highlighting the need for better characterization of the disease and the development of novel therapeutic strategies.

This thesis focuses on the central role of the endoplasmic reticulum (ER) and its stress response, particularly the Unfolded Protein Response (UPR), in GB pathophysiology. Special emphasis is placed on the UPR sensor IRE1, which regulates the secretory machinery in GB cells, influencing key processes such as protein maturation, trafficking, and cell migration. We propose that IRE1 may broadly affect protein secretion by directly influencing key molecular actors of the secretory pathway in GB cells. Specifically, we identified one IRE1 target, GOLIM4, a cis-Golgi protein involved in protein trafficking, whose silencing led to disrupted cell adhesion and enhanced migration, this suggests that IRE1 plays a crucial role in regulating protein transport and cell surface expression, which are vital for maintaining GB cell function and invasiveness.

Résumé

Le glioblastome (GB) est une tumeur cérébrale agressive et mortelle, avec une pathophysiologie très hétérogène, ce qui contribue à sa résistance aux thérapies actuelles. Malgré les avancées de la recherche, le pronostic des patients atteints de GB reste mauvais, soulignant la nécessité d'une meilleure caractérisation de la maladie et du développement de nouvelles stratégies thérapeutiques.

Cette thèse se concentre sur le rôle central du réticulum endoplasmique (RE) et de sa réponse au stress, en particulier la Réponse aux Protéines Mal Repliées (UPR), dans la pathophysiologie du GB. Une attention particulière est accordée au senseur IRE1 de l'UPR, qui régule la machinerie sécrétoire dans les cellules de GB, influençant des processus clés tels que la maturation des protéines, leur trafic et la migration cellulaire. Nous proposons qu'IRE1 puisse avoir un impact large sur la sécrétion des protéines en influençant directement des acteurs moléculaires clés de la voie sécrétoire dans les cellules de GB. En particulier, nous avons identifié GOLIM4, une protéine du cis-Golgi impliquée dans le trafic de protéines, comme une cible d'IRE1. La suppression de GOLIM4 a entraîné une perturbation de l'adhésion cellulaire et une migration accrue, ce qui suggère qu'IRE1 joue un rôle crucial dans la régulation du transport des protéines et de leur expression à la surface cellulaire, des processus essentiels pour maintenir la fonction des cellules de GB et leur invasivité.

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List of Abbreviations

A

ADAM9 - A Disintegrin and Metalloproteinase 9
ATF4 - Activating Transcription Factor 4
ATF6 - Activating Transcription Factor 6

B

BBB - Blood-Brain Barrier
BIP - Binding Immunoglobulin Protein (also known as GRP78, a heat shock protein)
BMDMs - Bone Marrow-Derived Macrophages

C

CAR T - Chimeric Antigen Receptor T Cells
CBTRUS - Central Brain Tumor Registry of the United States
CDK - Cyclin-Dependent Kinase
CDKN - Cyclin-Dependent Kinase Inhibitor
CHOP - C/EBP Homologous Protein
CNS - Central Nervous System
COP - Coat Protein
CSCs - Cancer Stem Cells
CT - Computed Tomography
CTLA-4 - Cytotoxic T-Lymphocyte Antigen 4
CXCL - C-X-C Motif Chemokine Ligand

D

DCs - Dendritic Cells

E

EGFR - Epidermal Growth Factor Receptor
eIF2a - Eukaryotic Initiation Factor 2 Alpha
EMT - Epithelial-Mesenchymal Transition
ER - Endoplasmic Reticulum
ERAD - Endoplasmic Reticulum-Associated Degradation
ERGIC - Endoplasmic Reticulum-Golgi Intermediate Compartment
EVs - Extracellular Vesicles

F

FLNA – Filamin A

G

GB - Glioblastoma
GFAP - Glial Fibrillary Acidic Protein
GOLIM4 - Golgi Integral Membrane Protein 4
GSC - Glioma Stem Cells
GTR - Gross Total Resection

H

HCC - Hepatocellular Carcinoma

HIF - Hypoxia-Inducible Factor

I

ICI - Immune Checkpoint Inhibitors

IDH - Isocitrate Dehydrogenase

IHC - Immunohistochemistry

IL - Interleukin

IL13Ra2 - Interleukin-13 Receptor Alpha 2

IR - Incidence Rate

IRE1 - Inositol-Requiring Enzyme 1

J

JNK - c-Jun N-terminal Kinase

K

KPS - Karnofsky Performance Status

M

MET - Mesenchymal Epithelial Transition Factor

MES - Mesenchymal

MDSCs - Myeloid-Derived Suppressor Cells

MGMT - O6-Methylguanine-DNA Methyltransferase

MMP - Matrix Metalloproteinase

MRI - Magnetic Resonance Imaging

mTOR - Mammalian Target of Rapamycin

MTTP - Microsomal Triglyceride Transfer Protein

N

NF1 - Neurofibromin 1

NOTCH - Notch Signaling Pathway

NRF2 - Nuclear Factor Erythroid 2-Related Factor 2

NSC - Neural Stem Cells

NSCLC - Non-Small Cell Lung Cancer

O

OS - Overall Survival

P

PD1 - Programmed Death 1

PDCD6IP - Programmed Cell Death 6-Interacting Protein

PDGFRA - Platelet-Derived Growth Factor Receptor Alpha

PDL1 - Programmed Death-Ligand 1

PER1 - Period Circadian Regulator 1

PERK - Protein Kinase RNA-Like Endoplasmic Reticulum Kinase

PFS - Progression-Free Survival

PI3K - Phosphoinositide 3-Kinase

PTEN - Phosphatase and Tensin Homolog

R

RIDD - Regulated IRE1-Dependent Decay

RNase - Ribonuclease
ROS - Reactive Oxygen Species

S

S1/2P - Site-1/2 Protease
SOX2 - SRY-Box Transcription Factor 2
SPARC- Secreted Protein Acidic and Rich in Cysteine
STX6 - Syntaxin 6

T

TAMCs - Tumor-Associated Myeloid Cells
TAMs - Tumor-Associated Macrophages
TCGA – The Cancer Genome Atlas
TERT - Telomerase Reverse Transcriptase
TGF- β - Transforming Growth Factor Beta
TKI - Tyrosine Kinase Inhibitors
TME – Tumor Microenvironment
TMED10 - Transmembrane Emp24 Domain-Containing Protein 10
TMZ - Temozolomide
TNBC - Triple-Negative Breast Cancer
TRAF2 - TNF Receptor-Associated Factor 2
TP53 - Tumor Protein p53
Tregs - Regulatory T Cells
TTF - Tumor Treating Fields

U

UPR - Unfolded Protein Response

V

VEGF - Vascular Endothelial Growth Factor
VEGFR - Vascular Endothelial Growth Factor Receptor
VMP1 - Vacuole Membrane Protein 1

W

WHO - World Health Organization

X

XBP1 - X-Box Binding Protein 1
XBP1s - Spliced Form of X-Box Binding Protein 1

Y

YIF1B - Yip1 Interacting Factor Homolog B

Preface

Cancer remains one of the most pressing global health concerns of this century, accounting for millions of deaths each year. Advances in cancer research have significantly improved our understanding of cancer mechanisms, leading to more accurate diagnostics and treatments. Despite the advancements, many cancers especially the more aggressive ones continue to pose substantial challenges to treatment, with poor survival outcomes and limited therapeutic options.

One example of such cancer is glioblastoma, which is going to be the focus of this thesis. It is the most aggressive and deadly primary brain tumor. Its highly invasive nature and resistance to conventional therapies, highlights the need for continued research into the disease. In recent years, the field of cancer research has placed increasing emphasis on understanding how tumors interact with their environment, adapt to stress and evade immune detection. This includes studying mechanisms like the unfolded protein response, which cells use to cope with stress in the endoplasmic reticulum. In cancers such as glioblastoma, these adaptive responses allow tumor cells to survive under unfavorable conditions.

The aim of this thesis is to explore the role of IRE1 axis of the UPR in regulating the secretory machinery in glioblastoma cells. Understanding how IRE1 influences protein secretion and its broader impact on tumor progression could reveal novel therapeutic avenues to combat this aggressive disease.

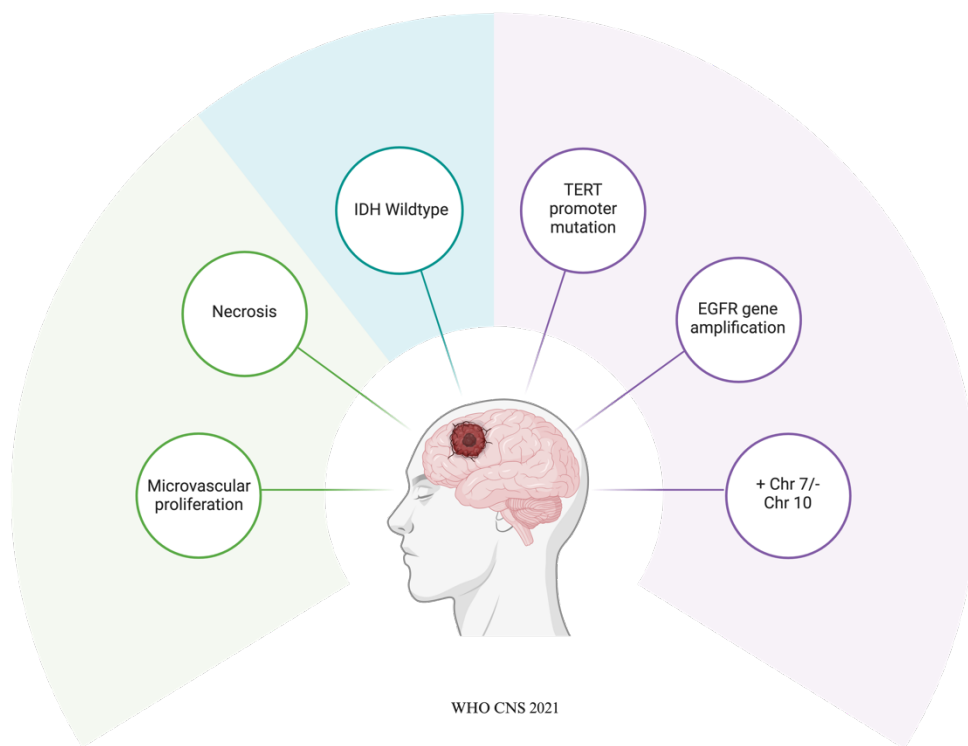
Notes: The results obtained during the three years of this thesis have been presented as posters and oral communications at several conferences, including the ER meeting in Paris, the 14th international Calreticulin workshop in Saint Malo, the first cancer and immunology symposium in Nantes, the conference SCAN in Rennes, the first IGDR symposium where it received the prize for the best poster, and the doctoral school days.

The review found in the manuscript has been submitted to JCB journal and is currently being reviewed. The article recapitulating the results has been submitted to BioRxiv as a preprint. The references for both the review and the article were incorporated into the general references at the end of the manuscript for more fluidity and easier reading.

Introduction

Chapter 1:

Glioblastoma: biology, treatment, and research advances



Chapter 1: Foreword

Glioblastoma is one of the most aggressive and challenging cancers, demanding significant efforts in both research and treatment strategies. This first chapter aimed to provide a comprehensive overview of glioblastoma, from its historical context to its epidemiology, pathophysiology and clinical management. We will then explore the most recent developments in the molecular classification and treatment approaches while incorporating research on therapeutic innovations. By exploring both the established and emerging concepts in glioblastoma biology and therapy, this chapter seeks to inform on the ongoing effort to develop more effective treatments and improve patient outcomes.

I. Definition, biology and diagnosis

Glioblastoma, also known as glioblastoma multiforme (GB), is the most common adult primary brain tumor, described as an aggressive tumor, relatively resistant to therapy correlated with poor prognosis. The history of GB involves significant milestones in understanding, diagnosing, and treating this challenging disease. Understanding the molecular basis of GB, particularly the key genetic mutations and pathways involved in its pathogenesis, has become essential for developing new therapies.

1. Historical background

Gliomas were first described in the 1800s. Berns and Abernety respectively reported of primary non-metastatic central nervous system (CNS) tumors and diffuse tumors formation in the CNS on gross morphological observations in 1800 and 1804 [1]. Different terms were used at that time for these tumors “medullary sarcoma” or “encéphaloïde” by the French or “fungus medullare” by Germans [2]. It is only in 1865 that Dr. Rudolf Virchow, a German physician performed a comprehensive histomorphological description of glial tumors, he was able to show the healthy clear brain tissue and the invading tumors. These glial tumors are malignant tumors originated from the glial cells of the CNS and for the first time were named “telangiectatic” or “hemorrhagic” glioma. Virchow observed histological contrast between the tumors and decided to divide them into two groups according to their cellularity and general contrast compared to the healthy tissue. Nearly 100 years after the first description of gliomas, the neuropathologist Dr. Percival Bailey and neurosurgeon Dr. Harvey Cushing, described what is now considered as the basis for modern classification of gliomas [1], [3]. The collaboration between Bailey and Cushing generated so many changes in the neurosurgery fields, they performed an extensive work of description of gliomas notably via the publication in 1926 of their book *A Classification of the Tumors of the Glioma Group on a Histogenetic Basis with a Correlated Study of Prognosis*, these different descriptions have served till date as a basis to modern neuro-oncology. Prior to these works all brain tumors were called gliomas [3]. During this era, the classification of brain tumors had only begun, and was considered inadequate and confusing. They then decided to perform a meticulous observation and examination of tumor samples and medical records of different cases observed over the years starting from the discovery of the tumor to the death of the patient. From these materials, they analyzed the

variability and histological differences between the different gliomas and whether it carried clinical implications.

In 1922, Bailey began the classification of gliomas from Cushing's collection of brain tumors specimens collected from his different surgeries. He began by examining specimens as they arrived and classified them into groups according to the patient's survival time. In addition, as new methods were arising, he was also able to highlight the neuronal and interstitial aspects of the nervous system using the method of impregnation. Bailey classified total of 414 cases of glioma in Cushing's samples and performed 254 histological tissue studies of these specimens. All in all, it took him three years to complete this in-depth work, which put an end to the logic at the time that microscopic examination of a sample taken after surgery could not be used to predict the clinical course of the disease. Based on this analysis, Bailey divided these tumors into 13 categories [3] then simplified it to 10 groups two years later (**Figure 1**). This was the first ever categorization and formed a basis for all the classification in gliomas. Glioblastoma was first called *Spongioblastoma multiforme* because of the *multiform* aspect of cells within the same tissue samples [1]. Cells from these tumors were morphologically different and atypical compared to other gliomas or even healthy glial cells which made them believe that this tumor type had a different cellular origin than the others. The name glioblastoma multiforme was finally coined with time and its common origin established.

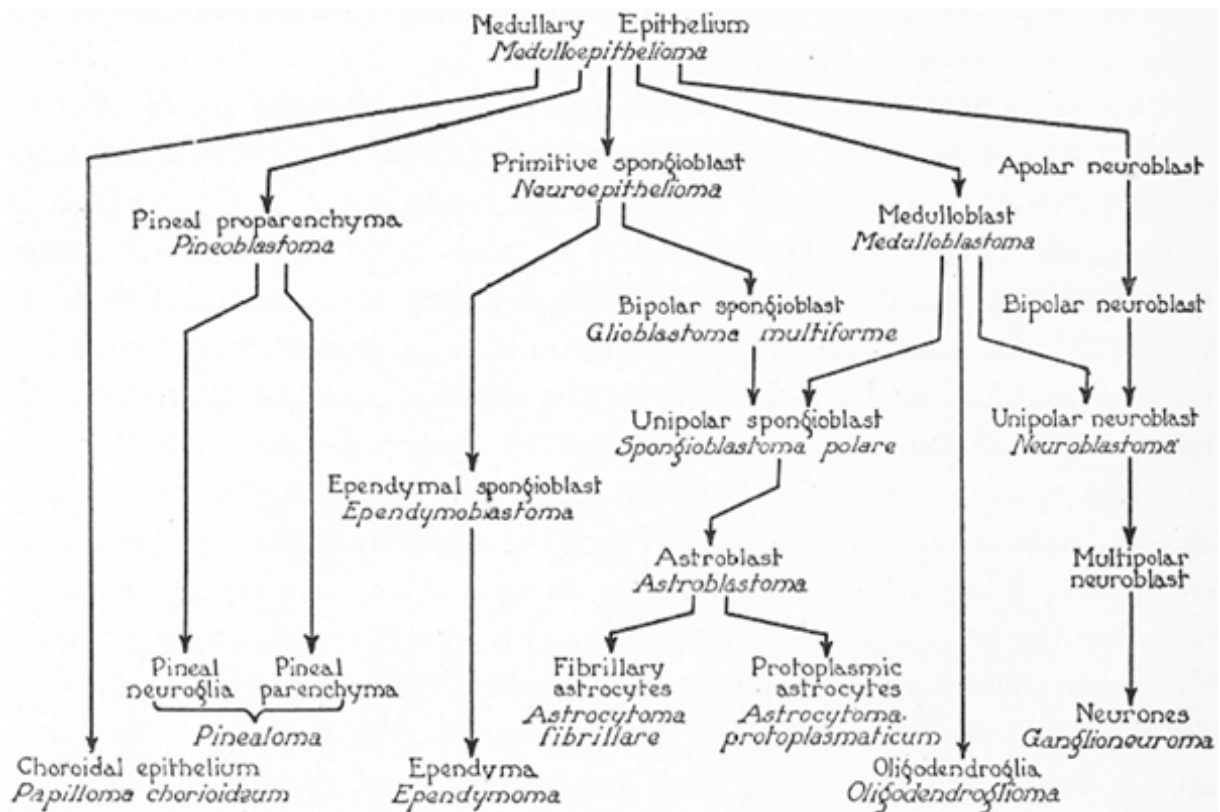


Figure 1. Glioma classification scheme of P. Bailey according to the cellular constitution of each group of tumors (from Ferguson et Lesniak, 2005).

In the 1940's Hans-Joachim Scherer, established that the histomorphological diagnosis should be based on the whole tumor sample[1,2]. Based on his works, he concluded that GB and astrocytoma are originated from the same precursor cell of origin, but that there also in some cases astrocytoma can progress in time to GB. Which then, coined the notion of primary *de novo* glioblastoma or secondary glioblastoma. According to Scherer's work these two types shared histomorphological hallmarks but diverse in their biological properties. The two types of GB differ in their evolution but also in their clinical manifestation and progression. Primary GB is very aggressive and has a dramatic prognosis. Secondary in the other hand has slower progression and better general prognosis.

After, a period of expansion and many discoveries, research on gliomas slowed down a bit in the second half of the 20th century. This deceleration was due in part to the complexities involved in studying these brain tumors, as well as the limited technological advancements available at the time. The terminology of glioblastoma has changed, the most malignant brain tumor was referred to simply as *glioblastoma* the term *multiforme* having been removed for a long period of time before coming back years later.

Despite the slowdown during this period, other fields significantly advanced, the fields of imaging and histopathology, which then played a crucial role in the diagnosis of GB and other gliomas. Techniques like cerebral angiography were developed in the 1930s, allowing the visualization of blood vessels in the brain providing valuable information about the vascular networks in tumors. The arrival of computed tomography (CT) scans and Magnetic resonance imaging (MRI) in the 1970-80s revolutionized neuroimaging. CT scans provided detailed images of the brain, allowing for the non-invasive detection of brain tumors leading to more diagnostic accuracy in gliomas. CT imaging allowed the identification of the location, size and effects of glioblastomas on surrounding brain structures. MRI in the other end, provided superior contrast resolution compared to CT, allowing for better visualization of brain tumors and differentiation from surrounding brain tissue. Techniques such as T1-weighted, T2-weighted, and fluid-attenuated inversion recovery (FLAIR) sequences improved the detection of tumor characteristics and peritumoral edema.

Different advances were also performed in histopathology, immunohistochemistry (IHC) techniques were developed, allowing the detection of specific proteins within the tissue samples using antibodies. IHC enabled the identification of key markers such as glial fibrillary acidic protein (GFAP), characteristic of glial cells. S100, Vimentin and others were also introduced to help in the differential diagnosis of GB and metastatic CNS diseases [4,5]. All in all, the advances in imaging and histopathology during the mid- 20th century significantly improved the diagnosis, classification, and understanding of GB but also other brain tumors in general. CT and MRI provided a non-invasive method to visualize brain tumors. Furthermore, advancements in histopathological techniques such as IHC but also electron microscopy, allowed for detailed examination of tumor cells and their molecular characteristics.

Globally GB and other CNS tumors have been included in clinical entity classifications by the World Health Organization (WHO) since 1956. This initiative started when the WHO Executive Board passed a resolution, which was later endorsed by the World Health Assembly in 1957, to create an international classification system for human tumors. It is only in 1979 that CNS tumors were incorporated into this classification system with the publication of the first histological classification of tumors in the “WHO Blue Book” (as it is commonly termed) [6,7]. There have been five editions and six versions in total of the international standard for the classification of brain and spinal cord tumors that reflect the evolving understanding of CNS tumors integrating new diagnostic technologies and genetic information to provide more

comprehensive and precise classification system. In fact, tumors were traditionally classified according to their histological features, but over the years, and particularly since the 4th edition (2016) of WHO classification of CNS tumors, classification has increasingly been based on molecular parameters such as genetic alterations (i.e., IDH mutation, 1p/19q codeletion) as well as histology to provide guidelines regarding tumors diagnosis. The last CNS WHO consortium took place in 2021 and brought different modifications again compared to the previous 2016 one. Whereas, the 4th edition of 2016 incorporated IDH-wildtype (primary GB) and IDH mutants strains (secondary GB) of the tumor, the 5th edition has defined glioblastomas solely as adult IDH-wildtype tumors which correspond to so-called primary or *de novo* glioblastoma [8,9].

2. Epidemiology

2.1. Incidence and Prevalence

GB is the most common malignant primary brain tumor with a proportion of 54% of all gliomas and 16% of all primary brain tumors [10–12]. The incidence of glioblastoma is approximatively 35 cases per million individuals annually, with a male-to-female ratio of 1.6:1 [13,14]. GB usually occurs after the age of 40 years with a peak incidence between 64 and 75 years old [11] [13]. It has been observed for instance by the Central Brain Tumor Registry of the United States (CBTRUS) that the incidence of GB increases with age with a peak around 75 years and a decrease after 85 years [15]. Among this category of people, the incidence rate (IR) reaches around 130 per million individuals. The prevalence is variable according to regions of the world; in Europe and North America for instance, the average annual incidence rate (IR) is around 3-4 per 100.000, whereas in Asia it is 0.59 per 100.000, making it the highest IR among brain and CNS tumors [10,16–21]. Regarding gender, there is a slightly preponderance in males than females (3:2 M:F ratio in the United States) [12]. In terms of ethnicities, white patients are more frequently affected than the others. Indeed, GB IR is 2.5 times higher in European Americans than in African Americans, it has been also shown that GB is more common in non-Hispanics ethnicities [16,22].

Traditionally, GBs had been divided into two types the primary *de novo* type (90%) occurring in older patients (mean age 64 years) and secondary type developing from a pre-existing lower grade tumor (10%) occurring in younger patients (mean age 45 years), with a high IR of primary in men and higher IR of secondary in women [10]. This nomenclature was

also correlated with mutations in isocitrate dehydrogenase-1 (IDH1) and IDH2, secondary GB contained 70-80% of IDH mutations. However, in the 5th edition (2021) WHO classification of CNS tumors, these terms have been suppressed and should no longer be used. Primary glioblastomas now equate to glioblastoma IDH-wildtype WHO CNS grade 4, whereas secondary glioblastomas now are referred to as astrocytoma, IDH-mutant WHO CNS grade 4 [9].

2.2. Risk factors

The only established risk factor for the occurrence of glioblastoma is a prior radiation exposure but this only happen in very rare cases [10,23–25]. There is an increased incidence in patients with rare hereditary tumor syndromes such as neurofibromatosis type 1 (NF1) [26] and Li-Fraumeni syndrome [27] but also other syndromes such as Turcot syndrome [28,29], Ollier disease [30], and Maffucci syndrome [31]. Otherwise, GB is a sporadic disease without genetic predisposition [10]. It has been identified that there is a lower risk of GB in people with asthma and other allergic conditions or atopic disease such as eczema, and psoriasis [10,32]. Furthermore, a short-term use of anti-inflammatory drugs has been associated with a protection against GB [33]. Different studies have been carried out to identify factors associated with GB in the environment or patients' lifestyle i.e. diet (foods containing N-nitroso compounds), cigarette smoking, alcohol consumption, drug use or mobile phones use but none of them carry strong evidence [10,23,24,32,34].

3. Diagnosis

3.1. Classification

The 5th edition (2021) of the WHO classification of CNS tumors incorporates molecular parameters into the diagnostic criteria, reflecting the latest advances in the understanding of tumor biology. In this updated classification, specific criteria must be met to diagnose a glioblastoma [9]:

- (i) The patient must be an adult
- (ii) The tumor must be a diffuse astrocytic tumor
- (iii) The tumor must be IDH-wildtype

Additionally, at least one of the following molecular or histological features is required:

- Necrosis
- Microvascular proliferation
- TERT promoter mutation
- EGFR gene amplification
- Combined gain of whole chromosome 7 and loss of chromosome 10 [+7/-10].

On top of it, all tumors in adults that are IDH-wildtype, which do not have the same histologic features (even if they appear histologically lower grade) of glioblastoma but instead have one or more of the 3 genetic parameters (TERT promoter mutation, EGFR gene amplification, or combined gain of whole chromosome 7 and loss of chromosome 10 [+7/-10]) will be classified as glioblastomas (Figure 5) [35,36].

Adult-type diffuse glioma classification (simplified)

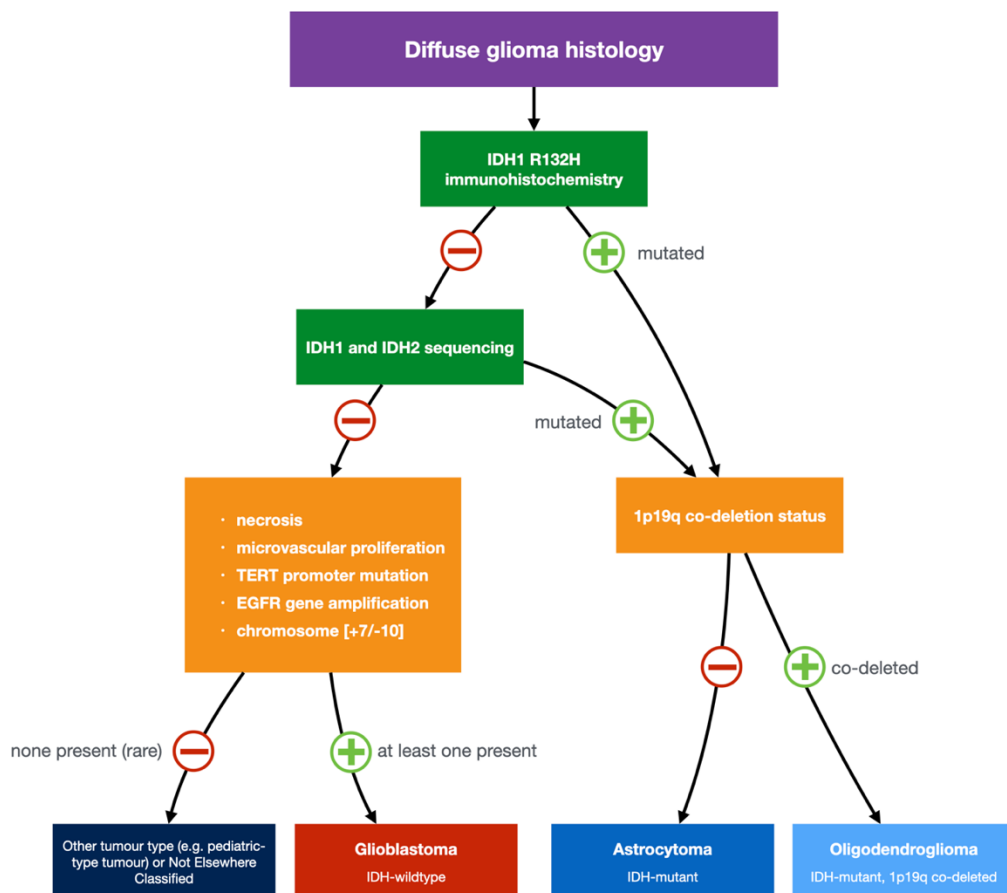


Figure 2. Adult-type diffuse glioma classification based on the 5th edition (2021) of WHO CNS (Case courtesy of Frank Gaillard, Radiopaedia.org, rID: 94212)

Glioblastoma was formerly known as glioblastoma multiforme, with the term “multiforme” highlighting the heterogeneity of the tumor. However, in the revised 4th edition (2016) [8] of the WHO classification, this term was removed, to simply refer to these tumors as glioblastomas. Although the abbreviation “GBM” was retained for clarity in that edition, it appears to have been removed in the 5th edition summary. Regarding the grading, glioblastomas are still considered grade 4, the only modification is the use of Arabic numerals instead of romans for all of WHO CNS tumors [9]. In addition, what were called glioblastoma variants in previous classifications are now called subtypes [37]. There are still three subtypes of GB aside from the “typical” GB: giant cell glioblastoma characterized by multinucleated giant cells [38], gliosarcoma that are very similar to GB but with an added sarcomatous component [39], and epithelioid glioblastoma which contains large epithelioid cells that have abundant eosinophilic cytoplasm and resemble melanoma cells, most of them have the BRAF V600E mutations [8].

3.2. Histopathology

Macroscopic appearance

Anatomically, GB is mostly found in the supratentorial white matter (85% of the cases) with a higher incidence in the frontal lobe (almost 25%). In very rare cases, it can be in the cerebellum, in the spinal cord and the brainstem. The supratentorial location is more prevalent in older patients [10,13,40–42]. The heterogeneous mass usually possesses irregular peripheral enhancement, central necrosis, and surrounding vasogenic edema. However, they can also have the same appearance as lower grade astrocytoma, IDH mutant.

Microscopic appearance

In terms of histopathology, GB displays pleomorphic astrocytes that are atypical and poorly differentiated. They possess a polygonal to spindle shape with acidophilic cytoplasm and indistinct cellular border. These cells are highly mitotic, necrotic and shows microvascular proliferation which are hallmarks of GB. Two types of necrotic regions can be encountered; the first one consists of a large necrotic area in the center of the tumor resulting from insufficient blood supply. The second type is multiple small irregularly shaped necrotic foci surrounded by “pseudopalisading” areas that are composed of tumor cells arranged in parallel to each other, also orthogonal to the necrotic center [9,13,40,42] (**Figure 4**). This feature is defining for glioblastoma and predicts an aggressive behavior. Indeed, “pseudopalisading” cells are highly

hypoxic and secrete pro-angiogenic factors such as VEGF and IL-8 [43]. Pleomorphism can also be observed in the nuclei of the cells. GB cells have an increased nuclear to cytoplasmic ratio. GB is also characterized with a high vascularization; the newly formed vessels appear like renal glomeruli and contain Weibel-Palade or tubular bodies which is unusual for normal brain endothelial cells. Vascular thrombi are also present and lead to endothelial damage and proliferation [13,40,44].

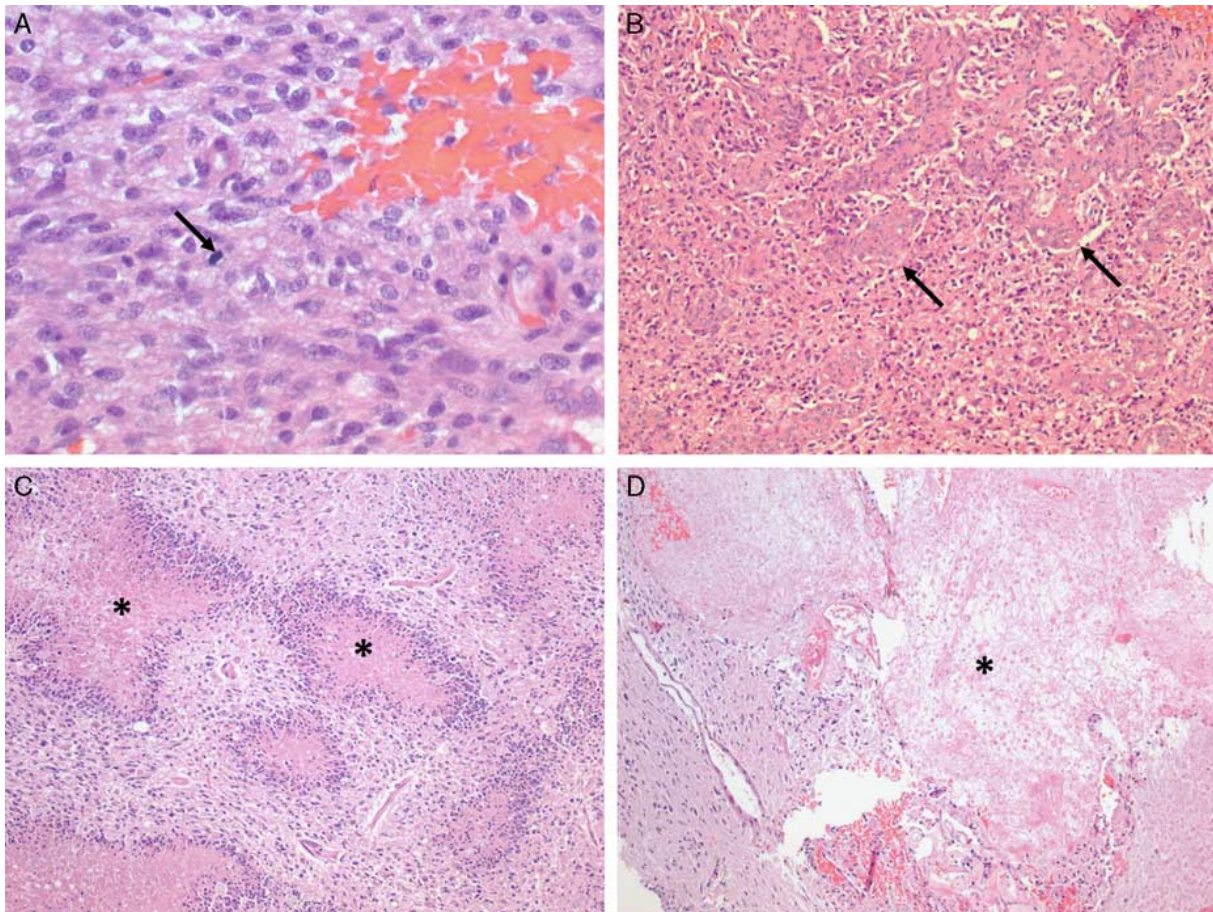


Figure 3. Pathologic features of Glioblastoma. Panel A, Cellular areas with mitotic activity (arrow) (x400). B, Vascular proliferation: Islands of blood vessels (arrows) (x100). C, Pseudopalisades surrounding geographic necrosis (*) (x100). D, Large areas of necrosis (*) (x100) (from Gokden, 2017).

3.3. Clinical presentation

Patients typically present neurological symptoms such as focal neurological deficit, symptoms of increased intracranial pressure, headaches, seizures, epilepsy, confusion, dizziness, speech and visual deficits (blurred vision, diplopia), motor deficit [13,40,45]. These unspecific symptoms may also suggest other inflammatory or infectious conditions, leading to

misdiagnosis. The symptoms can vary depending on the location and size of the tumor. The management of glioblastoma is multidisciplinary and requires different specialists including neurosurgeons, neuro-oncologists, radiologists, pathologists and radiation oncologists to formulate an optimal treatment plan.

4. Pathophysiology

Glioblastoma is a highly aggressive brain tumor characterized by complex and multifaceted pathophysiology. The tumor arises de novo however the mechanisms governing gliomagenesis remain elusive. Several studies hypothesized that GB may arise from a multipotent neural stem cells (NSC) that can differentiate into several progenitor such as glial precursor cells, oligodendrocyte precursor cells, astrocyte precursor cells and neural progenitor cells but further studies are still needed to validate this statement [46,47]. GB genesis and development are driven by a combination of different genetic mutations, abnormal signaling pathways, and interactions with the tumor microenvironment, making it a very heterogeneous tumor both molecularly and phenotypically within the tumor itself and even between patients [48]. These different parameters put together make glioblastoma a challenging tumor to treat [49,50].

4.1. Genetic basis

One of the key features of glioblastoma as stated before, is its high degree of cellular and molecular heterogeneity. This heterogeneity is due to several factors, one of the largest is the various genetic alterations that affect key regulatory pathways involved in key processes such as cell growth, apoptosis, and DNA repair [50]. The most frequent genetic alterations identified can be distributed across three major signaling pathways: receptor tyrosine kinase family RTK/RAS/PI(3)K signaling, p53 and RB tumor suppressor pathways, suggesting that this is an essential condition in the pathogenesis of GB [51,52] (**Figure 4**).

Glioblastoma, previously referred to as primary glioblastoma, is known to be IDH-wildtype. Isocitrate dehydrogenase enzymes (IDH1,2,3), play key roles in cellular metabolism, converting isocitrate to α -ketoglutarate (α -KG). IDH1 is in the cytoplasm and helps regulate glucose and lipid metabolism, as well as protect against oxidative stress. In the other hand, IDH2 and IDH3 are in the mitochondria, and are involved in tricarboxylic acid cycle and offer protection from reactive oxygen species (ROS). Mutations of IDH1 and IDH2 are generally associated with a better prognosis, because mutated IDH enzymes lead to the production of an

oncometabolite, 2-hydroxyglutarate (2-HG), which disrupts α -KG-dependent enzymes such as histone and DNA demethylases and induce a more stable tumor phenotype, which is less aggressive and more sensitive to several therapies. On the other hand, absence of IDH mutations also referred as IDH wild-type prevent tumors to produce 2-HG and retain a normal metabolic profile. This allows for more robust tumor growth and higher metabolic flexibility contributing to the aggressiveness of the tumor [53–55]

GB is characterized by amplifications of several genes including EGFR, PDGFRA, CDK4, and CDK6, as well as mutations in telomerase reverse transcriptase (TERT) promoter, and in tumor-suppressor genes such as PTEN, TP53 and the cyclin dependent kinase inhibitors CDKN2A/B [1,50,56,57]. The loss of heterozygosity (LOH) at chromosome 10 is also a common feature of glioblastoma. These genetic alterations contribute to uncontrolled cell proliferation and survival, playing a crucial role in gliomagenesis and driving the aggressive nature of the tumor. The methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) promoter an enzyme responsible for the dealkylation of DNA is also an important parameter for response to alkylating agents such as temozolomide.

Other genetic alterations can also be observed at a lower frequency, including mutations in NF1 (Ras endogenous inhibitor) as well as amplifications of MET, AKT and MDM2/4 genes. These contribute to continuous stimulation of cell proliferation and can promote tumor angiogenesis [58,59,51].

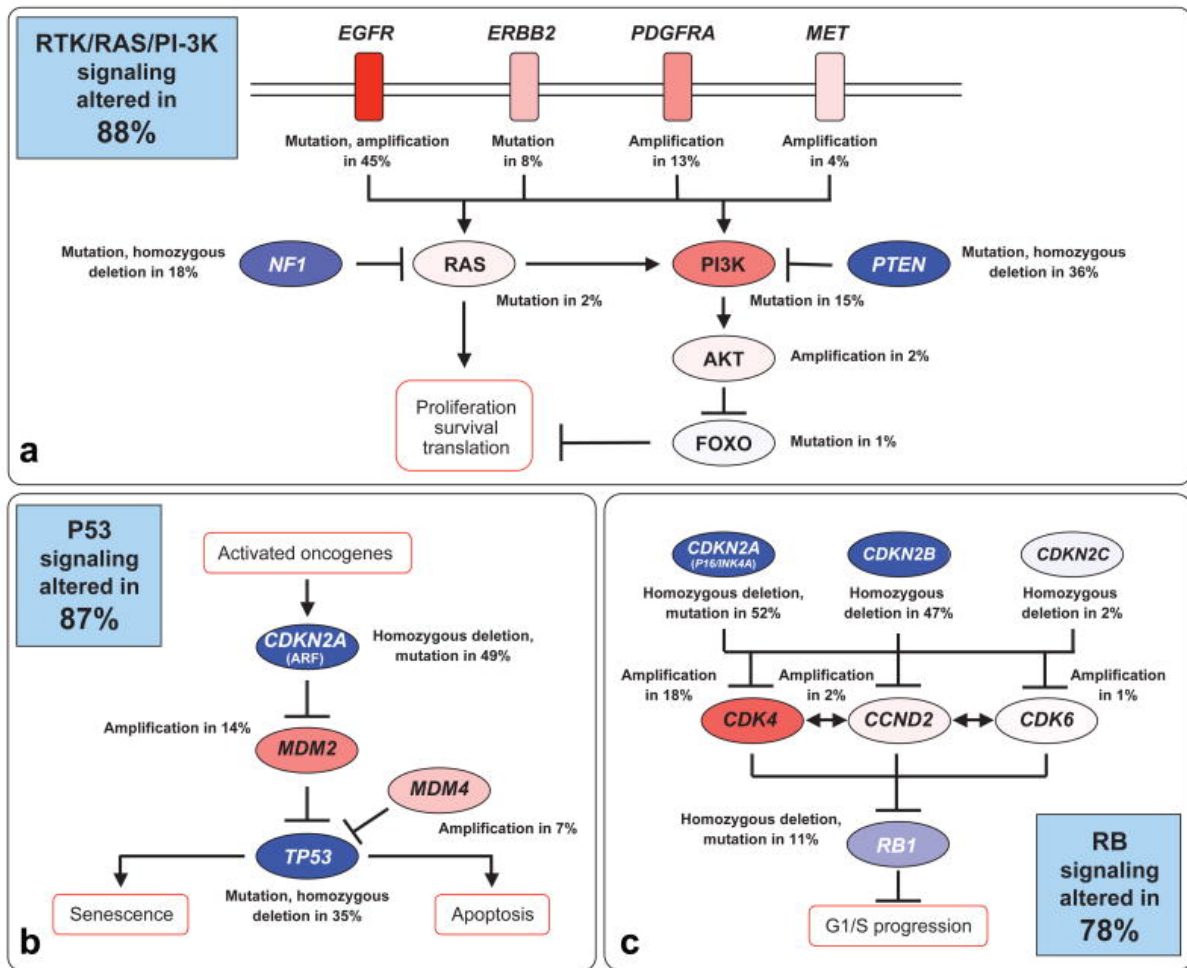


Figure 4. Summary of the frequent genetic alterations of GB in three critical signaling pathways. Red indicates activating genetic alterations, with frequently altered genes showing deeper shades of red. Conversely, blue indicates inactivating alterations, with darker shades corresponding to a higher percentage of alteration. The nature of each alteration and the percentage of affected tumors are indicated. Blue boxes contain the final percentages of GB with alterations in at least one known component gene of the designated pathway (from The Cancer Genome Atlas (TCGA) Research Network, 2023)

4.2. Molecular subclasses

The first genome-wide profiling studies performed by the Cancer Genome Atlas (TCGA) research network focused on glioblastoma. These studies enabled them to identify molecular subclasses within glioblastoma, thereby confirming its genomic heterogeneity [51]. The presence of different subclasses within the tumor contributes to the malignancy of GB. Specifically, these four subclasses are known as classical (CL), neural (NL), pro-neural (PN), and mesenchymal (MES), and they have been associated with distinct genetic alterations. For instance, the PN subclass is often linked to platelet-derived growth factor receptor alpha (PDGFRA) mutations while epidermal growth factor receptor (EGFR) alterations are more

frequently observed in the CL subclass [48,52,56]. Further research suggests that GB malignant cells exhibit a set of cellular states in their development in the tumor: neural-progenitor-like (NPC-like), oligodendrocyte-progenitor-like (OPC-like), astrocyte-like (AC-like) and mesenchymal-like (MES-like) states. These states can co-exist within a tumor, with the frequency of each state varying between tumors. Additionally, cellular plasticity allows glioblastoma cells to transition between these states under different conditions, intermediate “hybrid” states which are a combination of two states were also identified. Moreover, each state is characterized by specific genetic alterations [60,61]. AC-like, OPC-like and NPC-like are respectively associated with the amplifications of EGFR, PDGFRA, and CDK4 whereas MES-like is associated to neurofibromin 1 (NF1) alterations.

4.3. Tumor microenvironment (TME)

The microenvironment in GB is also highly heterogenous and plays a critical role in its pathophysiology. It involves the tumor extracellular matrix (ECM) and various non-cancerous cell types, including immune cells, endothelial cells, pericytes, fibroblasts, and other non-tumor glial cells, which surround and interact with the tumor and contribute to the establishment of the hypoxic and necrotic regions, tumor expansion and resistance to therapy [62].

The tumor landscape of GB can be divided into four main niches: the central necrotic and hypoxic niche of the tumor surrounded by the immune niche, the perivascular niche composed of various vessels, and the infiltrative niche composed of various other cells such as neurons, glial cells, glioblastoma stem cells (GSCs) etc. (**Figure 5**). This diverse microenvironment is responsible for the tumor proliferation, angiogenesis and invasiveness. Despite, the tumor immune infiltration, glioblastoma is considered a “cold” tumor due to the highly amounts of immunosuppressive regulatory and myeloid cells [63].

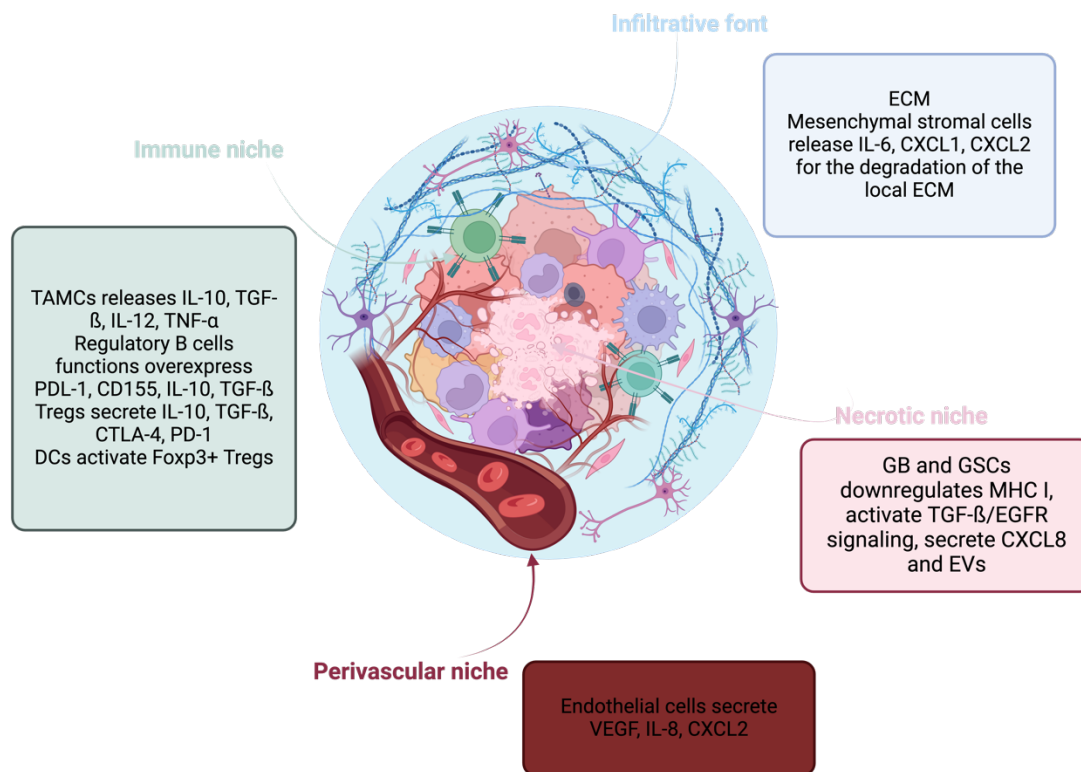


Figure 5. The four niches of tumor microenvironment in Glioblastoma and how they contribute to immunosuppression.

Extracellular matrix (ECM)

ECM constitutes up to 20% of brain volume, and has a major role in brain development, and homeostasis. It has been shown that the ECM composition in GB setting is different from a healthy human brain ECM [50]. In GB, an increased secretion of ECM components such as hyaluronic acid (HA), fibronectin (FN) and laminin, by tumor cells as well as an increased expression of integrins and other specific receptors on the tumor cell can be observed. ECM component HA and its receptor CD44 on GB cells are overexpressed and this is a requirement for GB invasion. Collagen levels are also elevated in GB compared to healthy brain tissue enhancing cell motility and invasion [64,65].

Glioblastoma microenvironment supports its invasiveness and ability to infiltrate surrounding brain tissue by setting up various mechanisms, including the upregulation of matrix metalloproteinases (MMPs) and the release of cytokines such as IL-6, CXCL1 and CXCL2 that degrade the ECM and facilitate tumor cell migration [66]. GB cells can modify ECM components to promote tumor infiltration. The architecture of ECM is also important. Indeed,

GB ECM is characterized by a disorganized structures which also participate to the invasiveness of the tumor.

Immune cells

The brain has traditionally been considered an immune-privileged site due to the existence of the blood brain barrier (BBB) and the absence of conventional lymphatic system, though a unique lymphatic system has recently been identified. Recent discoveries have shown that the BBB and the concept of immune privilege are particularly relevant to the brain parenchyma, a highly sensitive area of the brain. Normally, during inflammation in the CNS, immune cells can only migrate into the perivascular spaces. However, in severe inflammatory or disease conditions like GB, the BBB becomes compromised due to inflammation, physical distortion and increased vascularity, allowing immune cells to infiltrate the parenchyma and causing blood vessel leakage [50].

A variety of immune cells are present in the glioblastoma microenvironment starting with the tumor-associated myeloid cells (TAMCs) that can constitute up to 50% of the tumor mass. These include bone marrow-derived macrophages (BMDMs), microglia, myeloid-derived suppressor cells (MDSCs), neutrophils and dendritic cells (DCs). Tumor-associated macrophages (TAMs) (microglia and BMDMs) constitute one third of the immune cells in glioblastoma and can promote immunosuppression by releasing factors such as interleukin 10 (IL-10) and transforming growth factor beta (TGF- β) [50,63]. These TAMs are also involved in the secretion of several chemokines, angiogenic molecules and growth factors leading to more GB cells proliferation and infiltration [67]. Moreover, their quantity correlates inversely with overall survival in recurrent glioblastoma.

MDSCs also exert as strong immunosuppressive effects, and their increase is associated with poor outcomes. These cells can suppress CD8⁺ T cell activity and render T cell receptor non-functional [68].

Regulatory T cells (Tregs) expressing the transcription factor Forkhead Box P3 (FOXP3) are also recruited to the TME and contribute to immunosuppression by secreting IL-10 and TGF- β and expressing immune checkpoint molecules like CTLA-4 and PD-1 [69,70].

Natural killer (NK) cells are important for targeting tumor cells. However, in glioblastoma TME, they are almost absent and their function is suppressed, and their activity inhibited through contact with glioma cells [63,66]. Furthermore, DCs are also recruited to the TME but are influenced by the immunosuppressive environment leading them to activate Tregs.

Other cells in the TME

Glioblastoma stem cells (GSCs) are a subpopulation of GB cells that are capable of self-renewal and pluripotent differentiation. They play a crucial role in tumor initiation, growth, recurrence and treatment resistance [23,66]. GSCs, identified by markers like CD133 and CD44 can be found in the perivascular niche, the necrotic niche and at the proliferative front [71]. They exhibit enhanced migratory and invasive capabilities. Studies have shown that GSCs with high expression of CD133 are more invasive infiltrating healthy brain tissue, while CD44 is linked to migration at the tumor periphery. Several signaling pathways are critical to regulate GSC invasion and aggressive behavior (TGF- β , Wnt, Sonic Hedgehog). GSCs can evade the immune response by downregulating MHC class I, which prevent the activation of cytotoxic T cells [50].

Glioblastoma is one of the most vascularized cancers and the proliferation of endothelial cells is a hallmark of the tumor. Endothelial cells are in contact with GSCs and participate to the perivascular niche. GSCs' infiltration into the perivascular space is promoted by the secretion of vascular endothelial growth factor (VEGF) by endothelial cells which may induce the differentiation of GSCs into endothelial cells to generate the tumor vasculature [72]. Endothelial cells secrete various factors such as IL-8 and CXCL12 enhancing tumor growth and invasiveness [64].

GB TME includes various other non-cancerous cells, such as astrocytes, neurons, oligodendrocytes, which support tumor growth and invasion. Astrocytes for instance, can be activated by GB cells to enhance invasion through the secretion of IL-6 and proteins like MMPs. They also facilitate tumor cell communication and infiltration via extracellular vesicles [66].

Hypoxia

Hypoxia, or low oxygen level, is a common feature that exacerbates the malignancy of glioblastoma. Hypoxic conditions are typically associated with necrotic regions within glioblastoma particularly in the tumor's core. Hypoxia leads to the activation of transcription factors known as hypoxia-inducible factors (HIFs), which promote angiogenesis, the formation of new vessels from existing ones to supply the rapidly growing tumor with nutrients and oxygen [63,66,73]. In this oxygen-deprived environment, GSCs can differentiate into endothelial cells to generate tumor vasculature [74].

In this pathological condition, angiogenesis is abnormal and aberrant, resulting in a dysfunctional vascular network that contributes to tumor invasiveness and resistance to treatment. Additionally, these new blood vessels differ from normal vessels in terms of shape,

diameter and permeability leading to the alteration of blood flow and local edema. HIF-1 α induces the transcription of various factors involved in angiogenesis, including VEGF and its receptors VEGFR [71]. VEGF plays a key role in tumor neovascularization; its binding to VEGFR receptors activates downstream pathways that stimulate the formation of new blood vessels.

Extracellular vesicles

Recent research has highlighted the important role of secretion in glioblastoma pathophysiology. Indeed, this brain tumor is highly secretive of a variety of substances, including cytokines, growth factors and extracellular vesicles. Additionally, glioblastoma cells secrete extracellular vesicles which are small membrane-bound particles that carry proteins, lipids, DNA and RNA molecules. These vesicles are involved in intercellular communication and communication with the TME [67,75]. They can modulate the behavior of surrounding cells and TME, including promoting the invasiveness of the tumor and altering the immune environment [64,76]. For instance, exosomes released by glioblastoma cells have been shown to carry molecules that can suppress the activity of T-cells thereby promoting immunosuppression microenvironment, they can also interact with endothelial cells to promote angiogenesis [66]. Exosomal miRNAs have been described to contribute to the pathogenesis of glioblastoma leading to proliferation and invasion. Others like exosomal miR-1238 have been implicated in TMZ resistance [75].

Metastasis

Migration and cell invasion are key features of glioblastoma spreading. Glioblastomas are known for their ability to infiltrate surrounding brain tissue, making complete surgical resection impossible and causing tumor recurrence. GB malignant cells can spread very far from the primary tumor and invade the contralateral hemisphere, and this behavior is mediated by various molecular mechanisms such as the upregulation of MMPs [65]. Extracranial metastases are very rare (< 2%) and the mechanisms underlying remain unclear. Multiple factors have been presented to explain this including the presence of the BBB, but also the short patient survival [77,78]. Emerging rare cases are showing extracranial dissemination correlating with the increased survival [79]. Recent studies have been describing rare cases where metastasis was found in various organs with a preference with bone metastases. The dissemination routes are not well understood; hematogenous dissemination, infiltration of the

cerebrospinal fluid and the glial lymphatic system are suspected to be the main routes of extracranial invasion.

Overall, the pathophysiology of glioblastoma is a very complex and multifactorial process involving genetic mutations, aberrant signaling pathways, and the tumor microenvironment which is highly secretive of several factors that promote immunosuppression, proliferation, invasion and treatment resistance (**Figure 5**). This complexity is responsible for the aggressive nature of the tumor and the challenges in developing effective treatments.

II. Treatment and research advances

1. Treatment approaches

The aggressive and heterogeneous nature of glioblastoma makes it a highly resistant tumor, making it challenging to treat. A multimodal approach is typically employed, it is a combination of surgery, radiotherapy with daily dose of chemotherapy with temozolomide (TMZ) commonly known as Stupp protocol [80], and emerging treatments focused on targeted therapy and immunotherapy.

Surgery

Surgical resection allows the removal of as much of the tumor as possible without causing significant neurological damage. It is also very important to establish the definitive histological diagnosis of the tumor and help with the genotyping. Complete resection of the tumor is often difficult due to the invasiveness nature of GB making it difficult to distinguish tumor tissue from normal brain tissue. The guideline is the maximal safely feasible surgical resection. Gross total resection (GTR) is generally recommended, if safely feasible. Several studies have shown that GTR may improve patients' survival. Maximal tumor resection also leads to a better prognosis [81]. Despite the different challenges, surgery is crucial for reducing the tumor burden, easing symptoms, and improving the efficacy of other combined treatments for a better overall survival (OS).

Radiotherapy

The standard approach after surgery for newly diagnosed glioblastoma is radiotherapy which enhances patients' survival by targeting the tumor and surrounding areas to kill any remaining cancer cells. Conventional radiotherapy consists of fractionated focal irradiation over several weeks for a total of 60 Gy divided in 30 fractions, combined with chemotherapy with TMZ. Several strategies were developed to increase the radiation dose to the tumor without achieving much. Brachytherapy and stereotactic radiosurgery have been utilized in combination with surgery to enhance control of local metastases but have failed to improve patients' survival [23,82,83].

Chemotherapy

Several alkylating agents have been tested in the treatment of GB (carmustine, lomustine) [34,84]. However, Temozolomide (TMZ) is the only standard drug for glioblastoma. It is an oral alkylating agent that can cross the BBB. Chemotherapy is administered concurrently with radiotherapy for several weeks (Stupp protocol) followed by six cycles of adjuvant TMZ [80]. TMZ is responsible for DNA damage in cancer cells preventing them from replicating. However, the efficacy of TMZ can be limited by the methylation status of MGMT promoter which if non methylated enhance the tumor's ability to repair DNA damage [24,25]. Studies are on-going for alternatives to TMZ in cases where MGMT promoter is unmethylated.

Standard of care for patients aged ≥ 70 years can vary due to the differences in performance, general health and higher risk of toxicity. Indeed, for older patients with a good performance status and MGMT promoter methylation, the standard of care remains the first line treatment. However, for the most frail patients hypofractionated radiation (total of 40 Gy in 15 fractions) is preconized in conjunction with concurrent and adjuvant TMZ [81,85,86]. TMZ alone may also be an alternative to radiotherapy for older patients with methylated MGMT who cannot benefit the combination treatment [34,85].

Glioblastoma is notorious for its recurrence, even after aggressive treatment. Till now, there is no standard of care or systemic treatment for recurrence, management should therefore take into consideration the patient overall health and the previous treatment administered to avoid toxicity. Recurrent GB is even more complex in terms of genetic variability than the primary tumor which makes it even more complicated to understand and treat. Studies have

demonstrated that TMZ chemotherapy influences the characteristics and mutation rate of recurrent GB tumors [50]. Repeat surgery with GTR if feasible may improve survival outcomes however age, performance status and tumor volume are associated with its benefit. In case of recurrence in younger patients (<70 years) with good performance status, reirradiation can be performed and may show an improvement in survival and quality of life but have to be repeated at least 6 months after the first one [81,82]. Alkylating agents like lomustine can be used as single agent or combined treatment but showed a modest benefit. Bevacizumab, the VEGF blockade was rapidly approved by the US Food and Drug Administration (FDA) in monotherapy for the treatment of recurrent glioblastoma after phase II trials but have failed to improve OS. However, the European Medicines Agency (EMA) rejected the authorization because of the lack of positive benefit-risk [81,83].

Targeted therapies

The current experience of treatment in GB shows that we need to get closer to our objectives, and that rational combinations of established treatments and new approaches could offer the possibility of improving prognosis. Given the highly vascularization of glioblastoma, targeting angiogenesis has become a key area of focus. Several strategies have been developed to block the main mediators, VEGF and VEGFR such as VEGF trap, blockade or suppression of VEGFR signaling using tyrosine kinase inhibitors (TKIs) [87]. Bevacizumab, a humanized monoclonal antibody that blocks VEGF have been tested in combination with chemoradiotherapy. Although, progression-free survival (PFS) was improved, overall survival was not increased and there were an increased toxicity [40,87]. There have been clinical trials that tried to target EGFRvIII mutation, using vaccine (rindopepimut) or a combination of vaccine, or TKI like erlotinib without success [34,57]. Other therapies with TKIs have been explored to target signaling pathways commonly dysregulated in GB including PI3K/mTOR, MET, PDGFRA but they have all failed to demonstrate efficacy.

Immunotherapy

In the last few years, there is a reconsideration of immunotherapy as a promising strategy. Immunotherapy is very challenging due to the immunosuppressive environment, but also the standard therapies and corticosteroids used as supportive care that may enhanced immunosuppressive effects. However emerging therapies such as immune checkpoint inhibitors (ICI), brain tumor vaccines, chimeric antigen receptor (CAR) T-cell therapies,

immune monoclonal antibodies and adoptive cell transfer therapies are being actively developed [34,81,83]. To overcome this immunosuppressive environment, ICI such as anti-PD-1/anti-PD-L1 and anti-CTLA-4, which help to overcome the tumor's ability to evade immune detection have been ongoing although the initial results have not been favorable [57,66]. Finally, (CAR) T-cell therapies are recently being expanded to target EGFRvIII, and interleukin (IL)13Ra2 (IL13Ra2), a cytokine receptor overexpressed in GB compared to brain normal tissue. Further studies and data are needed to conclude on their efficacy [88,89].

Other approaches

Other approaches are also being explored, such as tumor-treating fields (TTF), which consist in wearing scalp transducers that use alternating electric fields to disrupt cancer cell division while working in synergy with concurrent chemotherapy [32,75]. This locoregional therapy has been approved as an adjuvant therapy with TMZ and has shown a benefit in both PFS and OS [34,81,90]. Recent approaches combining bevacizumab and TTF are suggesting a gain in recurrent glioblastoma but are still under study [91].

2. Prognosis

Glioblastoma is very well known for its poor prognosis, making it one of the most challenging cancers to treat despite the advances in the medical field and cancer research [62]. The outlook for patients diagnosed with this invasive brain tumor remains bleak, mainly because of its rapid progression, resistance to treatment and recurrence even after intensive therapy. The median survival time for patients is approximately 15 months from the time of diagnosis, even with the current standard of care and a 5-year survival of only 7.2% [14,84,92]. The survival rate drops abruptly with time; only about 40% of patients survive one year after diagnosis, and around 17% the second year [93]. Several factors can influence the prognosis of GB, such as the patient's age, the extent of surgical resection, the molecular and genetic profile of the tumor and the patient's overall health. Indeed, younger patients (around 40 years) tend to have a better prognosis than elderly patients (≥ 70 years) [81]. Older age is associated with a poorer overall survival, likely due to a decreased tolerance to aggressive treatments and the presence of other comorbidities. As stated above, the extent of surgical resection significantly impacts prognosis. GTR where, as much of the tumor as possible is removed, is associated with better outcomes compared to partial resection. The multifocality of the tumor is also a prognostic factor [10]. GB was previously inclusive of any IDH status, and the IDH

mutant variants were of better prognosis [86]. IDH-wildtype is a more aggressive phenotype associated with worse outcomes. Other mutations like EGFR amplification, especially mutant variant III (EGFRvIII) is associated with a poor prognosis and a shorter overall survival. The PTEN tumor suppressor gene as well as the TERT promoter are also negative prognostic factors [57]. Methylation of the MGMT gene promoter also plays a critical role in prognosis. Patients with a hypermethylation of MGMT promoter tend to respond better to alkylating agent like TMZ, leading to an improved survival [13,81]. Good performance status and overall health help improve prognosis, a good Karnofsky performance score (KPS) confers improved survival [13], this standardized tool help to assess cancer patients' functional status and their ability to perform daily activities. It provides a score ranging from 0 to 100, with higher scores indicating better functional status.

GB tumors are always recurrent, even after the heavy combination of treatment. The tumor often recurs in or near the original site within 6 months of initial treatment. Recurrent GB is particularly difficult to treat, as it is often more resistant to therapies than the initial tumor. The median survival after recurrence is generally a few months [34,84].

Different studies are now incorporating radiomics analysis extracted from MRI or CT scans in the prognosis of glioblastoma to predict the survival outcomes, the treatment response or even to characterize and quantify tumor heterogeneity. Radiomics plays an increasingly significant role in the prognosis of GB and other cancers by extracting and analyzing large amounts of quantitative features from medical images. These features can include details about the shape, texture, intensity, and spatial relationship within the tumor and surrounding tissues. The primary goal here is to correlate these imaging features with clinical outcomes, such as survival rates, treatment response and tumor behavior [93–96].

3. Future directions

Several clinical trials are ongoing and focusing on exploring new treatment strategies and combinations. These trials are investigating a variety of therapies that aim to improve patient outcomes. Recent advances in molecular research are also deepening our understanding of glioblastoma's biology, giving us the opportunity to uncover potential therapeutic targets that could pave the way for new treatments. Innovative technologies such as the utilization of nanocarriers to circumvent the BBB and allow drug delivery are being actively developed [83].

The discovery of anticancer effect of alkaloids and its implementation in chemotherapy to manage glioblastoma is also being studied [97].

Finally, the integration of multi-omics approaches into research is expected to reveal new therapeutic targets and biomarkers for better disease management.

Chapter 1: Conclusion

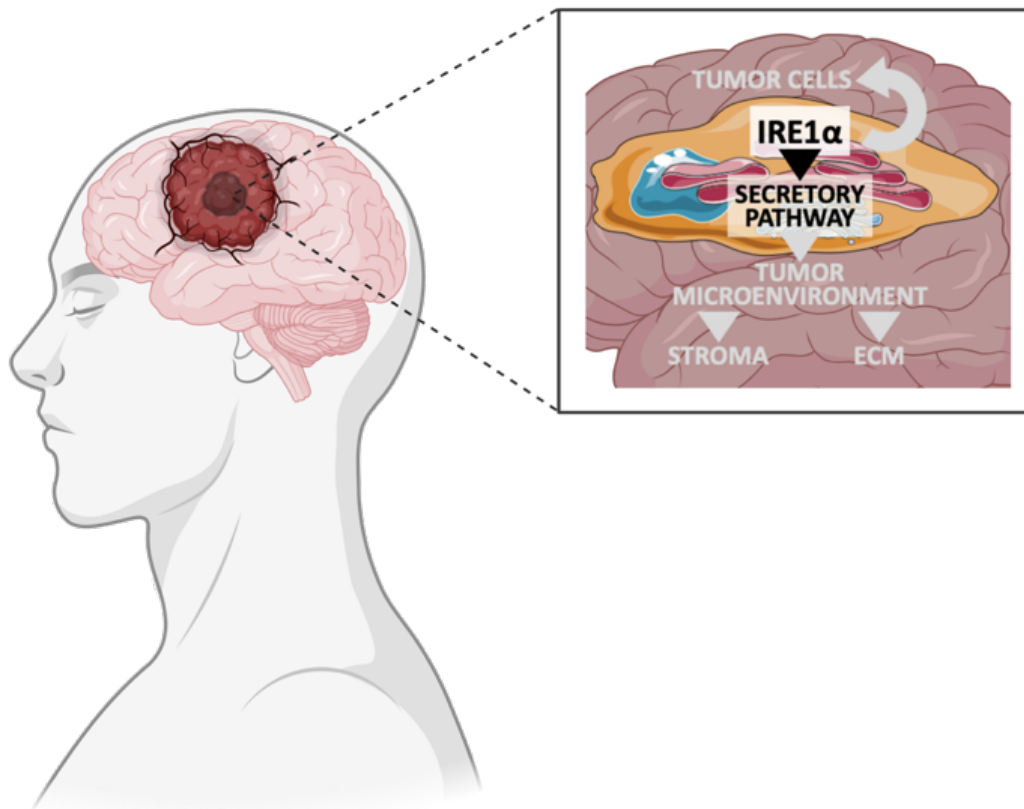
In this first chapter, we have provided a detailed overview of glioblastoma, one of the most aggressive and challenging brain tumors highlighting the critical importance of understanding and targeting this devastating disease in the field of cancer research. We covered key aspects of the disease including its complex pathophysiology, where molecular heterogeneity, genetic mutations and the diverse microenvironment, play a pivotal role in tumor rapid development, recurrence and resistance to therapy. The chapter also included the evolution of the glioblastoma classification particularly the significant updates in the 5th edition (2021) of WHO CNS, that redefined glioblastoma as solely IDH-wildtype, removing the idea of primary and secondary GB. Additionally, this new classification integrates molecular markers into diagnostic criteria, providing more precise prognostic and therapeutic insights.

This description has also allowed us to explore advances in imaging, such as the emerging role of radiomics, which offers detailed tumor characterization and can be used to predict patient outcomes more accurately. The treatment approaches for glioblastoma, though limited in improving long-term survival, were presented, with a focus on the standard of care also known as the Stupp protocol involving surgical resection, radiation, and chemotherapy, as well as emerging therapies like targeted therapies, immunotherapy and adjunct therapy like Tumor-treating fields.

Current experience of glioblastoma treatment shows that rational combinations between established treatments and new approaches still need to be explored and could offer the possibility of improving prognosis.

Chapter 2:

The Unfolded Protein Response and cancer – clinical implications



Chapter 2: Foreword

In the first chapter, we provided an overview of glioblastoma and the complex pathophysiology of this fatal disease and outlined the current therapeutic approaches. Despite extensive treatment, the prognosis for glioblastoma remains poor, emphasizing the need for further investigation into its molecular mechanisms and the development of new therapeutic strategies. In this chapter, we will focus on the role of cellular stress responses, particularly the Unfolded Protein Response (UPR), in glioblastoma. Tumor cells often experience various forms of stress, such as hypoxia and nutrient deprivation, which lead to the accumulation of misfolded proteins in the endoplasmic reticulum (ER). The UPR is a cellular mechanism designed to restore protein homeostasis by alleviating this stress. However, in cancer, including glioblastoma, the UPR can be co-opted to support tumor survival, progression, and resistance to treatment.

The second part of this chapter will delve into the involvement of the UPR in glioblastoma pathophysiology, with a specific focus on IRE1, a key sensor of the UPR. We will examine how IRE1 contributes to tumor adaptation and survival under stress conditions, and its potential as a therapeutic target for disrupting these survival pathways in glioblastoma cells.

I. UPR

This paragraph describing the UPR was extracted from the review below

The UPR is a cellular stress response related to the ER activated in response to an accumulation of misfolded or unfolded proteins in the ER, a situation that can arise due to various stress conditions including those associated with tumorigenesis.

The UPR is transduced by three ER-transmembrane sensors: the activating transcription factor 6 (ATF6), the inositol-requiring enzyme 1 α (IRE1, encoded by ERN1 gene), and the protein kinase RNA-like ER kinase (PERK, also known as EIF2AK3) (**Figure 6**). Under homeostatic conditions, these sensors are maintained in an inactive conformation by their binding to the chaperone BIP. Whereas, when the level of unfolded proteins exceeds the adaptive capacity of the ER, BIP dissociates from the sensors and binds the misfolded proteins. This dissociation leads to PERK and IRE1 homodimerization and activation by auto-phosphorylation of their kinase domain. ATF6 translocates into the Golgi apparatus to be cleaved by S1P and S2P molecules. Once activated, each sensor activates specific signaling pathways described below, that participates to restore homeostasis. Activated PERK phosphorylates the alpha subunit of the translation initiation factor, eIF2 α , resulting in inhibition of global protein synthesis. Activation of IRE1 and ATF6 promotes transcription of UPR target genes.

ATF6, an ER transmembrane sensor that delocalizes into the Golgi to signal - Upon ER stress, ATF6 dimerization facilitated by the protein-disulfide reductase ERP18 induces its export into the Golgi apparatus where it is cleaved by the Golgi resident proteases S1P and S2P, releasing its cytoplasmic domain (**Figure 6**). ATF6f is a potent transcription factor that induces expression of UPR genes whose promoters contain regulatory ER stress response elements ERSE, involved in ER homeostasis maintenance, protein degradation and cellular redox regulation.

IRE1, a central UPR component with kinase and endoribonuclease activities - IRE1 is the most documented ER stress sensor as the most conserved sensor across eukaryotic organisms and plays a pivotal role in sensing and responding to ER stress (**Figure 6**) [98–100]. IRE1 is also

the only ER stress sensor found in yeast. IRE1 is a type I ER resident transmembrane protein with a cytosolic serine/threonine kinase and endoribonuclease (RNase) domain, both activated by its oligomerization and autophosphorylation of its kinase domain [101]. Upon ER stress, IRE1 RNase activity is responsible for the unconventional splicing of X-box binding protein 1 (XBP1) mRNA, leading to generate a novel spliced form of XBP1 named XBP1s. XBP1s is a potent transcription factor that binds directly to the ERSEs and activates transcription of ER molecular chaperones to increase the protein-folding capacity or ERAD-related genes to facilitate the degradation of misfolded proteins [102]. Under prolonged ER stress, IRE1 RNase is also involved in the degradation of RNAs, either ER-proximal mRNAs encoding membrane and secreted proteins or microRNAs, through a process called regulated IRE1-dependent decay of RNA (RIDD). Those targeted RNAs are degraded as they present a consensus cleavage site similar to that of XBP1 in their sequences. RIDD activity leads to reduce the load of protein production. Finally, upon ER stress, the IRE1 kinase activates JNK (for c-Jun N-terminal kinase) signaling by interacting with TRAF2 (for TNF receptor-associated factor 2), which plays a role in autophagy activation [101].

PERK, an ER stress sensor that attenuates RNA translation - Upon ER stress, active PERK phosphorylates eIF2 α (**Figure 6**). The latter is part of the eIF2 complex that initiates protein synthesis [98–100]. However, PERK-mediated phospho-eIF2 α inhibits its function, thus attenuating mRNA translation, leading to the reduction of protein load in the ER. Remarkably, several mRNAs resist to the PERK-dependent global translation slow-down. Indeed, these mRNAs contain upstream open reading frames that are still active and short allowing their translation. Among those, ATF4 (for activating transcription factor 4) induces transcription of down-stream targets including CHOP, that, in concert with ATF4, induces expression of many UPR-related genes involved in protein synthesis, amino acid transport and metabolism, autophagy and resistance to oxidative stress [98–100].

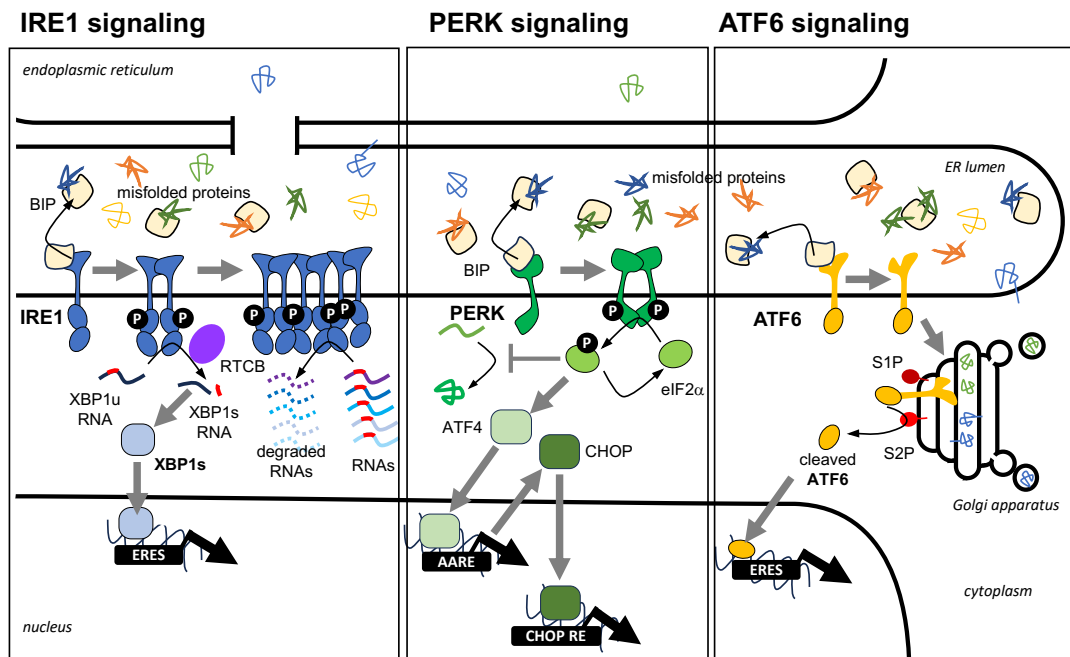


Figure 6. UPR stress sensors and their signaling pathways. Under normal homeostatic conditions, the UPR sensors ATF6, IRE and PERK are inactive and bound to the chaperone BIP. Accumulation of misfolded proteins into the ER trigger BIP dissociation that binds to the misfolded proteins, leading to the activation of the UPR sensors. This dissociation leads to PERK and IRE1 activation by auto-phosphorylation of their kinase domains. The release of BIP from the sensor ATF6 allows its export into the Golgi apparatus where it is cleaved by S1P/S2P proteases releasing the cleaved form of ATF6 that exhibits transcription factor function. IRE1 RNase activation triggers the unconventional splicing of the XBP1 RNA, together with the RTCB ligase, leading to the translation of the transcription factor XBP1s. Upon prolonged ER stress, IRE1 oligomers trigger the RIDD, decreasing the RNA translation load and therefore limiting new protein production. PERK activation triggers the phosphorylation of eIF2 α , that attenuates RNA translation but enhances the translation of the transcription factor ATF4 that increases the expression of several genes including the transcription factor CHOP. (From review 1; Bakambamba et al. 2024)

II. UPR involvement in cancer biology

Cancer cells encounter various environmental and metabolic stressors including hypoxia, oxidative stress, nutrient deprivation and other metabolic disruptions. These dysregulations disrupt cellular homeostasis, leading them to a persistent state of ER stress. The ER, becomes overwhelmed by the increased demand for protein production, folding, and the accumulation of misfolded proteins at the same time. Therefore, this persistent ER stress triggers the activation of a series of adaptive responses, termed the UPR to help the tumor cells adapt and survive these harsh conditions [103,104].

Studies have demonstrated that UPR components play a critical role in tumor growth and therapy resistance [105] of various cancers including breast, lung, liver, colorectal and glioma which makes the UPR a key target for new cancer treatments [106–110].

Neoplastic transformation is a multistep process in which normal cells acquire different biologic capabilities termed the hallmarks of cancer to be transformed into a tumor (**Figure 7**) [111]. Studies have shown that the UPR via its three sensors; IRE1 alpha (referred to IRE1 hereafter), PERK and ATF6 and chaperones is often upregulated in tumors and contributes to almost every step of the malignant transformation (**Figure 7**) [103].

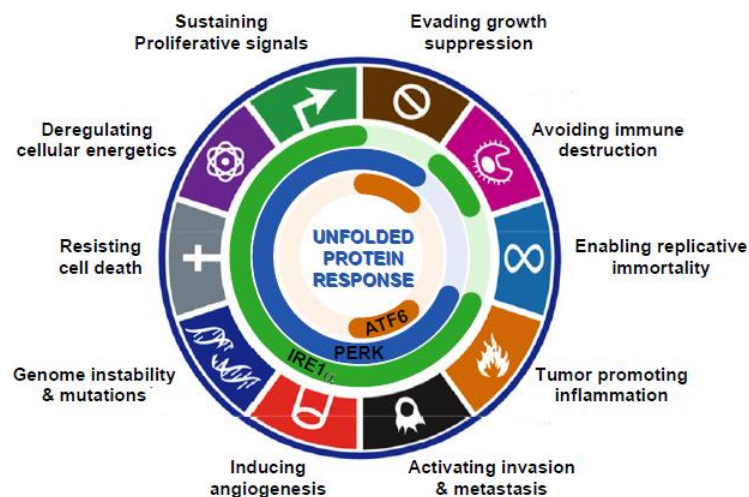


Figure 7. The UPR involvement in the hallmarks of cancer. IRE1 and PERK are involved in almost all the hallmarks whereas ATF6 is mainly involved in metastasis and escaping growth control (from Urrea et al. 2016)

1. Hypoxia

Under hypoxic conditions, cells do not only activate HIF-1 pathway but also other adaptive responses to help them survive these conditions. One of such response is the reduction in global protein synthesis through several pathways including the activation of the PERK branch and phosphorylation of the translation initiation factor eIF2 α [112,113]. These not only help to reduce the ER load but also lead to the upregulation of genes that support amino acid availability and maintain redox balance, further aiding in cell survival during hypoxia [114]. The IRE1/XBP1s branch also plays a significant role in helping cancer cells adapt to challenging conditions. For instance, in triple-negative breast cancer (TNBC), this pathway helps cells in surviving hypoxic conditions by interacting with HIF1 α and jointly regulating its transcriptional network [115], and is also involved in the production of pro-tumorigenic factors such as IL-6, IL-8, CXCL1 in TNBC cells [116]. Additionally, in prostate cancer cells, this pathway is directly activated by androgen receptor signaling, promoting cell survival [117].

2. Nutrient deprivation

It has been established that UPR activation due to glucose deprivation promotes angiogenesis by upregulating several pro-angiogenic factors such as VEGF, IL-6, IL-8, FGF2 [118], while simultaneously downregulating angiogenesis inhibitors like CXCL14, CXCL10 and THBS1. Indeed, in glucose deprivation conditions, ATF4 binds to VEGF promoter and directly control its expression [119]. In addition, XBP1s can bind to the VEGF-A promoter and upregulate its mRNA expression [120].

3. Oncogenes and tumor suppressor

The activation of the UPR, specifically PERK, IRE1 and ATF6 signaling pathways, has been observed in various cancers following oncogene activation [121]. For instance, in Burkitt's lymphoma, in B cells overexpressing the oncogene c-MYC there is increased phosphorylation of PERK, as well as elevated levels of XBP1s and ATF4 compared to B cells from healthy individuals [122]. Moreover, the BRAF^{V600E} mutation in melanoma has been shown to trigger the activation of IRE1 and ATF6 pathways [123]. UPR activation has also been reported in melanocytes and keratinocytes following the activation of the oncogene HRAS [124]. These

findings collectively indicate that UPR activation is a common response to oncogenic stress, helping in the survival and adaptation of cancer cells under these conditions. A study has highlighted that ATF6 is crucial in maintaining BRCA-1 expression thereby protecting colon cancer from the cytotoxic effects of ER stressors like thapsigargin. Inhibiting ATF6 led to BRCA-1 degradation, increased DNA damage and cell death [125]. In addition, several studies have shown that ATF6 is upregulated in hepatocellular carcinoma (HCC) [126–129], and polymorphism in ATF6 has been linked to a susceptibility to HCC. Further studies have shown that ATF6 promotes tumor progression in HCC by down-regulating tumor-suppressor genes [130,131].

4. Therapy resistance

PERK signaling is also involved in the activation of other pathways including the nuclear factor erythroid derived 2 (NRF2) which is key in the cellular antioxidant response. Indeed, PERK phosphorylates and stabilizes NRF2, and this PERK-NRF2 signaling pathway plays a crucial role in protecting de-differentiated cells from the effects of chemotherapy [132]. Besides, NRF2 also interacts with the HIF pathway, thereby influencing the HIF response. In lung and pancreatic cancer models, PERK-dependent activation of NRF2 enhances the activity HIF-1, contributing to cancer progression. The combined action of PERK-NRF2-HIF axis is particularly important in promoting tumor growth and inducing chemoresistance under hypoxic conditions [133]. To a lesser extent, the sensor ATF6 has been reported in the context of cancer. Indeed, its high expression has been correlated with poor prognosis in cancer such as colon cancer [134] and chemoresistance [135,136]. The chaperone BiP is overexpressed in tumors compared to normal tissue, and especially in metastatic cancer cell lines. This increased expression leads to stress tolerance and tumor cell invasion. Moreover, treating xenograft models with antiangiogenic agents promoted the induction of BiP in the viable chemoresistant cancer cells surrounding necrotic regions created by the treatment. Malignant glioma also overexpresses BiP and its knockdown leads to a better response to TMZ [137].

5. Other mechanisms

In colon cancer, IRE1 has been identified as playing a pivotal role in maintaining the stemness of colon cancer stem cells (CSCs) and influencing the expression of β -catenin, a major driver of colonic tumorigenesis [138]. IRE1/XBP1s is also involved in the pathogenesis of

multiple myeloma [139] and a recent study has shown that IRE1/RIDD targets several mRNA substrates that are involved in survival/proliferation of multiple myeloma cells [140]. Also, XBP1s was associated with cancer aggressiveness in non-small cell lung cancer (NSCLC) and the transcription of epithelial-to-mesenchymal transition (EMT) factors such as SNA1/2, ZEB2 and TCF3 [141,142]. Another study on NSCLC has revealed that XBP1s upregulates the expression of prostaglandin E synthase (PEGS1) which allows the production of prostaglandin E2, an immunosuppressive lipid mediator, reinforcing the emerging immunosuppressive role of IRE1 in cancer [143]. IRE1 plays a crucial role in GB that will be further discussed below.

These various studies have enabled us to investigate the involvement of UPR in different cancers (**Figure 8**). Given its different role in tumor growth and therapy resistance, the UPR has become a target for new cancer treatments [144]. Currently, inhibitors of UPR especially of PERK and IRE1 are being investigated as potential therapies with the aim of making cancer cells more vulnerable to stress and increase the efficacy of existing treatments [145–147].

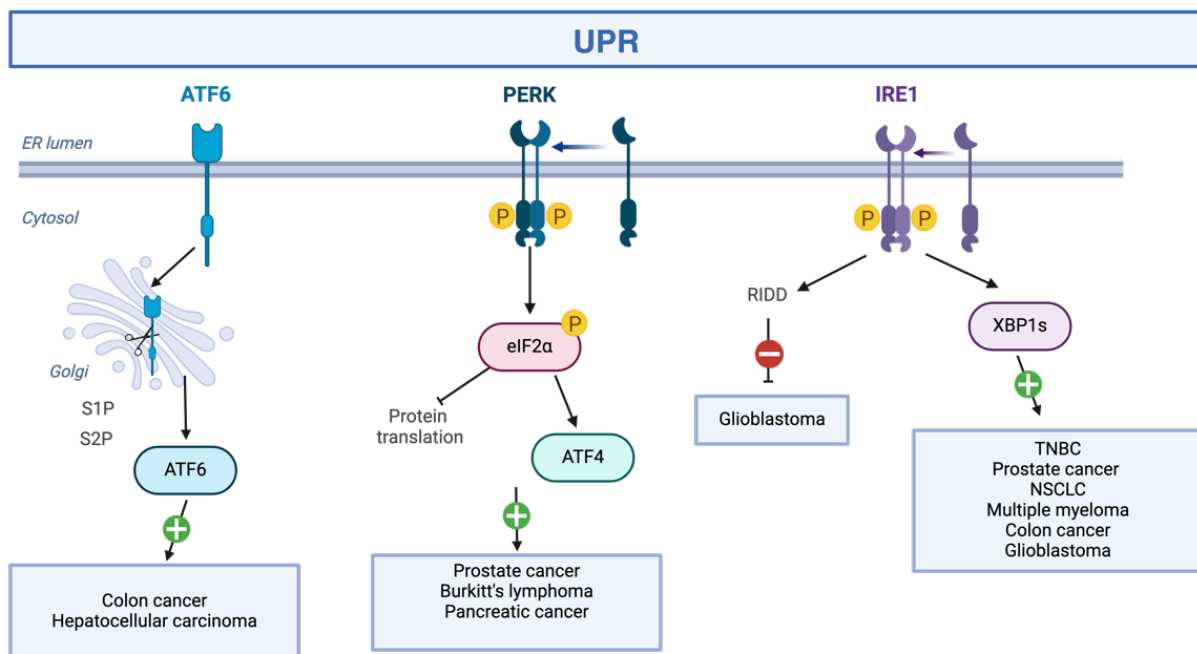


Figure 8. UPR sensors involvement in various cancers.

III. UPR involvement in Glioblastoma pathophysiology

1. PERK and Glioblastoma

PERK signaling pathway plays several critical roles in the pathophysiology of GB. Studies reported that PERK contributes to ECM stiffening, which is essential for GB progression. Indeed, a PERK/FLNA/F-Actin axis has been described to enhance GSCs adaptation to the harsh TME promoting proliferation, motility, to support tumor survival and invasion [148]. Additionally, PERK plays a role in controlling GSC stem cell maintenance and differentiation by regulating SOX2 [149]. These findings suggested that inhibiting PERK could reduce GB aggressiveness by promoting the differentiation of GSCs and lowering SOX2 expression. PERK is also associated with a malignant profile comprising invasive characteristics and poor survival in GB via ATF4 [149,150]. Under stress conditions like glucose deprivation, PERK promotes cell viability, tumor growth and progression to support GB [151]. Additionally, PERK activation, via ATF4, induces autophagy in GB cells. Upon administration of an autophagic cell death triggering drug Loperamide, ATF4 upregulated autophagic responses, leading to autophagic cell death in GB cells, indicating a dual role of PERK in promoting both survival or cell death depending on the context [152]. PERK activity has also been associated to chemotherapy and radiation resistance. PERK inhibition sensitized GB cells to apoptosis in several conditions [153,154].

The PERK branch of the UPR plays a multifaceted role in GB progression by promoting different features of GB progression and contributing to therapeutic resistance, making it a potential target for enhancing the effectiveness of chemotherapy and radiation in GB treatment.

2. ATF6 and Glioblastoma

ATF6 is not very well described in GB biology. However, one study has highlighted its pro-survival role in GB by resolving ER stress and enhancing resistance to radiotherapy [155]. Indeed, the study showed that the increased reactive oxygen species (ROS) generated by irradiation were activating ER stress response including ATF6. Moreover, ATF6 activation led to a specific increase expression of chaperones like BiP that helped cells manage misfolded proteins and prevented apoptosis contributing to a pro-survival phenotype in response to therapeutic stress. Another target of ATF6 was discovered, it is NOTCH1, which is important for radioresistance and tumor survival in GB. NOTCH1 has been associated to pathways

involved in cell survival, differentiation and angiogenesis in GB cells [156,157]. Targeting ATF6, could make GB cells more vulnerable to radiation by reducing survival factors like BiP and NOTCH1.

3. IRE1 and Glioblastoma

For nearly two decades, IRE1 has been described as a key player in GB biology. Early research has revealed that IRE1 plays a role in the ischemic response to hypoxia and glucose deprivation particularly by upregulating VEGF-A in GB cells [158]. Additionally, further studies in mouse brain models have shown that inhibiting IRE1 can alter the mode of glioma expansion. Particularly, blocking IRE1 reduced angiogenesis which led to tumor cell invasion along blood vessels. The study further reveals that IRE1 affects the expression of several pro- and antiangiogenic factors like VEGF-A, IL-1 β , IL-6 and IL-8, thereby reducing angiogenesis. In the other hand, the blockade of IRE1 activity increased the expression of ECM proteins and proteolytic enzymes, which are associated with increased tumor cell migration and invasion. On top of it, impairing IRE1 activity was associated with increased survival and a slower growth rate [107]. Overall, these findings suggest that IRE1 signaling is determining in tumor progression and vascularization. IRE1 is also a major regulator of the adaptation of GB cells to their hostile microenvironment by maintaining the structure of the cytoskeleton, particularly in the formation of stress fibers, which are essential for preserving cell shape and movement. On top of this, IRE1 also regulates the expression of ECM proteins, such as SPARC, impacting tumor growth, infiltration and invasion. In addition, disrupting IRE1 activity in this model was followed by significant changes in gene expression profiles, particularly those related to secreted proteins involved in ECM or cell adhesion, such as collagen and fibronectin [159]. It was highlighted in the first chapter, that EGFR plays a crucial role in the development of malignant glioma. One study has shown that epiregulin, an EGFR ligand, might contribute to glioma cell growth and migration, under the control of IRE1 [160].

While, IRE1 was shown to promote tumorigenesis in GB by degrading the period circadian regulator 1 (PER1) via its RIDD activity [161], a recent study has emphasized a dual role of IRE1 in glioblastoma according to its activity [162]. Here, patients' data analysis revealed the existence of an IRE1 signature separating the tumors into two groups, the IRE1 high and low activity, with the high activity correlated with a shorter OS. The IRE1 high group also showed a high XBP1s activity and was correlated to an increased angiogenesis, invasion and macrophage infiltration. This group was associated with the mesenchymal subclass which

is considered more aggressive whereas the IRE1 low group was associated with the pro-neural and classical subclasses. High RIDD activity in the other hand led to a decrease of angiogenesis and migration [162], no significant role on immune infiltration and a longer survival. Revealing that these two branches of IRE1 are antagonist in GB context. IRE1 is crucial for glioblastoma neovascularization, its RNase activity specifically has been described to drive tumor invasion [163]. Furthermore, it was recently demonstrated that IRE1 controls the recruitment of myeloid cells in GB microenvironment, by controlling the secretion of chemokines consequently promoting pro-tumoral inflammation [164].

These different studies have helped us to decipher the significant role in the progression of GB. In the stressful tumor microenvironment, IRE1 activation helps GB cells survival and proliferation by promoting angiogenesis and by facilitating cell migration and invasion via the modulation of various proteins. Because of its central role in these different processes, IRE1 is being investigated as a therapeutic target in GB, however further studies are still needed to better understand its activity to be an effective target in this challenging disease.

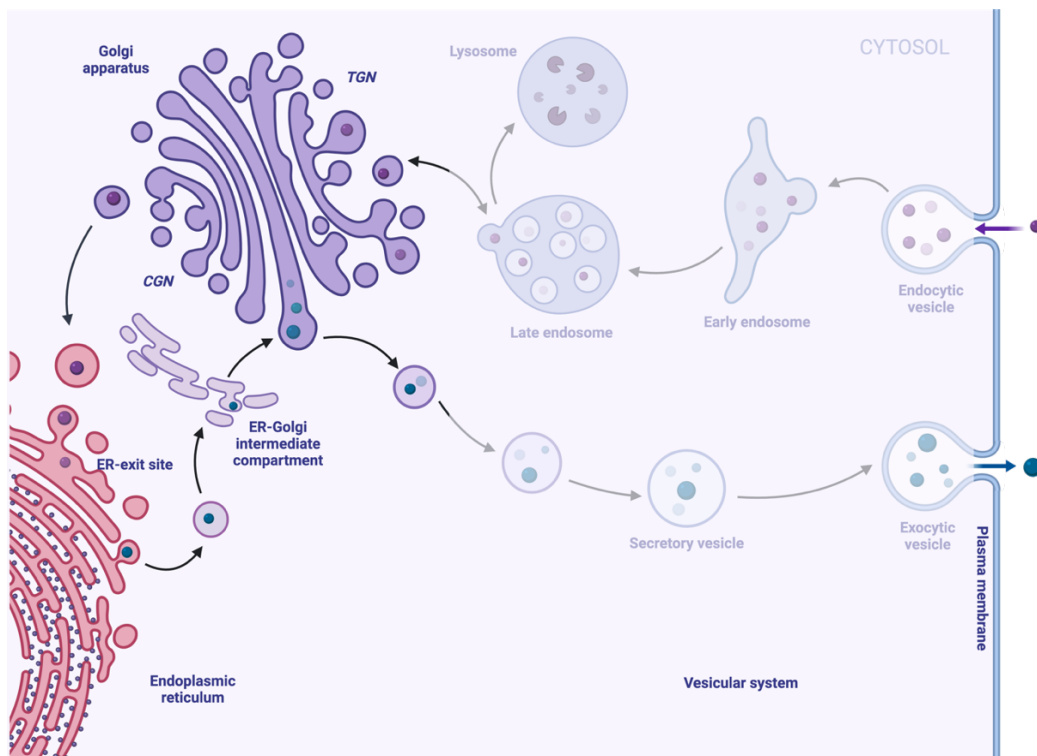
Overall, the UPR is deeply intertwined with cancer biology, acting as a helper of tumor growth and a contributor to therapy resistance. Ongoing studies aim to exploit this connection to develop more effective cancer treatments.

Chapter 2: Conclusion

This second chapter has helped us to have an overview of the UPR involvement in cancer. We have shown how the different sensors play critical roles in several GB features including angiogenesis, cell proliferation, migration, invasion, survival and resistance to treatment. As we explored the evident role of the UPR in GB pathophysiology, the IRE1 signaling axis, emerged as a central theme. The activation of IRE1 in GB cells influences multiple features of GB promoting its aggressiveness. Given its pivotal role, IRE1 represents a promising target in therapy and understanding its regulatory mechanisms could lead to novel treatments that inhibit tumor progression by disrupting the secretory process and improving the response to existing therapies.

Chapter 3:

The UPR in the regulation of the early secretory pathway



Chapter 3: Foreword

As described in the first chapter, secretion plays an important role in glioblastoma pathophysiology. Indeed, tumor cells are known to secrete several substances (cytokines, growth factors, EVs), which are involved in tumor progression, immune invasion and resistance to treatment.

The secretory pathway is a fundamental cellular process involved in the synthesis, modification, sorting and release of secretory proteins onto the plasma membrane and other membrane-bound organelles or into the extracellular space. Several organelles are involved in this process starting with the endoplasmic reticulum (ER), followed by the ER-Golgi intermediate compartment (ERGIC) and the Golgi complex. Finally, proteins are sorted into specific vesicles or specific transporters to be delivered to their final destination [165–167].

In this second chapter, we will describe by way of a review, the organelles involved in the early secretory pathway and their importance in maintaining cellular functions under both healthy and pathological conditions.

Review 1: “Endoplasmic reticulum and Golgi stress signaling-mediated regulation of the early secretory pathway in healthy and pathological cells” (Bakambamba et al., 2024)

Endoplasmic reticulum and Golgi stress signaling-mediated regulation of protein secretion.

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Key words: Secretion pathway, protein secretion machinery, endoplasmic reticulum stress response, golgi stress response, IRE1, cancers, neurodegenerative diseases.

Abstract

The eukaryotic secretory pathway (SP) is essential to ensure cellular functions and multicellular communication. The early SP is constituted mostly of the endoplasmic reticulum (ER), the ER Golgi intermediate compartment and the Golgi apparatus. These intracellular organelles achieve proper folding and modification of newly synthesized transmembrane and secretory proteins, prior their traffic to their final destination, e.g. plasma membrane, endosomes, lysosomes, and the extracellular space. They also integrate quality control systems to ensure export of mature proteins and trigger dysfunctional proteins to degradation. The ER as the first SP compartment is subjected to a precise control of its homeostasis through signaling of the Unfolded Protein Response, however the impact of this adaptive mechanism on the entire SP has not yet been described in an integrated manner. Herein, we address this issue and provide an overview of the early SP and its regulatory mechanisms, focusing on the ER and Golgi stress signaling dependent-regulation and contextualize this information regarding physiology and pathology.

Introduction

In eukaryotic cells, the early secretory pathway (SP) starts at the rough endoplasmic reticulum (ER) where newly synthesized proteins associated with ribosomal molecules are imported into the ER through the translocon complex. The rough ER is the main site of protein elongation and maturation including post-translational modifications, such as N-glycosylation and formation of di-sulfite bonds, leading to proper protein folding that regulates its function [168]. This process is tightly controlled by chaperones and enzymes involved in protein folding such as thiol-disulfide oxidoreductases and protein disulfide isomerases (PDIAs). Properly folded secreted proteins, that pass the ER quality control, are then exported from this compartment through protein addressing and trafficking molecular machines interconnected with complex vesicular tubular systems including the ER Golgi intermediate compartment (ERGIC) and the Golgi apparatus [169,170]. Protein folding is a highly regulated process ensuring that proteins adopt their native three-dimensional conformation, and enabling them to achieve their biological functions. Improper folding can lead to the production of proteins which are susceptible to aggregation and potentially toxic to the cell [169,170]. Therefore, both ER and Golgi combine complex machineries to promote proper protein folding and to control their homeostasis. The ER hosts a unique protein folding control system that detects its own homeostasis disruption. Indeed, in case of an accumulation of misfolded proteins, ER stress sensors trigger the unfolded protein response (UPR), a cellular signaling pathway that aims to restore ER homeostasis [98–100,171]. Similar to the ER, Golgi dysfunction prompted by an increased demand of protein production and transport, triggers a Golgi stress response [172]. In this review, we focus on the early SP from ER exit to the Golgi apparatus. We first present the different cellular organelles and the specialized molecular machineries involved in the early SP. After a brief description of the ER and Golgi stress responses, we document how those stress signaling pathways control the early SP in healthy and pathological contexts. Finally, we elaborate how on stress signaling pathways may (or may not) control protein trafficking in the early SP.

I. Cellular organelles and molecular machineries associated with the early SP

The endomembrane system comprises all membrane-bound organelles including those playing a key role in the protein synthesis and transport [168]. During

their transport, about one third of the proteins encoded by the mammalian genome pass through a series of cellular compartments composing the SP. These proteins are successively synthesized, modified and matured until their delivery to their final destination [173]. The different organelles constituting the SP are the ER, the Golgi apparatus, and all the transport intermediate structures (e.g. transport vesicles) (**Fig.1**) [174]. Some transport vesicles select specific cargo molecules and move them to the next organelles of the early SP (anterograde transport), while others retrieve escaped resident proteins and return them the previous compartment where they normally function (retrograde transport) [174].

I.1. The endoplasmic reticulum (ER), the first compartment of the SP

Besides its functions on lipid biosynthesis and intracellular calcium storage, the ER is the protein folding 'factory' of the SP that contributes to the synthesis of more than 10,000 secretory and membrane cargo proteins in the way to various cellular destinations including the Golgi apparatus, endosomes, lysosomes and the cell surface [175–178]. To enter the conventional SP and reach their final destination, newly synthesized proteins undergo through multiple processes such as translocation to the ER, folding and maturation. Efficient protein folding of nascent proteins is made possible by chaperones such as BIP (for immunoglobulin heavy-chain binding protein), calnexin, calreticulin and foldases such as PDIs localized in the ER lumen, that promote folding thus preventing protein aggregation and degradation [169]. Two types of proteins therefore associate with the ER, i) ER resident proteins well folded but retained in the compartment and ii) secretory proteins which traffic through this compartment [176,177].

I.1.1 Translocation of the nascent proteins into the ER

Resident and secreted proteins into the ER enter this compartment through protein translocation that occurs either during mRNA translation (co-translational translocation) or after synthesis (post-translational translocation) [179,180]. Co-translational translocation takes place in the rough ER, a particular region of ER contiguously to the nucleus characterized by dense areas of ER-membrane-bound ribosomes [180]. Well-described protein complexes have been involved in protein integration and translocation into the ER. Initially, hydrophobic amino acids of nascent secreted proteins emerging from ribosomal complexes RNC (for ribosome-nascent

chain complexes) are recognized by signal recognition particles (SRP) [179–181]. SRP are composed of protein subunits including SRP54 and the 7S RNA. The latter in complex with RNC interacts with SRP receptors to facilitate RNC binding to the protein translocation channel composed of multiple heterotrimers of SEC61 [179,180,182]. This process is dependent on a GTPase cycle and accessory molecules such as TRAM and TRAP. In yeast, RNC entry into the ER is also dependent on Sec62/63 complex [180] that works in concert with Sec61. As soon as the nascent proteins are exposed to the ER lumen, their signal peptides are cleaved by the signal peptidase complex SPC [182]. The incoming peptides could be modified by the oligosaccharyl transferase (OST), the GPI transamidase or PDIA5 [179,180,182]. They are also supported by the chaperone molecule BIP, that also helps in gating the SEC61 channel. The post-translational transport mechanism is facilitated by the cytoplasmic heat shock proteins of the HSP40 and HSP70 families that interact with the SEC62/63 complex to allow the ER entry [179,180]. In addition, the anchorage of transmembrane proteins into the ER membrane is mediated by the ER membrane (EMC) and the guided entry of tail-anchored proteins (GET) complexes [183,184]. The GET pathway is involved in the membrane insertion of post-translational tail-anchored proteins [185]. The EMC mediates the membrane insertion of multiple proteins, for instance, including some G-protein-coupled receptors (GPCRs) displaying multiple transmembrane domains [186].

1.1.2 ER protein quality control

During the maturation process in the ER, nascent proteins interact with multiple factors comprising chaperones and folding enzymes [187]. Once they achieve their full maturation, new synthesized proteins reach the ERES to be addressed to the following compartment of the SP. In case of improper folding, proteins are targeted to a degradation process described as the ER associated degradation (ERAD) linked to the proteasome [187]; or the ER-phagy associated to the lysosomal system. The ER quality control (ERQC) comprises all molecular pathways associated with chaperones, folding enzymes and degradation factors. In this part, we will succinctly describe the molecular actors of the ERQC, refer to [187] for a complete description of their modes of action.

ER chaperones and protein folding enzymes - Once the nascent proteins enter into the ER, they are supported by the ER chaperones to prevent any exposure of vulnerable regions, pre-maturation and non-folding of the proteins [187]. Specific chaperones and co-factors also facilitate disulfide bonds and glycan linkage. ER chaperones BIP (belong to the HSP70 family), GRP94 (associated to HSP90 family) and GRP170 (from the HSP110 family) possess a KDEL motif as an ER retention motif. They act on nascent proteins by consuming ATP for their folding functions. Glycosylation of secreted glycoproteins starts as soon as they enter into the ER. Attachment of glycans on asparagine of N-glycoproteins is mediated by ER resident glucosidases including GLU1, GLU2 and UDP-glucose:glycoprotein glucosyl transferases (UGGT1/2) [187,188]. This process exposes new synthesized glycoproteins to the lectin chaperones calnexin (CANX) and calreticulin (CRT) that also protect the premature glycoproteins. The ER chaperone BIP and lectin chaperones facilitate the recruitment of PDI enzymes P4HB and PDIA3 that catalyze oxidative folding and disulfide-bound structures [187,188].

ER degradation machines - In case of a defect in the protein maturation, misfolding proteins are triggered to a degradation process including the ERAD machinery. These misfolded proteins are addressed to the retrotranslocon, a membrane channel opened to the cytosol and coupled to the ubiquitination system leading to degradation through the proteasome [187]. This process involves BIP and lectin chaperones assisted by co-factors including members of the HSP40 family DNAJB9, DNAJC3 and DNAJC10 [187,189]. Alternatively, misfolded proteins could be addressed to an ER-phagy process involving specific molecules of the conventional autophagic production system where ER microvesicles are driven to the lysosomal compartment. Specific receptors of the ER-phagy including FAM134B and RTNL3 have been described [187,189].

1.1.3 ER exit sites, anterograde protein transport mediated by COPII

The export of proteins from ER by vesicle budding happens in specialized regions of smooth ER called ERES or transitional elements [168,173,174,190] (**Fig.1**). Over time, ERES change in number and size by *de novo* formation, fusion or division [191]. The buds formed at the ERES are coated with COPII (coatamer protein II) complexes. The COPII-coated buds transform into coated vesicles that can shed their coats and fuse with each other or with another compartment between the ER and the Golgi apparatus

in ERGIC structures [168,174,190]. Interestingly, the ERES are often juxtaposed to the ERGIC [175]. After the budding formation, the bud is separated from the donor membrane by scission of the neck. Released vesicles reach and dock to their target membrane of the next sorting organelle. Their coat is dissociated before the fusion (**Fig.1**).

The COPII machinery - At the molecular level, the small GTP-binding protein SAR1 acts as an initiating factor for the coat assembly. Additional coat components are further recruited with the recognized cargo proteins. Coat polymerization next allows the formation and release of the nascent vesicle from the donor membrane i.e. ER, ERGIC, or Golgi membranes. Other key regulators of the COPII coat assembly include the integral membrane protein SEC12 and the soluble protein SEC16 [190–192]. These molecules regulate the binding of SAR1 GTPase and the two major coat cytosolic subunits SEC23/SEC24 and SEC13/SEC31 [190–193]. The SEC23/SEC24 complex promotes the entrapment of cargo proteins via SEC24 and the recruitment of SEC13/31 hetero-tetramers. At the outer face of the vesicle coat, SEC13/SEC31 complexes form polymers around SEC23/SEC24/cargo complexes to produce cage-like structures to extract ERES membranes and bud COPII transport vesicles. Several paralogs of these molecules are expressed in the cell to fit with multiple cargo proteins. The process of budding resulting of a progressive membrane curvature is mediated by the physical interaction between SEC23/SEC24 and SEC13/SEC31 complexes. The cage formed by these COPII components can adopt multiple geometric structures to accommodate with the sizes and shapes of cargoes including large molecules such as procollagen. Once formed, the COPII vesicles are extruded though the GTPase function of SAR1 triggered by SEC23 [173].

1.1.2. Regulation of secreted protein exit from the ER

The newly synthesized secreted proteins produced in the ER follow several routes: the export from the ER via the COPII vesicles; the ER retention that prevents ER-resident proteins entry in the COPII system; or the ER-associated degradation that removes secreted proteins displaying the wrong conformation [175,193]. At the ERES, the secretory cargo sorting is mediated through two main processes according to the nature of the protein: the receptor-mediated transport (or cargo capture) and the bulk flow (**Fig.1**) [192].

The ER export signals - Protein exit signals allowing ER-to-Golgi trafficking is mostly not understood. During the cargo capture, cargo receptors, adapters, accessory molecules or components of the COPII vesicle coat contribute to the sorting and recruitment of secreted proteins into ER-derived transport COPII vesicles. Specific protein motifs of proper folded and mature cargo proteins are recognized by these receptors. A large number of different sorting signals have been described for COPII export, including di-acidic and hydrophobic/aromatic motifs [192]. The SEC23/SEC24 adapter complexes directly select cargo proteins through the binding of their export signal to SEC24 subunits [191]. SEC24A and SEC24B associate with cargoes containing LxxLE or DxER exit motifs, whereas SEC24C and SEC24D recognize IxM motifs [173]. For instance, two distinct sites of SEC24 allow recognition of two different export motifs: a specific YNNSNF-containing protein only present on SNARE SED5; and LxxLE present in SNARE BET1 and large number of cargo proteins including BET1, SYS1, GAP1. SAR1 could also directly bind to specific cargoes including mammalian glycosyltransferases by their C-terminal di-arginine motifs [168]. However, misfolded or mis-assembled proteins are associated with ER chaperones molecules that might hide these exit signals until the complete protein maturation [174].

Cargo receptors - As indicated above, adapter molecules capture diverse luminal cargo proteins that do not directly interact with COPII subunits. These adapters are key for selecting the secreted cargos. They are divided in two groups: the membrane receptors (named cargo receptors) that link cargo to the inner layer of the coat; and the accessory molecules, ER resident proteins involved in protein folding, that aid cargo proteins to incorporate the nascent COPII vesicles [175]. Cargo receptors are transmembrane proteins that physically link cargo to COPII coats and accompany their cargo until their release in the ERGIC. These cargo receptors only carry mature cargo proteins, some of them are lectins that bind to the glycosylated portion of their cargos. The function of these cargo receptors is completely dependent on the retention routes in which export signals of non-mature secreted proteins are still engaged with chaperones to allow proper protein folding [191]. Some cargo receptors could also recognize soluble secreted proteins. ERGIC-53 (also named LMAN1) is a transmembrane protein that binds to COPI and COPII complexes. Its luminal part presents a L-type lectin domain with high affinity for high-mannose oligosaccharides. ERGIC-53 mainly acts as a cargo

receptor for certain glycoproteins, such as pro-cathepsin, cathepsins C and Z, α 1-antitrypsin; and the blood-clotting factors V and VIII, working in concert with the adapter molecule MCFD2, allowing their transport from ER-to-ERGIC and the Golgi apparatus [168,175,191]. Importantly, ERGIC-53 oligomerization seems to be key for efficient COPII interaction and ER-to-Golgi cargo export, as ERGIC-53 binds to all SEC24 paralogues [168,175]. ERGIC-53 also has a glycoprotein quality control function [194]. Of note, ERGIC-53 belongs to a calcium-dependent L-type lectin family with ER-resident proteins ERGL, VIP36 and VIPL for which no client has been identified yet [191]. In the same line, the transmembrane emp24 domain (TMED) family members are also type I single-pass transmembrane cargo receptors. They regulate the protein transport involved in the early and late secretory pathways [195]. During anterograde and retrograde protein transport, TMED proteins dimerize and interact with COP protein complexes to facilitate cargo selection and vesicle formation [195]. The best-characterized cargos addressed by TMED molecules are glycosylphosphatidylinositol (GPI)-anchored proteins such as CD55 and CD59 [196,197]. The yeast ERV29 (corresponding to mammal SURF4 cargo receptor for surfeit locus protein 4), involved in COPII-vesicle formation, forms a complex with several soluble proteins such as vacuolar hydrolases, carboxypeptidase Y, proteinase K and a precursor of the soluble pheromone α -factor; and interacts with COPII coat through its hydrophobic I-L-V motif to allow cargo packaging in COPII vesicles [168,191]. ERV29 and SURF4 are cargo receptors for soluble proteins that exhibit ER-ESCAPE motifs (for exit by soluble cargo using amino-terminal peptide-encoding motif) that include amino-terminal tripeptides such as dentin sialophosphoprotein DSPP and amelogenin X-linked AMELX that readily aggregate in the ER lumen [198].

Other accessory molecules - In contrast to adapter molecules, accessory molecules are not exported into the ERGIC [175]. Beta-catenin (CTNNB1) and PX-RICS, a GTPase-activating protein, appear to be involved in ER export of N- and E-cadherins [175]. At the ERES, incorporation of large cargo proteins such as procollagen and prechylomicrons into the COPII vesicles requires the involvement of key accessory molecules assisting COPII proteins SAR1 and SEC13/SEC31. The membrane protein TANGO1/MIA3 acts with cTACE5 to promote formation of bigger COPII structures to carry large molecules such as procollagen VII [192]. TANGO1 is a transmembrane protein with a luminal SH3 domain that mediates interaction with procollagen through the ER

chaperone HSP47 [192]. TANGO1/MIA3 interacts with SEC23/SEC24 to staff COPII vesicle formation until the packaging of procollagen. This process is facilitated by the SEC13/SEC31-dependent flexibility of the COPII cage, modulating the membrane curvature, that is controlled by differential interactions of SAR1 paralogues with SEC31 [175]. TANGO1/MIA3 do not accompany the cargo proteins but facilitate the packaging in COPII vesicles. In addition, TANGO1/MIA3 binds to ERGIC membrane and brings it closer to ERES, providing additional membrane sources for ER microdomains [193]. cTAGE5 promotes collagen secretion by concentrating SEC12 at ERES and facilitating SEC23/SAR1-GTP interaction, triggering SEC23 GAP activity [173,175,193]. Luminal folding chaperones are also involved in cargo export.

Protein secretion by bulk - The second process is named the bulk flow in which soluble and membrane proteins are incorporated in COPII vesicles by default, without the involvement of cargo receptors, when they are properly folded and have no retention signal [173,175,192]. This transport type involves a non-selective mechanism in which secreted proteins are freely encapsulated into COPII vesicles by diffusion into a budding zone, through a passive transport [175,190]. When nascent proteins are ongoing folding, they are engaged with the ER package machinery composed of chaperones and folding enzyme that exclude them from COPII cargo vesicles simply due to their size. When properly folded, secreted proteins are released from these large folding complexes and are accessible for being incorporated in cargo vesicles by free diffusion [176].

1.2. The ERGIC, a sorting platform between the ER and the Golgi apparatus

Once loaded with cargo proteins, the COPII vesicles fuse either with other transport vesicles to form the ERGIC or fuse with pre-existing ERGIC structures. These novel vesicular tubular clusters (VTCs), in constant dynamic movements, are localized along microtubules between the ER and the *cis*-Golgi. ERGIC as the Golgi apparatus is the starting point for retrograde transport to allow membrane and cargo proteins trafficking back to the ER (**Fig.1**) [168,191]. Important proteins are present in the ERGIC structure such as the mannose-binding lectin ERGIC-53 and molecules of the p58 and p24 families which are implicated in cargo selection and/or structural maintenance of VTCs. ERGIC-53 and p58 have been proposed as cargo receptors at the ERES (see above).

1.2.1. ERGIC- and VTCs-associated proteins including the COPI machinery

At the ERGIC compartment, COPI complex is composed of the small GTPase ARF which, once activated, triggers the recruitment of pre-assembled coatomer complexes of seven subunits, allowing the COPI coat assembly [168]. The COPI coat binds to retrograde sorting signals of secreted proteins. These vesicles mediate the retrograde cargo retrieval to the ER. COPI vesicles seem also to mediate the anterograde transport within the Golgi apparatus [199].

The COPI coat subunits - ARF1 activation allows the recruitment of heptameric COPI complexes i.e. F-COPI (composed of β -, γ -, δ - and ζ -COP subunits) and B-COPI subcomplexes (including α -, β' - and ε -COP subunits) from the cytosol. These subunits share homologies with clathrin-binding AP complexes involved in vesicular cargo selection and transport. ARF1 directly interacts with β -, γ - and ε -COP subunits. Similar to SEC24, α - and β' - COP proteins are involved in cargo recruitment. Importantly, several GAPs are involved in the GTP hydrolysis and deactivation of ARF1-GTP but are not direct components of the COPI coat: yeast Glo3 and Gcs1 localized in the Golgi apparatus, and mammalian members of ARF GAP family [168]. The sorting peptide signals of a cargo protein determine its cellular localization by interacting directly with vesicles coat subunits.

1.2.2. Retrieval of ER and ERGIC resident proteins

The retrieval of resident proteins from the ERGIC or *cis*-Golgi back to the ER or the ERGIC is based on cargo capture. Recycling of soluble ER resident proteins is mediated by transmembrane cargo receptors that further are associated with COPI subunits. Therefore, soluble ER resident proteins such as chaperones BIP and protein di-sulfide isomerases PDIs are recognized through their H/KDEL motifs (in yeast/mammals) by the KDEL receptor. Multiple isoforms of KDEL receptors participate to the efficient retrieval of ER resident proteins [191]. The retrieval pathway is conditioned by the presence of the canonical ER retrieval peptide signal composed of two lysines i.e. KKxx or KxKx sequence at the C-terminus region of the cargo protein, allowing its interaction with the COPI coat in which γ -COP subunit plays a predominant role [168,174,191,192]. The KDEL receptor itself possesses a di-lysine retrieval motif for interacting with the COPI coat [168]. Although the interactions between KDEL

receptor and its clients need to be better characterized, this binding triggers KDEL receptor up-take into the COPI vesicles [168]. Other mechanisms have been recently described for the retrieval of ER proteins lacking the di-lysine motifs. For instance, the PDI family member ERp44 escorts incompletely assembled IgM subunits back to the ER by exposing its KDEL motif. One possible regulation of the cargo traffic is the control of those export and retrieval peptide signal accessibility to cargo receptors or COPI/II complexes [168].

1.2.3. The SNARE protein family and vesicle delivery

At the ERGIC as at the *cis*-Golgi sites, the vesicles delivery to the acceptor compartment is controlled by tethering and SNARE (for soluble N-ethylmaleimide-sensitive factor attachment protein receptor) activities. The vesicle composition conditioned by the coat programs the vesicle for fusion by selecting the appropriate tethers and SNAREs during vesicle budding [168]. Vesicle tethers contribute to SNARE rearrangement and priming. SNAREs are essential for further vesicle docking and fusion on donor membrane of the next acceptor organelles [174]. SNARE assembly is dependent on the target membrane (t-SNARE) and vesicle (v-SNARE) SNAREs. The t-SNAREs are composed of three subunits including syntaxin-like heavy chain and two light chains of SNAREs. The v-SNARE is a monomeric protein located at the opposite membrane from the t-SNAREs. The pleiotropic effect of SNAREs renders difficult the identification of the precise role of each SNARE. For instance, at the ERES, both BET1 and BOS1 are required on ER-derived vesicles; and SED5 on the Golgi acceptor membranes. Each individual vesicle undergoes homotypic fusion to form ERGIC [173]. Although a multitude of SNAREs molecules have been described, only few of them are involved in the ER-to-Golgi trafficking. For the COPII transport, key vesicular v-SNARE SED5, BET1 and SEC22 interact directly with SEC23/SEC24 complexes [173]. In addition of having a key role in vesicle fusions, SNAREs might help segregation of cargo proteins at the ER by interacting with SEC24. Indeed, BET1 and SEC22 interact with Sec24A/B through a YxxCe sequence whereas IxM is involved in SNARE SEC24C/D interactions. SED5 seems to be the only SNARE that interacts with SEC24C/D [173]. In the retrograde Golgi-ER route, v-SNAREs are both SEC22 and BET1 proteins. Regulatory factors play an important role in this process. SEC1/MUNC18 family proteins interact and positively or negatively regulate syntaxin-

like SNARE activity [190]. RAB proteins also interact with tethering complexes to dock vesicles to the target membrane [168].

1.3. The Golgi apparatus, the final platform of the early SP

The Golgi apparatus is the major site of protein maturation involving glycosylation, phosphorylation, proteolysis steps but it also serves as a sorting and dispatching platform for the ER-derived proteins [174,200]. In mammalian cells, the Golgi apparatus is composed of stacks of five to seven cisternae overlapping one another. These stacks often line-up and become interconnected by tubular structures to form a ribbon. These flattened cisternae form five polarized and functional regions: the *cis*-Golgi and the *cis*-Golgi network (CGN) immediately downstream of the ER constituting the entry side of the Golgi. Proteins and lipids enter the CGN in vesicular tubular clusters arriving from the ER. They next go through the medial cisterna, a region involved in various post-translational modifications, sorting and packaging of proteins for transport. Proteins finally exit the Golgi via the *trans*-Golgi network TGN, the central cargo sorting station of the cell. From there, proteins can move forward and be sorted according to their final destination to the cell surface or the next trafficking compartment through endosomes or lysosomes secretory vesicles. They can also return to an earlier organelle back into the ER via the retrograde pathway mediated by the COPI system (**Fig.1**) [174,200].

Golgi matrix proteins - Different families of proteins are responsible for maintaining the architecture and the function of the Golgi apparatus in protein secretion, among them Golgi reassembly stacking proteins (GRASPs) composed of GRASP65 & GRASP55 [174,200]. They are peripheral membrane proteins attached to the Golgi membranes, and are involved in the different steps of the formation of the Golgi structure including the stacking, ribbon-linking, cargo transportation, unconventional secretion, cell cycle regulation, apoptosis, and autophagy. GRASP65 is found on the *cis*-Golgi whereas GRASP55 localizes to the medial/*trans*-Golgi cisternae. Golgins are also part of the Golgi matrix proteins, GM130 being one of the first Golgi matrix protein identified. They comprise a family of Golgi-associated coiled-coil proteins required for vesicle tethering, membrane trafficking at the Golgi and the maintenance of Golgi integrity [174,200].

1.3.1. Retrograde protein transport from the Golgi apparatus to the ER

The retrograde transport of cargo proteins is dependent of two distinct mechanisms: the COPI vesicles and the RAB6-mediated tubular membrane elements [191]. COPI recruitment mediates separation between proteins destined for anterograde transport and those dedicated for retrograde transport back to the ER. Soluble proteins are excluded from COPI-mediated retrograde trafficking and accumulate in the tubules of VTCs. The mechanism responsible for this exclusion has not yet been fully elucidated. The KDEL receptor is involved in the retrograde process. KDEL receptor recognizes the retrieval signal i.e. KDEL sequence bearing by soluble ER resident proteins [168]. The COPI system is also the tool to regulate membrane trafficking. As the COPII machinery use the equivalent of one total volume of membrane every one or two hours, the COPI system also contributes to the ER homeostasis by allowing the retrieval of membrane elements back to the ER (**Fig.1**) [191]. The mechanism involving RAB6 GTPase is a tubulation of *cis*-Golgi regions without an apparent coat formation, independently to COPI system; and the full process remains to be better characterized [190,191]. Golgi-associated enzymes, some bacterial toxins and ERP44 are retained in the Golgi apparatus by this mechanism.

Figure 1

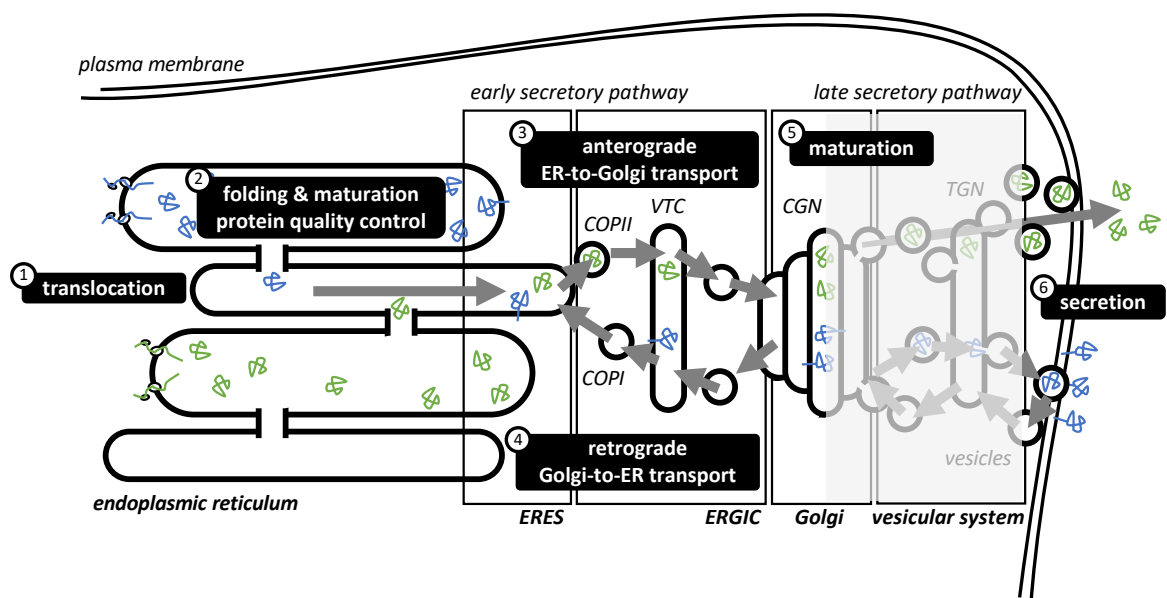


Figure 1: Cellular compartments involved in the early protein SP - The newly synthesized nascent proteins are translocated across the ER membrane (1). The proteins then undergo maturation processes leading to proper protein folding and glycosylation initiation (2). When properly folded, the secreted proteins are exported at

the ER exit site (ERES) and are packed into the COPII vesicles, leading to the anterograde ER-to-Golgi path that reaches the ER-Golgi intermediate compartment (ERGIC) (3). ER-resident proteins that escape the ER are retro-transported into the ER through COPI vesicles via the retrograde ER-to-Golgi path (4). Then the secreted proteins finish their maturation into the Golgi apparatus (5) and are finally exported through the vesicular system to the plasma membrane (6).

1.3.2 Golgi-associated proteins involved in protein secretion

Proteins associated with COPII vesicles arriving at the Golgi apparatus - As indicated above, vesicle tethering and SNARE activity are key to fusion vesicles to acceptor membrane, including both faces of the Golgi apparatus i.e. at the *cis*- and *trans*-Golgi sites. The SNARE BET1 is involved in vesicles fusion with the Golgi apparatus. The mammalian p115 is also important for this step and is recruited in COPII vesicles via the G protein RAB1 priming. Extended oligomeric structures composed of a large coiled-coil protein p115, the transport protein particle I TRAPPI (at the entry of the Golgi apparatus) or the conserved oligomeric Golgi COG (at the exit of the Golgi apparatus) complexes are involved in vesicle tethering and facilitate the SNARE assembly, leading to the membrane fusion and delivery of vesicle contents [168,173,190]. Interaction between TRAPPI and SEC23/24 complexes allow the fusion of COPII vesicles into the Golgi apparatus [173]. TRAPPI is a highly conserved multi-subunit protein complex that tethers the vesicles to the acceptor target membranes. TRAPP I acts as a guanine exchange factor towards the mammalian GTPase RAB1 [173]. In yeast, BET3 subunit of TRAPPI binds to SEC23, thereby allowing TRAPPI to specifically recognize COPII-coated vesicles. At the *cis*-Golgi, SEC23 phosphorylation controls its interaction with TRAPPI complex to allow the tethering of the COPII-vesicles [175]. BET3 binding depends on dissociation of SAR1-GTP to prevent tethering before vesicle scission. BET3 activates RAB1, which in turn recruits the tether p115, allowing the vesicle tethering to the target. At the target membrane, Hrr25/CK1 δ dissociated the TRAPPI complex and phosphorylates SEC23/SEC24 complex, catalyzing the disassembly of the inner membrane coat. This allows the pairing SNAREs on the vesicle and target membranes that catalyze the membrane fusion and delivery of vesicle contains in the target organelle.

1.3.3 Protein quality control in the Golgi apparatus

Similar to mechanisms developed in the ER, the Golgi apparatus possesses a quality control system to detect misfolded proteins to either re-address them back to the ER or directly to trigger them to the Golgi-associated degradation machineries [201,202]. The Golgi is an important site of protein maturation (including glycosylation, phosphorylation, proteolysis steps) and formation of protein complexes [201]. The protein retrieval process into the ER is mediated by the COPI machinery described above. For instance, unassembled molecular complexes like polymeric immunoglobulins M or molecules of the major histocompatibility complex (MHC) class I and II are detected by the protein quality control machinery in the Golgi apparatus to be retrieved in the ER [202,203]. This quality control process is mediated by the ERP44 molecule localized at the ERGIC and the *cis*-Golgi [203]. The retention in ER sorting receptor 1 RER1 is also involved in the COPI-mediated retrograde protein transport. RER1 recognizes transmembrane-based retrieval signals of mis-located proteins and unassembled subunits of protein complexes [201]. The Golgi apparatus also displays several degradation machineries that eliminate improperly matured proteins and are connected to either the cytoplasmic proteasome or the lysosomal system [201]. Degradation of membrane proteins is associated with the ubiquitination machinery involving NEDD4 and MARCH4 E3 ubiquitin ligases and the endosomal sorting complexes required for transport (ESCRT)-dependent lysosomal degradation through multivesicular body (MBV) [201,204]. For instance, in case of overload of the Golgi apparatus, SORT1 is involved in sorting a fraction of luminal proteins such as α 1-antitrypsin and apolipoprotein B100 into lysosomes for degradation. Defective membrane proteins can also be extracted from the Golgi to be targeted into the cytosolic proteasome in a process referred as endosome and Golgi associated degradation (EGAD) [201,205]. VCP extract ubiquitinated membrane proteins from the Golgi to the cytosol for proteasome degradation. Of note, upon Golgi stress, VCP is involved in the Golgi apparatus associated degradation, by extracting the Golgi-resident protein GM130, a tethering Golgi matrix protein for degradation [201,206]. Beside its function in the maturation of N-glycosylation proteins, the Golgi-resident α 1,2-mannosidase MAN1A also contributes to the retention, recycling and re-addressing of specific misfolded proteins that escape from the ER into the ERAD machinery [207].

II. The ER and Golgi stress responses

II.1. General aspects of the ER unfolded protein response

Under particular cell conditions such as an acute demand of protein synthesis (e.g., during oncogenesis) or deprivation of nutrients, a dysfunction of the protein production could lead to an accumulation of unfolded or misfolded proteins, inducing an ER stress. ER stress could affect multiple cellular function (e.g., metabolism, cell functions including cell migration or protein secretion). Therefore, accumulation of improperly folded proteins in the ER triggers a molecular signaling program leading to a transcriptional response to the nucleus to restore ER homeostasis. This response from the ER to the nucleus is called the unfolded protein response (UPR) [208] that leads to an upregulation of ER-resident proteins such as chaperones involved in protein folding and a general translational repression. In parallel, protein degradation processes like ERAD and autophagy help to remove misfolded proteins and recycle cellular components. Finally, if these actions fail to restore ER homeostasis, the UPR can induce various types of cell death [208,209]. The UPR is essential to prevent the aberrant secretion of misfolded proteins, which could become proteotoxic and cause several diseases. Indeed, such protein aggregates have been implicated in the pathogenesis of numerous neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's diseases. Additionally, disruptions in protein homeostasis have been associated with cancer, metabolic disorders, and cardiovascular diseases, underlining the impact of protein misfolding on human health [169].

II.2. The unfolded protein response and the ER stress sensors

The UPR is transduced by three ER-transmembrane sensors: the activating transcription factor 6 (ATF6), the inositol-requiring enzyme 1 α (IRE1, encoded by ERN1 gene), and the protein kinase RNA-like ER kinase (PERK, also known as EIF2AK3) [98–100] (**Fig.2**). Under homeostatic conditions, these sensors are maintained in an inactive conformation by their binding to the chaperone BIP. Whereas, when the level of unfolded proteins exceeds the adaptive capacity of the ER, BIP dissociates from the sensors and binds the misfolded proteins. This dissociation leads to PERK and IRE1 homodimerization and activation by auto-phosphorylation of their kinase domain. ATF6 translocates into the Golgi apparatus to be cleaved by S1P and S2P molecules [100]. Once activated, each sensor activates specific signaling pathways described below, that participates to restore homeostasis. Activated PERK

phosphorylates the alpha subunit of the translation initiation factor, eIF2 α , resulting in inhibition of global protein synthesis. Activation of IRE1 and ATF6 promotes transcription of UPR target genes [98–100].

ATF6, an ER transmembrane sensor that delocalizes into the Golgi to signal - Upon ER stress, ATF6 dimerization facilitated by the protein-disulfide reductase ERP18 induces its export into the Golgi apparatus where it is cleaved by the Golgi resident proteases S1P and S2P, releasing its cytoplasmic domain (**Fig.2**) [98–100,210]. ATF6f is a potent transcription factor that induces expression of UPR genes whose promoters contain regulatory ER stress response elements ERSE, involved in ER homeostasis maintenance, protein degradation and cellular redox regulation [100,211].

IRE1, a central UPR component with kinase and endoribonuclease activities - IRE1 is the most documented ER stress sensor as the most conserved sensor across eukaryotic organisms and plays a pivotal role in sensing and responding to ER stress (**Fig.2**) [98–100]. IRE1 is also the only ER stress sensor found in yeast. IRE1 is a type I ER resident transmembrane protein with a cytosolic serine/threonine kinase and endoribonuclease (RNase) domain, both activated by its oligomerization and autophosphorylation of its kinase domain [101]. Upon ER stress, IRE1 RNase activity is responsible for the unconventional splicing of X-box binding protein 1 (XBP1) mRNA, leading to generate a novel spliced form of XBP1 named XBP1s. XBP1s is a potent transcription factor that binds directly to the ERSEs and activates transcription of ER molecular chaperones to increase the protein-folding capacity or ERAD-related genes to facilitate the degradation of misfolded proteins [102]. Under prolonged ER stress, IRE1 RNase is also involved in the degradation of RNAs, either ER-proximal mRNAs encoding membrane and secreted proteins or microRNAs, through a process called regulated IRE1-dependent decay of RNA (RIDD). Those targeted RNAs are degraded as they present a consensus cleavage site similar to that of XBP1 in their sequences. RIDD activity leads to reduce the load of protein production. Finally, upon ER stress, the IRE1 kinase activates JNK (for c-Jun N-terminal kinase) signaling by interacting with TRAF2 (for TNF receptor-associated factor 2), which plays a role in autophagy activation [101].

PERK, an ER stress sensor that attenuates RNA translation - Upon ER stress, active PERK phosphorylates eIF2 α (**Fig.2**). The latter is part of the eIF2 complex that initiates protein synthesis [98–100]. However, PERK-mediated phospho-eIF2 α inhibits its function, thus attenuating mRNA translation, leading to the reduction of protein load in the ER. Remarkably, several mRNAs resist to the PERK-dependent global translation slow-down. Indeed, these mRNAs contain upstream open reading frames that are still active and short allowing their translation. Among those, ATF4 (for activating transcription factor 4) induces transcription of down-stream targets including CHOP, that, in concert with ATF4, induces expression of many UPR-related genes involved in protein synthesis, amino acid transport and metabolism, autophagy and resistance to oxidative stress [98–100].

Figure 2

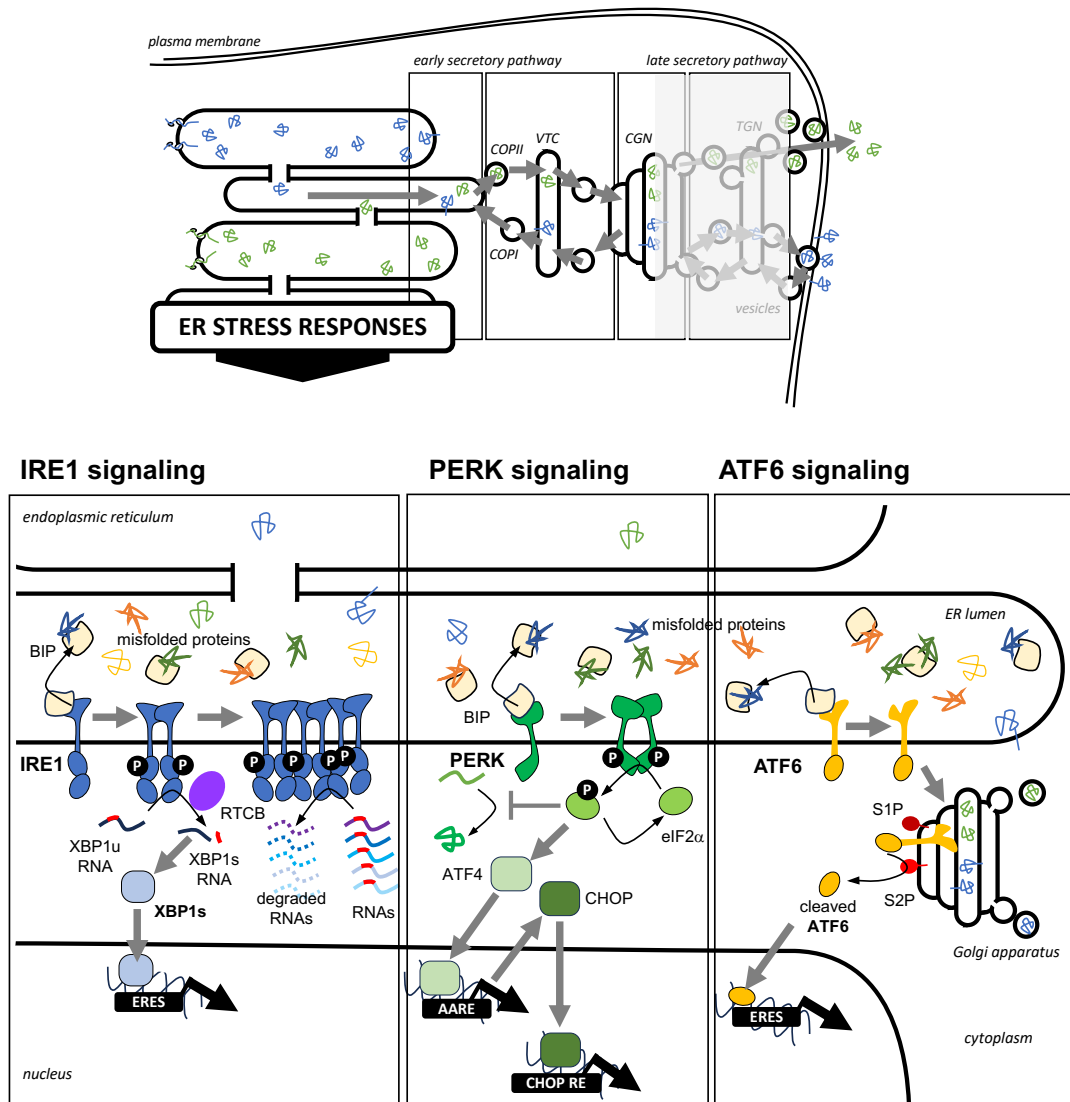


Figure 2: Molecules involved in the unfolded protein response - Under normal homeostatic conditions, the UPR sensors ATF6, IRE and PERK are inactive and bound to the chaperone BIP. Accumulation of misfolded proteins into the ER trigger BIP dissociation that binds to the misfolded proteins, leading to the activation of the UPR sensors. This dissociation leads to PERK and IRE1 activation by auto-phosphorylation of their kinase domains. The release of BIP from the sensor ATF6 allows its export into the Golgi apparatus where it is cleaved by S1P/S2P proteases releasing the cleaved form of ATF6 that exhibits transcription factor function. IRE1 RNase activation triggers the unconventional splicing of the XBP1 RNA, together with the RTCB ligase, leading to the translation of the transcription factor XBP1s. Upon prolonged ER stress, IRE1

oligomers trigger the RIDD, decreasing the RNA translation load and therefore limiting new protein production. PERK activation triggers the phosphorylation of eIF2a, that attenuates RNA translation but enhances the translation of the transcription factor ATF4 that increases the expression of several genes including the transcription factor CHOP.

II.3. The Golgi stress response and its sensors

As described with the ER, when the Golgi apparatus is overwhelmed by an increased demand of protein production and transport, these transmembrane and secretory proteins cannot be modified and exported properly leading to the Golgi stress [172]. The Golgi stress response is far less studied than the ER stress response. Although the Golgi stress sensors still needs to be identified, several Golgi stress-dependent pathways have been reported, involving various kinases and leading to the activation of transcription factors [172,212,213]. Among them, CREB3 is involved in Golgi stress-induced cell death, through ARF4 and DR4 induction (**Fig.3**). Similar to ATF6, upon Golgi stress, the transmembrane ER resident CREB3 is transported from the ER to the Golgi apparatus. CREB3 is then cleaved by S1P and S2P proteases to release its cytosolic region that translocates into the nucleus to upregulate Golgi-associated genes ARF4 and DR4 (**Fig.3**). Transcription factors of the ETS family including ELK1, ETS1 and GABPA/B are activated through the mitogen-activated proteins kinases (MAPK) MEK1/2 and ERK1/2 (**Fig.3**). These transcription factors are also involved in spliceosome function and Golgi stress-induced cell death induction via MCL1 splicing [172,212,213]. CREB3 also induces TRAPPC13, a component of the TRAPPIII complex involved in autophagy. Finally, TFE3 augments the global function of the Golgi apparatus by up-regulating expression of genes containing Golgi apparatus stress response element (GASE) including Golgi proteins involved in its structure such as GOLGA2 and GOLGB1 (also called GM130 and giantin respectively), in vesicular transport such as RAB20 and STX13, and enzymes of the N-glycosylation (**Fig.3**).

Figure 3

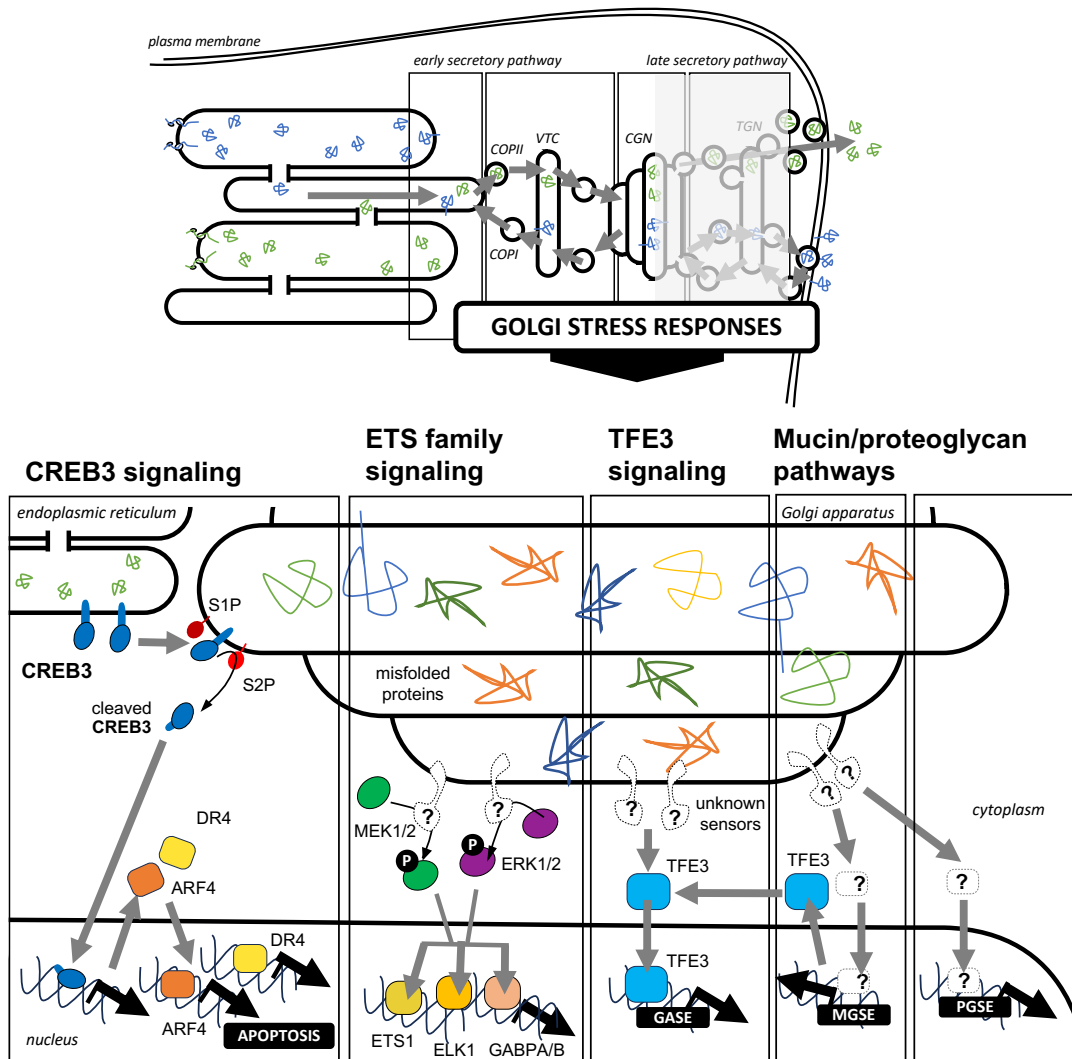


Figure 3: Molecules involved in the Golgi stress response - Overwhelming Golgi functions due to increased protein production and transport trigger the Golgi stress response. Unlike to the ER stress response, most of the Golgi stress sensors are unknown. Upon Golgi stress, the ER transmembrane protein CREB3 is exported into the Golgi to be cleaved by S1P/S2P proteases releasing the cleaved form of CREB3 that exhibits transcription factor function, regulating in particular ARF4 and DR4 that leads to Golgi stress-induced cell death. Golgi stress also activates MAPK MEK1/2 and ERK1/2 that trigger transcription factors of the ETS family. Through an unknown mechanism, Golgi stress also activates the transcription factor TFE3 that regulates genes involved in several Golgi functions. To reinforce glycosylation of mucin and proteoglycans, the Golgi stress response triggers additional signaling pathways

including mucin and proteoglycan pathways that lead to induce PGSE and MGSE motifs-containing genes. Linked transcription factors are not identified yet .

III. Control of the early SP upon ER and Golgi stress responses

III.1. ER and Golgi stress-dependent modification of organelles associated with the early SP

As described above, ER stress affects ER homeostasis and triggers UPR to restore proteostasis [98,99]. Importantly, ER stress and its UPR affect the morphology and function of organelles involved in protein secretion i.e. the ER, the ERGIC and the Golgi apparatus (**Fig.4**). Moreover, new cellular structures appear during ER stress such as stress vacuoles. Here we will summarize the organelles modifications described during ER or Golgi stress.

Altered ER morphology and ERES upon ER stress - ER stress is known to induce enlargement of ER cisternae [215,216] as well as ER membrane expansion generating new ER sheets and tubules (**Fig.4**) [217–220]. The latter is controlled by UPR-dependent regulation of lipid biosynthesis involving the IRE1/XBP1s branch [217]. Changes in ER morphology allow accumulation of newly-synthesized chaperones and PDIAs involved in protein folding, to alleviate ER stress and further resolve ER homeostasis [217–220]. For instance, in renal cells, hyperosmolarity induces lipid biosynthesis via ER stress/TonEBP-dependent activation of IRE1/XBP1s axis, that induces SREBP1/2 expression that in turn trigger lipogenic enzymes LPIN1/2 and DGAT1. XBP1s acts as an osmoprotective protein that contributes to lipid metabolism, membrane generation and restoration of ER homeostasis [221]. In addition, the number of ERES decreases during ER stress in drosophila cells to form SEC bodies described below [222,223]. In case of prolonged ER stress, ER forms multimembrane vesicles called ER whorls initiated by SAR1 and SEC22 molecules, involving PERK activity [224].

ER stress-dependent modification of ERGIC and Golgi apparatus - Upon ER stress, the architecture of the ERGIC and the Golgi apparatus is completely altered, leading to a slowdown in the ER-to-Golgi protein trafficking (**Fig.4**). Golgi ribbons and most of

the cisternae stacks disappear. In addition, reduced numbers of COPII-coated vesicles are observed as revealed by a decreased number of SEC31 puncta [225].

Novel cellular structures generated upon ER stress - Autophagy is part of the arsenal of response to adapt during several cellular stresses including ER stress. Autophagosomes are double membranes structures that contain a degradation system to recycle abnormal proteins and excess/damage organelles. UPR is known to be involved in this process. In pathological conditions such as cancer, cellular oxygen availability could be limited i.e. hypoxia. UPR enhances the capacity of hypoxic tumor cells to trigger autophagy, helping in cell survival. Cancer cell autophagy is also a protective mechanism to resist against anti-cancer drugs attack. ER stress induces expression of Beclin-1, important for the autophagosome formation; and ATG5/12, a key complex that in concert with LC3 is involved in formation and extension of membrane structures to initiate autophagosome formation [226]. Beclin-1 is induced by PERK/ATF4 activation [226]. ATG5 and ATG12 are induced by ER stress-dependent CHOP [227] and by PERK/ATF4 [228,229] respectively. Interestingly, UPR also triggers ER autophagic degradation, involving numerous proteins including VAP proteins that facilitate ER and autophagosome membrane contacts [230]. In yeast, upon ER stress, IRE1 induces EPR1 expression, to facilitate this process [230]. ERP1 is localized in the ER, interacting with ER resident VAP proteins and ATG8 to bridge ER fragments into the autophagosomes (**Fig.4**) [230]. In *Drosophila* embryonic cells, amino acids privation induces formation of SEC bodies, membrane-less granules that act as a protective reservoir for ERES components [231] and are associated with inhibition of the protein secretion but also survival [232]. In concert with the salt inducible kinases SIK1/2, IRE1 and PERK activation are involved in SEC bodies formation upon combined salt and ER stresses [232].

Altered Golgi morphology upon the Golgi stress - As indicated above, in physiological and/or pathological conditions, upon ER or Golgi stress, the Golgi structure can be altered due to the loss of Golgi integrity, resulting in the disruption of the ribbon-like structure and uncoupling of the stacks [172]. This change of morphology, mostly known as Golgi fragmentation, is reversible or irreversible depending on its nature and has several functional consequences more or less dramatic for cells depending on its extent and severity. In pathological conditions, this process is mostly irreversible and

causes cellular perturbations as described in different diseases (neurodegenerative diseases, cancer, inflammation) such as alteration of protein glycosylation.

Golgi fragmentation and consequences in protein secretion – As described above, GRASP molecules contribute to the Golgi structure. Importantly, GRASP proteins depletion impairs protein glycosylation triggering the Golgi stress response [233]. Surprisingly, GRASP65 inhibition prevents the Golgi stacking and leads to accelerated protein trafficking. Golgi fragmentation can also lead to a mis-sorting of proteins to the wrong compartment. For instance, during Golgi fragmentation, cathepsin D and mannose 6-phosphate receptor are addressed to the extracellular environment instead of lysosomes [233]. Interestingly, the multikinase inhibitor sorafenib used for hepatocellular carcinomas disrupts the protein secretion pathway by inducing the Golgi fragmentation via the inhibition of p97/VCP (for valosin containing protein) phosphorylation [234].

Figure 4

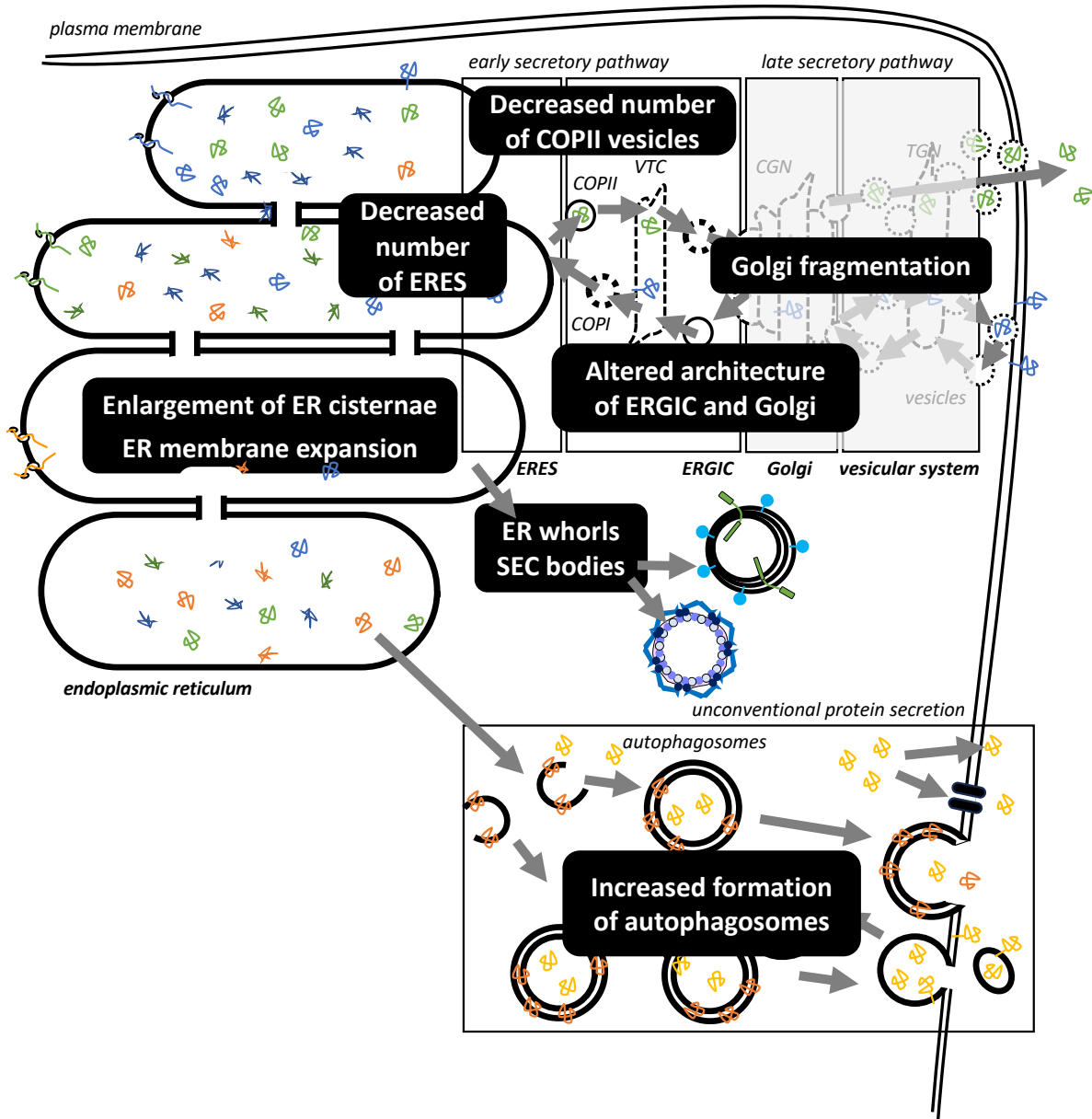


Figure 4: Alteration of cellular organelles involved in protein secretion upon ER and Golgi stress - ER and Golgi stress lead to modification of cellular organelles including the enlargement of ER cisternae, the decrease of ERES and COPII vesicles, the alteration of the ERGIC and the Golgi apparatus morphologies, and the appearance of new cellular structures such as ER whorls and SEC bodies. IRE1 and PERK branches have been involved in some of these phenomena.

III.2. UPR and Golgi stress-dependent modulation of the early SP machineries

In the past years, little has been described in the direct regulation of molecular actors of the protein secretion machineries by ER or Golgi stress sensors. However, several studies report the impact of ATF6, IRE1, and PERK activities on these machineries under healthy or pathological contexts such as inflammation, infection, chronic liver diseases and cancers (**Fig.5**). In this part, we will not describe ER or Golgi stress regulations of the initial steps of the SP i.e. protein translocation and quality control as well as misfolded protein degradation, which are well-documented in [187,201,235].

Figure 5

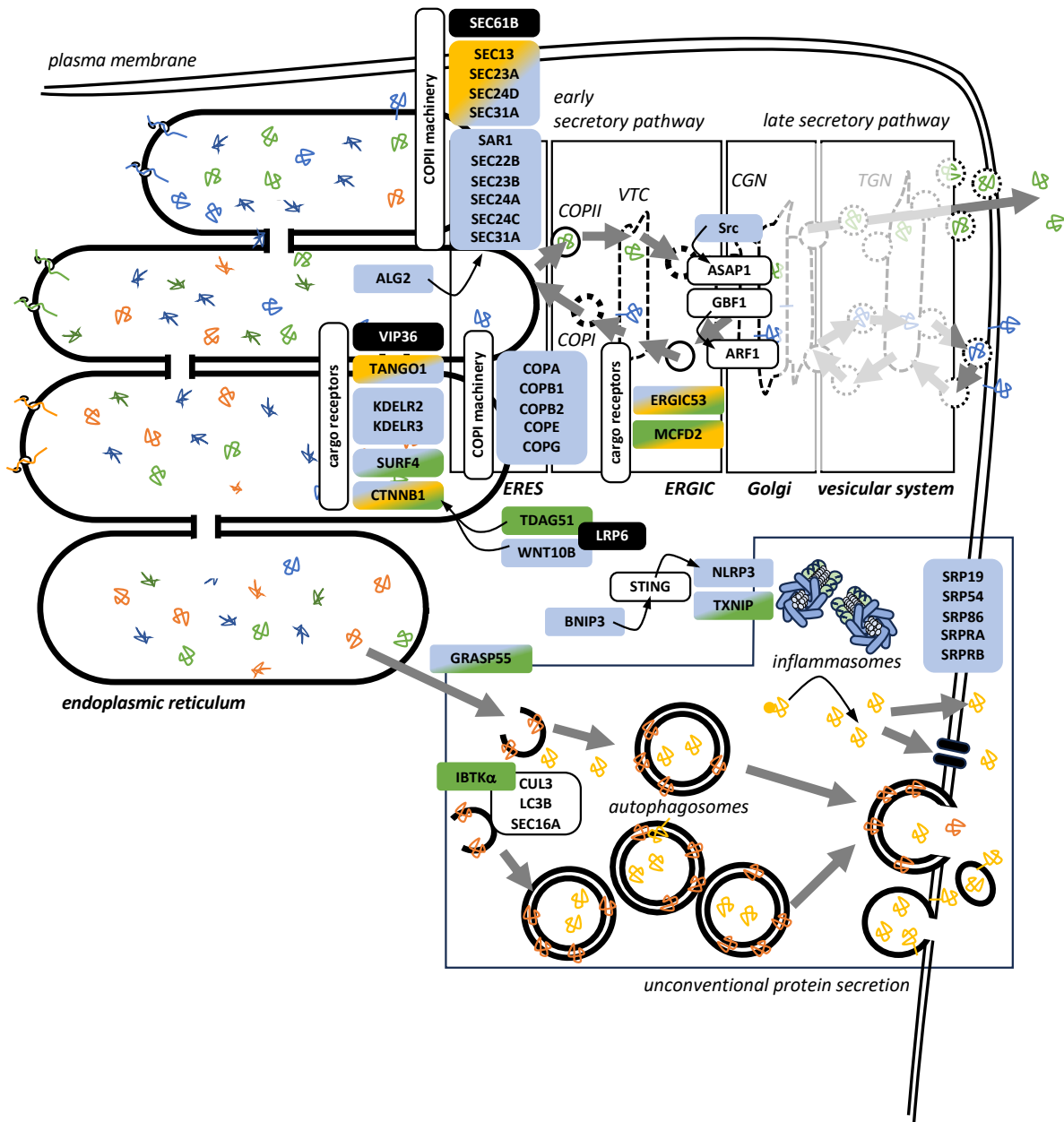


Figure 5: Molecular actors of the early SP controlled by the activation of the UPR sensors - Activation of the 3 ER stress sensors promotes the expression of cargo receptors, molecules of the COPI and COPII machineries, proteins associated with the Golgi apparatus and UPS such as inflammasome components. Molecules controlled by ATF6, IRE1 and PERK activation are framed respectively in yellow, blue and green.

UPR-dependent regulation of COPII machinery at the ERES - The COPII assembly is conditioned by the activation of SAR1 [168,173,175]. One study reports that ER stress

induces sar1 as well as nsf1 and pdi1 expression but not ypt1 (RAB) in *Trichoderma reesei* fungi [236]. Concerning the UPR-mediated regulation of the COPII machinery components, in modified HEK cells active for XBP1s and/or ATF6, SEC23A/B, SEC24A/C/D and SEC31A are regulated by XBP1s; and SEC13 by both ATF6 and XBP1s [237]. XBP1s also induces the regulator ALG2 that stabilizes SEC31 at the ERES [175]. Liver is remarkably able to adapt to physiological fluctuations of nutrient availability, particularly in regulating its ability to secrete proteins and lipids under those conditions. Maintenance of hepatic metabolic homeostasis is linked to a tight regulation of the COPII machinery [238]. Interestingly, in mouse liver, knock-down of IRE1 leads to a reduction in COPII-associated proteins SEC22B, SEC23B, SEC24D and SEC61B. Furthermore, in fasted mouse, a reduced XBP1s expression is associated with a reduced expression of SEC22B, SEC23B and SEC24A [238]. In nonalcoholic steatohepatitis (NASH) liver disease, saturated free fatty acids trigger UPR in human hepatocytes, involving PERK activation and eIF2 α phosphorylation [239], leading to CHOP-induced transcription and the phospho-eIF2 α -dependent preferential translation of IBTK α (for Inhibitor of Bruton's Tyrosine Kinase alpha) [240]. IBTK α participates in the autophagy process as a substrate adapter for the E3 ubiquitin ligase CUL3. In the ERES, IBTK α complexes with CUL3, LC3B, SEC16A, and SEC31A to initiate formation autophagy-associated phagophores [240]. In addition, IBTK α is associated to CUL3 and SEC31A to trigger COPII vesicles formation leading to cytokines secretion such as IL8 and TNF α . The latter contribute to the aggravation of the NASH disease by enhancing inflammation [240]. In plants, IRE1, through bZIP60 (homologous of metazoan XBP1s), increases SEC31A expression upon ER stress [241], contributing to the pollen coat secretion during heat stress [242]. Upon infection with pathogens including viruses or bacteria, ER stress is triggered in host cells leading to activation of ER stress sensors IRE1, PERK and ATF6 [243–248]. For instance, in *Brucella*-infected HeLa cells, IRE1 activation leads to XBP1s expression and enhanced expression of the COPII-associated proteins SAR1, SEC23 and SEC24D present at the ERES [249]. Intriguingly, YIP1A (for YPT-interacting protein 1A), a SEC23/24-associated molecule localized at the ERES, enhances IRE1 activation and also directly interacts with phosphorylated IRE1, leading by an unknown mechanism to the control of IRE1 oligomer assembly [249]. Interestingly, YIP1A also controls IRE1 and PERK activation in human cervical cancer cells, engaging two anti-apoptotic and

autophagic programs to preserve cell survival [250]. In medaka fish embryos, the ATF6-like ER sensor BBF2H7 is cleaved at the Golgi apparatus upon ER stress; and translocates into the nucleus [251]. BBF2H7 allows transcription of COPII-associated proteins SEC13/31 and SEC23A/24D to facilitate the formation of COPII-vesicles [251].

UPR-dependent regulation of cargo receptors, adapter and accessory molecules involved in protein secretion - Expression of a group of cargo receptors including ERGIC-53, MCFD2 and VIP36, involved in the protein transport from the ER to the Golgi apparatus, is controlled by the UPR [252], mainly involving the ATF6 branch for ERGIC-53 [253,254]. ERGIC-53 recycles between the ER and the ERGIC to sort ER resident proteins and to act as a cargo receptor for several N-glycoproteins through the COPII-associated vesicular system to the Golgi apparatus [168,175,191]. Therefore, ERGIC-53 binds to COPI and COPII. As described above, liver metabolism is linked to a fine regulation of the COPII machinery. Interestingly, in mouse liver, knock-down of IRE1 leads to a reduction in COPII-associated proteins as well as ERGIC-53. And again, in fasted mouse, a reduced XBP1s expression is associated with a decreased expression of ERGIC-53. Nitric oxide release is a cell mechanism triggering activation or apoptosis during the inflammatory response. As important actors of the inflammation, macrophages are known to resist to NO-induced apoptosis at the early phase of inflammation [255]. Interestingly, NO modifies protein transport in macrophages, triggers ER stress via ATF6 and eIF2 α , increasing the expression of ERGIC-53 and its associated protein MCFD2 [255]. The KDELR molecules are also well known cargo receptors, localized between the ER and the ERGIC to retrieve ER resident proteins [191]. Only two studies report a link between KDELRs and ER stress sensors. Indeed, KDELR2/3, but not KDELR1, are induced by the activation of the IRE1/XBP1s axis upon ER stress in cells [256]. In modified HEK cells active for XBP1s, KDELR3 is induced [237]. Beta-catenin (CTNNB1), involved in many cellular functions such as cellular activation (as part of the WNT signaling pathway) and cellular adhesion, also participates in ER export of N- and E-cadherins [175]. In adipocytes, XBP1s represses transcription of WNT10B, leading to decreased expression of CTNNB1 protein [257]. In endothelial cells, inhibition of IRE1/XBP1s suppresses CTNNB1 translocation into the nucleus [258]. In renal tubular epithelial cells, ER stress-dependent PERK/eIF2 α activation induces TCGA51 expression that controls

CTNNB1 translocation into the nucleus, participating to the EMT process [259]. In trophoblastic cells, deprivation of glucose triggers UPR-dependent activation of IRE1, leading to CTNNB1 increased expression, activating STARD7 expression which is involved in the maintenance of ER and mitochondria morphology [260]. In contrast, elevation in glucose induces hexosamine biosynthetic pathway activation associated with phospho-eIF2 α and XBP1s, also leading to CTNNB1 increased expression [260]. Intriguingly, in vascular smooth muscle cells, XBP1u interacts with CTNNB1 to promote its degradation, therefore inhibiting CTNNB1/TCF-mediated transcription of RUNX2 and MSX2, important transcription factors involved in vascular calcification in cardiovascular diseases [261]. In pathological context such as liver ischemia-reperfusion injury, hyperglycemia is a worse prognostic factor. In liver tissues of these patients, ER stress is activated in particular ATF6/CHOP axis. This leads to suppression of CTNNB1 expression and activation, accelerating inflammatory response in liver [262]. In breast cancer cells, PERK/eIF2a activation by betulinic acid inhibits CTNNB1 expression [263]. In colon cancer cells, repression of IRE1 expression leads to decreased expression of CTNNB1, a key molecule driving colon tumorigenicity impacting on tumor proliferation and stemness [264]. Hypoxic ER stress also reduces LRP6 expression, which decreases CTNNB1 accumulation and WNT/CTNNB1 signaling (86). Of note, CTNNB1 interacts with XBP1s and HIF1 α , thereby suppressing XBP1s activity i.e. induced expression of HIF α target gene expression [265]. In yeast, heat shock response is also activated by ER stress sensor IRE1, impacting on COPII cargo receptor ERV29 expression [266]. In mammary epithelial cells, IGF-1 involved in galactopoiesis induces ER extension dependent of XBP1s regulation of ER-biogenesis related genes CHKA, PCYT1A and SURF4 [267]. Other adapter and accessory molecules of the secretion machineries are also regulated upon ER stress. For instance, ERp29 is an ER luminal protein with putative escort function via COPII machinery through its interactions with KDELR1 [268,269]. ERp29 is elevated in cancer cells, after irradiation or genotoxic stress [270,271] and up-regulated during ER stress [269,272,273]. ERp29 overexpression enhances PERK expression in lung cancer cells [274] and thyrocytes [275]. Moreover, ERp29 directly interacts with PERK, leading to eIF2 α phosphorylation [274]. ERp29 is also described as an escort factor for ATF6 localization in the Golgi apparatus [276]. Interestingly, doxorubicin increases ERP29 expression, associated with drug-resistance process

linked to PERK activity [274]. Finally, in breast cancer cells, cytokeratin-19 induces ER stress via XBP1s leading to inhibition of ERp29 expression [277]. ERp44, a PDI protein involved in ER protein retrieval and retention in the ER, is also described as an escort protein for incompletely assembled IgM or insulin respectively in immune B cells and pancreatic beta cells. Indeed, under physiological conditions, the IRE1/XBP1s branch is highly and constitutively active in mammalian pancreatic beta cells. This activation is required for ERp44 (TXNDC4) and ERp46 (TXNDC5) expression, important chaperones for proinsulin folding and cargo receptors for insulin secretion [278]. TANGO1 helps ER export of large collagen proteins [279]. As indicated above, upon ER stress, the ATF6-like ER sensor BBF2H7 allows transcription of COPII-associated proteins as well as accessory proteins KHLHL12, SEDLIN and TANGO1, that facilitate the formation of COPII-vesicles [251]. In hepatic stellate cells, TGF β 1 induces UPR activation through XBP1s branch leading to increased TANGO1 expression [280].

UPR-dependent regulation of Golgi-associated secretion machinery - In modified HEK cells active for XBP1s, molecules of the COPI machinery, involved in the retrograde protein trafficking, are induced i.e. COPA, COPB1/2, COPE and COPG [237]. Upon ER stress, IRE1 binds and activates SRC kinase, leading to ASAP1 phosphorylation and its accumulation at the Golgi membrane. Hereby, phospho-ASAP1 forms a complex with GBF1, a GTP exchange factor, enhancing its GEF activity [281]. This leads to increased ARF1-GTP levels, an active form of ARF1, a major GBF1 substrate involved in the regulation of retrograde vesicle assembly. As a consequence, KDEL is dispersed from the Golgi apparatus revealing the disruption of the KDEL retrieval machinery, and the abrogation of the retrograde protein transport [282].

Golgi stress-dependent regulation of glycosylation of secreted proteins - In addition to what is described above, the Golgi stress response triggers additional pathways linked to the restoration of protein glycosylation [172,214]. For instance, the proteoglycan and mucin pathways reinforce glycosylation of proteoglycan and mucin, respectively (**Fig.3**). They lead to induce PGSE (for proteoglycan-type Golgi stress response element) and MGSE (for mucin-type Golgi stress response) motifs-containing genes including glycosylation enzymes such as glycosyltransferase and sulfotransferase although either Golgi stress sensors and transcription factors are not identified yet [172,214] (**Fig.3**).

III.3. New aspects of the regulation of the SP machinery upon ER and Golgi stress signaling

As described above, the UPR is the main adaptive response that has been described to regulate the early SP machineries. Little is known about the involvement of the Golgi stress response. Using publicly available resources, we anticipate novel putative regulation mechanisms of the molecular machineries of the SP by transcription factors induced by the UPR and Golgi stress pathways. Among the molecules involved in protein transport according to gene annotation with GO (3298 genes), almost 75% are putative UPR targets genes downstream of the three sensors ATF6, IRE1 and PERK (**Fig.6A**). Intriguingly, the IRE1/XBP1s branch seems to control genes of the protein transport located in the ER, whereas the PERK/ATF4/CHOP branch is rather involved in the control molecules involved in the late secretory pathway located in the Golgi and the vesicular system (**Fig.6C**). In the same line, more than 60% of the genes associated with protein transport are putative targets of the Golgi stress-dependent transcription factors CREB3, TFE3 and of the ETS family (ELK1, ETS1 and GABPA/B) (**Fig.6B** and **6C**). Again, differences in signaling pathways are observed with a large majority of targets potentially controlled by transcription factors of ETS family (**Fig.6C**). Importantly, more than half of these molecules are associated with the early SP (**Fig.6C**). Further investigations are urgently required to finely characterize these molecular regulations and to confirm that both UPR and Golgi stress response could impact on the early and late SP mechanisms.

Figure 6

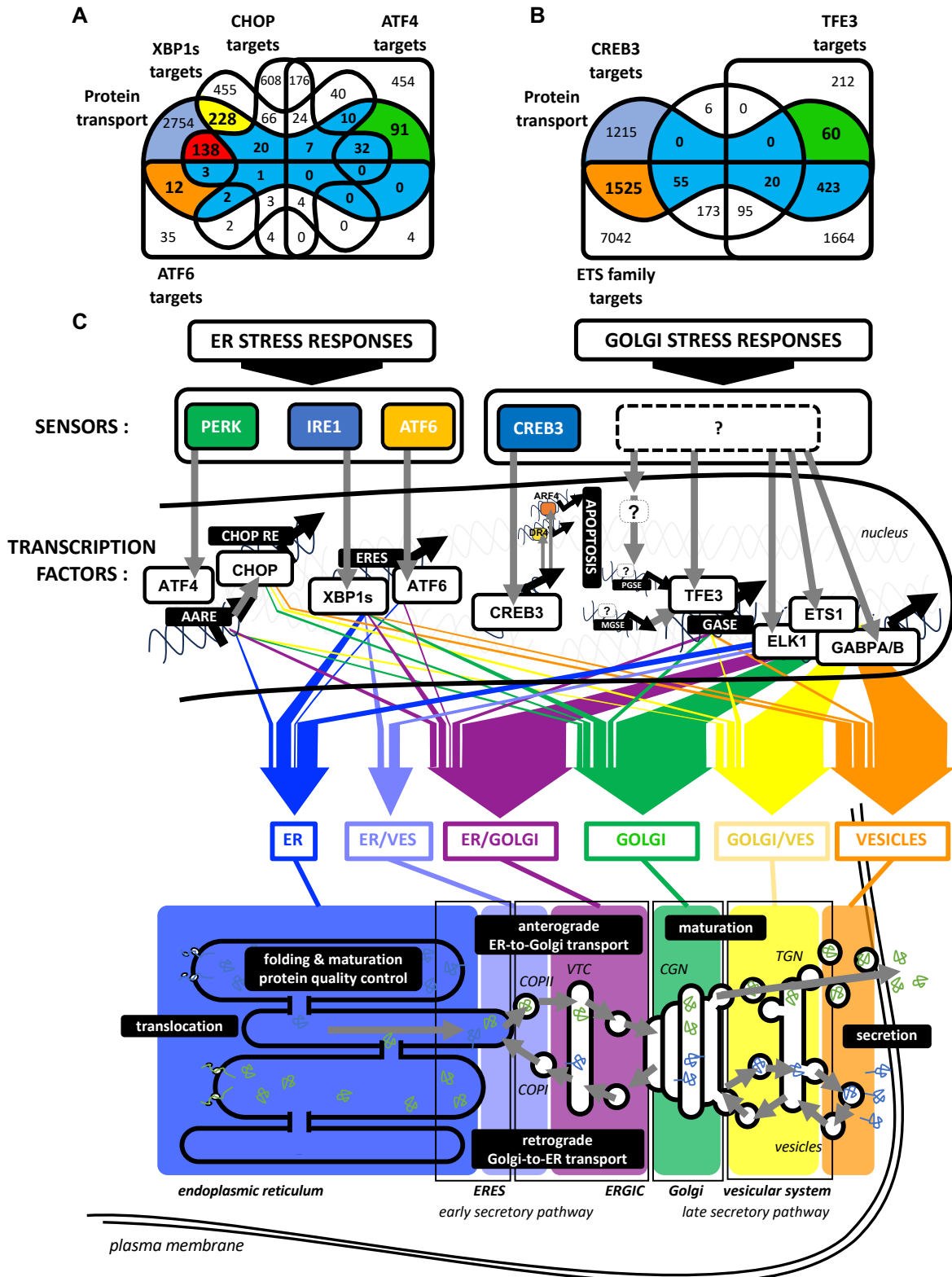


Figure 6: Molecular actors of the SP controlled by transcription factors induced by the UPR sensors and Golgi stress - (A and B) Genes involved in protein transport are

intersected with targets of the transcription factors associated with the UPR i.e. ATF6, XBP1s and ATF4/CHOP generated after ATF6, IRE1 and PERK activation **(A)**; or with transcription factors associated with the Golgi stress response i.e. ETS1, ELK1 and GABPA/B (form the ETS family) and TFE3 **(B)**. Gene lists were obtained from publicly available datasets from Gene Ontology (<https://amigo.geneontology.org/>) (GO: 0015031) and ChIP-Atlas (<https://chip-atlas.org/>) (score threshold: 100). **(B)** Cellular localization and compartment associated with the novel putative molecules involved in protein transport and controlled by the UPR sensors or the Golgi stress responses were accessed using STRING (<https://string-db.org/>). Integrated information from **(A)** and **(B)** unveils a major output of the Golgi stress response compared to UPR on the potential regulation of genes involved in protein transport from the ER to the post-Golgi organelles.

Conclusion & perspectives

The regulation of protein secretion by ER and Golgi stress signaling is a crucial and intricate process that plays a pivotal role in maintaining cellular homeostasis in both healthy and pathological situations. In healthy cells, the ER stress response allows for adaptation to changing conditions, such as increased protein synthesis demands to enable cells to cope with stressors and maintain normal functions. Dysregulation of ER stress signaling and protein secretion are implicated in the pathogenesis of various diseases. Indeed, aberrant secretion of misfolded proteins could become proteotoxic and cause several diseases. Such protein aggregates have been implicated in the pathogenesis of numerous neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's diseases [169,173]. Additionally, disruptions in protein homeostasis have been associated with cancer, metabolic disorders, and cardiovascular diseases, underlining the impact of protein misfolding on human health. For instance, ER-to-Golgi protein trafficking is a conserved process that fulfills numerous physiological functions. Mutations in genes encoding accessory ER-to-Golgi trafficking proteins as SNAREs but also in genes associated with unconventional protein secretion (UPS) pathways that bypass the Golgi for protein secretion have been linked to neurodegenerative diseases [283]. Our understanding of how dysfunction of ER-to-Golgi trafficking contributes to diseases, such as neurodegeneration, should facilitate the development of effective cures for these devastating diseases [173]. Better understanding and modulating these processes are essential for the development of targeted therapies and interventions. ER stress signaling and protein secretion targeted approaches could be designed to mitigate the progression of diseases linked to ER stress, offering new opportunities for treatments, and improving patient outcomes. Therapeutic approaches that target both the UPR and the cellular secretion machinery are actively under investigation, particularly in the context of disorders associated with protein misfolding and secretion anomalies. These strategies have a dual objective: enhancing protein folding, trafficking, and secretion processes, while also managing ER stress through UPR modulation. One avenue of investigation involves chaperone-based therapies. Indeed chemical chaperones, which assist in protein folding and stability, have been used to enhance the correct folding and trafficking of misfolded proteins in cystic fibrosis [284] or nephrogenic diabetes insipidus [285]. Another therapeutic approach includes modulators of protein trafficking

such as compounds derived from brefeldin A targeting the ARF protein family including ARFGEFs and ARF GTPases, affecting the formation of the COPI complex, the integrity of the Golgi apparatus and the endosomes [286]. Combining UPR-modulating therapies with existing treatments might also enhance their efficacy as demonstrated in breast cancer with the use of chemotherapy agent paclitaxel and the IRE1 inhibitor MKC8866 as an anti-tumor therapy, limiting the cancer resurgence after chemotherapy [144]. The Golgi stress inducer monensin confers cytoprotection of striatal cells in Huntington's disease by inducing the CSE (for cystathionine gamma-lyase) an important enzyme to restore cysteine metabolism [287]. The effectiveness of these therapies may also vary depending on the specific disease and its underlying mechanisms. While these therapeutic strategies show promise in preclinical studies and some clinical trials, the translation of these UPR and overall secretion therapies is still an ongoing process, and their safety and efficacy in humans are still carefully evaluated.

To conclude, the regulation of protein secretion by ER and Golgi stress signaling is a fundamental cellular process with far-reaching implications for both health and disease. Research in this field continues to uncover critical insights into the mechanisms underlying cellular function and dysfunction, offering potential possibilities for novel therapeutic strategies and diagnostic tools. Understanding how this regulation impacts cellular behavior is central for advancing our knowledge of biology and improving the management of various pathological conditions.

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Competing Interests Statement - EC is a founder of Thabor Therapeutics. The authors declare no conflicting interests.

Chapter 3: Conclusion

In this third chapter, we have shown the importance of the eukaryotic secretory pathway for maintaining cellular functionality focusing particularly on the roles of the ER, ERGIC and the Golgi apparatus in ensuring the correct folding, modification, and trafficking of proteins to their destined locations. This has also help us to shed light on how ER and Golgi stress signaling are intertwined with the regulation of the early SP, offering insights into their roles in both health and pathological conditions. It is thus evident, that understanding these mechanisms provides a foundation for exploring therapeutic targets within these pathways, especially in pathological circumstances such as cancer.

Hypothesis & Objectives

Glioblastoma is known to be a highly secretive tumor with a complex pathophysiology, that requires further understanding to develop novel therapeutic options. It has been established that the Endoplasmic reticulum stress and the Unfolded Protein response play a significant role in health and disease contexts including cancers. Among the UPR transducers, IRE1 is particularly important for maintaining the UPR functionality and has been implicated in the pathophysiology of GB by regulating the expression of various secreted proteins involved in tumor progression. Based on these insights, we propose the following hypothesis for testing:

“IRE1 may broadly affect protein secretion by directly influencing the molecular actors of the secretory pathway in GB cells”

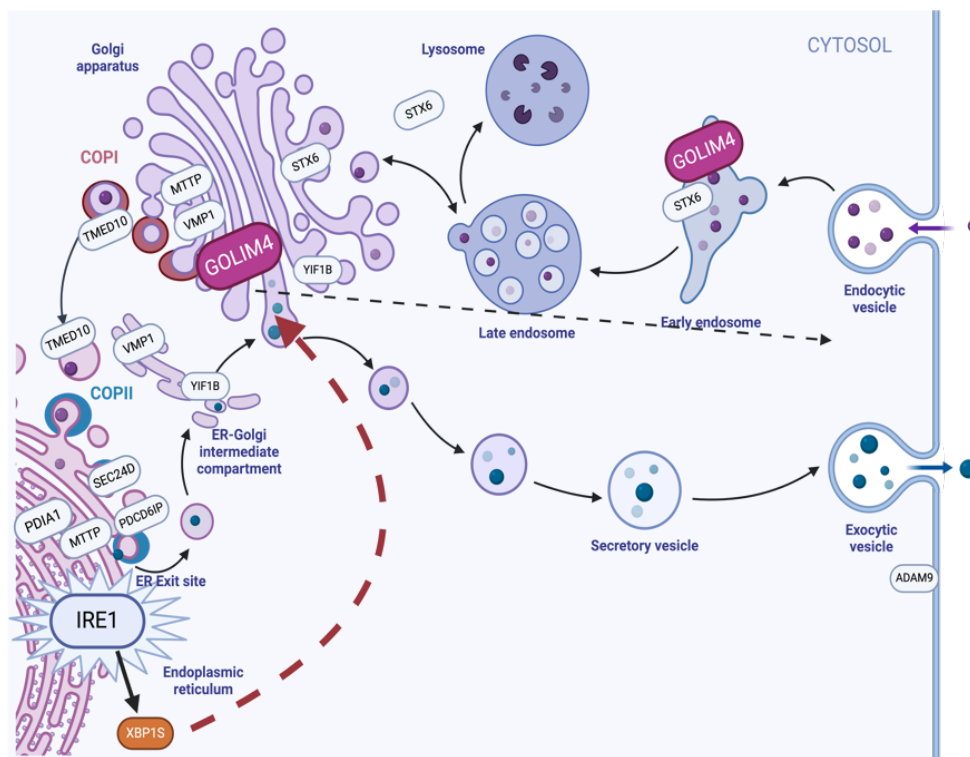
This hypothesis will be tested via four main objectives:

- 1) **Investigate the role of IRE1** in regulating the overall protein secretion machinery in GB cells, beyond its known involvement in the secretion of pro-tumoral factors and chemokines.
- 2) While several COPII and COPI molecules have already been described as regulated by XBP1s, we aimed to **identify additional molecular actors regulated by IRE1** that control early protein secretion in GB cells.
- 3) **Examine how these newly identified molecular actors influence** the secretory machinery in GB cells.
- 4) **Assess the functional impact** of these molecular actors GB biology by performing functional assays.

Results

Chapter 3:

Identification of IRE1-regulated molecules involved in Glioblastoma protein secretion



Chapter 3: Foreword

This chapter aims to present the results obtained so far in the thesis. In the first part, we presented by way of an article our different results that answer our working hypothesis. The article has been submitted to BioRxiv. Our investigation aims to uncover how IRE1 influences globally protein secretion within GB cells. While XBP1s has been previously reported to modulate COP molecules, we seek to identify additional molecular actors regulated by IRE1 that govern early protein secretion. Furthermore, this chapter will assess how these molecular actors affect GB biology. In the second part, we wanted to present the other targets that we discovered with another sorting method but didn't show any modulation upon IRE1 knockdown.

PART I: Article: “IRE1 controls protein secretion via GOLIM4 to modulate cell adhesion and migration in glioblastoma”

IRE1-dependent GOLIM4 expression controls protein secretion to modulate glioblastoma cell adhesion and migration

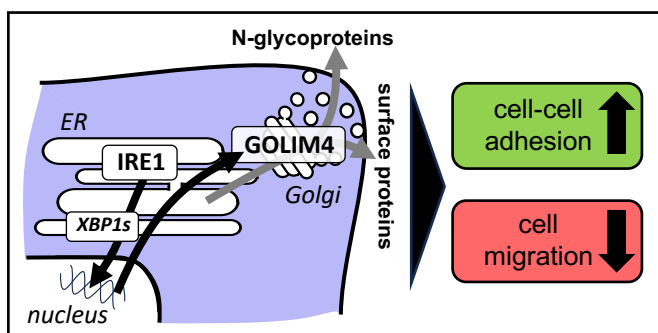
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Graphical abstract



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Competing Interests

EC is a founder of Thabor Therapeutics.

The other authors declare no conflicting interests.

Abstract

One of the main glioblastoma (GB) features is the diffuse migration of the tumor cells within the surrounding brain parenchyma, rendering almost impossible the complete tumor resection and irradiation, leading to inexorable lethal relapse of the disease. In the past years, we demonstrated that IRE1 α (hereafter IRE1), one of the Endoplasmic Reticulum (ER) stress sensors, plays a key role in GB biology by impacting on immune infiltration, angiogenesis and tumor cell migration/invasion, all these features being linked to an alteration of protein secretion. In the present study, we investigated if and how IRE1 could regulate the functionality of the secretory machinery in GB cells and identified GOLIM4, a Golgi-associated molecule whose expression is regulated downstream of IRE1 through the transcription of XBP1s. Interestingly, GOLIM4 silencing led to decreased surface expression of multiple molecules including MHC class I molecules, growth factor receptors (PDGFRA and IL13RA2) and proteins involved in cell-cell adhesion (CD44, CD54, NCAM1), adhesion to matrix (ITGB1) or cell migration (CD90) without alteration of their encoding transcripts' expression levels. Moreover, GOLIM4 silencing phenotypically affected GB cell-cell adhesion and cell migration in multiple models. Overall, we have described a novel IRE1/XBP1s/GOLIM4 operon that controls the secretion of specific proteins and impacts the tumor aggressiveness.

Key words: glioblastoma, ER stress, IRE1, GOLIM4, GPP130, GOLPH4, protein secretion, cell adhesion, cell migration.

Introduction

Upon oncogenesis, cells are exposed to acute demands of protein synthesis, that occur despite an unfavorable cell environment such as oxygen or nutrient deprivation. This defect in protein production in the Endoplasmic Reticulum (ER) could lead to an accumulation of unfolded or misfolded proteins inducing an ER stress [99,288]. To restore ER homeostasis, ER stress induced cells display a signaling program leading to a transcriptional response called the unfolded protein response (UPR) [99,208,288]. In mammals, three ER-resident proteins act as ER stress sensors ATF6, IRE1 α (referred to as IRE1 hereafter) and PERK to initiate the UPR [99,288]. One of these ER stress sensors IRE1 plays a key role in glioblastoma (GB) biology [289], affecting multiple cellular functions including immune recruitment [290,291], angiogenesis [292,293] or tumor cell migration [291,294].

The ER stress response, which triggers the activation of the Unfolded Protein Response (UPR) with IRE1 as one of its main transducers, aims at restoring ER homeostasis when perturbed thus ensuring proper function of the secretory pathway (SP). The SP is essential to maintain cellular functions and multicellular communications. The SP is executed through the intervention of several cell organelles (ER, Golgi apparatus, vesicles, ...) and multiple molecular machineries (ER translocation, ER quality control and folding, ERAD, export, ...) [295], all of those being challenged in cancer cells [296]. Previous studies describe modifications of SP-associated organelles morphology upon ER stress, i.e. enlargement of ER cisternae [215,216], generation of new ER sheets and tubules via ER expansion [217–220], ER Golgi intermediate compartment (ERGIC) and Golgi apparatus fragmentation [172,225]. The activation of the ER stress sensors also controls the expression of molecular actors of the SP. For instance, upon prolonged ER stress, ER forms multimembrane vesicles called ER whorls initiated by SAR1 and SEC22 molecules, involving PERK activity [224]. The cargo receptor ERGIC53 that recycles

between the ER and the ERGIC to sort ER resident proteins is regulated by ATF6 [254]. Moreover, IRE1, through the induction of the transcription factor XBP1s, induces the expression of the COPII components SEC22, SEC23 and SEC24 which are involved in the export of secreted proteins from the ER to the Golgi [237,238]. Cargo receptors such as KDEL_R are also induced by the activation of the IRE1/XBP1s branch [237,256]. Furthermore molecules of the COPI machinery i.e. COPA, COPB1/2, COPE and COPG involved in the retrograde protein trafficking are induced after XBP1s activation [237].

Recently, we have shown that IRE1-mediated via UBE2D3 expression control leads to NF κ B activation and triggers proinflammatory chemokine secretion and the subsequent recruitment of immune/inflammatory cells to the tumor site in GB [290]. In the present study, we aimed at investigating if and how IRE1 could more globally regulate the functionality of the secretory machinery in GB cells.

Materials and Methods

Antibodies and other reagents – Primary antibodies are listed in **Table S1**. Secondary antibodies used were horseradish peroxidase conjugated polyclonal goat anti-rabbit IgG, goat anti-mouse IgG and rabbit anti-goat IgG (all from Dako). Unless specified, all other reagents were from Sigma Aldrich.

Cell culture and treatments – The immortalized GB cell U251 and U87 lines (all from ATCC) were grown in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Lonza) in a 5% CO₂ humidified atmosphere at 37°C. Primary GB lines RADH85 and RADH87 were previously described [297,298]. For transient silencing of GOLIM4, IRE1 and XBP1, cells were transfected with specific siRNA (from Ambio, Santa Cruz and Dharmacon respectively) using Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific), according to the manufacturers' instructions as described in [290]. Forty-eight to seventy-two hours after transfection, cells were used in either flow cytometry, Q-PCR, western blot or functional experiments (proliferation, adhesion or migration assays). For treatment with the IRE1 inhibitor MKC8866 (Selleckchem, Cologne, Germany), cells were incubated in culture medium in the presence of MKC8866 at 30 µM for 72 hours.

Gene expression data analysis – Transcriptomes of parental and IRE1-modulated GB cell lines were previously described in [290,291]. For tumor transcriptome derived from GB patients, the TCGA cohort was analyzed (<https://www.cancer.gov/tcga>). Complete gene expression analysis of the GB TCGA was performed with R (R version 3.5.0) / Bioconductor software as described in [290,291]. Hierarchical clustering of GB patients labeled for their IRE1 activity (high or low), based on their ranked IRE1sign38 score obtained from [291], was coupled with GB patients survival and the gene of interest expression.

Quantitative real-time PCR – Total RNA from GB cells was extracted using the TRIzol reagent (Invitrogen). RNAs were reverse-transcribed with Maxima Reverse Transcriptase (ThermoFisher) according to the manufacturer's protocol. qPCR was performed with a QuantStudio™ 5 Real-Time PCR System and the PowerUp™ SYBR Green Master Mix (ThermoFisher). Experiments were performed at least in triplicate for each data point. Each sample was normalized based on the expression of the GAPDH or ACTB gene using the $2^{-\Delta\Delta CT}$ method. Primers pairs used in this study are listed in **Table S2**.

Mass spectrometry – Parental U251, RADH85, RADH87 treated or not with the IRE1 inhibitors MKC8866 or Z4 [299], and IRE1 dominant negative U251_DN, RADH85_Q* and RADH87_Q* cells were lysed with lysis buffer composed of 20 mM Tris pH 8, 1.5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 15 μ M MG132, 10mM NEM (N-ethylmaleimide), supplemented with proteases and phosphatases inhibitor cocktails (Roche). Total proteins were precipitated overnight with 80% ice-cold acetone. Protein pellets were then washed 3 times with 80% acetone, followed by centrifugation at 500 g for 30 mins at 4°C. Samples were alkylated and digested with trypsin at 37°C overnight. After Sep Pak desalting, peptides were analyzed using an Ultimate 3000 nano-RSLC (Thermo Fisher Scientific) coupled in line with an Orbitrap ELITE (Thermo Scientific). Each sample was analyzed in triplicate. Briefly, peptides were separated on a C18 nano-column with a linear gradient of acetonitrile and analyzed in a Top 20 CID (Collision-induced dissociation) data-dependent mass spectrometry. Data were processed by database searching against Human Uniprot Proteome database using Proteome Discoverer 2.2 software (Thermo Fisher Scientific). Precursor and fragment mass tolerance were set at 7 ppm and 0.6 Da respectively. Trypsin was set as enzyme, and up to 2 missed cleavages were allowed. Oxidation (M, +15.995), GG (K, +114.043) were set as variable modification and Carbamidomethylation (C) as fixed modification. Proteins were filtered with False Discovery

Rate <1% (high confidence). Lastly, quantitative values were obtained from Extracted Ion Chromatogram (XIC) and p-values were determined by ANOVA with Precursor Ions Quantifier node in Proteome Discoverer.

Western blotting – Cells were lysed in ice-cold lysis buffer (30 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% Triton X). Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane for blotting. The membranes were blocked with 5% BSA in 0.1% Tween 20 in PBS and incubated with the diluted primary antibodies (1/1000) (**Table S1**). Antibody binding was detected with the appropriate horseradish peroxidase-conjugated secondary antibodies (1/7000) and visualized with ECL (KPL, Eurobio) according to the manufacturer's instructions and chemiluminescence signal was detected using G:Box Chemi XX6 imager from Syngene. Protein expression levels were determined by analyzing the luminescence signals using Fiji [300].

Flow cytometry – Cells were washed in PBS 2% FBS and incubated with saturating concentrations of human immunoglobulins and fluorescent-labelled primary antibodies (**Table S1**) for 30 minutes at 4°C. Cells were then washed with PBS 2% FBS and analyzed using the Novocyte 3000 flow cytometer (Acea Biosciences).

Cell-cell adhesion – Cell-cell adhesion was tested in cell aggregation experiments. Parental, controls, and U251 and U87 cells silenced for GOLIM4 (5,000 cells) were incubated in a 25 µL-drop on a cover of a 24-well plate. Images of cell aggregates were taken after 72 hours and cell density was estimated using Fiji by calculating the aggregates' size. High aggregate's size corresponds to the low cell-to-cell adhesion.

Boyden chamber migration assay – Parental, controls, and U251 and U87 cell lines silenced for GOLIM4 were washed in DMEM, placed in Boyden chambers (10⁵ cells/chamber in DMEM)

that were placed in DMEM 20% FBS and incubated at 37°C for 24 hours. After 24 hours, Boyden chambers were washed in PBS and cells were fixed in PBS 0.5% paraformaldehyde. Cells that did not migrate and remained inside the chambers were removed and cells that migrated were then stained with Giemsa (RAL Diagnostics). After washes in PBS, pictures of five different fields were taken. Migration index was given by the mean of number of migrated cells observed per field.

Statistical analyses – Graphs and statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software). Data are presented as the mean \pm SD or SEM of at least three biological replicates. Statistical significance was determined using a paired, unpaired t-test or ANOVA as appropriate. Significant variations are represented by asterisks above the corresponding bar when comparing test and control conditions, and above the line when comparing the two indicated conditions.

Additional information can be found in the Supplemental Materials and Methods.

Results

IRE1 endoribonuclease activity modulates the secretion of N-glycosylated and cell surface proteins in GB cells

We recently demonstrated that IRE1 activity controls GB biology by secreting chemokines leading to the recruitment of inflammatory cells such as myeloid cells [290]. Herein, we tested whether this was also true for other secreted proteins that could affect other GB cell functions. To this end, we investigated whether IRE1 modulation impacted on protein secretion i.e. N-glycoproteins (**Fig.1A**) and surface proteins (**Fig.1B**) using two different proteomic approaches. We first compared global N-glycoproteome of parental and GB cells in which IRE1 was inhibited using a dominant negative form of IRE1 or the IRE1 inhibitors MKC8866 and Z4 (**Fig.1A**) we have previously described [290,291,299]. Genetic ablation of IRE1 in RADH85/87 and U251 cells led to expression attenuation of 208 N-glycoproteins mainly involved in cell adhesion and migration whereas treatment with MKC8866 in the same GB cells led to an attenuation of the expression of 490 of those proteins (**Fig.1B**). Less than 10% of them were found to be modulated when the total proteome was analyzed. Interestingly, these proteins whose expression is IRE1-dependent exhibited steady mRNA expression levels (**Fig.1C**). We next analyzed the expression of cells surface molecules in parental and RADH85/87 and U251 GB cells expressing a dominant negative form of IRE1 using a cell surface biotinylation approach (**Fig.1D**). Again, the cell surface expression of 223 proteins was down-regulated after inhibition of IRE1 activity, most of those were associated with cell adhesion and migration (**Fig.1E**), and exhibited an unaltered mRNA expression (**Fig.1F**). These results highlight that IRE1 RNase activity promotes the secretion of multiple cell adhesion and cell-migration-related proteins.

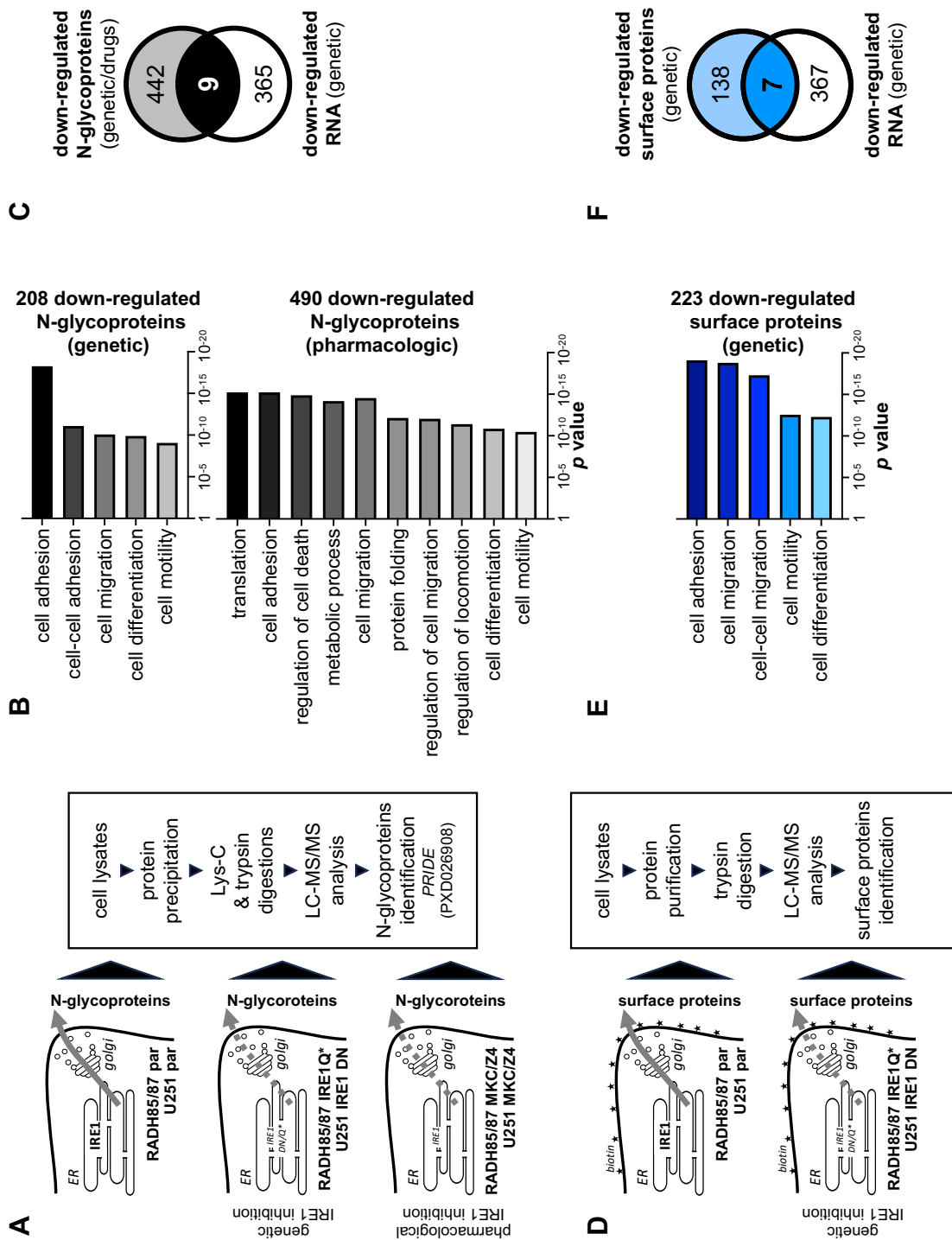


Figure 1. IRE1-dependent regulation of secreted proteins in GB cells

(A) A schematic representation of the proteomic approach used for analyzing N-glycoproteins present in IRE1-modulated GB cell lines U251, RADH85 and RADH87. IRE1 inhibition was performed genetically using overexpression of dominant negative IRE1 (IRE1_DN or IRE1_Q*) or pharmacologically using the IRE1 inhibitors MKC8866 or Z4. (B) Down-regulated N-glycoproteins were annotated using STRING resource (39). (C) Down-regulated N-glycoproteins were represented in a Venn diagram with genes which RNA expressions were down-regulated upon IRE1 inhibition. (D) A schematic representation of the proteomic approach used for analyzing cell surface proteins present in IRE1-modulated GB cell lines U251, RADH85 and RADH87. IRE1 inhibition was performed genetically using overexpression of dominant negative IRE1 (IRE1_DN or IRE1_Q*). (E) Down-regulated cell surface proteins were annotated using STRING resource. (F) Down-regulated cell surface proteins were represented in a Venn diagram with genes which RNA expressions were down-regulated upon IRE1 inhibition.

IRE1 endoribonuclease activity promotes the expression of the Golgi associated protein GOLIM4 in GB cells

As IRE1 invalidation modulated the expression of secretory proteins without affecting their mRNA levels, we postulated that IRE1 could, instead of regulating the expression of the proteins, rather control important molecular actors involved in protein secretion and/or traffic. We therefore defined potential candidates as being functionally involved in protein secretion (and localizing to organelle of the SP) that were potential XBP1s targets, based on the CHIP-Atlas resource [301], and whose expression was down-regulated upon IRE1 inhibition (genetic or pharmacologic) in GB cells (**Fig.2A**). Using these criteria, we identified 57 candidates (**Fig.2A**). We selectively focused on 5 genes that were also potentially regulated by IRE1 in GB specimens based to our iRE1 activity signature described in [291], and whose expression modulation was associated with GB patients' survival advantages or disadvantage (**Fig.2B**). Messenger RNA expression of these 5 candidates was tested in 4 GB cell lines (RADH85/87, U251 and U87) in which the expression of IRE1 was attenuated using siRNA-mediated silencing (**Fig.S1A**). Amongst the tested candidates, only GOLIM4 mRNA expression was down-regulated in all GB cells treated with siRNA targeting IRE1 (**Fig.2C**). We confirmed that GOLIM4 protein expression was reduced in GB cells upon IRE1 silencing (**Fig.S1B, 2D**) excepted in the RADH85 GB line (**Fig.S1C**). In addition, both IRE1 inhibitor MKC8866 (**Fig.S1D, 2D**) or XBP1 siRNA (**Fig.S1E, 2D**) led to reduced GOLIM4 protein expression in RADH87, U87 and U251 cells. GOLIM4 (for Golgi integral membrane protein 4, also named GPP130) is localized in the cis-Golgi, and is constitutively cycling between early endosomes, the Golgi and the plasma membrane. Overall, these data show that the expression of GOLIM4 protein is induced upon activation of the IRE1/XBP1s signaling axis in most of the GB cells tested in the present study.

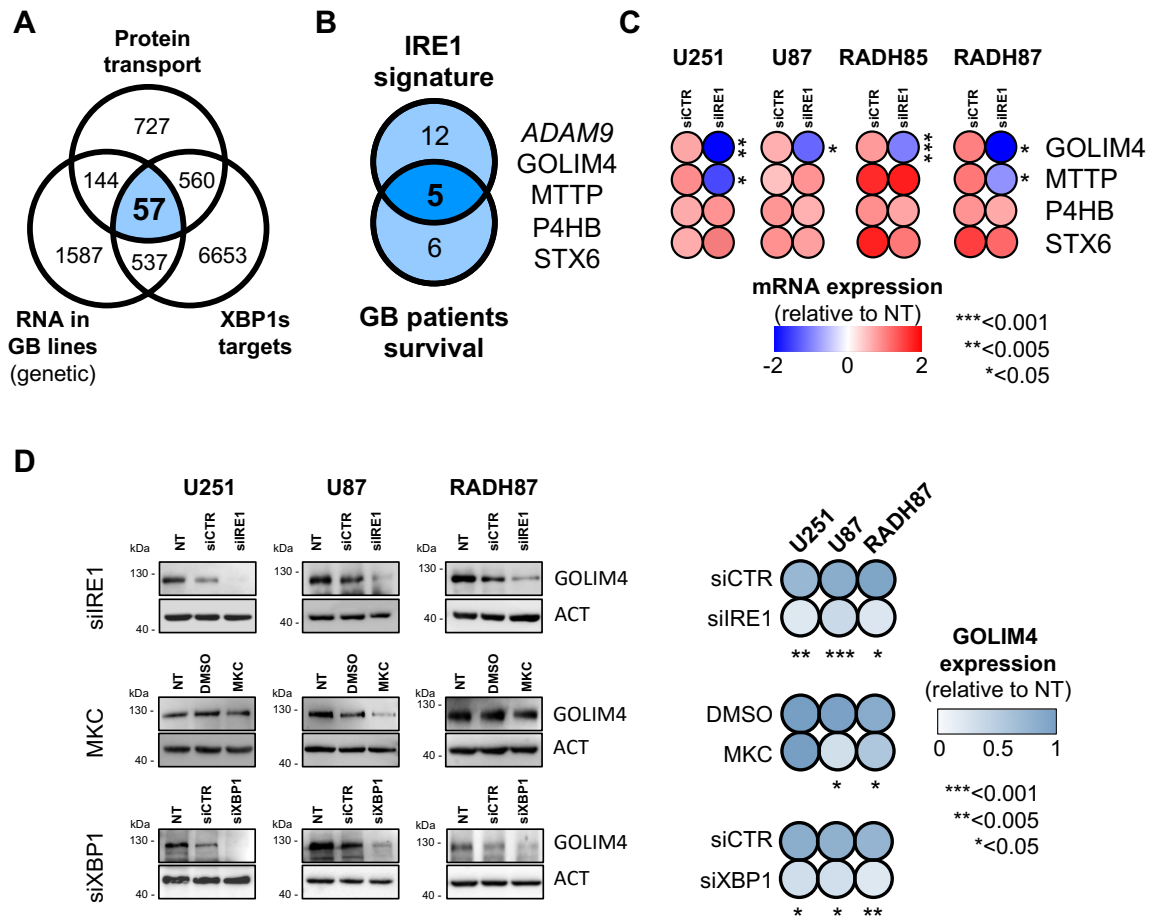


Figure 2. IRE1/XBP1s-dependent regulation of GOLIM4 in GB cells

(A) Genes involved in protein transport (from AMIGO gene ontology resource [302] GO:0015031) were represented in a Venn diagram with genes which RNA expressions were down-regulated upon IRE1 inhibition and putative genes regulated by XBP1s (from ChIP-Atlas resource [301]). (B) The selected genes were represented in a Venn diagram with genes modulated by IRE1 in GB specimens from the TCGA GB cohort [291] and GB patients survival. (C) mRNA expression of GOLIM4, MTTP, P4HB and STX6 from parental (NT), control (siCTR) and IRE1-silenced (siIRE1) GB cells U251, U87, RADH85 and RADH87 were analyzed by RT-Q-PCR. Expression levels were relative to parental cells (n=3); (*): $p < 0.05$, (**): $p < 0.005$ and (***) : $p < 0.001$. (D) Protein expression of GOLIM4 from parental (NT), control (siCTR or DMSO) and IRE1-modulated (siIRE1 or siXBP1) GB cells U251, U87 and RADH87 were analyzed by western-blot. IRE1 and XBP1s inhibition was performed using specific siRNA or the IRE1 inhibitor MKC8866 (MKC). Expression levels were relative to parental cells (n=3); (*): $p < 0.05$, (**): $p < 0.005$ and (***) : $p < 0.001$.

IRE1 and GOLIM4 promote the secretion of N-glycosylated and cell surface proteins in GB cells

To evaluate the impact of GOLIM4 on protein secretion in our experimental systems, we first identified whose expression at the cell surface (cell surface biotinylation and mass spectrometry sequencing of the biotinylated proteins) was affected by IRE1 genetic or pharmacologic inhibition. This led to the identification of 25 cell surface proteins with reduced presence at the cell surface in at least 2 out of 3 GB cell lines tested (**Fig.3A**), whereas their mRNA expression remained unchanged. We then tested whether GOLIM4 silencing could also affect cell surface expression of those candidates. Importantly, siRNA-mediated GOLIM4 silencing (**Fig.3B**) led also to reduce surface expression of MHC class I, IL13RA2, ITGB1 (a membrane partner of ITGA1/2), NCAM1 (CD56) and PDGFRA (CD140a) molecules, as determined by flow cytometry (**Fig.3C**). Furthermore, others surface molecules involved in cell adhesion and migration including CD44, CD54 (ICAM1), CD90 and CD109 were also affected by the modulation of GOLIM4, at least in one of the two of the GB cell lines tested (**Fig.S2**).

GOLIM4 modulation affects cell-cell adhesion and migration of GB cells

As GOLIM4 controlled the presence of specific proteins at the cell surface and that those proteins were shown to be involved in cell adhesion and migration (without affecting their mRNA expression), we next analyzed the impact of the reduction of GOLIM4 on GB cell functions including cell proliferation, cell adhesion to extracellular matrix, cell-to-cell adhesion and cell migration.

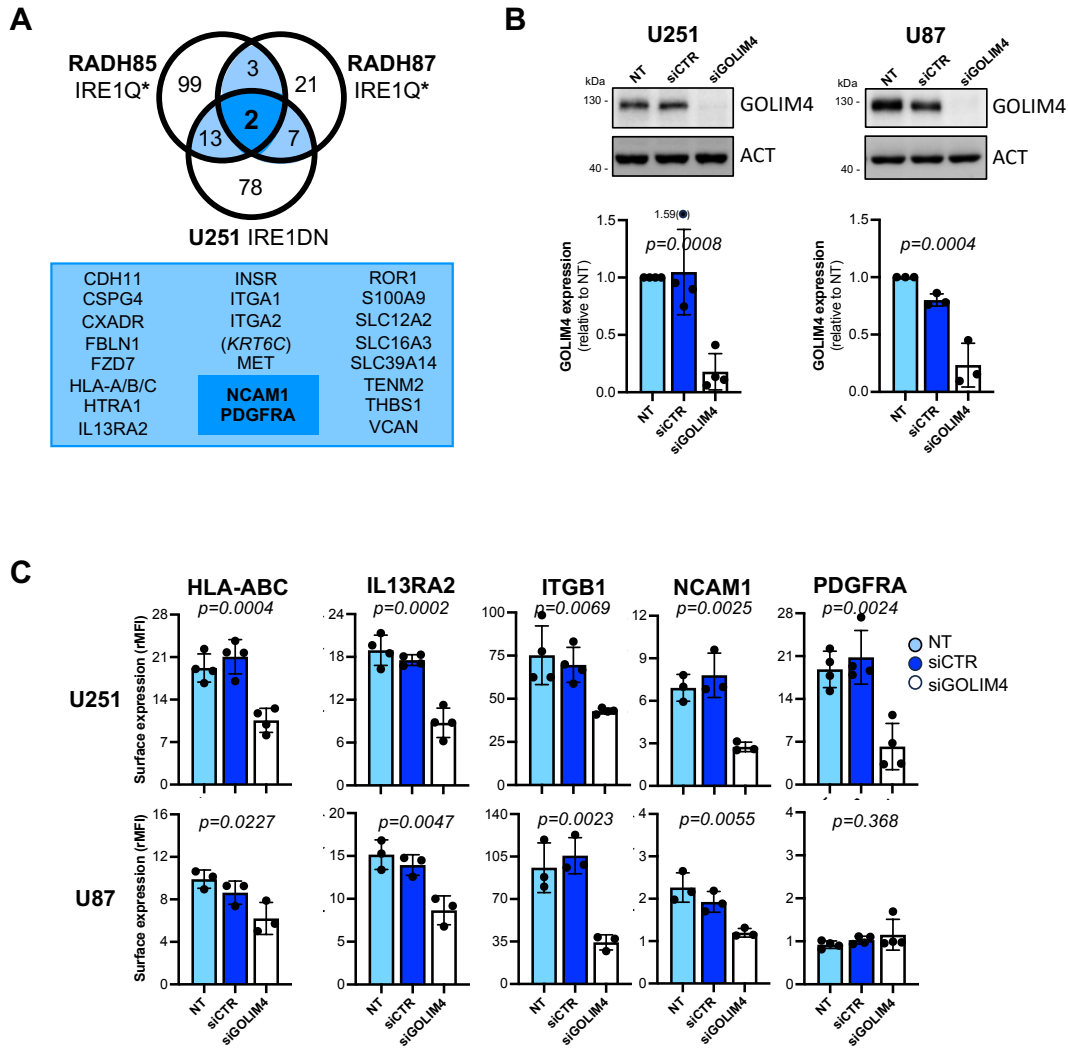


Figure 3. IRE1 and GOLIM4-dependent regulation of N-glycoproteins and surface proteins in GB cells

(A) Down-regulated surface proteins upon IRE1 genetic inhibition obtained from proteomic analyses of U251, RADH85 and RADH87 GB cells were represented in a Venn diagram. (B) GOLIM4 protein expression in parental (NT), control (siCTR) and GOLIM4-silenced (siGOLIM4) U251 and U87 GB cells was analyzed by western-blot. Protein expression levels were determined using Fiji; (n=3); *p* values were indicated on the top of the graph. (C) Surface expression of HLA-ABC, IL13RA2, ITGB1, NCAM1 and PDGFRA of parental (NT), control (siCTR) and GOLIM4-silenced (siGOLIM4) U251 and U87 GB cells was analyzed by flow cytometry. Protein expression levels were determined by the ratio of fluorescence intensity mean (n=3 to 4); *p* values were indicated on the top of the graph.

GOLIM4 modulation with specific siRNA did not affect GB cell proliferation (**Fig.S3A**); cell adhesion to collagen, fibronectin and matrigel composed of multiple extracellular matrix proteins (**Fig.S3B**); or cell migration using a wound healing assay (**Fig.S3C**). However, down-regulation of GOLIM4 expression reduced cell-cell adhesion accessed by a cell aggregation assay (**Fig.4A**) and increased GB cell migration in a Boyden chamber assay (**Fig.4B**).

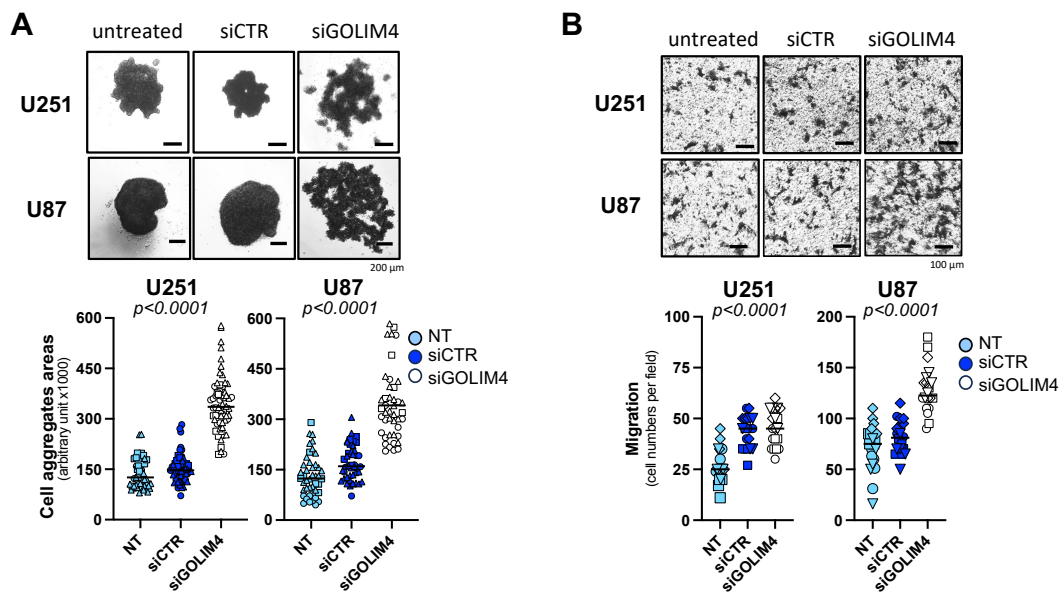


Figure 4. GOLIM4-dependent regulation of GB cell-to-cell adhesion and migration

(A) Parental (NT), control (siCTR) and GOLIM4-silenced (siGOLIM4) U251 and U87 GB cells was analyzed in a cell aggregation assay for testing cell-to-cell adhesion. Surface of cell aggregates were calculated using Fiji. High aggregate's size corresponds to the low cell-to-cell adhesion (n=3); *p* values were indicated on the top of the graph. **(B)** Parental (NT), control (siCTR) and GOLIM4-silenced (siGOLIM4) U251 and U87 GB cells was using in a Boyden chamber migration assay. Migration was quantified by the mean of number of migrated cells observed per field (n=3; 5 fields per experiments); *p* values were indicated on the top of the graph.

Overall, these results demonstrate a novel IRE1/XBP1s/GOLIM4 regulon that controls cell surface and secreted proteins including adhesion molecules leading to affect cell-to-cell adhesion and migration in GB.

Discussion

In the past years, we have demonstrated the important role of the ER stress sensor IRE1 in GB (18,19,23), in particular in the tumor microenvironment remodeling by affecting the angiogenesis [292] and immune infiltration [290], favoring the secretion of growth factors, cytokines and chemokines. In this context, we have hypothesized that IRE1 could more generally influence the protein secretion by directly impacting on the molecular machinery of the SP. In this study, we show that the activation of the IRE1/XBP1s branch of the UPR induces GOLIM4 expression, a Golgi-associated protein, that in turn modulates the presence of specific cell surface proteins involved in adhesion/migration of GB cells, hence identifying a new molecular link between IRE1 activation of cell adhesion/migration.

The molecule GOLIM4 (for Golgi integral membrane protein 4), also named GPP130 (for phosphor-protein 130 kDa) localizes to the cis-Golgi, and constitutively cycles between early endosomes, the Golgi and the plasma membrane [303,304]. Luminal pH of cellular compartments controls GOLIM4 distribution, since acidic pH causes GOLIM4 localization into early endosomes [303,305,306]. GOLIM4 is involved in sorting both invasive toxins from *Shigella genus* [307] and endogenous secreted proteins into the early endosome-to-Golgi route [303]. Importantly, manganese induces GOLIM4 oligomerization via its sortilin-mediated addressing into lysosomes for degradation [303,308,309]. More recently, GOLIM4 has been described as a novel oncogene in human head and neck cancer by impacting on tumor cell division and resistance to apoptosis [310]. GOLIM4 is also part of the chromosomal 3q amplicon found in multiple tumor types including head and neck, lung, and esophagus cancers; that encodes key regulators of secretory vesicles driving the tumor secretory addiction [311]. In this work, we demonstrate that GOLIM4 down-regulation affects surface expression of molecules including those involved in cell adhesion and migration. One could speculate that

GOLIM4 might contribute to the recycling of cell surface proteins by extracting them from the degradative route. This trafficking route from the early endosomes back into the Golgi apparatus might facilitate the maintenance of a certain protein level at the cell surface. Alternatively, GOLIM4 could directly act at the Golgi-to-secreted vesicles interface to mediate protein export to the plasma membrane. The precise mechanisms by which these targets are selected by GOLIM4 need to be further explored to better understand consequences on GB cell biology. Of note at the Golgi lumen, GOLIM4 bridges the calcium channel ATP2C1 to the Golgi phosphoprotein 3 GOLPH3 to allow the loading of calcium/CAB45-associated cargoes and the vesicle scission from the Golgi membrane [311]. These molecular partners could participate to the selectivity of surface proteins recycled by GOLIM4.

In previous studies, the ER stress sensor IRE1 has been involved in regulating molecular actors of the SP associated with the ER and the ERGIC compartments. Through XBP1s induction, IRE1 induces COPII components SEC22, SEC23 and SEC24 [237,238], the ERGIC associated cargo receptor KDELR [237,256] and COPI molecules COPA, COPB1/2, COPE and COPG [237]. This work adds new inputs on how IRE1 could affect protein secretion by modulating the protein trafficking between the plasma membrane, the early endosomes and the Golgi apparatus. Furthermore, this novel IRE1-dependent level of protein secretion of surface molecules such as ITGB1 and NCAM1 and directly impacts important GB functions including cell adhesion and migration. The current work implies that targeting IRE1 signaling might impede GB aggressiveness by attenuating tumor secretory functions that influence tumor cell invasion, inflammation and immunity [290].

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Author Contributions

KB, MN, SM, LN – methodology, investigation, formal analysis; EL, EC – conceptualization, funding acquisition, writing (review and editing); TA – supervision, conceptualization, methodology, investigation, formal analysis, funding acquisition, project administration, writing (original draft, review and editing) (<https://www.casrai.org/credit.html>).

SUPPLEMENTARY INFORMATION

IRE1-dependent GOLIM4 expression controls protein secretion to modulate glioblastoma cell adhesion and migration. Ketsia Bakambamba et al.

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SUPPLEMENTAL METHODS AND MATERIALS

Cell proliferation – Parental, control and GB cells silenced for GOLIM4 were cultured in 96-well plates at the concentration of 1×10^3 cells/well and the growth rate was measured daily for 6 days using the Incucyte apparatus (SX5 Live-Cell Analysis System, Essen BioScience, Germany). Phase images were taken every 24 hours and were recorded (400 ms exposure, 10x lens) for each condition in triplicate. Images were analyzed using the Adherent Cell-by-cell on phase algorithm from Incucyte 2022B Rev2 software. The proliferation index was given by the ratio of the specific index observed with tested cells and the specific index observed with cells used at the beginning of the experiment.

Cell adhesion to extracellular matrix – Ninety-six-well plates were coated with a filtered solution of 400 $\mu\text{g/mL}$ rat tail collagen I, 1 mg/mL fibronectin and 1% matrigel solution (BD Biosciences) in PBS. Parental, control and GOLIM4-silenced U251 and U87 cells (25,000 cells) were plated for time points 0, 15, 30 60 and 120 minutes. Medium and unattached cells were aspirated. Wells were washed with PBS and attached cells were stained with the WST1 reagent. The percentage of cell attachment was calculated by the ratio of the specific OD observed with tested cells and the specific OD observed with total cells used at the beginning of the experiment.

Wound healing migration assay – Parental, control and GB cells silenced for GOLIM4 were cultured in 96-well plates at the concentration of 30,000 cells/well for 24 hours. Wound on the cell monolayers was applied using a wound maker (Essen BioScience). The cell migration rate was measured daily for 48 hours using the Incucyte apparatus. Phase images were taken every 2 hours and were recorded (400 ms exposure, 10x lens) for each condition in triplicate. Images were analyzed the Schrach Wound algorithm from Incucyte 2022B Rev2 software.

SUPPLEMENTAL TABLES

Table S1. Antibodies used in the study

Targets	Species / isotype		Clone / RRID	Compagny
<i>for western-blot</i>				
ACTIN	mouse mAb		AC-74 / AB_476743	Sigma Aldrich
GOLIM4	mouse mAb		XY-2 / AB_2247835	Santa Cruz
IRE1	rabbit mAb		14C10 / AB_823545	Cell Signaling
Targets	Species / isotype	Fluo.	Clone / RRID	Compagny
<i>for flow cytometry</i>				
controls	mouse mAb (IgG1)	FITC	MOPC21 / AB_2891079	BioLegend
	mouse mAb (IgG2a)	FITC	MOPC173 / AB_2884007	BioLegend
	mouse mAb (IgG2b)	FITC	27-35 / AB_396085	BD Biosciences
	mouse mAb (IgG1)	PE	X40 / AB_400130	BD Biosciences
	mouse mAb (IgG2a)	PE	G155-178 / AB_11151914	BD Biosciences
	mouse mAb (IgG1)	APC	MOPC21 / AB_2888687	BioLegend
	mouse mAb (IgG2b)	APC	27-35 / AB_398612	BD Biosciences
	mouse mAb (IgG1)	BV421	A85-1 / AB_2737664	BD Biosciences
CD44	mouse mAb (IgG2b)	APC	G44-26 / AB_398683	BD Biosciences
CD109	mouse mAb (IgG1)	PE	TEA 2/16 / AB_396311	BD Biosciences
HLA-ABC	mouse mAb (IgG2a)	FITC	W6/32 / AB_2566253	BioLegend
ICAM1 (CD54)	mouse mAb (IgG1)	APC	HA58 / AB_395901	BD Biosciences
IL13Rα2 (CD213a2)	mouse mAb (IgG1)	APC	SHM38 / AB_2562583	BioLegend
ITGB1 (CD29)	mouse mAb (IgG1)	BV421	MAR4 / AB_2741751	BD Biosciences
NCAM1 (CD56)	mouse mAb (IgG2b)	FITC	NCAM16.2 / AB_397180	BD Biosciences
PDGFRA (CD140a)	mouse mAb (IgG2a)	PE	α R1 / AB_2737804	BD Biosciences
THY1 (CD90)	mouse mAb (IgG1)	FITC	5E10 / AB_893429	BioLegend

Table S2. Primers used in the study

Gene	Forward primer	Reverse primer
ACTIN	5'-CATGGGTGGAATCATAATGG-3'	5-AGCACTGTGTTGCGCTACAG-3'
GAPDH	5'-AAGGTGAAGGTCGGAGTCAA-3'	5'-CATGGGTGGAATCATAATGG-3'
GOLIM4	5'-ATGAGCCTCGTGAACAAGGACC-3'	5'-CTCTCACTTGCTCGGCTTCTTC-3'
IRE1	5'-GCCACCCTGCAAGAGTATGT-3'	5'-ATGTTGAGGGAGTGGAGGTG-3'
MTTP	5'-AGGCTGTCAGAACTTCCTGGC-3'	5'-GTCTGAGCAGAGGTGACAGCAT-3'
P4HB	5'-AGGCTGATGACATCGTGAAC-3'	5'-GGTATTTGGAGAACACGTCCTG-3'
STX6	5'-CACGAATTGGAGAGCACTCAGTC-3'	5'-GAGGATGAGCACAACCAACAGG-3'
XBP1s	5'-TGCTGAGTCCGCAGCAGGTG-3'	5'-GCTGGCAGGCTCTGGGGAAG-3'

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. IRE1/XBP1s-dependent regulation of GOLIM4 in GB cells

(A) Parental (NT), control (siCTR) and IRE1 silenced (siIRE1) U251, U87, RADH85 and RADH87 GB cells were tested for IRE1 mRNA down-regulation by RT-Q-PCR. mRNA expression levels were relative to parental cells (n=3 to 4); *p* values were indicated on the top of the graph. **(B)** Parental (NT), control (siCTR) and IRE1 silenced (siIRE1) U251, U87 and RADH87 GB cells were tested for IRE1 protein down-regulation by western-blot. Protein expression levels were relative to parental cells (n=3); *p* values were indicated on the top of the graph. **(C)** Parental (NT), control (siCTR) and IRE1 or GOLIM4 silenced (siIRE1 or siGOLIM4 respectively) RADH85 GB cells were tested for IRE1 and GOLIM4 protein down-regulation by western-blot. Protein expression levels were relative to parental cells (n=3); *p* values were indicated on the top of the graph. **(D and E)** Parental (NT), control (DMSO and siCTR) IRE1-modulated (MKC and siXBP1) U251, U87 and RADH87 GB cells were tested for XBP1s mRNA down-regulation by RT-Q-PCR. IRE1 inhibition was performed using MKC8866 (MKC) **(D)** or XBP1s silencing using specific siRNA **(E)**. mRNA expression levels were relative to parental cells (n=3 to 4); *p* values were indicated on the top of the graph.

Figure S2. GOLIM4-dependent regulation of surface proteins in GB cells

Surface expression of CD44, CD54 (ICAM1), CD90 (THY1) and CD109 of parental (NT), control (siCTR) and GOLIM4-silenced (siGOLIM4) U251 and U87 GB cells was analyzed flow cytometry. Protein expression levels were determined by the ratio of fluorescence mean (n=3 to 4); *p* values were indicated on the top of the graph.

Figure S3. GOLIM4-dependent regulation of GB cell proliferation, cell adhesion to extracellular matrix and cell migration

(A) Parental (NT), control (siCTR) and GOLIM4 silenced (siGOLIM4) U251 and U87 GB cells were tested for cell proliferation using the Incucyte apparatus. The proliferation index is given by the ratio of the specific index observed with tested cells and the specific index observed with cells used at the beginning of the experiment (n=3); *p* values were indicated on the top of the graph. **(B)** Parental (NT), control (siCTR) and GOLIM4 silenced (siGOLIM4) U251 and U87 GB cells were used in a cell adhesion to extracellular matrix assay with collagen (COL), fibronectin (FN) and matrigel substrates. Results were expressed as percentage of adherent cells (n=3); *p* values were indicated on the top of the graph. **(B)** Parental (NT), control (siCTR) and GOLIM4 silenced (siGOLIM4) U251 and U87 GB cells were tested in a wound healing assay for cell migration using the Incucyte apparatus. Results were expressed as percentage

of migrating cells obtained with the Incucyte software (n=3); *p* values were indicated on the top of the graph.

FIGURE S1

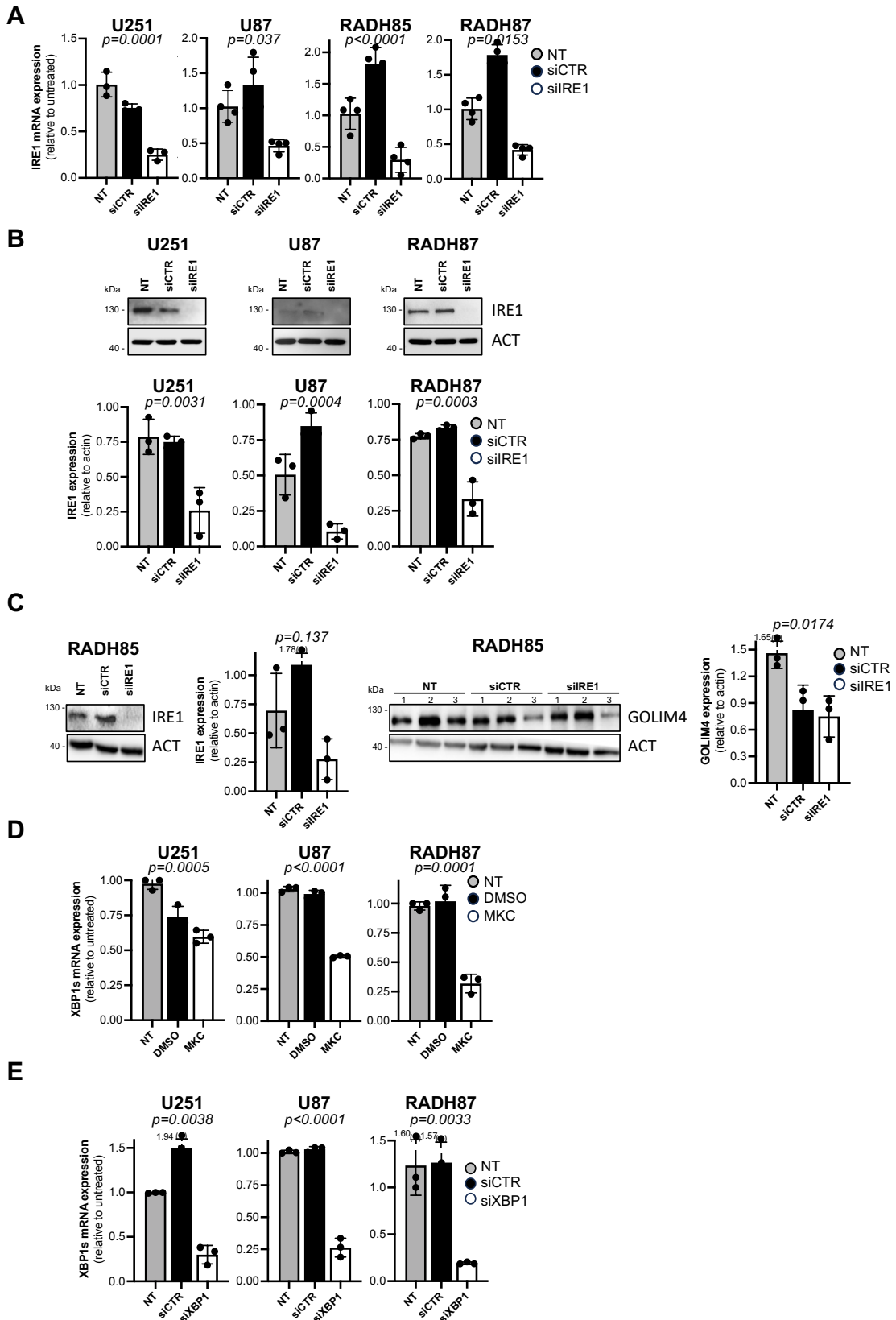


FIGURE S2

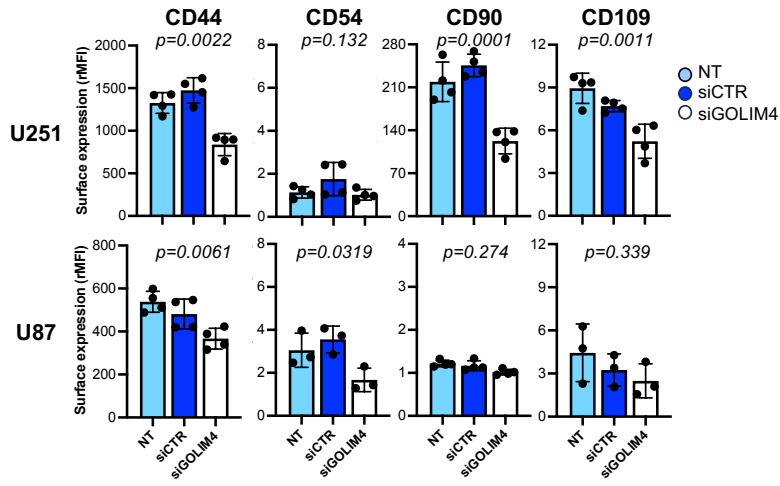
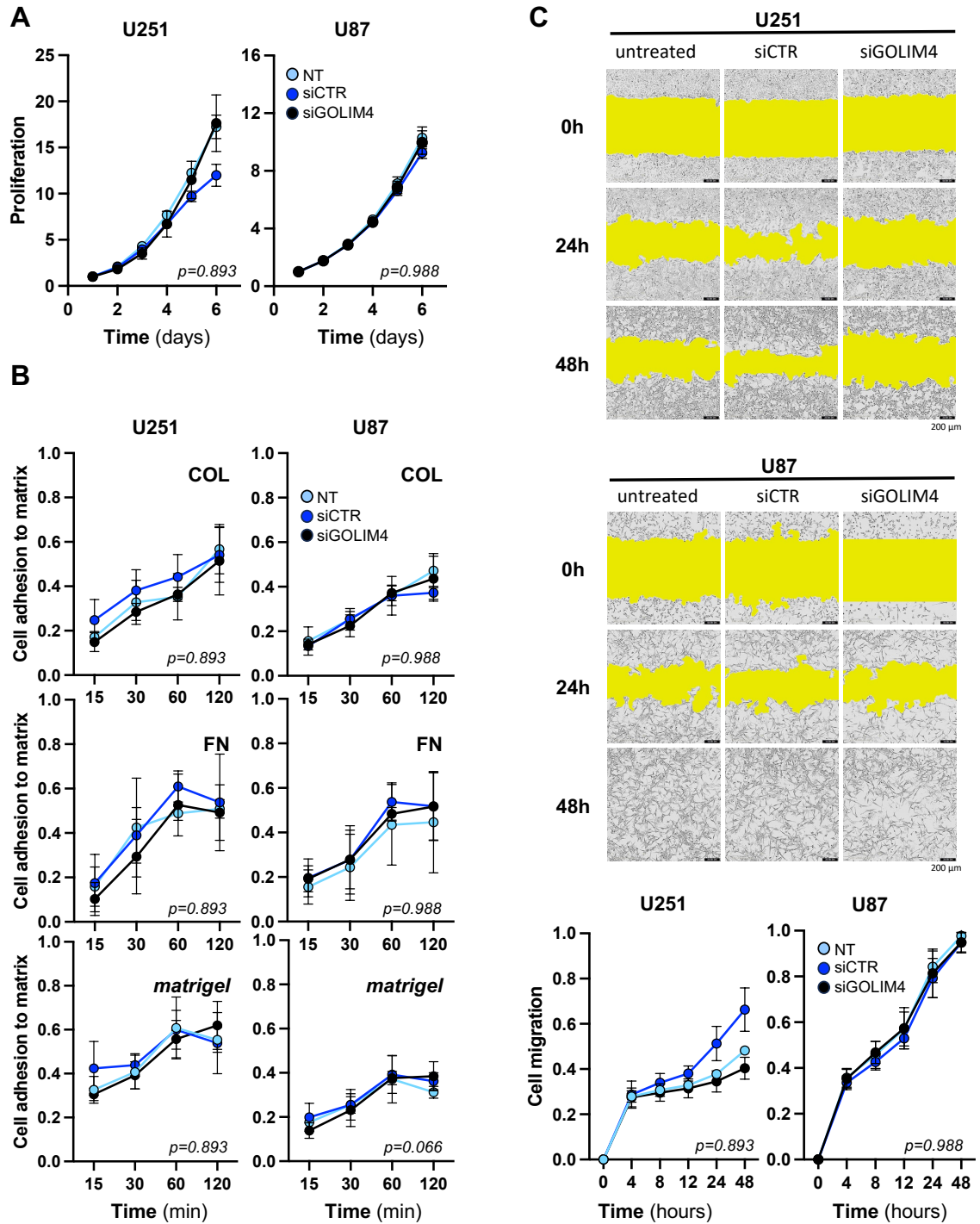


FIGURE S3



PART II: Other molecular targets studied

Identification of molecules involved in the early secretory machinery potentially regulated by IRE1

In the beginning of the thesis, we tried a different approach to identify the role of IRE1 in the regulation of molecular actors of the early secretory pathway in GB cells but after spending almost a year and a half on this approach. It became evident that this methodology was not rigorous enough to respond to our fundamental hypothesis, here is what we did: Molecules involved in the early machinery of protein secretion were extracted from using the gene ontology website AmiGO developed by the Gene Ontology (GO) consortium. In total, 246 human genes were listed including genes from ‘COPI-coated vesicle budding’ GO term (GO:0035964, named COPI), genes from ‘COPII-coated vesicle budding’ GO term (GO:0090114, named COPII), genes from the GO term ‘endoplasmic reticulum exit site’ (GO:0070971, named ERES) and 132 genes from the ‘endoplasmic reticulum-Golgi intermediate compartment’ GO term (GO:0005793, named ERGIC). In addition, 4 other molecules described in the bibliography made on the secretory pathway have been included such as GOLIM4, SORT1, CYTH1 and ARF1 (**Figure 9A**).

To investigate the possible role of IRE1 on regulating molecular actors of the early secretory pathway, we generated a list of genes potentially regulated by IRE1 activity using a series of ‘in-house’ datasets. These datasets were previously generated from transcriptome and proteome analysis of primary GBM (RADH85 and RADH87), and immortalized (U87) cell lines modified for IRE1 activity (i.e., cell lines over-expressing IRE1 that increases IRE1 activity) and a dominant negative form of IRE1 (that lacks the RNase domain, and strongly reduces IRE1 activity). In addition, datasets from mRNA directly cleaved in vitro by a recombinant IRE1 and from proteins that directly interact with IRE1 (in BioID experiments using HeLa cells treated or not by ER stressors) were used. In total, 5347 molecules regulated by IRE1 identified in these datasets were intersected with the list of molecules involved in the early protein secretory pathway (**Figure 9A**). Overall, 66 molecules in which half of them were exclusively involved in the ERGIC GO term were identified as candidate molecules (**Figure 9A**).

To fine-tune our selection, we assessed their relevance in GB biology, by first analyzing their expression in GB specimens compared to non-tumor tissues; their correlation with IRE1 and XBP1s signature and their impact in GB patient survival using TCGA datasets (Gliovis). Importantly, 5 molecules including PDIA1, PDCD6IP, TMED10, VMP1 and YIF1B had a significant impact on GBM patients’ survival and were further analyzed. As summarized in **Figure 9B**, all the targets showed no significant changes at their mRNA level when IRE1 was silenced by siIRE1 or treated with its RNase inhibitor MKC8866 (results were not shown here). These results compelled us to reconsider our methodology and pursue another approach, which resulted in the findings presented in the article.

A.



B.

Molecular Target	Effect of IRE1 treatment on mRNA Expression
TMED10	No significant change
VMP1	No significant change
YIF1B	No significant change
PDCD6IP	No significant change

Figure 9. (A) Identification of molecular actors of the early secretory pathway potentially regulated by IRE1. Related genes extracted from AmiGO and linked to secretory pathway were intersected with 'in-house' datasets of genes possibly regulated by IRE. **(B) Candidate molecules** modulation upon IRE1 knockdown by silRE1 and MKC8866 treatment.

Primer pairs used for qPCR: **TMED10** (For: 5'-CTCAAAGAGGATGCAACCAAGG-3';

Rev: 5'-TCACGAGTTGGTCAGGTATCCG-3'), **VMP1**(For: 5'-

TTGGAACAGGGCTGCACACCTT-3'; Rev: 5'-

TCAGGATAGGGTGGTTCGGGAA-3'), **YIF1B**(For: 5'CATCCTGCTCAGCCTCTATCTG-3'; R

ev: 5'-CACCAGGTAGTAGCCAATCTTCC-3'), **PDCD6IP**(For: 5'-

GCTCAGATGAGAGAAGCCACCA-3'; Rev: 5'-AGTCTGGATGCCTCCCTGTTCA-3')

Chapter 3: Conclusion

Previous studies have highlighted the role of IRE1 in regulating cell migration and invasion in GB cells. Notably, through the modulation of key molecules like SPARC and PER1 [159,161], which are associated with enhanced tumor growth, migration and invasion in GB cells. Building on these findings, our study has identified GOLIM4 as a novel candidate regulated by IRE1, further extending IRE1 influence over the secretory machinery and features like migration and cell adhesion. Through our investigation, we discovered that GOLIM4, known for its role in protein trafficking, also significantly promotes the migratory capacities of GB cells. This finding broadens our understanding of how IRE1 modulates multiple pathways to enhance GB cell invasion. Further studies still need to be performed to better understand how this novel IRE1/XBP1s/GOLIM4 operon contributes to GB progression.

Discussion

Given that IRE1 activity has been shown to control important features of GB biology such as angiogenesis, cell migration, remodeling of the TME, and the recruitment of myeloid cells by regulating key factors responsible for these processes [107,159,163,164], we aimed to investigate a broader role of IRE1 in the protein secretory machinery of GB cells. Specifically, our goal was to understand whether IRE1 regulates components of the secretory pathway that might affect other cellular functions beyond the ones already described. By identifying key effectors within the secretory pathway machinery that are directly modulated by IRE1, we hoped to uncover new insights into how IRE1 might influence additional tumor-promoting features in GB cells.

IRE1 RNase activity is a key regulator of protein secretion in glioblastoma cells

The study of the N-glycoproteome and surface proteins under IRE1 inhibition showed a significant downregulation of several N-glycoproteins and surface proteins even though their mRNA expression levels were not reduced in most cases. On top of that, analysis of the total proteome revealed no major impairments in global protein synthesis. These results suggest that IRE1 knockdown most likely disrupts critical post-translational modifications necessary for proper protein folding and trafficking within the secretory pathway rather than affecting protein synthesis and degradation. This disruption could impair either the maturation or the transport of proteins, causing them to fail in reaching their intended cellular destinations. The IRE1-XBP1s axis is known to regulate genes encoding molecular chaperones and enzymes critical for protein folding and quality control, such as DNAJs and PDIs [312–315]. Although the direct relationship between IRE1 and N-glycosylation is less well-characterized, one study showed that XBP1s activation can shift the distribution of N-glycans on membrane-bound and secreted proteins [316]. Additionally, the IRE1-XBP1s pathway has been shown to influence N-glycosylation through its impact on the hexosamine biosynthesis pathway [317], thereby affecting protein trafficking. Furthermore, these results suggest that the key effectors of the secretory pathway, such as those involved in vesicle formation and transport (e.g., COPII complex), may be impaired due to IRE1 knockdown. This could explain the significant reduction in surface proteins. Indeed, studies have already demonstrated that IRE1, via XBP1s, regulates several COPII and COPI components, as well as ERGIC associated cargo receptor KDELR [318–320].

Taken together, these findings align with the rationale that IRE1 could directly impact protein secretion by modulating the molecular actors of the secretory pathway and indicate that IRE1 is crucial for the processing and trafficking of several proteins in GB cells and could affect cellular processes that rely on these proteins such as cell adhesion and migration as seen in these results which are particularly relevant in GB progression.

Identification of molecular actors potentially regulated by IRE1 and involved in GB cells secretion machinery

In this thesis, we aimed to investigate whether IRE1 directly regulates molecular components of the early secretory pathway in GB cells. To achieve this, we first extracted a list of genes involved in the secretory pathway using the Gene Ontology website (AmiGo) [321]. This initial list was cross-referenced with several ‘in-house’ datasets previously generated in our lab, comprising transcriptomic and proteomic analyses of primary GB cell lines (RADH85 and RADH87 generated as described in [322]), and immortalized GB cell lines (U87, U251 described in [14]), all of which were modified for IRE1 activity. Specifically, these cell lines were either treated with a pharmacological IRE1 RNase inhibitor MKC8866 or genetically modified to overexpress a dominant-negative form of IRE1 that impairs RNase function as described in [162]. Furthermore, we cross-referenced the GO-derived list with additional datasets, including those involving mRNAs directly cleaved *in vitro* by recombinant IRE1 and proteins that interact with IRE1, based on BioID (proximity proteomics [323]) experiments. This integrative approach resulted in the identification of 66 candidate molecules, of which five (PDIA1, PDCD6IP, TMED10, VMP1, and YIF1B) were prioritized based on their expression profiles in GB compared to non-tumor tissues and their impact on patient survival, as analyzed through public TCGA datasets available on the website GlioVis [324].

However, when we assessed experimentally the regulation by IRE1 of these targets using siRNA of IRE1, XBP1, and the inhibitor MKC8866, we observed that none of these molecules were significantly regulated at mRNA level. These unexpected results prompted us to question our initial method of candidate selection, as our initial approach, which combined multiple data source, had generated a list that was too broad and potentially irrelevant to IRE1’s regulation in GB. Based on this, we revised our strategy and narrowed our focus by intersecting only transcriptomic data from our datasets with the GO list. Recognizing the role of XBP1s in the protein secretory pathway, we also retrieved XBP1s targets based on CHIP-Atlas resource

[325]. This refined approach resulted in a list of 57 candidates. After further filtering based on IRE1 signatures identified in [162] and patient survival data from TCGA, we identified five key molecules: GOLIM4, STX6, MTTP, PDIA1 and ADAM9 as described in the article.

One limitation of this approach became apparent with the identification of ADAM9, which, despite being a candidate, was not relevant as an effector or modulator of the early secretory pathway. This highlights the importance of fine-tuning selection criteria and demonstrates the challenges inherent in identifying meaningful targets using large datasets. All in all, this refined methodology has allowed us to identify novel molecular candidate regulated by IRE1 that could be relevant to GB pathology.

IRE1 controls GOLIM4 a molecular actor involved in the secretory pathway in GB cells

GOLIM4 has been identified as an IRE1 target in the secretory machinery of GB cells. In this study, we have demonstrated its role in regulating protein trafficking and its impact on cell-cell adhesion and migration in GB cells. Our findings showed that GOLIM4 knockdown decreases cell-cell adhesion thereby increases migration, suggesting that GOLIM4 plays a suppressive role in GB cell motility.

GOLIM4, also known as GPP130 or GOLPH4, is a cis-Golgi protein that cycle between the Golgi, early endosomes, and the plasma membrane, maintaining the surface expression of critical proteins [326]. In GB cells, GOLIM4 may act by recycling cell surface proteins involved in adhesion, ensuring that they are retained at the membrane to support intercellular contacts. When inhibited, these proteins might be misrouted towards degradation pathways, leading to reduced adhesion and enhanced migratory capacity. This suggests that GOLIM4 plays a protective role against the migratory behavior that is characteristic of GB, by facilitating the retention of adhesion molecules at the cell surface. Interestingly, this suppressive role contrasts with previous studies identifying GOLIM4 as an oncogene in other cancers, such as head and neck cancer [327], where it promotes tumor cell division and resistance to apoptosis. Moreover, GOLIM4 is part of the chromosomal 3q amplicon, a region amplified in multiple cancers, including lung and esophageal cancers [328]. This amplicon encodes regulators of the secretory vesicles that drive tumor progression, and GOLIM4's role in secretory trafficking could similarly contribute to GB behavior by maintaining surface protein levels required for cell migration and invasion. However, a contrasting observation was made in breast cancer, where GOLIM4 has been shown to function as tumor suppressor [329]. Its reduction by miR-

105-3p promoted cancer cell proliferation, migration and invasion. This dual role of GOLIM4 acting as an oncogene in some cancers and tumor suppressor in others highlights the complexity of its function, which seems to be cancer dependent.

In the case of GB, GOLIM4's inhibitory role in migration may be related to its function in controlling the trafficking of adhesion molecules. One possibility is that GOLIM4 could mediate the transport of these proteins from early endosomes back to the Golgi apparatus, where they are sorted for recycling to the plasma membrane, thus preventing their degradation. This trafficking route would facilitate the maintenance of adhesion proteins at the cell surface, supporting the cohesive properties of tumor cells and limiting their migratory capacity. Conversely, when GOLIM4 is inhibited, adhesion proteins may be misdirected toward lysosomal degradation, reducing cell-cell adhesion and enabling tumor cells to migrate more freely.

At the molecular level, GOLIM4 interacts with partners such as ATP2C1 and GOLPH3 in the Golgi lumen, which are involved in the loading of calcium/CAB45-associated cargoes and vesicle scission from the Golgi membrane. These molecular interactions may contribute to the specificity of proteins recycled by GOLIM4, including those involved in cell adhesion. Understanding how these partners influence GOLIM4's role in protein trafficking could offer new insights into its function in GB biology. Finally, while GOLIM4 appears to limit cell migration in GB, its complex role in other cancers, such as breast cancer and head and neck cancer, suggests that its function is highly dependent on the tumor microenvironment and molecular context.

IRE1/XBP1s/GOLIM4 axis regulates migration and cell adhesion in GB cells

We hoped to uncover new insights into how IRE1 might influence additional tumor-promoting features in GB cells. Firstly, the N-glycoproteome and surface proteins studies have highlighted that the proteins down-regulated were mainly involved in cell adhesion and migration. Upon identifying GOLIM4 as one effector of the secretory pathway that is regulated by IRE1, we investigated its functional role in GB cells by silencing it and analyzing its impact on several surface proteins. Our surface analysis revealed that this effector modulates several cell surface proteins that are directly involved in cell adhesion and migration processes.

This suggested a potential link between IRE1-regulated secretory machinery and cellular behavior, particularly related to adhesion and motility, which are critical for GB progression and invasion. To explore this further, we performed a series of functional assays to

assess the consequences of silencing GOLIM4 in GB biology. Interestingly, we found that while cell proliferation was unaffected, cell-cell adhesion was notably reduced. This suggests that GOLIM4 may specifically regulate proteins that facilitate intercellular connections, which are crucial for maintaining tissue structure and communication between tumor cells. A decreased level of cell-cell adhesion could promote the dissemination of tumor cells within the brain parenchyma, contributing to GB's notorious invasiveness. In contrast, despite observing a reduction in surface proteins associated with cell adhesion, our adhesion assays revealed no significant impairment in the cells' ability to adhere to the ECM. This indicates that while cell surface proteins are altered, there may be alternative pathways that preserve matrix adhesion. Moreover, the wound healing assay demonstrated no changes in overall migration rates when cells were observed in a two-dimensional space. However, in the Boyden chamber migration assay, which assesses migration through a membrane barrier and better mimics *in vivo* invasion, we observed a significant increase in migratory capacity on one of our cell lines. This points to the possibility that reduced cell-cell adhesion could facilitate greater individual cell motility, enhancing the ability of GB cells to penetrate through tissue barriers. Taken together these results suggest that GOLIM4 silencing, rather than IRE1 itself, specifically alters cell-cell adhesion in ways that enhance migration in GB cells. Since GB cells are already highly migratory, losing cell-cell adhesion further promotes their ability to invade surrounding tissues, which is a hallmark of invasiveness in GB. Furthermore, we encountered certain experimental limitations. U87 GB cells which are typically more migratory *in vitro* [330], exhibited the expected increase in migration upon silencing GOLIM4, whereas U251 cells lines, shows, did not show significant in migration, highlighting the cell-type-specific responses in GB models. Exploring additional cell lines like the RADH87 primary GB lines validated in this study will help us have more insights on this mechanism.

Further experiments are required to fully understand the downstream mechanisms through which IRE1 and GOLIM4 influence cell-cell adhesion and migration. Rescue experiments, overexpressing GOLIM4 in the IRE1 knockdown cells could help determine if this rescues the migratory phenotype. If restoring GOLIM4 reverses the increased migration, it would strengthen the argument that the target is mediating the effects of IRE1 on migration.

Conclusions & Perspectives



Glioblastoma as presented throughout this thesis manuscript is a deadly disease that requires further studies and better characterization to be able to offer hope to the patients in the future. One key feature responsible for the complexity of the disease is the very heterogenous pathophysiology that renders the disease more aggressive and incurable till date. Studies have shown that glioblastoma thrives in harsh conditions such as hypoxia, nutrient deprivation and increased protein synthesis to constitute the aggressive microenvironment and activates different adaptive responses to be able to survive and proliferate under these conditions. We have shown that glioblastoma cells are able to secrete several molecules including cytokines, growth factors and pro-angiogenic factors, which promote the formation of new blood vessels to supply the tumor with nutrients and oxygen. GB cells are also able to modulate their extracellular matrix by secreting several proteins that are involved in cell adhesion and movement to influence cell migration and invasion. Through a review, we have explored the organelles responsible for the early protein secretion and especially the ER, its structure, functions, and perturbations, detailing the effects and consequences of ER stress. We introduced the UPR, the major cellular stress response related to the ER and its different transducers such as IRE1 and their roles in cancer biology. Over the years, the role of IRE1 in GB has been established particularly in how it reshapes its microenvironment and promotes the secretion of several pro-tumoral factors.

This thesis has highlighted the important role of IRE1 in regulating the secretory machinery in GB cells, particularly in modulating key effectors responsible for proteins maturation and trafficking. Through our exploration, we identified GOLIM4, a cis-Golgi protein. When silenced, GOLIM4 led to the alterations in cell-cell adhesion and enhanced migration. This shows the involvement of IRE1 in maintaining proper protein transport and cell surface protein expression but also in controlling important GB features such as migration.

However, further studies are needed to fully elucidate the molecular mechanisms linking IRE1 to secretory pathway regulation, cell adhesion, and migration. Understanding these connections may reveal new therapeutic avenues to target IRE1 in GB, in combination with strategies aimed at reducing tumor invasiveness and resistance to conventional therapies.

Several studies have shed light on the importance of IRE1 in GB pathophysiology suggesting why targeting it may offer a promising therapeutic approach, particularly when combined with conventional treatments. Previously in the lab, a novel preclinical murine model of GB that mimics the standard of care used for human patients has been developed and combined with the IRE1 inhibitor MKC8866 which resulted in an increased efficacy of combined radiotherapy and chemotherapy [331]. Due to the challenge of crossing the BBB,

novel inhibitors are currently being developed in our lab to overcome this limitation [146,147]. When combined with standard treatments, these new inhibitors have shown a significant reduction in tumor volume and an extension of overall survival in mouse models. These findings emphasize the potential therapeutic benefits of incorporating IRE1 inhibitors into existing GB therapies. Furthermore, previous studies from our lab have demonstrated that IRE1 is overexpressed in the MES subclass of GB [162]. This suggests that identifying patient subgroups based on their IRE1 signaling profiles, may help tailor therapies and ultimately improve treatment outcomes for GB patients.

APPENDIX

Appendix: foreword

Since 1994, a group of researchers initiated a workshop focusing on the ER chaperone calreticulin. Since then, the workshop has expanded its thematic to explore all ER-related functions. The 14th workshop which took place in Saint-Malo once again brought together international experts to discuss the latest findings and advances concerning ER related topics. A comprehensive report summarizing the main topics covered during this event can be found just after.

Contributions: all the authors of this paper coordinated the writing process by being in constant communication with each other. The principal investigators Tony Avril, Éric Chevet, Frédéric Delom and Elodie Lafont commented and reviewed the original draft.

I wrote sections 5, the conclusion and the appendix of this review.

The next document of this appendix is a pre-review that Elodie Lafont and I co-wrote. It is a commentary on the preprint “**Identification of Glucosidase II that regulates STIM1 activation dynamics**”

This pre-review was written following our discussions during the Journal club within the PROSAC group and was published on the PreReview platform.

Review 2: “Endoplasmic reticulum homeostasis- from molecules to organisms”



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REVIEW

WILEY

Endoplasmic reticulum homeostasis—From molecules to organisms: Report on the 14th International Calreticulin Workshop, Saint Malo, France

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Abstract

The Calreticulin Workshop, initiated in 1994 by Marek Michalak in Banff (Alberta, Canada), was first organized to be an informal scientific meeting attended by researchers working on diverse biological questions related to functions associated with the endoplasmic reticulum (ER)-resident lectin-like chaperone and applied to a wide range of biological systems and models. Since then, this workshop has broadened the range of topics to cover all ER-related functions, has become international and has been held in Canada, Chile, Denmark, Italy, Switzerland, UK, USA, Greece and this year in France. Each conference, which is organized every other year (pending world-wide pandemic), generally attracts between 50 and 100 participants, including both early career researchers and international scientific leaders to favour discussions and exchanges. Over the years, the International Calreticulin Workshop has become an important gathering of the calreticulin and ER communities as a whole. The 14th International Calreticulin Workshop occurred from May 9–12 in St-Malo, Brittany, France, and has been highlighted by its rich scientific content and open-minded discussions held in a benevolent atmosphere. The 15th International Calreticulin Workshop will be organized in 2025 in Brussels, Belgium.

KEYWORDS

AGR2, cancer, chemotherapy, Calreticulin, degenerative diseases, endoplasmic reticulum, ER stress, immune regulation, unfolded protein response

1 | INTRODUCTION

An international workshop dedicated to the study of calreticulin and other endoplasmic reticulum (ER) chaperones has been taking

place every 2 years since 1994. The Calreticulin workshop derives its name from the calreticulin protein (CALR), an ER-resident lectin-like chaperone initially identified in 1974 as a high-affinity Ca²⁺ binding protein in the muscle sarcoplasmic reticulum.¹ With a profound

Federico Di Modugno, Ketsia Bakambamba, Marianne Guilbard, Simon Le Goupil are equally contributed.

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interest in this protein and the belief in its fundamental role within the ER, Pr. Marek Michalak organized the inaugural International Calreticulin Workshop in Banff, Canada, thus marking the beginning of a biannual tradition. Subsequent workshops were hosted in various countries, including Italy, Switzerland, the USA (twice), the UK, Chile, Canada (four times), Denmark and Greece. Since the first meeting, the workshop emphasized the discussion on the structure and function of the ER, as well as the protein/lipid quality control mechanisms.²⁻⁴ After 29 years, the 14th meeting took place for the first time in France in Saint Malo, May 9–12, 2023 (Figure 1). The workshop hosted researchers from 12 different countries mostly located in North and South America, Middle East and Europe. They presented their latest findings and engaged in extensive discussions with fellow colleagues. The presentations covered a broad range of ER-related topics, including the molecular mechanisms of secretory autophagy, the role of Anterior GRadient proteins, structural characteristics of calreticulin and its functional aspects in immunity and diseases, the impact of ER stress and the unfolded protein response (UPR) in cancer and neurodegenerative diseases, and novel mechanisms controlling the functions of the early secretory pathway. A significant focus of the workshop regarded the relationship between ER biology and various diseases. This included many types of cancers (e.g. blood, brain, breast, colorectal, pancreas and lung cancers), degenerative diseases (such as age-related macular degeneration (AMD), Alzheimer's and Parkinson's diseases, multiple sclerosis, amyotrophic lateral sclerosis, Wolfram syndrome) and COVID-19 (see sections below). Collectively, this edition of the International Calreticulin Workshop presented a vision of ER functions at multiple scales from the molecule (e.g. AGR2, CALR and Spike) to the cell (e.g. protein secretion, calcium homeostasis and translation) and at last evaluated their impact in diseases such as cancer or degenerative disorders. Of note, therapeutically actionable mechanisms related

to ER functions were also described in a number of presentations thereby highlighting the enormous potential of exploring ER biology beyond fundamental science.

The Keynote lecture was given by Dr Thierry Galli (Inserm, Paris, France) on the role of unconventional protein secretion (UPS) in brain diseases. The UPS allows the secretion of cargos lacking a signal peptide or proteins that manage to escape the Golgi apparatus despite entering the ER. Cargos subjected to UPS can be further classified into different types based on their mode of transport. Types I and II are independent of vesicles or SNARE proteins, while Types III and IV do rely on those. VAMP7, a vesicular SNARE, plays a significant role in exosome, lysosome, and autophagy-mediated secretion and is an essential part of axon growth fine-tuning. This mechanism is partly dependent on the secretion of VGF (not an acronym, rather 'Victory' Growth Factor) or pro-VGF through the UPS. Pro-VGF appears to be associated with extracellular vesicles (EVs) and endo-lysosomes. Moreover, the overexpression of LRRK2 disrupts the peripheral localization of VGF in primary neurons. Taken together, these findings suggest that LRRK2 regulates the secretion of VGF/pro-VGF by interacting with VAMP7, thus influencing its trafficking and release.

2 | SESSION I—AGR/PDI

The first session aimed to clarify the functions of Protein Disulfide-Isomerase (PDIs) with a specific focus on the Anterior GRadient (AGR) protein family. There is a growing interest in understanding the novel functions acquired by the three members AGR1-3 in many diseases. It is well established that AGR proteins are ER-resident, showing both N-terminal signal peptide and C-terminal pseudo-KDEL, with foldase/holdase and ER protein homeostasis maintenance



FIGURE 1 Picture of the 14th International Calreticulin Workshop participating members in the Rotonde at the Palais du Grand Large in Saint Malo with a fantastic background.

functions. AGR proteins display variable tissue expression patterns, and their expression is regulated by different signalling mechanisms. While primarily localized to the ER, AGR proteins have also been detected in other cellular compartments such as the cytosol and extracellular environment where they exert non-canonical gain of functions. Among the AGR proteins, AGR2 has been the most studied member. It has been found to be overexpressed in most epithelial cancers, which generally correlated with poor patient outcome.⁵ ER-associated functions of AGR proteins were discussed by Dr Arvin Pierre (Glasgow University, Glasgow, UK) who highlighted the importance of AGR1 (ERp18) in the ER as a regulator of the activation of the UPR sensor ATF6 α (hereafter referred to as ATF6).⁶ ATF6 luminal C-terminal region contains two cysteine residues (467 and 618) able to form either intra- or inter disulfide chains. In unstressed cells, ATF6 predominantly exists as a monomer with some disulfide-linked oligomers detected. Upon stress, the C467-C467 interchain dimer is increased while the C467-C618 intrachain form is decreased, giving rise to the suggestion that only this dimeric form exits the ER towards the Golgi. In accord, the cleaved ATF6 luminal domain is a dimer. Of note, ERp18 controls ATF6 redox status, and thus its dimerization. Indeed, ERp18 selectively associates with ATF6 upon stress, reduces interchain disulfide and thus ERp18 overexpression impairs ATF6 trafficking to the Golgi. The interaction between ERp18 and ATF6 may prevent the premature exit from the ER in the absence of stress. Dr Roman Hrstka (Masaryk University, Brno, Czech Republic) focused on investigating the AGR2 interactome in breast cancer models. Using reversible crosslinking followed by a pull-down of AGR2 complexes and high-resolution LC-MS/MS, the team identified a complex comprising AGR2, PDIA3 and PDIA6, which was further confirmed through co-immunoprecipitation.⁷ Induction of ER stress in breast cancer cells enhanced the AGR2-PDIA3 complex formation and increased extracellular levels of AGR2, thus suggesting that the AGR2-PDIA3 interaction might contribute to AGR2 secretion. Then Dr Delphine Fessart (Inserm, Bordeaux, France) presented evidences revealing the involvement of extracellular AGR2 (eAGR2) in tumorigenic processes, prompting her group to explore the role of eAGR2 in cell plasticity. She demonstrated that eAGR2 represents a pro-oncogenic regulator of epithelial morphogenesis and tumorigenesis⁸; and that AGR3 (which presents ~70% homology with AGR2) also shows the ability to promote cell migration in cell culture models.⁹ Dr Fessart also discussed the role of AGR2 in chemoresistance demonstrating that Doxorubicin-resistant tumour cell lines (MCF7-R) displayed increased expression and secretion of AGR2. To overcome this resistance, the addition of an AGR2 blocking antibody to the culture media sensitized resistant tumour cells to chemotherapeutic treatment, suggesting that targeting eAGR2 within the tumour microenvironment might represent a promising strategy to overcome chemotherapy resistance. Dr Aeid Igbaria (Ben Gurion University, Beer Sheva, Israel) focused on the reflux of ER-resident proteins to the cytosol, a mechanism known as ER to Cytosol Signaling (ERCYS¹⁰). In stressed cells, as well as in cancer cells, PDIA1, PDIA3 and AGR2 were found to be present in the cytosol. Provided that cytosolic AGR2 (cAGR2) inhibits p53 and that cytosolic PDIA1

can interact with the cleaved form of Caspase 3 (ongoing studies on the putative biological effects of the interaction on apoptosis), this might indicate that cytosolic PDIs could contribute to stress resistance mechanisms commonly observed in chemoresistant cells. In an effort to characterize the molecular mechanisms underlying ERCYS, Dr Igbaria identified HSC70 and DNAJB12 as key players in this process. Dr Olivier Pluquet (University of Lille, Lille, France) discussed the involvement of AGR2 in gastroesophageal junction (GEJ) adenocarcinoma chemoresistance. The analysis of healthy tissues, as well as pre- and post-treatment tumour samples, unveiled the upregulation of several ER-related genes in chemotherapy-treated samples, among which AGR2 was identified. Notably, low expression of AGR2 was associated with a more favourable response to treatment. Thus, these findings raised the question of whether AGR2 could sensitize tumour cells to chemotherapy. Importantly, AGR2 was silenced in OE19 cells (adenocarcinoma of gastric cardia/oesophageal gastric junction) which resulted in increased sensitivity to chemotherapies. These findings indicate that reducing the expression of AGR2 could potentially improve the responsiveness of tumour cells to chemotherapy. Lastly, Andrea Martisova (Masaryk University, Brno, Czech Republic) presented data on the implication of AGR2 in epithelial-to-mesenchymal transition (EMT). A transcriptomic approach in lung cancer cells (A549) knocked-out for AGR2 or not and treated or not with TGF- β treatment unveiled significant changes in transcripts related to focal adhesions and arachidonic acid metabolism, two pathways involved in EMT. Notably, the knockdown of AGR2 led to a significant decrease in PGE2 biosynthesis whereas PGE2 addition resulted in an upregulation of AGR2 expression.¹¹ These results establish a connection between AGR2 expression, arachidonic acid metabolism and EMT in lung cancer cells.

3 | SESSION II—CALRETICULIN (SFC SESSION)

Many presentations in this session, sponsored by the Société Française de Cancer, focused on the structural and functional aspects of calreticulin (CALR) mutants in myeloproliferative neoplasms (MPN).¹² Pr Stefan Constantinescu and Mr Nicolas Papadopoulos, PhD Student (Ludwig Institute for Cancer Research and University of Louvain, Belgium) discussed the properties of a class of frameshift mutations in CALR, with a prevalent exemplar, namely CALR del52 (deletion of 52bp), occurring in JAK2 V617F-negative and MPL/TpoR mutant-negative MPNs. Hydrogen-deuterium exchange mass spectrometry (HDX-MS) analysis showed that CALR del52 exhibited a global modification of its conformation, resulting in increased accessibility for its chaperone role. Remarkably, changes in the C-terminal domain of CALR induced by the del52 mutation led to an alteration in the structure of the N-terminal domain. This alteration is of importance as it unmask the N-glycan binding domain, enhancing the binding capacity of immature N-glycans, thus conferring the ability of CALR del52 to further bind to the luminal/extracellular domain of the Thrombopoietin-Receptor (Tpo-R). Data also showed that

mutant CALRs are secreted from clonal cells that do not express TpoR and the plasma form behaves like a cytokine, oligomerizing with endogenous mutant CALR at the surface of TpoR expressing cells; in this way the circulating form activates the TpoR on cells belonging to the MPN clone.¹³ The specificity of binding to the TpoR extracellular domain comes from interactions between the positively charged repetitive sequences in the new tail of mutant CALR and several negatively charged patches on TpoR D1 domain.¹⁴ This CALR mutant binding to Tpo-R activates the receptor, thereby acting as a cytokine to sustain aberrant proliferation of haematopoietic cells. These findings shed light on the mechanistic role of CALR del52 in promoting the aberrant proliferation of haematopoietic cells through its interaction with Tpo-R. Dr Malini Raghavan (University of Michigan, MI USA) further described the role of CALR mutations in cell transformation focusing on the relevance of the novel C-terminal domain of CALR mutants in TPOR binding and the different modes of mutant CALR multimerization relevant to Tpo-R activation.¹⁵ In this context, enhanced degradation of CALR mutants could lead to reduced cell proliferation in some MPN subtypes. Remarkably, lysosomal pathways synergistically increased mutant CALR and Tpo-R degradation, and their induction decreased cell proliferation induced by mutant CALR in cell lines and proliferation of CD34 positive cells from MPN patients. Next, Dr Ann Mullaly (Harvard University, Boston, USA) continued on CALR mutation, by examining CALR del52 and the epigenetic regulator ASXL1 (Additional Sex Combs Like Transcriptional Regulator 1, a protein that functions as a ligand-dependent co-activator for retinoic acid receptor in cooperation with nuclear receptor co-activator 1). Whereas CALR del52 is a common mutation in MPN patients, ASXL1 mutations arising in myelofibrosis reduce the survival of CALR del52 patients. This was confirmed using CALR del52/ASXL1^{mut} mice which exhibited a more severe MPN phenotype and aberrant megakaryocyte profile compared to CALR del52 MPN. At the molecular level, ASXL1 mutation led to a gain of function of the ASXL1-BAP1 deubiquitination complex and an increase in Histone H3 Ser10 phosphorylation, a marker of proliferation. The subsequent two presentations focused on calreticulin's role in immunity. Macrophage phagocytosis requires emission of signals by apoptotic or cancer cells to initiate programmed cell removal. CALR was long thought to be a pro-phagocytic signal expressed on the surface of cancer cells. Allison Banuelos (Stanford University, CA USA) described the secretion of CALR by macrophages which binds to asialoglycans on target cells, providing the 'eat me' signal for phagocytosis. Investigating the aberrant secretion of CALR containing a KDEL ER-retention motif, she demonstrated that TLR activation played a role in CALR translocation and subsequent secretion. This was further discussed by Allison Zhang (Stanford University, CA USA) who specified that CALR secretion by macrophages was associated with phagocytic activity and that both were higher in M1 macrophages than in M2. The last presentation of the session focused on age-related macular degeneration (AMD), which causes a blurry central vision. In the past years, the prevalence of AMD has been increasing, and the current standard treatment, VEGF intravitreal injection, is invasive and may cause side-effects. Dr Rimantas Slibinskas (Vilnius

University, Vilnius, Lithuania) discussed topical applications of CALR as a potential therapeutic strategy against AMD using laser-induced choroidal neovascularization (CNV) models. This relies on the anti-angiogenic properties of CALR fragment vasostatin (corresponding to the 180N-terminal amino-acids of CALR).¹⁶ Mice developing CNV were treated by either intraocular injection or topical application of full-length CALR, consisting in application of drops able to penetrate into the eye. In vivo angiography demonstrated that both application methods effectively inhibited angiogenesis and slowed disease progression, although topical application showed a delay in resolving vascular lesions. Remarkably, CALR topical applications at lower doses were more effective than higher doses, suggesting a dose-dependent effect and the possibility of off-target effects at high concentrations. Furthermore, the physico-chemical analysis of the CALR drop solution indicated high stability at room temperature and below (whereas instability was observed at 37°C), which could be of interest to control the bioavailability of the protein. However, the precise mechanism of action by which CALR inhibits angiogenesis in vivo still remains unclear.¹⁷

4 | SESSION III—CANCER (FONDATION ARC SESSION)

This third session, sponsored by the Fondation ARC pour la Recherche sur le Cancer, started with a presentation on protein translocation in the ER as one of the early steps of canonical protein secretion and as such, is an important component of ER proteostasis in cancer. Dr Caroline Demangel (Pasteur Institute, Paris, France) studies an infectious disease caused by *Mycobacterium ulcerans*, leading to ulcer development. This bacterium secretes the mycolactone polyketide, a non-selective inhibitor of the Sec61 translocon that prevents nascent proteins from entering the ER.¹⁸ This inhibition potentially affects IFN-inducible proteins, such as cytokines/chemokines and their receptors (e.g. IL-6R downregulation affecting IL-6 signalling pathway), TCR response, and antigen presentation, overall leading to an immunosuppressive effect of mycolactone. Taken together, these observations led to the consideration of mycolactone for therapeutic use. In vivo experiments have confirmed that both mycolactone and a synthetic derivative protect against chronic skin inflammation and inflammatory pain. Additionally, mycolactone synergizes with proteasome inhibitors and immunomodulators (such as Lenalidomide) in multiple myeloma cellular and mouse models, enhancing cell death likely through terminal UPR signalling. Next, Dr Jean-Ehrland Ricci (Inserm, Nice, France) presented new insights into metabolic effects on anti-tumour immunity. Using mouse models with colon cancer xenografts, it was observed that a 25% reduction in protein consumption (but not carbohydrates) led to reduced tumour growth upon treatment with chemotherapy. However, this effect was not observed in mice deficient in CD8+ T cells, indicating that a low protein diet promotes anti-tumor immunity through at least the activation of T cells. Further investigations revealed that a low protein diet activates the IRE1 branch of the UPR in tumour cells and that

IRE1 inhibition reduced the benefits from the diet. Conversely, IRE1 overexpression in colon cancer cells resulted in reduced tumour progression and increased tumour-infiltrating lymphocytes, thus providing an anti-tumour role of IRE1, which triggers the immune response based on metabolic cues. An *in situ* interactome analysis of IRE1 identified a component of a mannose-Transferase complex responsible for providing Mannose substrate for N-glycosylation. This protein is overexpressed in colon adenocarcinoma. Cells KO for this gene exhibited increased oligomerization of IRE1 and hypoglycosylation of PD-L1. As a result, tumour cells lose their ability to trigger this immune checkpoint. Analysis of immune infiltration revealed that KO tumours displayed less pro-tumoral M2 macrophages (which is consistent with reduced display of mannose) and more CD3+/CD8+ cells. These findings link metabolic regulation to the N-glycan biosynthesis and the subsequent ER stress regulation of tumour immune control. Next, Dr Jody Groenendyk (University of Alberta, Edmonton, Canada) pursued the discussion on cancer metabolism and presented data on fructose, one of the main sources of energy of cancer cells, transported by the SLC2A5 (GLUT5) transporter, often upregulated in cancer.^{19,20} Using boyden chamber and scratch assays in pancreatic cancer cell lines knocked out for GLUT5, impaired cell migration was observed. However, this was rescued when full-length GLUT5 was reintroduced, but not when a transport-defective mutant was used, indicating that the impaired migration is likely due to fructose transport. *In vivo* experiments corroborated these results, with lower metastasis potential of GLUT5 knockout fibrosarcoma cells compared to wild-type, accompanied by a decrease in primary tumour growth. Microscopy analysis revealed that GLUT5 knockout cells had a less pro-migratory morphology with few protrusions and exhibited impaired mitochondria morphology and transport. Loss of GLUT5 thus prevents transport of mitochondria at leading edges of cells, where they are supposed to sustain migration. The last three presentations focused on glioblastoma (GB), a tumour associated with increased matrix stiffness within and around the tumour, which triggers signalling pathways through cytoskeleton rearrangement.²¹ Pr Frank Kruyt (University of Groningen, Groningen, Netherlands) presented data on the mechano-transducer role of the UPR sensor, PERK. PERK KO in GB cell lines showed impaired cell adaptation to matrix stiffness due to a defect in F-actin polymerization. These data linked PERK to its association with the actin-binding protein filamin A (FLNA).²² By scaffolding FLNA, PERK induces remodeling of the actin cytoskeleton and promotes the formation of focal adhesions, which contributes to the adaptation of matrix stiffness. Continuation of these findings could lead to considering PERK as a relevant therapeutic target in GB treatment. This notion was supported by the presentation of Dong Liang (University of Groningen, Groningen, Netherlands), working on PERK modulation as a therapeutic tool for GB. Using the PERK activator MK-28, GB viability was reduced, and cell cycle arrest in the G1 phase was observed. Another therapeutic approach in GB was presented by Dr Diana Pelizzari-Raymundo (Inserm, Rennes, France) on her work regarding the development of a novel blood brain barrier (BBB) permeable IRE1 inhibitor. GB growth is supported by IRE1 activation, and the design

of a BBB permeable IRE1 kinase inhibitor named Z4P inhibited IRE1 RNase activity. *In vivo* experiments confirmed the potential of this approach, since combination of these IRE1 inhibitors with temozolomide synergistically reduced GB tumour relapse. Currently, a structure-activity relationship study is ongoing to optimize physico-chemical properties of the compounds, as well as to identify more potent analogues for inhibiting IRE1.

5 | SESSION IV—DEGENERATIVE DISEASES

In this session, the intricate relationship between degenerative diseases and ER functions was examined. Dr Benjamin Delprat (Inserm, Montpellier, France) presented his work on Wolfram syndrome (WS), an autosomal recessive genetic disorder characterized by diabetes, optic nerve atrophy, deafness and neurodegeneration. The progression of the disease ultimately leads to premature death of the patients around 35 years of age. While WS has long been considered a mitochondrial disease, recent hypotheses suggest the involvement of the ER in the syndrome. Variants in the WFS1 gene, coding for the Wolframin ER Transmembrane Glycoprotein, lead to the synthesis of proteins localized to the ER, leading to WS type 1, which represents 99% of WS cases. WFS1 plays a crucial role in regulating the transfer of mitochondrial calcium through the mitochondria-associated ER membranes (MAMs). The association of WFS1, NCS1 and the inositol-1,4,5-trisphosphate receptor (IP3R) prevents NCS1 degradation and sustains ER-mitochondria calcium transfer. In WS, this calcium transfer is disrupted, and connections between the ER and mitochondria are reduced, a phenotype also observed upon NCS1 knockdown. The team then developed a zebrafish model of WS by disrupting both *Wfs1a* and *Wfs1b* genes and investigated the potential therapeutic effect of *Ncs1* overexpression. Overexpression of *Ncs1* using mRNA microinjection restored mitochondrial alterations and locomotor hyperactivity in the mutant larvae. Based on these results, the researchers propose *Ncs1* as a potential therapeutic target for WS. The zebrafish model offers a useful platform for drug screening in WS and complements other animal models like rodents, in furthering our understanding of this disease. Dr Myriam Pujol (University of Alberta, Edmonton, Canada) gave a short talk on the interaction between calnexin (CANX) and fatty acid binding protein 5 (FABP5), which facilitates T-cell movement through the BBB. These findings suggest that the regulatory protein CD200 plays a significant role in reducing T-cell permeability in the absence of CANX or FABP5. These data highlight the potential of targeting this protein complex as a treatment approach for neuro-inflammatory diseases. To conclude the session on degenerative diseases, Pr Claudio Hetz (University of Chile, Santiago, Chile) delivered a presentation on the pivotal role of PDIs in maintaining proteostasis in the nervous system. Specifically, Pr Hetz focused on the significance of PDIA3 in amyotrophic lateral sclerosis (ALS), a progressive paralytic disorder characterized by the selective degeneration of motor neurons in the brainstem and cerebral cortex. Overexpression of PDIA3 in neurons

promotes axon elongation, while inactivating mutations of PDIA3 in zebrafish caused detrimental effects in the animals. The identification of a mutation in the catalytic site of PDIA3 (C57Y), associated with severe intellectual disability and neurodevelopmental problems in humans, led to its insertion in zebrafish and mouse models to study the associated pathogenic mechanisms. Zebrafish embryos carrying the mutation exhibited severely impaired development and abnormal morphologies. In mice, synaptic activity in the hippocampus was significantly impaired, resulting in altered neurogenesis and behavioural changes. Additionally, the C57Y PDIA3 mutants formed aggregates that abnormally interacted with the chaperones CANX and CALR. Moreover, the PDIA3-C57Y mutant also affected the biogenesis and signalling of integrins by disrupting the actin cytoskeleton and impairing neuritogenesis. Biochemical investigations finally revealed that the mutant exhibited lower enzymatic activity and formed aggregates due to increased disulfide bonds.

6 | SESSION V—ER FUNCTION (SBCF SESSION)

The last session of the workshop, sponsored by the Société de Biologie Cellulaire de France, focused on investigating other ER functions. The session was opened by Dr Laurence Abrami (EPFL, Lausanne, Switzerland) who introduced her work on SARS-CoV-2 and its impact on increased Spike protein S-acylation (or S-Palmitoylation), which enhances infectivity providing new information about infection mechanisms of the virus. S-acylation is a post-translational modification consisting in addition of an acyl lipid (composed of 12–20 carbons) to a cysteine residue through the action of enzyme ZDHHC S-acyltransferases. Spike protein S-acylation stabilizes its structure. The efficiency of SARS-CoV-2 infection dropped upon S-acylation mutants of Spike protein, suggesting that the S-acylation itself is necessary for stabilizing the Spike protein trimers. A siRNA screening of acyltransferase responsible for Spike protein S-acylation unveiled four different enzymes, notably ZDHHC20 and ZDHHC9. Of note, SARS-CoV-2 infection resulted in the expression of a variant ZDHHC20 (upstream translation start site), leading to the production of a more stable enzyme that appears in stressed or infected cells only in the ER. In conclusion, SARS-CoV-2 could promote its own infective potential by triggering the expression of a more stable form of the enzyme responsible for Spike protein activation. Next, Andrew Bazley (ISG, NYU Langone Health, New York, NY USA) presented valuable insights into the regulation of protein diffusion in ER. The movement of particles within an environment is influenced by molecular crowding. In the specific context of ER lumen, the study of diffusion under different stimuli was made possible through the use of single particle tracking techniques, using ER-genetically encoded multimeric nanoparticles. The diffusion of these particles upon ER stress varied depending on the stressor used. Higher diffusion rates were observed upon thapsigargin treatment (an ER calcium homeostasis disruptor) whereas lower diffusion rates were observed upon tunicamycin treatment (an N-glycosylation

inhibitor), suggesting that different causes of ER stress impact ER rheology. Ilaria Pontisso (Inserm, Saclay, France) presented the tight relationship between incremental ER calcium depletion and UPR activation. They combined experimental and mathematical modelling approaches to investigate how moderate calcium depletion can impact the activation of the different UPR sensor arms, to better understand the mechanisms underlying the progression of several diseases associated with ER stress. Treatment with tBubHQ (a reversible sarco/endoplasmic reticulum calcium-ATPase (SERCA) inhibitor) resulted in BiP diffusion reduction, suggesting an accumulation of misfolded proteins in the ER, a condition known to activate UPR. This was consistent with a dose-dependent phosphorylation of IRE1 and PERK, along with the accumulation of ATF6 α (cleaved form of ATF6) in the nucleus. The computational model, supported by in vitro experiments, predicted the possibility of UPR reversion, which was indeed observed. Dr Alison Forrester (University of Namur, Namur, Belgium) presented her work on ER export and its relationships with autophagy. Indeed, autophagy is required for efficient ER-Golgi trafficking, and it has been shown to promote the degradation of intracellular procollagens, to prevent their accumulation in the ER. ER-phagy of pro-collagen type I and II requires the interaction of CANX with FAM134B (also known as Reticulophagy Regulator 1 (RETREG1) protein), leading to their association with the phagophore. Dr Forrester then described a new target in retrograde transport. Shiga toxin can promote haemolytic-uremic syndrome through its trafficking in the ER using a mechanism of retrograde transport from the plasma membrane. Through screening, a small molecule named Retro-2 was identified, which exhibits high specificity in targeting the ER exit site protein Sec16A. By targeting this protein, Retro-2 slows down Shiga toxin retro-transport, a mechanism that could easily be transferred to other toxin-derived pathologies. The last presentation of the workshop was delivered by Dr Celine Philippe (Barts Cancer Institute, London, UK) introducing her work on UPR and how it can affect the Alternative Splicing Landscape. Both the transcriptome and translome are shaped upon ER stress and UPR activation, even within the initial minutes. However, our understanding of these processes is limited due to their dynamics. To investigate the de novo translation of mRNA upon ER stress, O-Propargyl puromycin labelling of nascent peptides was used, followed by LC-MS/MS analysis. This approach revealed an enrichment of splicing factors, indicating their preferential translation in response to ER stress. Among the three arms of the UPR response to stress, PERK, was identified as responsible for the ER alternative splicing signature. The mechanisms by which these splicing events occur depend on the increased phosphorylation of specific mRNA binding proteins.

7 | POSTER SESSIONS

During this event, two engaging poster sessions were conducted, featuring a diverse range of topics consistent with the sessions presented above. Over 15 poster presenters provided a concise overview

of their data and findings (Appendix A). The posters covered a wide array of subjects, ensuring a stimulating and varied scientific discourse throughout the event and evaluated by an independent jury composed of invited speakers. Following the conclusion of the poster sessions, a recognition ceremony took place, where prizes were awarded by the generous sponsors of the event. The SBCF (Société de Biologie Cellulaire de France) presented awards to Hussein Issaoui and Daniela Ricci for their contributions. In addition, the Company of Biologists Ltd awarded travel prizes to Jody Groenendyk, Marianne Guilbard, Nardin Georges, Jérôme Archambeau and Arvin Pierre in recognition of their presentations. Furthermore, Diana Pelizzari-Raymundo and Manon Nivet were awarded with the SFC (Société Française du Cancer) and the FEBS journal prizes, respectively. The awards ceremony not only celebrated the diverse achievements but also highlighted the collaborative and supportive nature of the scientific community.

8 | CONCLUSION

The 14th International Calreticulin workshop offered a fantastic platform for scientists from around the world to share their research and foster collaborations in understanding the complex biology of the ER and its implications in various diseases. Moreover, this workshop provided an ideal opportunity for scientists from diverse disciplines and different career stages to come together with a shared objective of advancing the understanding of the functions of CALR and other ER proteins as potential therapeutic targets. The 15th International Calreticulin Workshop will be organized in Brussels, Belgium, in 2025.

AUTHOR CONTRIBUTIONS

Ketsia Bakambamba: Writing – original draft (supporting). **Federico Di Modugno:** Writing – original draft (supporting). **Marianne Guilbard:** Writing – original draft (supporting). **Simon Le Goupil:** Writing – original draft (supporting). **Stéphanie Lhomond:** Writing – original draft (supporting). **Diana Pelizzari-Raymundo:** Writing – original draft (supporting). **Tony Avril:** Writing – original draft (lead); writing – review and editing (supporting). **Eric Chevet:** Writing – original draft (lead); writing – review and editing (lead). **Fredéric Delom:** Writing – original draft (lead); writing – review and editing (supporting). **Elodie Lafont:** Writing – original draft (lead); writing – review and editing (supporting).

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CONFLICT OF INTEREST STATEMENT

EC is founder of Thabor Therapeutics.

DATA AVAILABILITY STATEMENT

There is no data included in the article.

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APPENDIX A

Poster Session#1

F. Di Modugno	AGR2 pro-oncogenic gain-of-function associated with its non-canonical localization: a role in cholangiocarcinoma
M. Guilbard	AGR2: A link between ER proteostasis and cancer proliferation
A. Pierre	Investigating the role of ERp18 during Activation of UPR sensor ATF6 α
M. Baez	Endoplasmic reticulum-to-Golgi trafficking of Calreticulin is required for its translocation and secretion by macrophages
A. Banuelos	Investigation of Calreticulin processing and secretion by macrophages for programmed cell removal
N. Georgeos	Identification of Calreticulin-binding partners on the cell surface of activated macrophages
Dr. J. Archambeau	TMEM214: a new player in the regulation of ER morphology

Poster Session#2

Dr. D. Ricci	The sensor IRE1 couples stress detection to protein synthesis
K. Bakambamba	Control of the secretion machinery by IRE1 in human glioblastoma
Dr. X. Guillory	Targeting the IRE1 pathway for adjuvant therapies in cancers
Dr. J. Groenendyk	Loss of the Fructose Transporter SLC2A5 inhibits cancer cell migration
Dr. H. Issaoui	UPR activation in cancer cells promotes anti-cancer immune response
S. Le Goupil	Characterization of the IRE1 BioID proximitome that controls metastatic melanoma migration and invasion
M. Nivet	A novel IRE1 target ITGA6 controls glioblastoma aggressiveness
Dr. D. Pelizzari-Raymundo	A novel blood brain barrier-permeable IRE1 kinase inhibitor for adjuvant glioblastoma treatment in mice

Pre-review: "Identification of Glucosidase II that regulates STIM1 activation dynamics"



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PREreview of "Identification of Glucosidase II that regulates STIM1 activation dynamics"

Elodie Lafont ; Eric Chevet ; Ketsia Bakambamba

Research group: Proteostasis and Cancer Team INSERM U1242

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Pre-review of "Identification of Glucosidase II that regulates STIM1 activation dynamics"

by Ketsia Bakambamba, Elodie Lafont and Eric Chevet of the Proteostasis and Cancer team INSERM U1242

Summary of the article

In this article by Du et al, the authors identify glucosidase II (GANAB and PRKCSH) as partners of STIM1. They then explore the impact of STIM1 affinity for Ca²⁺ and store-operated Ca²⁺ entry and propose glucosidase II as a new modulator of STIM1 activation.

General comments

The results obtained by the authors are interesting, yet we think that the study could benefit from additional experiments, reanalysis of existing ones and text refinement (see suggestions in specific points below).

Specific comments

Figure 1 and corresponding Figure S1:

1A. How can STIM1 be biotinylated in its cytosolic part?

1B. How long is the Tg treatment?

1C, D It is not clear whether this is obtained upon Tg stimulation (which may have introduced a bias).

Figure 1 and S1 in general: Comparison of all 4 conditions for the proteomic results (+/- Tg for STIM1 and STIM2) results would be informative here. Given the topology of the constructs used, it is a bit surprising that actin filament organization-related proteins are enriched here. What is the author's take on this result?

Figure 2 and corresponding Figure S2:

<https://zenodo.org/records/13077445>

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Résumé de thèse

Le cancer reste l'un des problèmes de santé mondiale les plus pressants de ce siècle, responsable de millions de décès chaque année. Les progrès de la recherche sur le cancer ont considérablement amélioré notre compréhension des mécanismes de cette maladie, conduisant à des diagnostics et des traitements plus précis. Malgré ces avancées, de nombreux cancers, en particulier les plus agressifs, continuent de poser des défis majeurs en matière de traitement, avec de faibles taux de survie et des options thérapeutiques limitées.

Un exemple de ce type de cancer est le glioblastome, qui sera le sujet central de cette thèse. Il s'agit de la tumeur cérébrale primaire la plus agressive et la plus mortelle. Sa nature hautement invasive et sa résistance aux thérapies conventionnelles soulignent la nécessité de poursuivre les recherches sur cette maladie. Ces dernières années, le domaine de la recherche sur le cancer a accordé une attention croissante à la compréhension de la manière dont les tumeurs interagissent avec leur environnement, s'adaptent au stress et échappent à la détection par le système immunitaire. Cela inclut l'étude de mécanismes tels que la réponse aux protéines mal repliées (UPR), utilisée par les cellules pour faire face au stress dans le réticulum endoplasmique. Dans des cancers comme le glioblastome, ces réponses adaptatives permettent aux cellules tumorales de survivre dans des conditions défavorables.

L'objectif de cette thèse est d'explorer le rôle de l'axe IRE1 de l'UPR dans la régulation de la machinerie de sécrétion des cellules de glioblastome. Comprendre comment IRE1 influence la sécrétion de protéines et son impact plus large sur la progression tumorale pourrait révéler de nouvelles voies thérapeutiques pour lutter contre cette maladie agressive.

Chapitre 1 : Biologie, traitement et avancées dans la recherche sur le glioblastome

Ce chapitre introduit le GB en abordant sa définition, son historique, son épidémiologie et ses facteurs de risque. Le GB représente environ 54 % des gliomes et est associé à un pronostic très défavorable avec une survie médiane de 15 mois.

- **Classification** : La classification des tumeurs du système nerveux central de l'OMS (5^e édition, 2021) a redéfini le GB comme exclusivement IDH de type sauvage (IDH-wildtype), éliminant la distinction entre GB primaires et secondaires. Cette nouvelle classification intègre désormais des marqueurs moléculaires clés dans les critères diagnostiques comme les mutations de TERT et l'amplification de l'EGFR.

- **Physiopathologie complexe :** Le GB se distingue par des mutations génétiques fréquentes (EGFR, PTEN, TP53) et une hétérogénéité moléculaire qui favorisent un développement rapide de la tumeur et une forte résistance aux traitements. Le microenvironnement tumoral, comprenant des cellules immunitaires suppressives et une matrice extracellulaire désorganisée, joue un rôle crucial dans l'invasion et la récurrence tumorale.
- **Traitements actuels et perspectives :** Le protocole de Stupp qui comprend la résection chirurgicale suivie de la radiothérapie et la chimiothérapie au témozolomide reste le traitement standard. Les nouvelles approches incluent les thérapies ciblées, l'immunothérapie (anti-PD-1/PD-L1) et des stratégies innovantes comme les champs de traitement des tumeurs (TTF).

Chapitre 2 : La réponse aux protéines mal repliées (UPR) et son implication dans le cancer

La voie de sécrétion des protéines est un processus cellulaire clé qui implique plusieurs organelles. Cette voie commence dans le réticulum endoplasmique (RE) puis se poursuit dans l'appareil de Golgi. Les protéines qui empruntent cette voie sont soigneusement repliées et maturées avant d'être transportées vers leur destination finale grâce à un système bien régulé de vésicules. La première organelle de cette voie est donc le RE, qui prend en charge les protéines naissantes dans un environnement rigoureusement contrôlé, permettant ainsi de maintenir l'homéostasie cellulaire. Lorsque les protéines pénètrent dans la lumière du RE, elles sont immédiatement assistées par plusieurs protéines chaperonnes notamment la protéine BIP, qui joue un rôle central dans ce processus. Les différentes protéines permettent le bon repliement du peptide et sa maturation avant qu'il ne soit transporté dans l'appareil de Golgi. Les cellules tumorales subissent souvent diverses formes de stress, telles que l'hypoxie et la privation de nutriments, qui entraînent l'accumulation de protéines mal repliées dans le réticulum endoplasmique (RE) et donc le stress du RE. L'UPR (Unfolded Protein Response) est une voie de signalisation cellulaire activée en réponse au stress du RE, principalement dû à l'accumulation de protéines mal repliées. Trois senseurs principaux régulent cette réponse : IRE1, PERK et ATF6.

- **Fonctionnement de l'UPR :**
 - **IRE1 :** Lorsqu'il est activé, IRE1 subit une autophosphorylation et clive spécifiquement l'ARNm de XBP1 pour produire une forme active (XBP1s). Ce facteur de transcription induit l'expression de gènes impliqués dans le

repliement des protéines et la dégradation des protéines mal repliées (ERAD). En situation de stress prolongé, IRE1 peut aussi déclencher la dégradation de certains ARNm via la voie RIDD (Regulated IRE1-Dependent Decay), réduisant ainsi la charge protéique globale.

- **PERK** : Phosphoryle le facteur d'initiation de la traduction eIF2 α , réduisant la synthèse protéique globale tout en activant la traduction de certains ARNm comme ATF4, impliqué dans l'autophagie et la résistance au stress oxydatif.
- **ATF6** : Se déplace vers le Golgi où il est clivé pour devenir un facteur de transcription actif, régulant des gènes de chaperons protéiques et de dégradation des protéines mal repliées.

- **Implication dans le cancer et le glioblastome :**

Dans les cellules tumorales, y compris celles du GB, l'UPR joue un rôle paradoxal. Bien qu'elle puisse induire l'apoptose en cas de stress prolongé, elle favorise généralement la survie tumorale en conditions de stress comme l'hypoxie ou la déprivation en nutriments et la résistance aux traitements antitumoraux. IRE1 est particulièrement impliqué dans l'adaptation des cellules de GB à ces conditions défavorables :

- **Résistance thérapeutique** : En régulant des gènes impliqués dans la survie cellulaire, IRE1 permet aux cellules tumorales de résister aux traitements classiques.
- **Progression tumorale** : L'activation de l'axe IRE1/XBP1s favorise l'angiogenèse, l'invasion et la formation d'un microenvironnement immunosuppresseur.
- **Cible thérapeutique** : Des inhibiteurs d'IRE1 sont en cours de développement pour limiter ces réponses adaptatives et rendre les cellules tumorales plus vulnérables. Compte tenu son rôle central, IRE1 représente une cible thérapeutique prometteuse et la compréhension de ses mécanismes de régulation pourrait déboucher sur de nouveaux traitements qui inhibent la progression tumorale en perturbant le processus de sécrétion et en améliorant la réponse aux thérapies existantes.

Chapitre 3 : IRE1 et la régulation de la voie de sécrétion protéique

Ce chapitre présente les résultats expérimentaux démontrant le rôle central d'IRE1 dans la régulation de la sécrétion protéique dans les cellules de GB.

- **Hypothèse de travail :**

Parmi les transducteurs de l'UPR, IRE1 est particulièrement important pour maintenir la fonctionnalité de l'UPR et a été impliqué dans la pathophysiologie du glioblastome en régulant l'expression de diverses protéines sécrétées impliquées dans la progression tumorale. Sur la base de ces informations, nous proposons l'hypothèse suivante à tester : « IRE1 pourrait affecter de manière générale la sécrétion des protéines en influençant directement les acteurs moléculaires de la voie sécrétoire dans les cellules de glioblastome. »

- **Résultats principaux :**

- **Identification de GOLIM4 :** Nous avons identifié GOLIM4, une protéine du cis-Golgi impliquée dans le trafic des protéines, comme une cible majeure régulée par IRE1.

- **Effets de la suppression de GOLIM4 :**

- La suppression de GOLIM4 perturbe l'adhésion des cellules de GB, ce qui suggère son rôle crucial dans le contrôle de l'expression des protéines membranaires nécessaires à l'adhésion.
 - Une migration accrue des cellules tumorales a été observée après inhibition de GOLIM4.

- **Implications fonctionnelles :** L'étude a montré que l'inhibition de GOLIM4 réduit l'expression de protéines de surface comme **CD44** et **NCAM1**, essentielles à l'invasion tumorale. Ces protéines sont importantes pour la reconnaissance cellulaire et la migration dans le microenvironnement tumoral.

- **Voie IRE1/GOLIM4 :**

La régulation de la sécrétion protéique par IRE1 via GOLIM4 pourrait constituer une voie clé pour l'invasion et la progression du GB.

- **Perspectives thérapeutiques :** En ciblant l'axe IRE1/XBP1s/GOLIM4, il serait possible de perturber la capacité des cellules tumorales à maintenir leur adhésion et leur interaction avec l'environnement, réduisant ainsi leur potentiel invasif. Des inhibiteurs spécifiques d'IRE1 pourraient ainsi constituer une nouvelle approche pour limiter la progression du GB.

“But the path of the just is as the shining light, that shineth more and more unto the perfect day”

Titre: Identification des acteurs moléculaires régulés par IRE1 qui contrôlent la sécrétion de protéines dans les cellules de glioblastome

Mots clés : Glioblastome, stress du RE, UPR, sécrétion protéique, IRE1, GOLIM4

Résumé: Le glioblastome (GB) est une tumeur cérébrale agressive et mortelle, avec une pathophysiologie très hétérogène, ce qui contribue à sa résistance aux thérapies actuelles. Malgré les avancées de la recherche, le pronostic des patients atteints de GB reste mauvais, soulignant la nécessité d'une meilleure caractérisation de la maladie et du développement de nouvelles stratégies thérapeutiques. Cette thèse se concentre sur le rôle central du réticulum endoplasmique (RE) et de sa réponse au stress, en particulier la Réponse aux Protéines Mal Repliées (UPR), dans la pathophysiologie du GB. Une attention particulière est accordée au senseur IRE1 de l'UPR, qui régule la machinerie sécrétoire dans les cellules de GB, influençant des processus clés tels que la maturation des protéines, leur trafic et la migration cellulaire.

Nous proposons qu'IRE1 puisse avoir un impact large sur la sécrétion des protéines en influençant directement des acteurs moléculaires clés de la voie sécrétoire dans les cellules de GB. En particulier, nous avons identifié GOLIM4, une protéine du cis-Golgi impliquée dans le trafic de protéines, comme une cible d'IRE1. La suppression de GOLIM4 a entraîné une perturbation de l'adhésion cellulaire et une migration accrue, ce qui suggère qu'IRE1 joue un rôle crucial dans la régulation du transport des protéines et de leur expression à la surface cellulaire, des processus essentiels pour maintenir la fonction des cellules de GB et leur invasivité.

Title: Identification of molecular actors regulated by IRE1 that control protein secretion in glioblastoma cells

Keywords : Glioblastoma, ER stress, UPR, protein secretion, IRE1, GOLIM4

Abstract: Glioblastoma (GB) is an aggressive and deadly brain tumor with a highly heterogeneous pathophysiology, contributing to its resistance to current therapies. Despite advances in research, the prognosis for GB patients remains poor, highlighting the need for better characterization of the disease and the development of novel therapeutic strategies. This thesis focuses on the central role of the endoplasmic reticulum (ER) and its stress response, particularly the Unfolded Protein Response (UPR), in GB pathophysiology. Special emphasis is placed on the UPR sensor IRE1, which regulates the secretory machinery in GB cells, influencing key processes such as protein maturation, trafficking and cell migration.

We propose that IRE1 may broadly affect protein secretion by directly influencing key molecular actors of the secretory pathway in GB cells. Specifically, we identified one IRE1 target, GOLIM4, a cis-Golgi protein involved in protein trafficking and whose silencing led to disrupted cell adhesion and Enhanced migration, this suggests that IRE1 plays a crucial role in regulating protein transport and cell surface expression., which are vital for maintaining GB cell function and invasiveness.