



HAL
open science

Epigenetic control of ER stress-mediated cellular reprogramming: role of the AAA+ ATPase p97/VCP

Kim Barroso

► **To cite this version:**

Kim Barroso. Epigenetic control of ER stress-mediated cellular reprogramming: role of the AAA+ ATPase p97/VCP. Human health and pathology. Université de Bordeaux, 2016. English. NNT: 2016BORD0209 . tel-01968034

HAL Id: tel-01968034

<https://theses.hal.science/tel-01968034>

Submitted on 2 Jan 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTÉ
SPÉCIALITÉ GÉNÉTIQUE MOLÉCULAIRE ET CELLULAIRE

Par Kim BARROSO

**Contrôle Epigénétique du Stress du Réticulum Endoplasmique : un nouveau
rôle pour p97/VCP dans la regulation de l'homéostasie protéique**

Sous la direction de : Eric Chevet
co-directeur : Martin Fernandez-Zapico

Soutenue le 1^{er} Décembre 2016

Membres du jury :

Pr. Michel Moenner
Pr. Claire Vourc'h
Dr. Rémy Pedeux
Dr. Frédéric Delom
Dr. Eric Chevet
Pr. Martin Fernandez-Zapico

Président
Rapporteur
Rapporteur
Examineur
Directeur de Thèse
Co-directeur de Thèse

THESIS

PRESENTED AT THE
UNIVERSITÉ DE BORDEAUX

DOCTORAL SCHOOL OF HEALTH AND LIFE SCIENCE

By **Kim BARROSO**

Epigenetic control of ER stress-mediated cellular reprogramming: role of the AAA⁺ ATPase p97/VCP

Supervisors of the Research:
Dr. Eric Chevet
Pr. Martin Fernandez-Zapico

Defended on the 1st of December 2016

In front of a board of examiners composed of:

Pr. Michel Moenner	Professor at the University of Bordeaux	President of the jury
Pr. Claire Vourc'h	Professor at the University of Grenoble	Reporter
Dr. Rémy Pedeux	Researcher at INSERM	Reporter
Dr. Frédéric Delom	Lecturer at the University of Bordeaux	Member of the jury
Dr. Eric Chevet	Director of Research	Thesis director
Pr. Martin Fernandez-Zapico	Director of Research	Thesis co-director

ACKNOWLEDGEMENT

The first part of my thesis work was realized under the direction of Dr. Eric Chevet in the Stress, Organites and Cancer Team at the INSERM unit U1053 directed by Jean Rosenbaum. I would like to thank all the members of the INSERM unit 1053 with whom I had the pleasure of working during the first 2 years of my thesis and particularly the people from my team: Stéphanie, Nicolas, Saïd, Nestor and Eric who all have been really supportive and friendly.

Later I joined Dr Martin Fernandez-Zapico's laboratory in Rochester, USA and I would like to thank him for giving me the chance to have this very rewarding experience. I would also like to thank all the members of my team that have been extremely welcoming and friendly throughout all my stay, particularly: Martin, Lucianna, Ezequiel and Elisa.

When I came back to France I joined Eric Chevet's new team in Rennes, at the INSERM Oncogenesis Stress and Signaling directed by Patrick Legembre. I would like to thank all the members of my laboratory for this last year for their support and the good moments we have shared and particularly the members of my team: Asia, Tony, Eric, Gwen, Marie, Dimitrios, Alex.

I would like to thank Eric Chevet for giving me the opportunity to do this Ph.D, for motivating me when I needed, for always being available and enthusiastic about our work.

I would also like to thank my friends and family who have supported me outside of work and particularly my father and the person who shares my life Marie.

I also want to thank the members of the jury for taking the time to evaluate my work.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	1
TABLE OF CONTENTS	2
TABLE OF ILLUSTRATIONS	3
TABLE OF ABBREVIATIONS	4
ABSTRACT	6
INTRODUCTION	10
I Endoplasmic Reticulum	10
1 Endoplasmic Reticulum structure and functions.	10
a) Structure	10
b) Calcium storage and regulation	11
c) Lipid synthesis	12
d) Protein folding and maturation	13
e) Protein export from the ER	14
2 Endoplasmic Reticulum signaling	16
Epigenetic Regulation of Endoplasmic Reticulum Stress	17
3 Endoplasmic Reticulum Stress and cancer	33
Novel roles of the unfolded protein response in the control of tumor development and aggressiveness	34
II p97/VCP/CDC-48	42
1 Structure of p97/VCP.	43
2 Functions of p97/VCP/CDC-48.	44
a) Regulation of Protein homeostasis	45
b) Membrane fusion and trafficking	47
c) Chromatin-associated functions	49
3 p97/VCP/CDC-48 role in transcription regulation	53
4 p97/VCP in diseases	54
a) IBMPFD and ALS	54
b) Cancer	55
HYPOTHESIS AND OBJECTIVES	57
EXPERIMENTAL APPROACH	58
Adaptation of the Secretory Pathway in Cancer Through IRE1 Signaling	62
RESULTS	81
Genome-wide screen identifies a novel p97/CDC-48-dependent pathway regulating ER-stress-induced gene transcription	83
A p97/VCP-mSin3A-HDAC1/2 complex antagonizes non-canonical activation of the Hedgehog-GLI1 pathway by Endoplasmic Reticulum Stress	105
DISCUSSION	134
REFERENCES	139
APPENDIX	145

TABLE OF ILLUSTRATIONS

	Page
Figure 1	8
Figure 2	10
Figure 3	12
Figure 4	13
Figure 5	14
Figure 6	42
Figure 7	43
Figure 8	43
Figure 9	44
Figure 10	46
Figure 11	47
Figure 12	49
Figure 13	50
Figure 14	79
Figure 15	122
Figure 16	123
Figure 17	133

ABBREVIATIONS:

AAA+	ATPase Associated with various cellular Activity
ALS	Amyotrophic Lateral Sclerosis
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BIP	Binding immunoglobulin protein
Ca ²⁺	Calcium
CANR	Calreticulin
CBP	CREB binding protein
CBF/NFY	CCAAT-binding factor/nuclear factor-Y
CDC-48	Cell Division Cycle 48
CHOP	C/EBP homologous protein
CNX	Calnexin
COP II	Coat protein complex II
CRE	cAMP response element
CREB	CRE binding protein
DNA	Deoxyribonucleic acid
DSN	Double Strand Break
EDEM	ER degradation enhancing 1,2 mannosidase like protein
eIF2 α	Eukaryotic initiation factor 2 α
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
Erdj	Endoplasmic reticulum dna J
ERES	Endoplasmic reticulum exit site
ERSE	ER stress response element
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma
GRP	Glucose related protein
HDAC	Histone deacetylase
HCC	Hepatocellular carcinoma
HSP	Heat shock protein
IBM	Inclusion Body Myopathy with
IP3R	Inositol triphosphate receptor
IRE1 α	Inositol required enzyme 1 α
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
KO	Knock out
LC3	Microtubule associated protein 1 light chain 3
LTP	Lipid transfer proteins
MAM	Mitochondria-associated ER membrane
Nrf 2	NF-E2 related factor 2
ORP150	Oxygen-regulated protein 150

OST	Oligosaccharyl transferase
PFD	Prion disease and Frontotemporal Dementia
PDI	Protein disulfide isomerase
PERK	Protein kinase (PKR)-like ER kinase
PTM	Post Translational Modification
QC	Quality control
RER	Rough ER
RIDD	RNA regulated IRE1 dependent decay
RNA	Ribonucleic acid
RuvBL2	RuvB Like AAA ATPase 2
RyR	Ryanodine receptor
S1P	Site-1 protease
S2P	Site-2 protease
SER	Smooth ER
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
Sin3A	Sin3 Transcription Regulator Family Member A
SNP	Single nucleotide polymorphism
SPARC	Secreted protein acidic and rich in cysteine
Sp1	Stimulating protein 1
SREBP1/2	Sterol regulatory element binding protein 1 and 2
SRP	Signal recognition particle
σ 1R	Sigma-1 receptor
TP53	Tumor protein p53
TRAP	Translocon associated protein
uORF	Upstream open reading frames
UPR	Unfolded protein response
UPRE	UPR response element
UPS	Ubiquitin/proteasome system
VCP	Valosin-containing protein
VDAC	Voltage dependant anion channel
XBP-1	X-box binding protein 1
XBP1s	Spliced form of XBP1

ABSTRACT

p97/VCP is a member of the AAA⁺ ATPase family that plays major roles in various cellular processes including control of protein homeostasis and chromatin-associated functions (transcription, replication, DNA damage, cellular cycle progression). Moreover, p97/VCP is involved in a growing number of diseases including cancers in which it has been shown to contribute to protein homeostasis and adaptation to oncogenic stresses. Indeed, p97/VCP expression is increased in numerous cancers and in some cases correlates with tumor recurrence and poor prognosis for patients. However, the precise mechanism by which p97/VCP regulates tumor cell proteostasis remains unclear. To address this, we demonstrated a role of p97/VCP in gene expression control upon endoplasmic reticulum (ER) stress. We found that in basal conditions, RuvBL2 is part of chromatin remodeler complex that included HDAC1 and mSin3A and act as a repressor of ER stress genes. However under ER stress, ubiquitinated RuvBL2 is degraded by p97/VCP thus causing activation of ER stress genes. Moreover, we have identified GLI1, a known effector of Hedgehog signaling, as a target of the p97/VCP and RuvBL2-HDAC1-mSin3A complex. As a result under ER stress conditions, the Hedgehog pathway which have been linked to cancer development is non-canonically activated. Overall, our work indicated that p97/VCP acts as a molecular switch to inactivate RuvBL2-HDAC1 repressor complex under ER stress thus activating ER stress genes and Hedgehog genes in a non-canonical manner.

Keywords: AAA⁺ ATPase, p97/VCP, Endoplasmic Reticulum Stress, Proteasis, Gene expression control

Chemistry Oncogenesis Stress Signaling, INSERM unit 1242, Centre Eugène Marquis, Avenue de la bataille Flandres Dunkerque, 35042 Rennes cedex

RESUME

La protéine p97/VCP est un membre de la famille des ATPase AAA⁺ et joue un rôle majeur dans de nombreux processus cellulaires tel que le contrôle de l'homéostasie protéique ou de fonctions associées à la chromatine (transcription, réplication, dommage à l'ADN, progression du cycle cellulaire). De plus, la protéine p97/VCP est impliquée dans un nombre croissant de maladies dont les cancers où il a été montré qu'elle contribue à l'homéostasie protéique et l'adaptation au stress oncogéniques. En effet, l'expression de la protéine p97/VCP est augmentée dans de nombreux cancers et dans certains cas corrèle avec une récurrence de la tumeur et un mauvais pronostic pour les patients. Cependant, le mécanisme moléculaire précis par lequel la protéine p97/VCP régule l'homéostasie protéique des cellules tumorales reste incertain. Pour remédier à cela, nous avons démontré un rôle de la protéine p97/VCP dans le contrôle de l'expression des gènes lors du stress du Réticulum Endoplasmique (RE). Nous avons trouvé que en conditions basales, la protéine RuvBL2 fait partie d'un complexe remodeleur de la chromatine qui contient les protéines HDAC1 et mSin3A et agit comme un répresseur des gènes de stress du RE. De plus, nous avons identifié le gène Gli1, un effecteur connu de la voie de signalisation Hedgehog comme cible de la protéine p97/VCP et du complexe RuvBL2-HDAC1-mSin3A. Ainsi en condition de stress du RE, la voie de signalisation Hedgehog qui a été impliqué dans le développement de cancers est activée. Globalement, nos travaux indiquent que p97/VCP agit comme un interrupteur moléculaire pour inactiver le complexe répresseur RuvBL2-HDAC1 en condition de stress du RE et ainsi activer les gènes de stress du RE et de la voie de signalisation Hedgehog de façon non-canonique.

Mots clefs: AAA⁺ ATPase, p97/VCP, Stress du Réticulum Endoplasmic, Homeostasie Protéique, Contrôle de l'expression des genes

Chemistry Oncogenesis Stress Signaling, INSERM unité 1242, Centre Eugène Marquis, Avenue de la bataille Flandres Dunkerque, 35042 Rennes cedex

RESUME SUBSTANTIEL

Le cancer est aujourd'hui la première cause de mortalité en France avec près de 150 000 décès par an représentant un enjeu de santé publique majeur. Les cancers gastrointestinaux représentent une des causes les plus courantes de cancer en France et dans le monde : près d'un quart des cancers. Alors que chaque année le taux de survie des cancers s'améliore, les cancers gastrointestinaux sont parmi les plus mortels, avec notamment les cancers du foie, du pancréas qui présentent une survie à 5 ans après diagnostic inférieur à 10%. Il y a donc une réelle nécessité d'étudier ces types de cancers notamment le cancer du foie auquel mon équipe s'intéresse afin de trouver des traitements plus efficaces.

La protéine p97 est connue pour être impliquée dans de nombreuses pathologies neurologiques et des cancers. En effet, la surexpression de cette protéine a été associée à un mauvais pronostic de survie dans un très grand nombre de cancers : poumon, prostate, œsophage, colon, pancréas et foie. Bien que l'implication directe de la protéine p97 dans le développement et la progression du cancer du foie ait déjà été prouvée, les mécanismes moléculaires par lesquels la protéine p97 contribue à la progression et au développement de ce cancer reste peu claire. Nos récents travaux ont permis de montrer que le Sorafenib, le seul traitement qu'il existe aujourd'hui contre le cancer du foie agissait sur cette protéine. *In vitro* le Sorafenib provoque la mort des cellules cancéreuses et nous avons montré que cet effet était augmenté en associant le Sorafenib à des molécules inhibitrices de la protéine p97, ouvrant la voie à de possibles nouveaux traitements. En effet, nous avons des preuves que la protéine p97 participerait au développement du cancer du foie en régulant de nombreux autres gènes dont certains oncogènes. Nous avons cette année découverte une nouvelle façon pour la protéine p97 de réguler à distance certains autres gènes notamment les gènes de stress du Réticulum Endoplasmique. Cette régulation impliquerait la translocation de p97 au noyau en condition de stress du Réticulum Endoplasmique et son association avec des complexes

remodeleurs de la chromatine. En effet en condition de stress du Réticulum Endoplasmique la protéine p97 s'associerait de façon préférentiel avec les protéines RuvBL2 et HDAC1 et entrainerait leur déstabilisation et par conséquence l'activation transcriptionnel des gènes qu'ils répriment. Il y a donc une réelle nécessité d'étudier la façon dont la protéine p97 participe à la régulation de ces gènes pour comprendre comment elle promouvoit le développement du cancer. Il est fort probable que le rôle et l'implication de la protéine p97 dans le cancer continue de croître dans les années à venir. Cette protéine pourrait en effet devenir une cible thérapeutique très intéressante. Ainsi, la découverte de molécules régulant cette protéine pourrait permettre en association avec des traitements courants de soigner le cancer du foie, mais aussi s'appliquer à d'autres cancers et pathologies.

INTRODUCTION

I) Endoplasmic Reticulum

1) Endoplasmic Reticulum structure and functions

a) Structure

The secretion pathway was studied in yeast and mammalian since the early 60's, which led to the identification of its different compartments and their hierarchy (1). The Endoplasmic Reticulum (ER) is the first compartment of the secretory pathway, it is composed of a network of membranes called cisternae organized in membrane-enclosed sacs or tube-like structures. The ER membranes are continuous with the outer nuclear membrane and two types of ER can be differentiated: the Rough and the Smooth Endoplasmic Reticulum. The Rough ER (RER) is covered with ribosomes on its cytosolic face which are the site of active protein translation. Future secreted or transmembrane proteins synthesized at the ribosomes bound to the ER can enter the ER lumen proteins to be folded and matured, if these proteins reach their correct conformation they can escape the quality control machinery of the ER to be exported to their final destination.

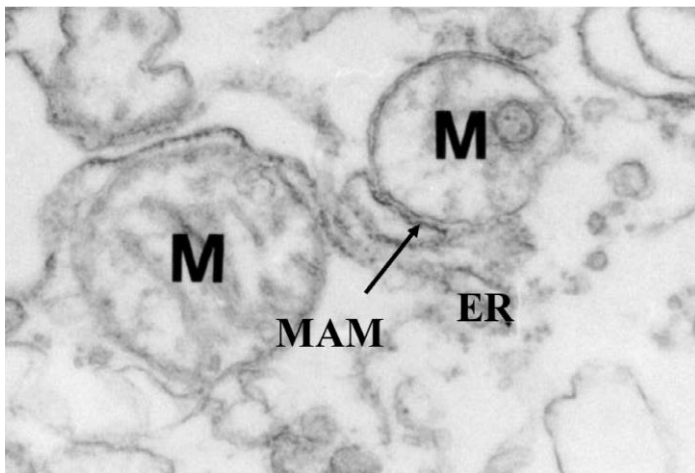


Figure 1: Electron microscopy image of the physical interaction between the ER and Mitochondria: Mitochondria Associated ER Membrane (MAM).

The smooth ER (SRE) is identifiable by the lack of ribosomes on its surface and functions in metabolic processes such as the synthesis of lipids, phospholipids and steroids. Beyond being the first compartment of the secretory pathway and the site of lipid synthesis, the role of the ER extends to the regulation of calcium, detoxification and maintenance of protein homeostasis (2). To ensure specific functions the ER is sub-compartmentalized and this is not only determined by

the type of ER (e.g. smooth or rough) but also by the localization of the ER membrane that can interact with the plasma membrane, the nucleus or other organelles such as: Mitochondria at Mitochondria-Associated ER Membrane (MAM) domains (**Figure 1**), lysosomes and Golgi apparatus.

b) Calcium storage and regulation

The calcium ion (Ca^{2+}) is essential to human physiology as it is involved in various biological processes such as bone formation, hormone regulation, heart contraction or embryonic development as well as playing a major second messenger role in signal transduction pathways (3). Therefore cellular calcium concentration must be tightly controlled in a space- and time-dependent manner. This is achieved by pumps and exchangers such as IP3R receptors (Inositol tri-phosphate Receptor 1-3) or SERCA proteins (Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase). In the cell, the ER is the main calcium storage compartment. Indeed the average ER calcium concentration is of 300 μM and it can go up to 1000 μM whereas the average cytosolic calcium concentration is of about 0.1 μM (3000-fold lower) (4). ER calcium homeostasis is essential, as too much Ca^{2+} in the cytosol can impair the normal functions of the cell, and too little Ca^{2+} in the ER lumen can impair ER functions and lead to ER stress. In addition this important difference in Ca^{2+} concentration between the ER and the rest of the cell allows for its fast and controlled release in response to stimulus and as such Ca^{2+} can act as a signal in many cellular processes such as secretion or motility. The release of the Ca^{2+} from the ER to the cytosol is controlled by 2 receptor families: the RyR (Ryanodine receptor) and the IP3R receptors (Inositol tri-phosphate Receptor 1-3) (**Figure 2**) (5). Moreover, Ca^{2+} can also be exchanged from the ER to the mitochondria at Mitochondria-Associated ER Membrane (MAM) domains thanks to VDAC (Voltage-dependent Anion Channel) pump and IP3R receptor which is regulated by the protein receptor $\sigma 1\text{R}$ (Sigma-1 receptor) The entrance of the Ca^{2+} into the ER relies on the SERCA proteins (Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase): ionic pumps that use the energy released by ATP hydrolysis to import the Ca^{2+} (6) In human cells there are 3 SERCA genes (SERCA1-3) which encode up to 10 isoforms by alternative splicing, these isoforms are differentially expressed between cell types to answer specific needs (e.g.: skeletal muscle, cardiac muscle). In the ER lumen the Ca^{2+} retention is mediated by the high capacity of many ER

resident proteins to bind it such as: calreticulin (CALR), calnexin (CANX) or GRPs (Glucose Related Protein) (7).

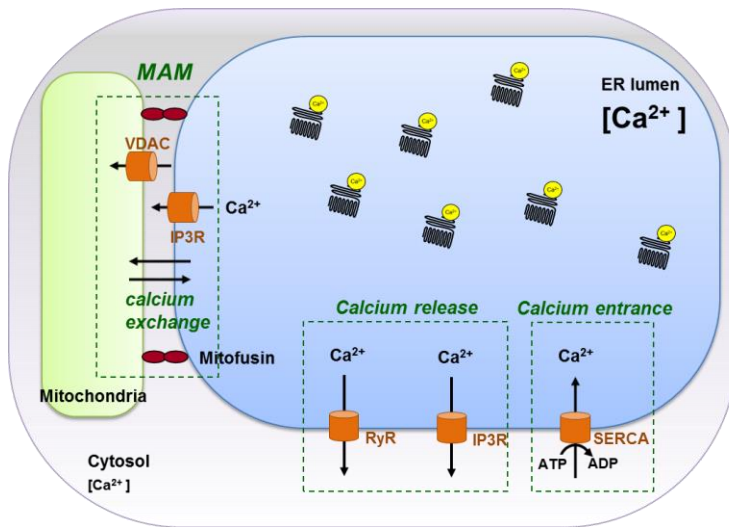


Figure 2: Schematic representation of calcium storage and regulation by the ER in the cell - The ER can release calcium in the cytosol through the RyR or IP3R receptors. To store calcium the ER relies on the SERCA pump. In the ER lumen the calcium is bound to ER resident proteins. The ER can also exchange calcium with the close mitochondria at MAM domains. These MAM domains are stabilized by Mitofusin which are mitochondria membrane proteins that can tether the Mitochondria to the ER.

c) Lipid synthesis

In the Eukaryotic cell the phospholipid synthesis mainly takes place in the Golgi apparatus and the ER, more precisely in the Smooth Reticulum Endoplasmic (SER) characterized by the low density of ribosomes surrounding the ER membrane. The phospholipids are the main components of all membranes and are used for the cell membrane and organites membranes such as endosomes, lysosomes, Golgi apparatus (8). Soluble cytosolic precursors are assembled on the cytosolic face of the SER, then flippase enzymes help the newly synthesized phospholipids to form a lipid bilayer (9). These lipids are then integrated into exocytosis vesicles for transportation before their release and merging with the destination membrane. However, thanks to the action of Lipid Transfer Proteins (LTP) if the destination of a lipid is a membrane close enough from the ER, like mitochondria at MAM domains there is no need for vesicle transportation (10). The SER is usually abundant in cells specialized in lipid metabolism: cells that synthesize steroid hormones from cholesterol. This is mainly achieved by the activation of transcription factors including SREBPs (Sterol Regulatory Element Binding Proteins). These transcription factors are synthesized as inactive precursors and are anchored in the ER membrane into protein complexes that are able to also bind to sterol lipids (SCAP, INSIG1, INSIG2) (11-12). Upon cholesterol depletion, the SCAP/SREBP complex is recruited by COPII (Coat Protein Complex II) vesicles

and exported to the Golgi apparatus where SREBP is cleaved by the S1P and S2P proteases thus releasing an active transcription factor in the cytosol (13). Alternatively, SREBP release can be induced by caspases 3 and 7. As a result, SREBP with the help of co-activators such as CBP (CREB Binding Protein), CBF (CCAAT-Binding Factor), NFY (Nuclear Factor Y) and Sp1 (Stimulating protein 1) activates the transcription of its target genes whose products are involved in lipid synthesis including cholesterol. The cholesterol synthesized in the ER can be exported in transport vesicles to any cellular membrane of which it is an essential component. If the cholesterol level at the membrane is diminishing, feedback loop exist to activate SREBP and re-synthesize cholesterol.

d) Protein folding and maturation

Proteins that are destined for the secretory pathway possess a signal peptide at the beginning of their sequence (**Figure 3**). During their synthesis at the ribosomes bound to the ER when this peptide is synthesized the translation is halted by the binding of the Signal Recognition Particle (SRP) to the signal sequence (14a). The complex composed of the mRNA, SRP, the ribosome and newly synthesized peptide bind to the cytosolic face of the ER by interacting with membrane proteins such as the SRP receptor and the Translocation Associated Proteins (TRAP). At this stage SRP dissociates from the nascent chain and translation resumes. Then the newly synthesized proteins can enter the ER lumen through a protein channel called the translocon. This channel is composed of SEC61 proteins and act as an aqueous channel through the hydrophobic membrane of the ER (14b). Inside the ER the signal peptide is cleaved by a signal peptidase that belongs to the serine protease family and the newly synthesized protein is then taken in charge by post translational modification (PTM) enzymes including PDIs (Protein disulphide isomerase) and Oligosaccharyl-transferase (OST). PDIs catalyze the formation of disulfide bonds between cysteine residues within the protein and OST is a multimeric complex containing eight different proteins located at the ER membrane that can transfer a preassembled oligosaccharide to selected asparagine residues, a process also called N-glycosylation. These PTMs are crucial for the protein to find its correct conformation (15), but some also require the help of foldases or chaperones. Foldases such as Grp58, PPI (Peptidyl prolyl cis-trans-isomerase) use the energy of ATP hydrolysis to catalyze structural transitions in the polypeptide chain. In contrast, chaperones such as BiP, GRP78 or GRP170 create a suitable environment for the peptides by masking the

hydrophobic sequences to prevent potential aggregation which could lead to a non-functional protein or by bringing closer different sequences of the peptide. If the protein is correctly folded and modified it is not held in the ER by the Quality Control (QC) machinery and can be exported to other compartments (15,16). However if the folding and maturation process fails, the protein is held in the ER by the QC machinery to get further folding chances, if the protein is terminally misfolded it is directed to the ERAD (Endoplasmic Reticulum Associated Degradation) pathway for degradation.

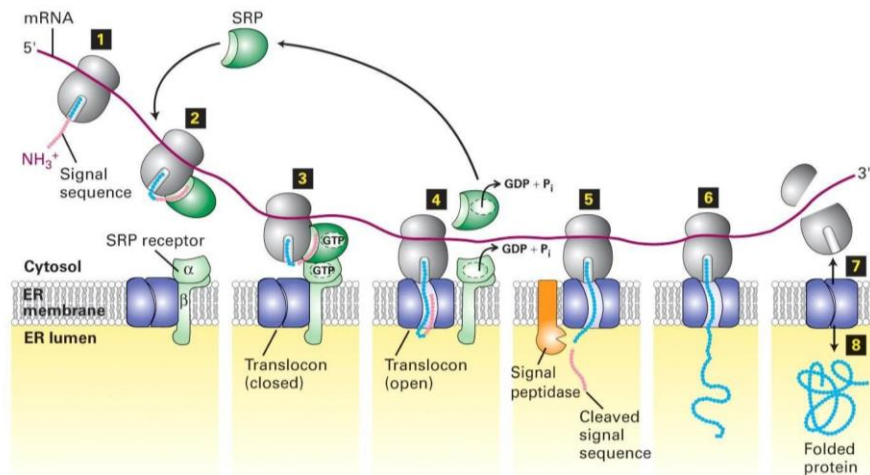


Figure 3: Co-translational translocation of a newly synthesized protein in the ER – 1) The mRNA is translated by the ribosome until the signal sequence is synthesized. The signal sequence is recognized by SRP. 2) The complex form by the mRNA/ribosome/nascent peptide and SRP is recruited at the ER membrane. 3) The nascent peptide is introduced inside the opened translocon. The signal peptidase cleaves the signal sequence. 4) Once the translation is finished the ribosome is released and the newly synthesized protein is taken in charge by PTM enzymes in the ER lumen..

e) Protein export from the ER

Proteins that enter the ER to be secreted can account for about 30-50% of all the protein synthesized by the cell (**Figure 4**) (17). Proteins that have passed the QC machinery are released from the ER at ER exit sites (ERES). These ERES are small membrane clusters that are contiguous with the ER membrane and that can bud to move to the Golgi (18). When these ERES start budding they are coated with COPII to form a vesicle, after their detachment from the ER these vesicles will flow to the Golgi where they will be recruited by surface receptor proteins. The vesicles will then be uncoated and fuse with Golgi membrane thus releasing their cargo proteins which will progress into the secretory pathway.

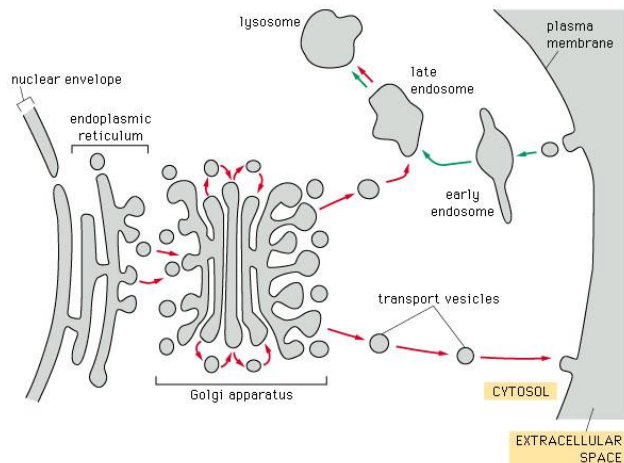


Figure 4: Schematic representation of the secretory pathway - The first compartment of the secretory pathway is the ER, proteins that are destined to be secreted are first synthesized at the ER membrane before they are folded in the ER lumen. They then exit the ER at ER exit sites and are transferred to the Golgi apparatus where they will complete their maturation. Finally proteins are exported to their final destination either to the cell membrane through transport vesicles or lysosomes for degradation (adapted from 19).

On the contrary terminally misfolded proteins are directed to the ERAD pathway for degradation (**Figure 5**) (20). The first step of the ERAD is substrate recognition: terminally misfolded proteins contains substructures such as exposed hydrophobic regions, unpaired cysteine residues or immature glycans which are recognized by ER sensor proteins of the ER quality control machinery. In mammalian cells two distinct ERAD pathways exist for glycoproteins and nonglycoproteins: Glycoproteins are recognized by lectin chaperones calnexin and calreticulin which can interact with ERp57 a glycoprotein specific thiol-disulfide oxidoreductase whereas nonglycoproteins are recognized and recruited to ERdj5 by BiP (21).

Evidences suggest that these misfolded proteins account for 30% of the proteins that enter the ER (22). Next the misfolded protein escorted by the chaperones is transported to a protein channel called retrotranslocon so it can be exported into the cytosol. The driving force required to extract the protein is provided by the AAA+ ATP p97/VCP/CDC-48 and its cofactors at the exit of the retrotranslocon (23). As the protein exits the dysloncon it is ubiquitinated (ubiquitin is a small 76 amino acid peptide) mainly by SYVN1 and MARCH6 two ER membrane anchored ubiquitin ligases. This polyubiquitin tag is then recognized by the 19S subunit of the proteasome and the polypeptide chain is fed into the central chamber of the 20S core region for proteolytic degradation.

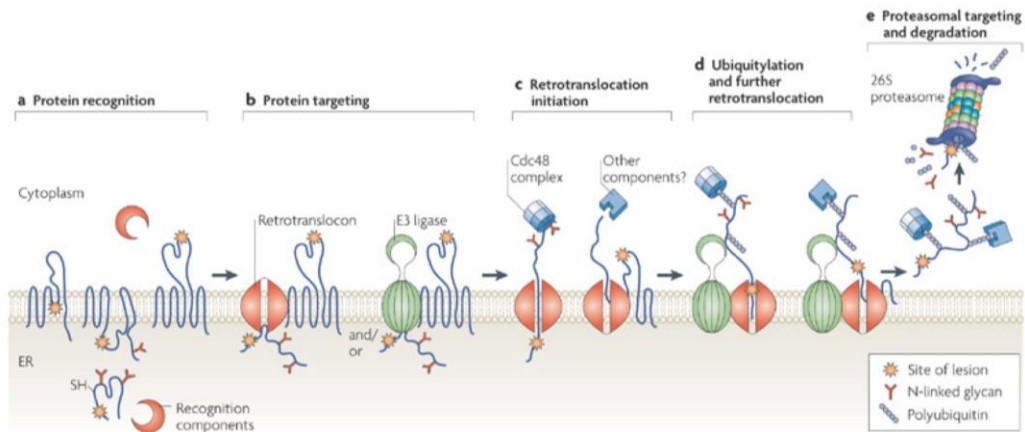


Figure 5: Schematic representation of Endoplasmic Reticulum Associated Degradation. A) Misfolded proteins contain substructures that are recognized by ER chaperones of the ER quality control machinery. B) The misfolded protein is addressed to the retrotranslocon by ER chaperones. C) P97/VCP/CDC-48 thanks to the energy of ATP hydrolysis pulls the protein out of the ER through the retrotranslocon. D) As the protein exits it is polyubiquitinated by E3 ubiquitin ligases. E) Once the ubiquitin modified protein is in the cytosol it is addressed to the proteasome for proteosomal degradation (adapted from 23).

2) Endoplasmic Reticulum signalling

As previously described, the ER homeostasis relies on several fine-tuned processes: protein folding, quality control, protein export or degradation and certain conditions such as change in ATP, calcium, redox state of the cell, or glucose can affect the folding of proteins in the ER. If the protein folding demand exceeds ER folding capacity thus disturbing the ER homeostasis it leads to a situation called ER stress. To restore its homeostasis the ER triggers an adaptive signalling response called the Unfolded Protein Response (UPR) (24). The following book chapter describes the signalling that emanates from the ER in response to ER stress: the UPR at the transcriptional, post-transcriptional, post-translation and epigenetic levels. This book chapter also highlights that the UPR signalling regulates pathways whose roles go beyond restoring the ER homeostasis. Finally, it provides an overview of the importance of ER stress induced transcription in diseases.

BOOK CHAPTER

Epigenetic Regulation of Endoplasmic Reticulum Stress

K. Barroso, E. Chevet

Centre de Lutte Contre le Cancer Eugène Marquis, Rennes, France

OUTLINE

Introduction	271	<i>Transcription Factor Complex Recruitment and Chromatin Modifications in the UPR</i>	277
<i>Response to Endoplasmic Reticulum Stress:</i>		<i>Relevance of ER Stress-Induced Transcription in Diseases</i>	279
<i>The Unfolded Protein Response</i>	272	Cancer	279
<i>Unfolded Protein Response Signaling</i>	272	Neurodegenerative Disorders	280
PERK Signaling	272	Inflammatory and Metabolic Diseases	281
ATF6 Signaling	273	Conclusions	281
IRE1 α Signaling	273	Acronyms	282
<i>The UPR Transcriptional Control Network</i>	274	Acknowledgments	282
Non-Canonical Regulation of Transcription	274	References	282
Posttranslational Modifications and Transcription Factor Complexes in UPR-Induced Transcription	275		
Crosstalk Between UPR and Other Signaling Pathways	275		
<i>ER Stress Posttranscriptional Signaling Networks</i>	276		
Gene Regulation by RIDD	276		
Gene Regulation by miRNAs	277		

INTRODUCTION

Cells in the body are often exposed to changes in their own nature (intrinsic) or in their environment (extrinsic) that can create stresses to which they must adapt to survive. As such, cells have evolved many adaptive mechanisms, some of which involve epigenetic regulation of gene expression leading to specific cellular protective responses [1]. In this chapter, we discuss the importance of epigenetic regulations occurring in response to protein homeostasis (also named proteostasis) imbalance in the endoplasmic reticulum (ER) [2]. We first describe the consequences of proteostasis disruption in the ER, which leads to a situation called ER stress and the subsequent activation of an adaptive response named unfolded protein response (UPR) [3], whose main function is to restore ER homeostasis. We then overview the molecular mechanisms involved in the UPR and the major transcriptional mechanisms controlling cell reprogramming. Moreover, we describe the dynamic interplay between transcription factors and chromatin changes that occur upon activation of the UPR. Finally, we define the relevance of these epigenetic changes in diseases.

Response to Endoplasmic Reticulum Stress: The Unfolded Protein Response

The ER is the first compartment of the secretory pathway and is responsible for the folding, maturation, quality control, and export of secreted or transmembrane proteins. Secretory proteins or proteins that are destined to the ER, plasma membrane, Golgi apparatus, or lysosomes represent one-third of all proteins. These proteins are translated on ER membrane-bound ribosomes and transit through the ER lumen. Before reaching their final destination, these proteins are folded and modified by chaperones, oxidoreductases, and glycosylation enzymes. Proteins that do not acquire their correct conformation are removed from the ER and ubiquitinated for subsequent degradation by the proteasome, a process called ER-associated degradation (ERAD). Evidence suggests that at least one-third of proteins that transit into the ER fail to satisfy the requirement of the ER quality control [4]. It is also noteworthy that the ER folding capacity varies among cell types; indeed, specialized secretory cells have a well-developed ER and therefore display increased protein-folding capacity.

When the protein load exceeds the protein-folding capacity of the ER, accumulation of improperly folded proteins causes ER stress. This accumulation can result from various conditions such as nutrient deprivation, hypoxia, calcium homeostasis disruption, or increased protein synthesis. To survive, cells activate the UPR [5], an adaptive mechanism that aims at restoring ER protein homeostasis. However, if the stress cannot be resolved, UPR signals trigger apoptosis. This adaptive response is known to involve many epigenetic changes including chromatin modifications, expression, and/or activation of transcription factors or regulations of non-coding RNAs. Most importantly, it has been reported that ER stress is involved in a wide range of human diseases such as diabetes, neurodegenerative disorders, stroke, pulmonary fibrosis, viral infection, inflammatory and metabolic disorders, cancers, and heart disease [6]. As a result, UPR signaling has been intensively studied to understand its contribution in diseases and to identify new therapeutic avenues.

Unfolded Protein Response Signaling

Since the discovery of an ER stress-induced adaptive response in the yeast *Saccharomyces cerevisiae* by Kozutsumi and colleagues [7], the UPR has been well described, and today much is known about how the canonical UPR works. In humans, the detection of misfolded proteins in the ER lumen relies on three ER transmembrane sensors: PERK (protein kinase RNA-like ER kinase), ATF6 (activating transcription factor 6 alpha), and IRE1 α (inositol-requiring enzyme 1 α ; Fig. 15.1). This is regulated by the dissociation of the ER-resident chaperone binding immunoglobulin protein (BiP), also known as glucose-regulated protein 78 kDa (GRP78), from the three sensors. Indeed, in basal conditions, BiP binds to the luminal domain of these three sensors, which prevents their activation. Accumulation of misfolded proteins in the ER causes the dissociation of BiP from the sensors, leading to their respective activation.

In the next sections, we describe the canonical UPR signaling, which comprises expression and activation mechanisms of the following transcription factors: X-box binding protein 1 (XBP1), activating transcription factor 4 (ATF4), activating transcription factor 6 (ATF6), and their target genes. Changes in posttranscriptional regulation due to miRNAs or mRNA stability will also be discussed. Finally, we define the specific and dynamic chromatin modifications associated with these changes in ER stress-induced transcriptional regulation.

PERK Signaling

Dissociation of BiP from PERK allows PERK oligomerization and *trans*-autophosphorylation, leading to activation of this kinase. Activated PERK phosphorylates the translation initiation factor eIF2 α (eukaryotic initiation factor 2 α), thus preventing the formation of the translation complex and causing a global attenuation of translation in the cell. This mechanism aims at limiting the amount of newly synthesized proteins that enter the ER. Although phosphorylation of eIF2 α diminishes the overall translation, mRNAs that contain a μ ORF upstream of the start codon are selectively translated, including ATF4, which is the major transcription factor activated downstream of PERK and belongs to the basic leucine zipper (bZIP) family [8]. This transcription factor binds to endoplasmic reticulum stress element (ERSE) sequences that are found on most ER stress-inducible genes to promote their transcription. ATF4 target genes include molecular chaperones (such as oxygen regulated protein 150 (ORP150), BiP, glucose-regulated protein 94 (GRP94), protein disulfide isomerases), and CHOP (C/EBP homologous protein). Interestingly, a growing number of reports (detailed in the UPR transcriptional control network section) are showing that ATF4 also participates to the control of genes from other pathways, including antioxidant response, hypoxia [9], autophagy [10], amino acid metabolism, apoptosis, and angiogenesis (vascular endothelial growth factor (VEGF),

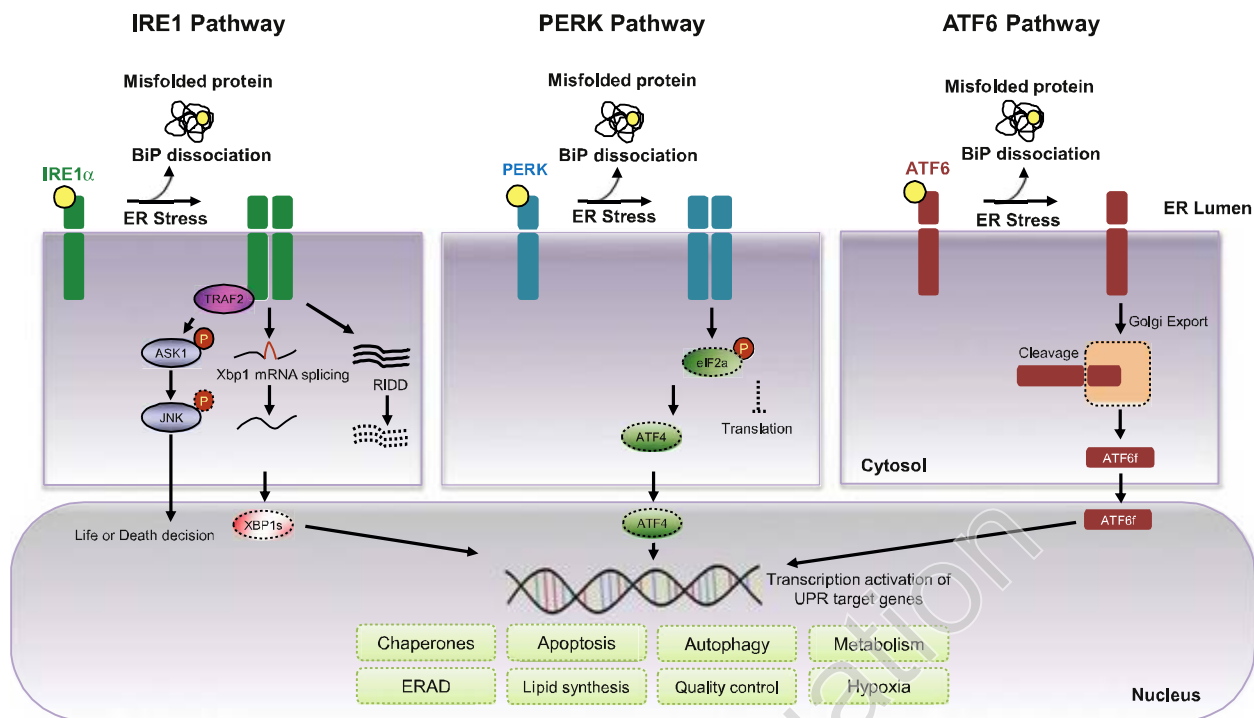


FIGURE 15.1 The UPR canonical pathway. Major proteins of the three arms of the UPR are indicated. Under basal conditions, BiP association with IRE1 α , PERK, and ATF6 prevent their activation. Accumulation of unfolded proteins in the ER lumen causes BiP dissociation and subsequent activation of IRE1 α , PERK, and ATF6 export to the Golgi. Activated IRE1 α controls Xbp1 mRNA splicing, TRAF2/ASK1/JNK, and RIDD pathways. Activated PERK phosphorylate eIF2 α causing a global translation attenuation that allows ATF4 mRNA translation. After ATF6 is export to the Golgi apparatus, it is cleaved to become an active transcription factor. Ultimately, all three arms of the UPR result in the synthesis of transcription factors that promotes transcription of UPR target genes to restart protein homeostasis.

fibroblast growth factor 2 (*FGF2*), and interleukin 6 (*IL-6*, [11])). The second major transcription factor of the PERK signaling pathway is CHOP, but it is also a transcriptional target of ATF6 and XBP1 [12]. During ER stress, CHOP translocates to the nucleus, where it controls programmed cell death genes and also the expression of *GADD34* (growth arrest and DNA damage-inducible protein 34) [12,13]. *GADD34* in association with protein phosphatase 1 (PP1c) controls the dephosphorylation of eIF2 α to reverse the translational inhibition caused upon PERK activation [14].

ATF6 Signaling

Under basal conditions, ATF6 is an inactive ER-localized transmembrane transcription factor. During ER stress, dissociation of BiP causes ATF6 export to the Golgi apparatus, where it is cleaved by the proteases site-1 protease (S1P) and site-2 protease (S2P) to liberate its transcriptionally active cytosolic domain [15]. This export also depends on ATF6 cysteine oxidation status [16] and protein disulfide isomerase A5 (PDIA5) [17]. The ATF6 cytosolic domain (ATF6f) is a bZIP transcription factor that translocates to the nucleus, where it regulates the expression of genes coding for proteins of the folding machinery such as chaperones of the glucose-regulated protein family and ERAD genes mostly by binding to ERSE motifs [18].

IRE1 α Signaling

During ER stress, activation of IRE1 α involves its oligomerization and *trans*-autophosphorylation downstream of the dissociation from BiP. IRE1 α exhibits two distinct cytosolic enzymatic activities: a kinase and an RNase activity. IRE1 α RNase activity is mainly known for removing a nonconventional intron from the XBP1 mRNA [19], causing a shift in the reading frame, which results in the translation of a potent transcription factor: XBP1s [20]. XBP1s is the main transcription factor of the IRE1 α signaling branch and promotes the transcription of genes whose products are involved in the folding machinery and ERAD, as it can also bind to ERSE motifs. Additionally, XBP1s participates in a wide range of UPR-independent cellular processes as it controls the expression of genes coding for proteins that are

involved in lipid synthesis [21], protein secretion [22], DNA damage and repair [23], angiogenesis (eg, VEGF pathway [24]) and even cell differentiation [23,25]. Regulation of genes from other pathways by XBP1 or other UPR transcription factors is herein referred to as the non-canonical UPR transcriptome (detailed in the UPR transcriptional control network section). Two roles were also reported for the un-spliced XBP1 protein called XBP1u as (1) it regulates its mRNA by delivering it to the ER for processing, and (2) it participates to the degradation of XBP1s [26]. IRE1 α RNase activity is not only important for the splicing of XBP1 mRNA, but it is also involved in the degradation of several RNA including mRNAs [27,28], ribosomal RNA [29], and microRNAs; this mechanism is known as regulated IRE1-dependent decay (RIDDC [30]). Finally, IRE1 α can also interact with the scaffold protein TNF receptor-associated factor 2 (TRAF2) to recruit and activate apoptosis signal-regulating kinase 1 (ASK1) and Jun N-terminal kinase (JNK). The ASK1/JNK1 pathway is crucial for triggering apoptosis if the cell fails to restore protein homeostasis [31].

The UPR Transcriptional Control Network

Microarray studies on ER stressed cells/tissues have revealed that the transcriptional output of the UPR is far from simple, as many genes unrelated to the ER proteostasis control machinery are upregulated. For instance, genes involved in metabolism, inflammation, and cell differentiation are also upregulated upon ER stress [32–34]. The simplistic vision of the UPR as a linear pathway is currently getting challenged. Indeed, the recent discovery of novel functions ensured by the UPR that are not directly related to protein-folding and exerted through the activation of UPR transcription factors in a wide range of cellular processes provides a new perspective on the global role of the UPR. Transcripts that are not regulated by the canonical UPR or that do not carry ER stress-related functions are considered non-canonical. Indeed, it appears that the transcriptional reprogramming depends on the input or stimuli and on the cell type. Here, we report several examples where UPR transcription factors target a different set of genes between the canonical and non-canonical pathways.

Non-Canonical Regulation of Transcription

As described earlier, phosphorylation of eIF2 α during ER stress causes a global translation inhibition; however, the extent and consequences on gene expression are not fully known. The translation inhibition affects all proteins including transcription factors according to their half-life and the duration of eIF2 α phosphorylation, which is regulated by PP1c and GADD34 [14]. In this context, proteins with short half-life decay faster than other proteins. Therefore, if a transcription factor has a longer half-life than its inhibitor protein and if the translation inhibition lasts long enough, loss of the inhibitor could free the transcription factor to activate transcription of its target genes. This mechanism was shown to be involved in pro-inflammatory signaling through regulation of nuclear factor kappa B (NF- κ B) a key player of the immune response, cellular growth, developmental processes, and apoptosis. Inhibitor of kappa B (I κ B), a repressor of NF- κ B, has a shorter half-life than NF- κ B. Therefore, ER stress and the resulting translation inhibition cause a decrease in I κ B protein level that liberates NF- κ B to activate the transcription of its target genes [32]. The global translation inhibition also affects mRNA differently; while most mRNA (90%) are not translated when eIF2 α is phosphorylated, the translation of 2–8% of cellular transcripts is increased [35]. These transcripts include some transcription factors, such as ATF3, CHOP, JUN, JUNB, FBJ murine osteosarcoma viral oncogene homolog (FOS), FOS-B, and cAMP responsive element binding protein 1 (CREB1), and their translation leads to transcription of their target genes. It is likely that it is the presence of a μ ORF (like ATF4 mRNA) on these mRNA that enables them to be selectively translated, but it has not been proved experimentally yet [36]. PERK and IRE1 α activation could also cause non-canonical transcription outputs. In theory, because they are both kinase, there is a possibility that they could have unknown substrates and activate (directly or not) a transcription factor that could partly explain the complexity of the UPR transcriptome.

Recently, a novel non-canonical pathway has been uncovered and regulates the UPR transcriptional program. Indeed, the AAA⁺ ATPase valosin-containing protein (VCP), also known as p97, is a key component of the ERAD machinery [37] that promotes the degradation of misfolded proteins by directing them to the proteasome. Beyond its role in ERAD, a novel function was discovered for p97/VCP as it regulates transcription regulation upon ER stress [38]. This was shown to act through the posttranslational regulation of RUVBL2, another AAA⁺ ATPase. Indeed, under basal conditions, RUVBL2 represses XBP1 mRNA splicing and ATF6 activation and, as a consequence, the transcription of their target genes. However, upon ER stress, RUVBL2 is degraded through a p97/VCP-dependent mechanism, allowing XBP1 mRNA processing, ATF6 activation, and subsequent transcription

activation of select target genes. These results suggest a novel mechanism of regulation of ER stress genes mediated by the two AAA⁺ ATPase p97/VCP and RUVBL2.

Posttranslational Modifications and Transcription Factor Complexes in UPR-Induced Transcription

The major transcription factors of the UPR are bZIPs: ATF4, ATF6, XBP1, CHOP, and JUN. It is known that members of this family can form homotypic and heterotypic dimers; therefore these complexes could each regulate different sets of genes [32]. Additionally, it is well known that posttranslational modifications can alter transcription factor stability and, as a consequence, the genes they regulate. Indeed, ATF4 phosphorylation, ubiquitination, and acetylation affect its stability and, as a consequence, its activity, leading it to regulate transcription differently [39]. ATF6f activity is also regulated by its phosphorylation status and by its association with specific cofactors: nuclear transcription factor Y (NF-Y), YY1, and TATA-binding protein (TBP [20,40]). Similarly, XBP1s is also regulated by posttranslational modifications: acetylation by p300 and SUMOylation by protein inhibitor of activated STAT-2 (PIAS2), which enhances its activity [41,42], whereas its phosphorylation is required to translocate to the nucleus [43]. Although, XBP1s posttranslational modifications have not been directly linked to the regulation of different target genes, many studies have described a role for XBP1s outside ER folding machinery and particularly in cell differentiation and survival. Indeed, XBP1s was shown to control the expression of the transcription factor *Mist1* involved in differentiation [23]. Another study reported a role for XBP1s in B-lymphocyte differentiation through events that depend on XBP1 mRNA splicing [25]. XBP1s may also control cell survival in estrogen receptor–positive breast cancer cells through modulation of *NF-κB* expression [44].

Additionally, it was shown that ATF6f and XBP1s can heterodimerize to control genes involved in folding, trafficking, and degradation of ER destabilized client proteins [45,46], which they could not control individually. Similarly, ATF4 and CHOP were also shown to heterodimerize during ER stress, and this interaction was required to control genes involved in protein synthesis and UPR. Also, if the stress persists, this increase of proteins leads the cell to apoptosis [47].

Crosstalk Between UPR and Other Signaling Pathways

Several reports have linked the UPR to signaling pathways induced by other cellular challenges (eg, hypoxia, DNA damage and repair, oxidative stress response, epithelial-to-mesenchymal transition). This was shown to occur often in the context of malignant cells where ER stress is triggered. Once again, activation of these distant pathways relies on epigenetic modifications including activation and synthesis of specific transcription factors.

In tumors, UPR and hypoxia pathways are often found co-activated for their cytoprotective properties, and there is a growing amount of evidence that those pathways are connected. Interestingly, hypoxia-inducible factor 1 α (HIF-1 α), a key player of the hypoxia pathway, is found more phosphorylated during ER stress, which is known to enhance its activity. Moreover, the proangiogenic factor *VEGF* is a common target of both pathways as XBP1, ATF4, and HIF-1 α can bind to its promoter, suggesting that simultaneous activation of both pathways in tumors may result in greater vascularization and tumor progression [48,49]. This represents another mechanism by which the UPR can promote malignancy.

Another example of such crosstalk is between ER stress and DNA damage and repair. Indeed, recent studies have suggested that ER stress may affect genomic stability and DNA repair pathways, which may contribute to oncogenic transformation. Various experiments in different systems [50–52] have shown a bidirectional regulation between UPR and DNA damage response. This was supported by studies in the budding yeast *Saccharomyces cerevisiae*, where IRE1p deficiency led to chromosome loss under basal conditions, a phenomenon that was further enhanced when DNA damage was generated by UV exposure. However, the exact contribution of ER stress in DNA damage and repair is unclear.

Oxidative stress, another common feature of tumor cells, is characterized by a disturbance in redox state that can be a threat for the cell through reactive oxygen species and is also related to ER stress. Indeed, PERK, upon activation, can recruit and phosphorylate transcription factor NRF2 (nuclear factor erythroid 2-related factor 2), triggering its dissociation from its repressor Keap1 (Kelch-like ECH-associated protein 1) and allowing its nuclear import where it controls the antioxidant response pathway [53,54]. ATF4 plays a dual role during oxidative stress: (1) it contributes to its worsening with the induction of ER oxidase 1 α (*ERO1* α) by CHOP, which increases formation of the disulphide bond and generates ROS, but also (2) it promotes amino acid import and synthesis, which limit the harmful impact of oxidative stress. Interestingly, XBP1u was shown to interact with histone deacetylase 3 (HDAC3) in endothelial cells [55], and it was part of a larger complex including mammalian target of rapamycin (mTOR) and

Akt1 that protects the cells from oxidative stress through the regulation of heme oxygenase-1 (*HO-1*) expression. This again raises the question of the XBP1u role that remains unclear.

The epithelial-to-mesenchymal transition (EMT) is a key process in both normal development and cancer progression. During this process, epithelial cells lose their cell polarity and cell–cell adhesions, and they gain migratory and invasive properties to become mesenchymal stem cells. These changes imply dramatic epigenetic regulations, as epithelial markers need to be downregulated and mesenchymal markers upregulated [56]. Recently, several reports have linked UPR and EMT; in this section, we describe diverse mechanisms by which ER stress leads to EMT phenotype [57]. For instance, in thyroid cells, the use of pharmacological ER stressors (thapsigargin or thapsigargin) triggered the UPR, the SRC pathway, and the transcriptional downregulation of the transcription factors that normally maintain thyroid cells differentiated: transcription terminator factor 1 (TTF1), transcription terminator factor 2 (TTF2), and paired box 8 (Pax8), causing dedifferentiation. ER stress also causes an EMT phenotype characterized by E-cadherin transcriptional downregulation, transcriptional upregulation of several mesenchymal markers (eg, vimentin, α -smooth actin muscle [α SMA], α 1 type I collagen [COL1A1], and SNAIL) and a change in cell morphology [58]. Similar observations were made in alveolar epithelial cells, where the UPR triggers the SRC and β -catenin pathways, causing an EMT-like phenotype [59]. Induction of a prolonged but nonlethal UPR by the overexpression of squamous cell carcinoma antigen 1 (SCCA1) in mammary epithelial cells allows NF- κ B to activate transcription of its target genes, including *IL-6*, also resulting in an EMT-like phenotype [60]. Finally, ER stress caused by disruption of p97/VCP induces change in expression levels of key EMT regulators: downregulation of E-cadherin (epithelial marker) and increase of SNAIL, zinc finger E-box binding homeobox 1 (ZEB1), vimentin (mesenchymal markers, resulting in an SRC-mediated EMT phenotype in A549), H358, and HPLD cells [61]. In conclusion, it seems that activation of UPR by disruption of the ERAD pathway, pharmacological ER stressors, or overexpression of a protein can lead to EMT through several signaling pathways (eg, SRC, IL-6, WNT), and this transition is accompanied by transcriptional silencing of epithelial markers and upregulation of mesenchymal markers. These observations raise the hypothesis that ER stressed cells could enter EMT to change their phenotypes, therefore attenuating the ER stress burden to survive.

In light of these observations, it appears that the UPR is a complex and interconnected network that depends on the stress intensity, cell condition, and/or the cell type. Moreover, the UPR and its network seems to be a very powerful tool for cancer cells to survive from DNA damage, oxidative stress, hypoxia, or even to acquire malignant traits such as invasion. Therefore, treatment with molecules that target the UPR could lower tumor cell survival, and combination with cancer treatments could be promising.

ER Stress Posttranscriptional Signaling Networks

A study by Kawai et al. [62] has revealed that during ER stress, around 75% of mRNA are regulated at least in part at the level of stability. In this section we describe two mechanisms of mRNA regulation: Regulated IRE1 Dependent Decay (RIDD) and miRNA-dependent posttranscriptional control.

Gene Regulation by RIDD

Recent evidences have shown that the stress intensity is controlling IRE1 α RNase activity. Indeed, under low stress, IRE1 α is in lower order oligomers (dimer/tetramer) and regulates XBP1 splicing, but under high stress, it associates in higher oligomers, enabling its RNase activity to target other mRNAs, leading to RIDD [63]. In contrast with those observations, an in vitro study suggests that one dimer is sufficient for RIDD activity, whereas higher level of oligomerization is needed for XBP1 splicing [64]. However, IRE1 α activity was analyzed under saturating protein concentrations, which may not be representative of the in vivo physiology. IRE1 α RIDD targets are involved in diverse processes such as transcription, signaling cascades, energy production, and lipid metabolism [27,65]. It appears that the RIDD activity not only degrades mRNA encoding ER translocated proteins but also mRNA encoding cytosolic proteins. This might be due to the fact that specific mRNAs are localized close to the ER membrane. An example of how RIDD activity affects UPR transcription is in glioblastoma. Period1 is a transcriptional repressor that plays a key role in the circadian rhythm, and its mRNA is a substrate of IRE1 α . Its degradation leads to increased expression of chemokine (C-X-C Motif) ligand 3 (CXCL3), an emerging key player in cancer development and subsequent tumor growth [66]. Similarly, secreted protein, acidic, cysteine-rich mRNA is also an IRE1 α RIDD substrate; its degradation leads to a change in the collective versus individual migration of glioblastoma cells and reduces cell migration. Additionally, IRE1 α cleaves the pro-oncogenic

glypican-3 (GPC3) mRNA in hepatocellular carcinoma [67], and it could dramatically affect transcription as GPC3 regulates signaling pathways mediated by WNTs, hedgehogs, and fibroblast growth factors.

In conclusion, little is known about RIDD substrate selectivity that may depend on the IRE1 α oligomeric state, its partner proteins, but also on the environmental conditions and cell type. More studies are needed to determine the RIDD substrate specificity and its extent and consequences on gene expression.

Gene Regulation by miRNAs

MiRNAs are short (~22 nt) single-stranded non-coding RNA that can promote degradation of their mRNA binding patterns. This mechanism represents another common way of mRNA regulation. A growing amount of evidence has demonstrated that this regulation occurs during UPR activation (reviewed in Ref. [68]). Although, several miRNAs have already been described to regulate positively or negatively the ER stress response, global approaches have shown that expression of many miRNAs is modified during ER stress [69,70]. In this section, we describe the miRNAs that are regulated by the three arms of the UPR and the consequences on gene expression.

Thus far only one miRNA was shown to be regulated by ATF6. In the pathological heart, ATF6 is activated and downregulates miR-455. Calreticulin (CALR) is a target of miR-455; therefore downregulation of this miR causes an increase in CALR expression, which in turn decreases hypertrophic growth [70]. IRE1 α RIDD activity is involved in degradation of several pre-miRNAs, including pre-miRs-17, -34a, -96, and 125b. Interestingly, these pre-miRs are not cleaved at the same sites when cleaved by the DICER complex or IRE1 α . Caspase 2 (CASP2), a protease that plays a key role in the execution-phase of apoptosis, is a target of these miRNAs. Therefore, during ER stress, their cleavage leads to increased expression of CASP2 and subsequent apoptosis [71]. Additionally, miR-17 can also target thioredoxin-interacting protein (TXNIP) mRNA. Decay of miR-17 by RIDD causes increased TXNIP expression, which controls NLR family Pyrin domain containing 3 (NLRP3) inflammasome activation, and the subsequent cleavage of pro-caspase-1 and interleukin-1 β (IL-1 β) secretion. This cascade of events leads to increase in systemic or local inflammatory response and promotes cell death [72]. In the IRE1 α pathway, XBP1 can also promote miR-346 expression that binds to transporter 1, ATP-binding cassette (TAP1) mRNA. Decrease of TAP1 expression causes a reduction in major histocompatibility complex class I-associated antigen presentation [73].

The PERK signaling pathway promotes several miRNAs that in turn regulate the expression of UPR pathways. Indeed, PERK activation promotes miR-30-c-2 and miR-211 that can repress XBP1 [74] and CHOP [75] expression, respectively. During UPR activation, this mechanism could allow the cell to specifically shut down a transcriptional program of another branch (IRE1 α /XBP1s) or within the same branch (PERK/ATF4/CHOP). Interestingly, miR-211 does not regulate CHOP expression at the posttranscriptional level by binding to its mRNA but rather at the transcriptional level by binding to its promoter and its nascent RNA. Presence of miR-211 at the CHOP promoter was associated with increase tri-methyl H3K27 (a repression of transcription mark), a sign of heterochromatin. Analysis of tumors also revealed that miR-211 is overexpressed in tumors and inversely correlated with CHOP expression. These data suggest that during early ER stress miR-211 attenuates CHOP induction by the UPR, thereby promoting cell survival [75]. In the PERK pathway, CHOP was shown to control the expression of miR-708, contributing to brain metastasis [76]. Last, in mouse embryonic fibroblasts, ATF4 and NRF2 downregulate miR-106b-25, which promotes BIM expression and subsequent apoptosis [77].

These examples illustrated another level of regulation of the UPR that could be by inhibiting or promoting only certain genes control specific transcription programs. This kind of fine-tuning regulation could be involved in modulation of the survival/death balance.

Transcription Factor Complex Recruitment and Chromatin Modifications in the UPR

As we previously mentioned, the UPR promotes activation and/or synthesis of several transcription factors that regulate the expression of many genes. In this section, we discuss the less studied chromatin modifications and the dynamic of transcription factors recruitment to ER stress gene promoters that accompany the UPR transcriptional regulation. Most ER stress gene promoters contain an ERSE box where the transcription factor NF-Y is constitutively bound, even in basal conditions. NF-Y is not a strong activator, but it helps the recruitment of transcription factors such as ATF6, XBP1s, and other cofactors [23,78,79]. Regarding transcription factor recruitment and histone modifications, *BiP* gene is better characterized than the other ER stress-inducible genes. Indeed, it was shown that tri-methylated H3K4 (H3K4me3) marks (activation of transcription mark) are constitutively presented on *BiP* promoter, while H4 acetylation (activation of transcription mark) increase only when ER stress is induced [80].

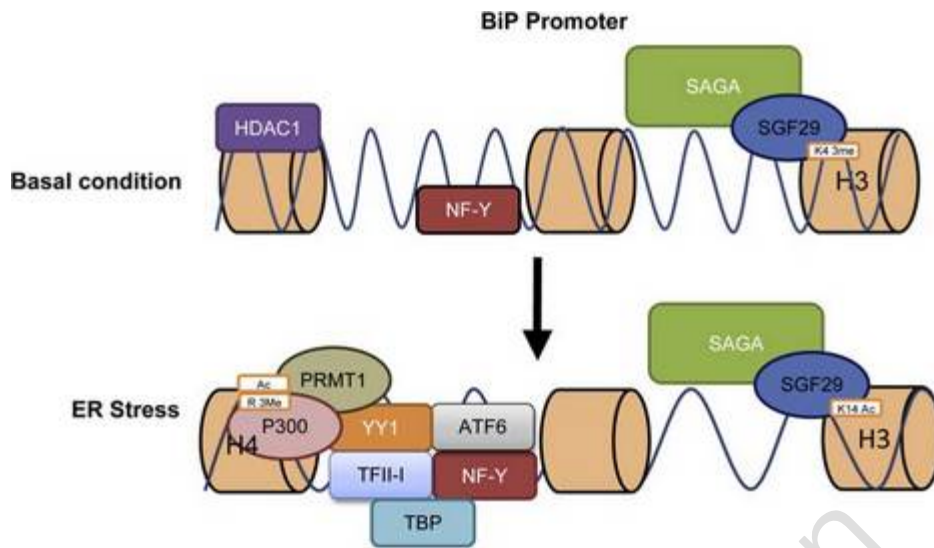


FIGURE 15.2 Dynamic recruitment of transcription factors and cofactors at the *BiP* promoter. In basal conditions, transcription factor NF-Y is found constitutively bound to *BiP* and other ER stress gene promoters; HDAC1 is also found and acts as a repressor of transcription, while SAGA and its subunit SGF29 maintain the level of tri-methyl H3K4, keeping the chromatin poised, and ensuring a rapid start of transcription if needed. Under ER stress, HDAC1 is dissociated from the chromatin, SAGA/SGF29 switch roles to promote acetylation of H3K14, and NF-Y allows the recruitment of various cofactors and transcription factors, such as ATF6, TBP, TFII-I, and YY1, that interact with two other chromatin remodelers: p300 and PRMT1. PRMT1 methylates the arginine of H4, and p300 acetylates H4. Altogether, those chromatin modifications and recruitment of transcription factors and cofactors cause the chromatin to unwind and promote transcription.

The same study also showed that the constitutively expressed transcription factor YY1 is key for optimal *BiP* transcription. YY1 was shown to physically interact with ATF6 and the chromatin modifiers protein arginine methyltransferase 1 (PRMT1) and the acetyltransferase p300 (Fig. 15.2), but these are only found on *BiP* promoter during ER stress. A later study from the same group showed that histone deacetylase 1 (HDAC1) acts as a repressor on *BiP* promoter before ER stress induction. Although HDAC inhibitors are emerging as effective therapies in the treatment of cancer, in this case, they activate *BiP* transcription, thus protecting the cell from apoptosis [81].

In an *in vitro* study, Donati and colleagues [82] investigated the dynamic processes that take place at ER stress gene promoters (including *BiP*, homocysteine-inducible endoplasmic reticulum stress-inducible ubiquitin-like domain 1 (*HERPUD1*), endoplasmic reticulum resident protein 70 (*ERP70*) and *XBPI*). RNA polymerase II (Pol II) was found at some promoters even before induction of ER stress, but TBP and p300 were not ubiquitously distributed and were recruited to these regions only after induction. Regarding histone modifications, they observed that on ER stress gene promoters H4 acetylation and H3K4me3 (two active transcription marks) increase drastically during ER stress, while a minor change was noticed in H3 acetylation compared to basal conditions. Increase of H3K4me2 (an active transcription mark) was also found, especially on the *XBPI* promoter. Because those marks were found at a high level on the coding sequence of these genes in unstressed cell, these data highlight a level of "preactivation" of ER stress genes. Also noteworthy, they noticed a depletion of histone H3 under maximal induction, reflecting important chromatin remodeling. Regarding transcription factor dynamic, they showed that *XBPI* rapidly binds to most ER stress genes tested, while ATF6 and CHOP bind to a few selective genes only. These observations highlight a very complex dynamic for both ER transcription factor binding and chromatin remodeling.

Schram and colleagues [83] further characterized the dynamic processes of chromatin modifications that occur upon ER stress gene promoters, and their results are consistent with previous reports. SAGA-associated factor 29 (SGF29) is a subunit of the histone acetyltransferase module of the SAGA and ATAC co-activator complexes that binds to H3K4me3. This study showed that SGF29 is both necessary to induce ER stress genes and for cells to survive ER stress. Indeed, SGF29 play two key roles: (1) prior to ER stress, SGF29 maintains H3K4me3 levels, conserving the chromatin in a poised state on ER stress target gene promoters, and (2) after ER stress induction, SGF29 is required to increase H3K14 acetylation levels on these genes, thereby promoting transcription and cell survival. In an *in vivo* study, on ethanol-fed cystathionine beta synthase-deficient mice, *BiP*, ATF4, CHOP, caspase 12 (CASP12) and sterol regulatory element binding transcription factor 1 (SREBP-1c) were found upregulated compared to control mice

reflecting induction of hepatic ER stress. Although there was no change in H3K4me3, a significant decrease of H3K9me3 (a repression of transcription mark) was observed on the promoters of *BiP*, *SREBP-1c*, and *CHOP* in ethanol-fed mice compared to the control group. This suggested the subsequent upregulation of the expression of these genes, all ER stress markers. Consistent with these observations, mRNA expression of the histone H3K9 methyltransferase G9a was decreased in ethanol-fed mice [84]. Finally, another link between UPR and epigenetic regulation has been established in *Caenorhabditis elegans*. Indeed, heterochromatin protein-like 2 (HPL-2, homolog of heterochromatin protein 1 [HP1]), an important protein for heterochromatin formation and therefore gene repression, was shown to downregulate UPR signals. Moreover, inactivation of HPL-2 is associated with increased expression of XBP1s that, in turn, activated autophagy genes and caused resistance to ER stress [85].

These reports indicate that there is a dramatic change on ER stress inducible gene promoters between basal conditions and after ER stress induction. Indeed, some transcription factors (NF-Y), chromatin remodeling proteins (HDAC1, SAGA), histone marks (H3K4me3), and Pol II seem to be constitutively present to ensure a rapid initiation of transcription, and after ER stress induction, other transcription factors (ATF6, XBP1), co-activators (YY1), and chromatin remodeling proteins (p300, PRMT1) are mobilized to ensure maximal transcription activation.

Relevance of ER Stress-Induced Transcription in Diseases

It is well known that ER stress caused by aging, genetic mutations, or environmental factors can result in various diseases such as diabetes, inflammation, and neurodegenerative disorders including Alzheimer disease, Parkinson disease, and bipolar disorder. In this section, we focus on the relevance of the change in transcription caused by ER stress in these diseases.

Cancer

Many reports have experimentally linked ER stress to cancer (reviewed in Ref. [86]). Indeed, tumor cells often experience nutrient deprivation and/or dysregulation of protein synthesis causing protein misfolding and subsequent ER stress. The activation of the UPR in tumor cells, together with the transcriptional changes that accompany it, have deep effects on cell proliferation and survival. All three arms of the UPR have been reported to participate in tumor malignancy by enhancing cell transformation, tumor cell dormancy, tumor growth, and EMT. Herein, we describe some examples of UPR epigenetic regulations that are involved in cancer.

Many reports have shown that PERK signaling is crucial for tumor survival in different challenging conditions. Indeed, in nutrient deprived-tumor cells, ATF4 is known to activate transcription of gene coding for proteins involved in amino acid import and synthesis. Moreover, under glucose deprivation, the PERK pathway plays a key role in angiogenesis. Indeed, transcriptome analyses have revealed that this pathway promotes upregulation of several pro-angiogenic factors (VEGF, FGF2, IL-6) and downregulation of angiogenesis inhibitors (thrombospondin 1 [THBS1], CXCL14, CXCL10) [87]. Reactive oxygen species, another common threat to survival for tumors, can be managed via activation of PERK signaling, as NRF2 phosphorylation by PERK leads to transcription of antioxidant genes that protect the cells. In human lymphomas, c-Myc-dependent increase of protein synthesis triggers the PERK pathway, which, by inducing autophagy, protects the cell by recycling long-lived proteins and damaged organelles to provide amino acids [88]. In several human cancer cell lines, during hypoxia, a similar mechanism was observed, as ATF4 and CHOP activate transcription of genes related to autophagy including *LC3* and autophagy-related gene 5 (*ATG5*) to promote survival [89].

Several reports also implicate the IRE1 α pathway (see Fig. 15.1) in tumor survival. Indeed, XBP1 mRNA splicing was proved to increase tumor tolerance to hypoxia, while loss of XBP1 prevents tumor cell survival and inhibits tumor growth [90]. In triple-negative breast cancer, XBP1s was shown to form a complex with HIF-1 α (one of the main transcription factor of the response to hypoxia) that activates transcription of HIF-1 α target genes, thus promoting survival in hypoxic tumors [49]. Moreover, activation of the UPR increases HIF-1 α activity through a phosphorylation-dependent mechanism, thereby resulting in increased transcription of its target genes including *VEGF*, which have been reported to promote angiogenesis in several cancers [24]. In tumors, this mechanism could promote survival, angiogenesis, and tumor growth.

Beyond its role in tumor survival, it was also shown that the UPR also contributes to tumor malignancy through modulating tumor stroma. Indeed, the IRE1 α pathway protects tumor cells from immune destruction [91]. Specifically, dendritic cells (whose main function is to present antigens at their surface to T-cells of the immune system) infiltrating ovarian cancers elicit an activated UPR. As such, prolonged activation of the IRE1 α pathway and downstream XBP1 transcriptional reprogramming modulate lipid homeostasis, which in turn impairs antigen

presentation [91]. Another feature of malignancy is tumor invasion, which can be acquired by ER stressed cells if they undergo EMT. The PERK signaling pathway seems crucial in this process, as it was reported to activate the SRC and/or β -catenin pathways, which in turn cause downregulation of epithelial markers and upregulation of mesenchymal markers and dedifferentiation [57–61].

MicroRNAs regulated by the UPR could also be involved in tumor survival as they often influence the apoptosis pathway. Only miR-211, which promotes formation of heterochromatin at the *CHOP* loci, and miR-708, which is transcriptionally regulated by CHOP, were shown to participate in cancer [75,76], but in theory the other miRNAs regulated by the UPR could also determine cell fate depending on how they are regulated (up- or downregulated). Indeed, miRNAs regulated by ATF6 (miR-455, [70]) and IRE1 α (miR-346 [73], miR-17 [73], miR-34a, miR-96, and miR-125b [71]) pathways are favorable to pro-survival, whereas the microRNAs regulated by the PERK pathway are either pro-survival (miR-211 [75]) or pro-death (miR-30c-2 [74]). Beyond cell death/survival, miRNAs could also be involved in specific tumor features, such as angiogenesis and proliferation, since miR-30c-2 and miR-211 regulate XBP1 and CHOP, while IRE1 α and PERK pathways are key in determining cell survival and cell death decisions.

Many reports have involved transcriptional and posttranscriptional regulations by the UPR in cancer. But little is known concerning involvement of chromatin modifications mediated by the UPR in cancer. However, mice with a liver knock-down for BiP and fed with alcohol that caused constitutive hepatic ER stress were more susceptible to develop liver tumors [92]. Moreover, the promoter regions of genes included in the ERAD pathway (Derlin 3 (*DERL3*), Cysteine-rich with epidermal growth factor-like domain 2 (*Creld2*), *Herpud1*, Wolfram syndrome gene (*Wfs1*) and *Yod1*) were hypermethylated. These results suggest that alcohol consumption may affect DNA methylation and gene expression of the ERAD pathway and promote tumorigenesis as a result.

These reports highlight that the UPR can promote proliferation/survival against threats such as reactive oxygen species, hypoxia, and nutrient deprivation by activating a specific transcriptional program. Moreover, the UPR can also help to the establishment of malignant traits (invasion, evading the immune system). However, the contribution of ER stress to malignancy is complex and different in each case, and this is in part due to the difference of cell types and the wide range of genes that can be regulated by the UPR.

Neurodegenerative Disorders

Although most neurodegenerative diseases are caused by aging and/or genetic background, a link has been established between ER stress and several neurodegenerative disorders. In this section, we describe some of the neurodegenerative disorders where ER stress transcriptional regulation is involved. Alzheimer disease (AD) is caused by the accumulation of amyloid β -peptide. Thus far, three genes have been identified as responsible for the disease, namely amyloid precursor protein (*APP*), presenilin 1 (*PS1*), and presenilin 2 (*PS2*). Interestingly, UPR signaling may transcriptionally control several AD-related genes. Indeed, a genome-wide screening has revealed that XBP1s target genes include proteins involved in APP trafficking and a component of the γ -secretase that cleaves APP [23]. Therefore therapeutic targeting of XBP1 could be interesting, as it could impair APP processing.

Moreover, it seems that in AD the UPR transcriptional program is affected. Indeed, expression of mutated PS1, found in familial AD, downregulated UPR signaling by impairing IRE1 α phosphorylation, and as a result, the transcription of UPR target genes was downregulated, including *BiP*, thereby causing an increased vulnerability to ER stress [93]. Although wild-type PS1 did not affect UPR signaling, expression of mutant PS1 displayed an attenuated ER stress response [94]. However, in this case, expression PS1 mutant affected ATF6 activation, as well as IRE1 α and PERK phosphorylation, possibly through a gain-of-function. Only *BiP* and *CHOP* transcription levels were tested and decreased, but because all three branches of the UPR are impaired, it is likely that more ER stress genes were downregulated in this context. Interestingly, although the UPR is activated in AD patients [95], BiP and GRP94 protein levels are decreased when compared to healthy controls, maybe due to a diminished activation of transcription of these genes. These reports suggest a strong link between ER stress transcriptional regulation and AD, although the exact contribution of the ER genes involved is unclear (reviewed in Ref. [96]).

Several reports have also linked the UPR to Parkinson disease (PD). Indeed, some patients display an increased UPR activation [79]. In addition, *Parkin* and ubiquitin carboxy-terminal hydrolase L1 (*UCH-L1*), two of the genes responsible for PD progression, are involved in the ERAD pathway: *Parkin* is an ubiquitin-protein ligase (E3), while *UCH-L1* can deubiquitinate misfolded proteins [79,97]. Recently, a functional link between *Parkin*, *DJ-1*, and ER stress has been established [98]. *DJ-1*, another gene responsible for PD, is transcriptionally regulated by XBP1. In basal conditions, XBP1 is transcriptionally repressed by p53; however, during ER stress, *Parkin* transcriptionally

represses *p53*, thereby leading to XBP1 transcription and the subsequent DJ-1 transcription. The alteration of this transcriptional cascade could be involved in some cases of PD.

These diseases are characterized by an impaired ER folding machinery; therefore it could be an interesting field to develop therapeutics that could either boost the ER folding capacity or inactivate a specific arm of the UPR to cure these diseases.

Inflammatory and Metabolic Diseases

Inflammation is one of the first responses of the innate immune system in which the UPR plays an important role. Indeed, in dendritic cells, toll-like receptor 2 (TLR2) and TLR4 can activate IRE1 α by phosphorylation leading to XBP1 mRNA splicing and transcription of its target genes, which are required for the production of pro-inflammatory cytokines such as IL-6 in macrophages [99]. Therefore, impairment of this pathway compromises dendritic cells and, as a result, impairs the global immune response. ER stress was shown to be involved in two intestinal bowel inflammatory diseases: Crohn syndrome and ulcerative colitis [100]. Indeed, *XBP1* and *ORMDL3*, two ER stress genes, have been genetically related to inflammatory bowel disease. Functional studies on mutated XBP1 found in patients with inflammatory bowel diseases revealed a hypomorphic UPR induction [101]. Inflammatory bowel diseases has also been linked to autophagy dysfunction, and it is well established that the UPR can control transcription of several autophagy-related genes by the PERK/ATF4/CHOP pathway [10,88,89].

Additionally, ER stress can worsen certain diseases (reviewed in Ref. [102]), such as cancer, where inflammation plays a pro-tumorigenic role, chronic inflammatory airway disease, obesity, and type II diabetes by promoting transcription of inflammatory genes via, among other transcription factors, NF- κ B and STAT3 [32,34]. We previously saw that upon ER stress, translation is inhibited, and the difference in stability between NF- κ B and its repressor I κ B can lead to transcription of NF- κ B target genes. IRE1 α can also promote NF- κ B transcription by activating I κ B kinase (IKK), which in turn phosphorylates I κ B and causes its degradation. It was also reported that PERK activated by the kinase Janus kinase 1 (JAK1) could in turn phosphorylate signal transducer and activator of transcription 3 (STAT3), leading to the transcription of inflammatory genes mediated by IL-6.

ER stress is also associated to metabolic disorders including insulin resistance, diabetes, fatty liver, and dyslipidemia. It is also an active field for the research of novel pharmacological molecules, as some of them are currently in clinical trials (reviewed in Ref. [103]). In certain of these metabolic disorders, changes in transcriptional regulation mediated by ER stress are directly involved. For instance, insulin resistance is associated with obesity and type 2 diabetes and is caused by impaired insulin signaling in organs, mostly the liver. ER stress can affect hepatic insulin responsiveness in different ways. Indeed, IRE1 α activation and subsequent kinase cascade (JNK/IKK) block the insulin receptor substrate by phosphorylation. ER stress can also cause hepatic IR through specific transcriptional regulation: (1) CREB3, a UPR transcription factor, can promote glycogenesis and inflammatory genes in hepatocyte [104], or (2) ATF6 can downregulated CREB-regulated transcription coactivator 2 (CRTC2), which control gluconeogenesis. Decrease of insulin responsiveness is caused by another transcriptional program regulated by the UPR in *Drosophila melanogaster*. Indeed, PERK can phosphorylate the transcription factor forkhead box O (FOXO) to prevent its nuclear migration and transcription of its target genes, which include the insulin receptor (*INSR*, [105]). Concerning type II diabetes, it involves β -cell dysfunction and/or apoptosis associated with ER stress. The IRE1 α signaling pathway plays an important role in the disease since its hyperactivation causes apoptosis in β -cells. Additionally, IRE1 α is required for the synthesis, folding, and maturation of proinsulin [74] and can regulate insulin mRNA with its RIDD activity.

ER stress aggravates or participates in many human diseases; to treat them, several kinds of molecules are being developed or tested [103]: (1) chemical chaperones that prevent protein misfolding by stabilizing ER chaperones; (2) molecules that enhance ER folding activity or prevent apoptosis by targeting a specific protein of the UPR; (3) molecules that regulate ER calcium homeostasis, creating a better environment for protein folding; and (4) molecules that alleviate ER stress by activating antioxidant or autophagy pathways. Although, targeting chromatin remodelers has shown success in treating other diseases, too little is yet known in this area to be considered for ER stress-related diseases. As our knowledge increases, it might become a viable option.

CONCLUSIONS

The UPR is an adaptive response conserved throughout evolution that aims at restoring proteostasis when the ER is overload with unfolded proteins. The canonical UPR depends on the activation of three sensors (IRE1 α , PERK, ATF6), which each produce a transcription factor (XBP1s, ATF4, ATF6f, respectively), resulting in activation of

transcription of ER stress-inducible genes. But it has become clear that the UPR transcriptome is more complex and has to be put into context as it may vary between cell types, environmental conditions, and stress stimuli intensity. As we saw, the UPR involves epigenetic modifications, including activation/synthesis of transcription factors, recruitment of transcription complex, and changes in chromatin histone marks. We are still failing to have a dynamic vision of transcription factor complex recruitment and chromatin modification, and in the few studies available, only a few histone marks and proteins were assessed. However, our knowledge concerning the possible transcriptional outputs of the UPR increases with these studies, which have revealed that the UPR is part of a complex transcriptional network. Moreover, there are numerous non-canonical mechanisms by which the UPR can regulate gene expression of distant pathways. This control network seems key in diseases, especially cancer, where regulation of these pathways helps cancer cells to survive or acquire malignant traits.

All these studies could help treating ER stress-related diseases (cancers, neurodegenerative disorder, metabolic and inflammatory diseases) but we also need to have a better understanding of the coordination and specific contribution of each arm of the UPR in each disease. Although, mice phenotypes upon deletion of ER functions or UPR signaling are very informative, more mechanistic and physiological studies are needed to ensure the efficacy and safety of pharmacological small molecules.

Acronyms

AD Alzheimer disease
bZIP Basic leucine zipper
ER Endoplasmic reticulum
ERAD Endoplasmic reticulum-associated degradation
PD Parkinson disease

Acknowledgments

The authors apologize to all colleagues whose work could not be cited owing to space limitations. This work was funded by grants from Institut National du Cancer and La Ligue Contre Le Cancer (LARGE, Comité des Landes) to EC.

References

- [1] Smith KT, Workman JL. Chromatin proteins: key responders to stress. *PLoS Biol* 2012;10(7):e1001371.
- [2] Hetz C, Chevet E, Oakes SA. Proteostasis control by the unfolded protein response. *Nat Cell Biol* 2015;17(7):829–38.
- [3] Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 2011;334(6059):1081–6.
- [4] Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 2000;404(6779):770–4.
- [5] Cox JS, Walter P. A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* 1996; 87(3):391–404.
- [6] Wang S, Kaufman RJ. The impact of the unfolded protein response on human disease. *J Cell Biol* 2012;197(7):857–67.
- [7] Kozutsumi Y, Segal M, Normington K, Gething MJ, Sambrook J. The presence of malformed proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature* 1988;332(6163):462–4.
- [8] Blais JD, Filipenko V, Bi M, Harding HP, Ron D, Koumenis C, et al. Activating transcription factor 4 is translationally regulated by hypoxic stress. *Mol Cell Biol* 2004;24(17):7469–82.
- [9] Ye J, Koumenis C. ATF4, an ER stress and hypoxia-inducible transcription factor and its potential role in hypoxia tolerance and tumorigenesis. *Curr Mol Med* 2009;9(4):411–6.
- [10] B'Chir W, Maurin AC, Carraro V, Averous J, Jousse C, Muranishi Y, et al. The eIF2alpha/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res* 2013;41(16):7683–99.
- [11] Pereira ER, Liao N, Neale GA, Hendershot LM. Transcriptional and post-transcriptional regulation of proangiogenic factors by the unfolded protein response. *PLoS One* 2010;5(9).
- [12] Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ* 2004;11(4):381–9.
- [13] Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, et al. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev* 1998;12(7):982–95.
- [14] Novoa I, Zeng H, Harding HP, Ron D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J Cell Biol* 2001;153(5):1011–22.
- [15] Shen J, Prywes R. Dependence of site-2 protease cleavage of ATF6 on prior site-1 protease digestion is determined by the size of the luminal domain of ATF6. *J Biol Chem* 2004;279(41):43046–51.
- [16] Nadanaka S, Okada T, Yoshida H, Mori K. Role of disulfide bridges formed in the luminal domain of ATF6 in sensing endoplasmic reticulum stress. *Mol Cell Biol* 2007;27(3):1027–43.
- [17] Higa A, Taouji S, Lhomond S, Jensen D, Fernandez-Zapico ME, Simpson JC, et al. Endoplasmic reticulum stress-activated transcription factor ATF6alpha requires the disulfide isomerase PDIA5 to modulate chemoresistance. *Mol Cell Biol* 2014;34(10):1839–49.

- [18] Adachi Y, Yamamoto K, Okada T, Yoshida H, Harada A, Mori K. ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. *Cell Struct Funct* 2008;33(1):75–89.
- [19] Calton M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 2002;415(6867):92–6.
- [20] Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 2001;107(7):881–91.
- [21] Basseri S, Austin RC. Endoplasmic reticulum stress and lipid metabolism: mechanisms and therapeutic potential. *Biochem Res Int* 2012; 2012:841362.
- [22] Hetz C, Martinon F, Rodriguez D, Glimcher LH. The unfolded protein response: integrating stress signals through the stress sensor IRE1alpha. *Physiol Rev* 2011;91(4):1219–43.
- [23] Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH, Arias C, et al. XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. *Mol Cell* 2007;27(1):53–66.
- [24] Pereira ER, Frudd K, Awad W, Hendershot LM. Endoplasmic reticulum (ER) stress and hypoxia response pathways interact to potentiate hypoxia-inducible factor 1 (HIF-1) transcriptional activity on targets like vascular endothelial growth factor (VEGF). *J Biol Chem* 2014;289(6): 3352–64.
- [25] Hu CC, Dougan SK, McGehee AM, Love JC, Ploegh HL. XBP-1 regulates signal transduction, transcription factors and bone marrow colonization in B cells. *EMBO J* 2009;28(11):1624–36.
- [26] Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 2012;13(2):89–102.
- [27] Hollien J, Lin JH, Li H, Stevens N, Walter P, Weissman JS. Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. *J Cell Biol* 2009;186(3):323–31.
- [28] Hollien J, Weissman JS. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* 2006;313(5783): 104–7.
- [29] Iwawaki T, Hosoda A, Okuda T, Kamigori Y, Nomura-Furuwatari C, Kimata Y, et al. Translational control by the ER transmembrane kinase/ ribonuclease IRE1 under ER stress. *Nat Cell Biol* 2001;3:158–64.
- [30] Maurel M, Chevet E, Tavernier J, Gerlo S. Getting RIDD of RNA: IRE1 in cell fate regulation. *Trends Biochem Sci* 2014;39(5):245–54.
- [31] Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, et al. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 2000;287(5453):664–6.
- [32] Arensdorf AM, Diedrichs D, Rutkowski DT. Regulation of the transcriptome by ER stress: non-canonical mechanisms and physiological consequences. *Front Genet* 2013;4:256.
- [33] Fu S, Watkins SM, Hotamisligil GS. The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. *Cell Metab* 2012; 15(5):623–34.
- [34] Garg AD, Kaczmarek A, Krysko O, Vandenabeele P, Krysko DV, Agostinis P. ER stress-induced inflammation: does it aid or impede disease progression? *Trends Mol Med* 2012;18(10):589–98.
- [35] Ventoso I, Kochetov A, Montaner D, Dopazo J, Santoyo J. Extensive translational remodeling during ER stress response in mammalian cells. *PLoS One* 2012;7(5):e35915.
- [36] Morris DR, Geballe AP. Upstream open reading frames as regulators of mRNA translation. *Mol Cell Biol* 2000;20(23):8635–42.
- [37] Lilley BN, Ploegh HL. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 2004;429(6994):834–40.
- [38] Marza E, Taouji S, Barroso K, Raymond AA, Guignard L, Bonneu M, et al. Genome-wide screen identifies a novel p97/CDC-48-dependent pathway regulating ER-stress-induced gene transcription. *EMBO Rep* 2015;16.
- [39] Ameri K, Harris AL. Activating transcription factor 4. *Int J Biochem Cell Biol* 2008;40(1):14–21.
- [40] Luo R, Lu JF, Hu Q, Maity SN. CBF/NF-Y controls endoplasmic reticulum stress induced transcription through recruitment of both ATF6(N) and TBP. *J Cell Biochem* 2008;104(5):1708–23.
- [41] Lee J, Sun C, Zhou Y, Gokalp D, Herrema H, Park SW, et al. p38 MAPK-mediated regulation of Xbp1s is crucial for glucose homeostasis. *Nat Med* 2011;17(10):1251–60.
- [42] Wang FM, Chen YJ, Ouyang HJ. Regulation of unfolded protein response modulator XBP1s by acetylation and deacetylation. *Biochem J* 2010;433(1):245–52.
- [43] Chen H, Qi L. SUMO modification regulates the transcriptional activity of XBP1. *Biochem J* 2010;429(1):95–102.
- [44] Hu R, Warri A, Jin L, Zwart A, Riggins RB, Clarke R. NFkappaB signaling is required for XBP1 (U and S) mediated effects on antiestrogen responsiveness and cell fate decisions in breast cancer. *Mol Cell Biol* 2014;35(2):379–90.
- [45] Shoulders MD, Ryno LM, Genereux JC, Moresco JJ, Tu PG, Wu C, et al. Stress-independent activation of XBP1s and/or ATF6 reveals three functionally diverse ER proteostasis environments. *Cell Rep* 2013;3(4):1279–92.
- [46] Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshida H, et al. Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev Cell* 2007;13(3):365–76.
- [47] Han J, Back SH, Hur J, Lin YH, Gildersleeve R, Shan J, et al. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol* 2013;15(5):481–90.
- [48] Koumenis C. ER stress, hypoxia tolerance and tumor progression. *Curr Mol Med* 2006;6(1):55–69.
- [49] Chen X, Iliopoulos D, Zhang Q, Tang Q, Greenblatt MB, Hatzia Apostolou M, et al. XBP1 promotes triple-negative breast cancer by controlling the HIF1alpha pathway. *Nature* 2014;508(7494):103–7.
- [50] Yamamori T, Meike S, Nagane M, Yasui H, Inanami O. ER stress suppresses DNA double-strand break repair and sensitizes tumor cells to ionizing radiation by stimulating proteasomal degradation of Rad51. *FEBS Lett* 2013;587(20):3348–53.
- [51] Epple LM, Dodd RD, Merz AL, Dechkovskaia AM, Herring M, Winston BA, et al. Induction of the unfolded protein response drives enhanced metabolism and chemoresistance in glioma cells. *PLoS One* 2013;8(8):e73267.
- [52] Feng R, Zhai WL, Yang HY, Jin H, Zhang QX. Induction of ER stress protects gastric cancer cells against apoptosis induced by cisplatin and doxorubicin through activation of p38 MAPK. *Biochem Biophys Res Commun* 2011;406(2):299–304.
- [53] Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol* 2003;23(20):7198–209.

- [54] Cullinan SB, Diehl JA. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem* 2004;279(19):20108–17.
- [55] Martin D, Li Y, Yang J, Wang G, Margariti A, Jiang Z, et al. Unspliced X-box-binding protein 1 (XBP1) protects endothelial cells from oxidative stress through interaction with histone deacetylase 3. *J Biol Chem* 2014;289(44):30625–34.
- [56] Dejeans N, Barroso K, Fernandez-Zapico ME, Samali A, Chevet E. Novel roles of the unfolded protein response in the control of tumor development and aggressiveness. *Semin Cancer Biol* 2015;33.
- [57] Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009;139(5):871–90.
- [58] Ulianich L, Garbi C, Treglia AS, Punzi D, Miele C, Raciti GA, et al. ER stress is associated with dedifferentiation and an epithelial-to-mesenchymal transition-like phenotype in PC Cl3 thyroid cells. *J Cell Sci* 2008;121(Pt 4):477–86.
- [59] Tanjore H, Cheng DS, Degryse AL, Zoz DF, Abdolrasulnia R, Lawson WE, et al. Alveolar epithelial cells undergo epithelial-to-mesenchymal transition in response to endoplasmic reticulum stress. *J Biol Chem* 2011;286:30972–80.
- [60] Sheshadri N, Catanzaro JM, Bott A, Sun Y, Ullman E, Chen E, et al. SCCA1/SerpinB3 promotes oncogenesis and epithelial-mesenchymal transition via the unfolded protein response and IL-6 signaling. *Cancer Res* 2014;74(21):6318–29.
- [61] Shah PP, Beverly LJ. Regulation of VCP/p97 demonstrates the critical balance between cell death and epithelial-mesenchymal transition (EMT) downstream of ER stress. *Oncotarget* 2015;6(19):17725–37.
- [62] Kawai T, Fan J, Mazan-Mamczarz K, Gorospe M. Global mRNA stabilization preferentially linked to translational repression during the endoplasmic reticulum stress response. *Mol Cell Biol* 2004;24(15):6773–87.
- [63] Ghosh R, Wang L, Wang ES, Perera BG, Igarria A, Morita S, et al. Allosteric inhibition of the IRE1alpha RNase preserves cell viability and function during endoplasmic reticulum stress. *Cell* 2014;158(3):534–48.
- [64] Tam AB, Koong AC, Niwa M. Ire1 has distinct catalytic mechanisms for XBP1/HAC1 splicing and RIDD. *Cell Rep* 2014;9:1–9.
- [65] So JS, Hur KY, Tarrio M, Ruda V, Frank-Kamenetsky M, Fitzgerald K, et al. Silencing of lipid metabolism genes through IRE1alpha-mediated mRNA decay lowers plasma lipids in mice. *Cell Metab* 2012;16(4):487–99.
- [66] Pluquet O, Dejeans N, Boucheccareilh M, Lhomond S, Pineau R, Higa A, et al. Posttranscriptional regulation of PER1 underlies the oncogenic function of IRE1alpha. *Cancer Res* 2013;73(15):4732–43.
- [67] Maurel M, Dejeans N, Taouji S, Chevet E, Grosset CF. MicroRNA-1291-mediated silencing of IRE1alpha enhances Glypican-3 expression. *RNA* 2013;19(6):778–88.
- [68] Maurel M, Chevet E. Endoplasmic reticulum stress signaling: the microRNA connection. *Am J Physiol Cell Physiol* 2013;304(12):C1117–26.
- [69] Behrman S, Acosta-Alvear D, Walter P. A CHOP-regulated microRNA controls rhodopsin expression. *J Cell Biol* 2011;192(6):919–27.
- [70] Belmont PJ, Chen WJ, Thuerauf DJ, Glembotski CC. Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response. *J Mol Cell Cardiol* 2012;52(5):1176–82.
- [71] Upton JP, Wang L, Han D, Wang ES, Huskey NE, Lim L, et al. IRE1alpha cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. *Science* 2012;338(6108):818–22.
- [72] Lerner AG, Upton JP, Praveen PV, Ghosh R, Nakagawa Y, Igarria A, et al. IRE1alpha induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress. *Cell Metab* 2012;16(2):250–64.
- [73] Bartoszewski R, Brewer JW, Rab A, Crossman DK, Bartoszewska S, Kapoor N, et al. The unfolded protein response (UPR)-activated transcription factor X-box-binding protein 1 (XBP1) induces microRNA-346 expression that targets the human antigen peptide transporter 1 (TAP1) mRNA and governs immune regulatory genes. *J Biol Chem* 2011;286(48):41862–70.
- [74] Byrd AE, Aragon IV, Brewer JW. MicroRNA-30c-2* limits expression of proadaptive factor XBP1 in the unfolded protein response. *J Cell Biol* 2012;196(6):689–98.
- [75] Chitnis NS, Pytel D, Bobrovnikova-Marjon E, Pant D, Zheng H, Maas NL, et al. miR-211 is a prosurvival microRNA that regulates chop expression in a PERK-dependent manner. *Mol Cell* 2012;48(3):353–64.
- [76] Ryu S, McDonnell K, Choi H, Gao D, Hahn M, Joshi N, et al. Suppression of miRNA-708 by polycomb group promotes metastases by calcium-induced cell migration. *Cancer Cell* 2013;23(1):63–76.
- [77] Gupta S, Read DE, Deepti A, Cawley K, Gupta A, Oommen D, et al. Perk-dependent repression of miR-106b-25 cluster is required for ER stress-induced apoptosis. *Cell Death Dis* 2012;3:e333.
- [78] Caretti G, Salsi V, Vecchi C, Imbriano C, Mantovani R. Dynamic recruitment of NF- κ B and histone acetyltransferases on cell-cycle promoters. *J Biol Chem* 2003;278(33):30435–40.
- [79] Yoshida H. ER stress and diseases. *FEBS J* 2007;274(3):630–58.
- [80] Baumeister P, Luo S, Skarnes WC, Sui G, Seto E, Shi Y, et al. Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. *Mol Cell Biol* 2005;25(11):4529–40.
- [81] Baumeister P, Dong D, Fu Y, Lee AS. Transcriptional induction of GRP78/BiP by histone deacetylase inhibitors and resistance to histone deacetylase inhibitor-induced apoptosis. *Mol Cancer Ther* 2009;8(5):1086–94.
- [82] Donati G, Imbriano C, Mantovani R. Dynamic recruitment of transcription factors and epigenetic changes on the ER stress response gene promoters. *Nucleic Acids Res* 2006;34(10):3116–27.
- [83] Schram AW, Baas R, Jansen PW, Riss A, Tora L, Vermeulen M, et al. A dual role for SAGA-associated factor 29 (SGF29) in ER stress survival by coordination of both histone H3 acetylation and histone H3 lysine-4 trimethylation. *PLoS One* 2013;8(7):e70035.
- [84] Esfandiari F, Medici V, Wong DH, Jose S, Dolatshahi M, Quinlivan E, et al. Epigenetic regulation of hepatic endoplasmic reticulum stress pathways in the ethanol-fed cystathionine beta synthase-deficient mouse. *Hepatology* 2010;51(3):932–41.
- [85] Kozlowski L, Garvis S, Bedet C, Palladino F. The *Caenorhabditis elegans* HPI family protein HPL-2 maintains ER homeostasis through the UPR and hormesis. *Proc Natl Acad Sci USA* 2014;111(16):5956–61.
- [86] Clarke HJ, Chambers JE, Liniker E, Marciniak SJ. Endoplasmic reticulum stress in malignancy. *Cancer Cell* 2014;25(5):563–73.
- [87] Wang Y, Alam GN, Ning Y, Visioli F, Dong Z, Nor JE, et al. The unfolded protein response induces the angiogenic switch in human tumor cells through the PERK/ATF4 pathway. *Cancer Res* 2012;72(20):5396–406.
- [88] Hart LS, Cunningham JT, Datta T, Dey S, Tameire F, Lehman SL, et al. ER stress-mediated autophagy promotes Myc-dependent transformation and tumor growth. *J Clin Invest* 2012;122(12):4621–34.

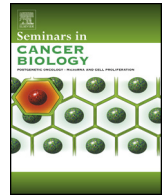
- [89] Rouschop KM, van den Beucken T, Dubois L, Niessen H, Bussink J, Savelkoul K, et al. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *J Clin Invest* 2010;120(1):127–41.
- [90] Romero-Ramirez L, Cao H, Nelson D, Hammond E, Lee AH, Yoshida H, et al. XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. *Cancer Res* 2004;64(17):5943–7.
- [91] Cubillos-Ruiz JR, Silberman PC, Rutkowski MR, Chopra S, Perales-Puchalt A, Song M, et al. ER stress sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. *Cell* 2015;161(7):1527–38.
- [92] Han H, Hu J, Lau MY, Feng M, Petrovic LM, Ji C. Altered methylation and expression of ER-associated degradation factors in long-term alcohol and constitutive ER stress-induced murine hepatic tumors. *Front Genet* 2013;4:224.
- [93] Katayama T, Imaizumi K, Sato N, Miyoshi K, Kudo T, Hitomi J, et al. Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. *Nat Cell Biol* 1999;1(8):479–85.
- [94] Katayama T, Imaizumi K, Honda A, Yoneda T, Kudo T, Takeda M, et al. Disturbed activation of endoplasmic reticulum stress transducers by familial Alzheimer's disease-linked presenilin-1 mutations. *J Biol Chem* 2001;276(46):43446–54.
- [95] Saltini G, Dominici R, Lovati C, Cattaneo M, Michelini S, Malferrari G, et al. A novel polymorphism in SEL1L confers susceptibility to Alzheimer's disease. *Neurosci Lett* 2006;398(1–2):53–8.
- [96] Cornejo VH, Hetz C. The unfolded protein response in Alzheimer's disease. *Semin Immunopathol* 2013;35(3):277–92.
- [97] Kahle PJ, Haass C. How does parkin ligate ubiquitin to Parkinson's disease? *EMBO Rep* 2004;5(7):681–5.
- [98] Duplan E, Giaime E, Viotti J, Sevalle J, Corti O, Brice A, et al. ER-stress-associated functional link between Parkin and DJ-1 via a transcriptional cascade involving the tumor suppressor p53 and the spliced X-box binding protein XBP-1. *J Cell Sci* 2013;126(Pt 9):2124–33.
- [99] Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, Tilg H, et al. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell* 2008;134(5):743–56.
- [100] Meares GP, Liu Y, Rajbhandari R, Qin H, Nozell SE, Mobley JA, et al. PERK-dependent activation of JAK1 and STAT3 contributes to endoplasmic reticulum stress-induced inflammation. *Mol Cell Biol* 2014;34(20):3911–25.
- [101] Iwakoshi NN, Pypaert M, Glimcher LH. The transcription factor XBP-1 is essential for the development and survival of dendritic cells. *J Exp Med* 2007;204(10):2267–75.
- [102] Fritz T, Niederreiter L, Adolph T, Blumberg RS, Kaser A. Crohn's disease: NOD2, autophagy and ER stress converge. *Gut* 2011;60(11):1580–8.
- [103] Cao SS, Kaufman RJ. Targeting endoplasmic reticulum stress in metabolic disease. *Expert Opin Ther Targets* 2012;17(4):437–48.
- [104] Lee MW, Chanda D, Yang J, Oh H, Kim SS, Yoon YS, et al. Regulation of hepatic gluconeogenesis by an ER-bound transcription factor, CREBH. *Cell Metab* 2010;11(4):331–9.
- [105] Zhang W, Hietakangas V, Wee S, Lim SC, Gunaratne J, Cohen SM. ER stress potentiates insulin resistance through PERK-mediated FOXO phosphorylation. *Genes Dev* 2013;27(4):441–9.

3) Endoplasmic Reticulum Stress and cancer

Cancer cells proliferate quickly and have to cope with intrinsic stresses (oncogene expression, high DNA replication, high synthesis rates, oxidative stress) and extrinsic stresses (hypoxia, nutrient deprivation, change of their micro-environment, chemotherapeutic agents) which they need to overcome to survive. In most cancer cells due to the high protein folding demand and challenging conditions, the UPR is activated (25), although in normal cells if ER homeostasis cannot be restored the cell signals toward apoptosis, cancer cells have reduced their sensitivity to stress-mediated cell death through forced adaptation mechanisms (26).

In the previous book chapter we have described how ER stress-induced transcription affects proliferation and survival, two key features of cancers cells. The following review further discusses the impact of the UPR activation in different tumors and through examples we illustrate how cancer cells use the secretory pathway to acquire malignant traits such as inflammation, immunogenicity or angiogenesis.

ARTICLE 1



Review

Novel roles of the unfolded protein response in the control of tumor development and aggressiveness



Nicolas Dejeans^{a,b}, Kim Barroso^{a,b,c,d,e}, Martin E. Fernandez-Zapico^c, Afshin Samali^f, Eric Chevet^{a,b,d,e,*}

^a INSERM, UMR-1053, Team "Endoplasmic Reticulum Stress and Cancer", 33000 Bordeaux, France

^b Université de Bordeaux, 33000 Bordeaux, France

^c Schulze Center for Novel Therapeutics, Division of Oncology Research, Mayo Clinic, Rochester, MN, USA

^d ER440 Université Rennes 1, "Oncogenesis, Stress, Signaling", Rennes, France

^e Centre de Lutte Contre le Cancer Eugène Marquis, Rennes, France

^f Apoptosis Research Centre, School of Natural Sciences, NUI Galway, Galway, Ireland

ARTICLE INFO

Keywords:

Endoplasmic reticulum
Cancer
Stress
Tumor
EMT

ABSTRACT

The hallmarks of cancer currently define the molecular mechanisms responsible for conferring specific tumor phenotypes. Recently, these characteristics were also connected to the status of the secretory pathway, thereby linking the functionality of this cellular machinery to the acquisition of cancer cell features. The secretory pathway ensures the biogenesis of proteins that are membrane-bound or secreted into the extracellular milieu and can control its own homeostasis through an adaptive signaling pathway named the unfolded protein response (UPR). In the present review, we discuss the specific features of the UPR in various tumor types and the impact of the selective activation of this pathway on cell transformation, tumor development and aggressiveness.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Tumor phenotypes including development and aggressiveness features can dramatically vary depending on the origin of tumor cells and context. The hallmarks of cancer defined by Hanahan and Weinberg [1] have helped to define these characteristics, which were also connected to the status of the secretory pathway (SP) [2,3]. As a consequence this essential cellular component has taken significant importance in the acquisition of cancer cell features. The SP ensures the biogenesis of proteins that are membrane-bound or secreted into the extracellular milieu. It is well accepted that approximately one-third of the polypeptides synthesized by a cell, enter the endoplasmic reticulum (ER), the first compartment of the SP [2,3]. However, the quantity of proteins entering the secretory pathway fluctuates, depending on the cell physiology, function and specific microenvironment. For instance, the synthesis of antibodies, extracellular matrix proteins, membrane receptors or secretory cyto/chemokines is cell-type specific and can impact the

workload of the secretory machinery. Moreover, cell migration, differentiation or proliferation features can also create the demand for a higher need for protein secretion. Protein secretion fluctuations affect cell homeostasis, particularly cell amino acid, lipid and sugar metabolism and energy consumption. As such, a strong and reliable adaptive system is central for the cell to cope with the increased demand for protein folding in the ER. This adaptive system is named the unfolded protein response (UPR). In this review, we provide specific examples illustrating how the diversification of UPR signals in many human cell types, particularly in secretory cells, could impact typical cancer initiation, tumor development and cancer cell aggressiveness.

The UPR transmits stress signals from the ER lumen to the rest of the cell by three different proteins called PERK, ATF6 and IRE1. PERK (PKR-like endoplasmic reticulum kinase) is a transmembrane protein with a specific kinase activity in its cytosolic domain. Its main substrate is the translation initiation factor eIF2 α . Phosphorylation of eIF2 α results in a decrease in translation as well as a preferential translation of key proteins such as CHOP and GADD34, two factors directly involved in the cellular decisions of life or death. The transmembrane protein ATF6 (Activating Transcription Factor 6) is an ER transcription factor. Under stress conditions, ATF6 is exported to the Golgi apparatus, cleaved and released from its membrane attachment by the proteases S1P and S2P, to play its role as nuclear

* Corresponding author at: ER440 Université Rennes 1, "Oncogenesis, Stress, Signaling", Centre de Lutte Contre le Cancer Eugène Marquis, 35000 Rennes, France. Tel.: +33 0223237258.

E-mail address: eric.chevet@inserm.fr (E. Chevets).

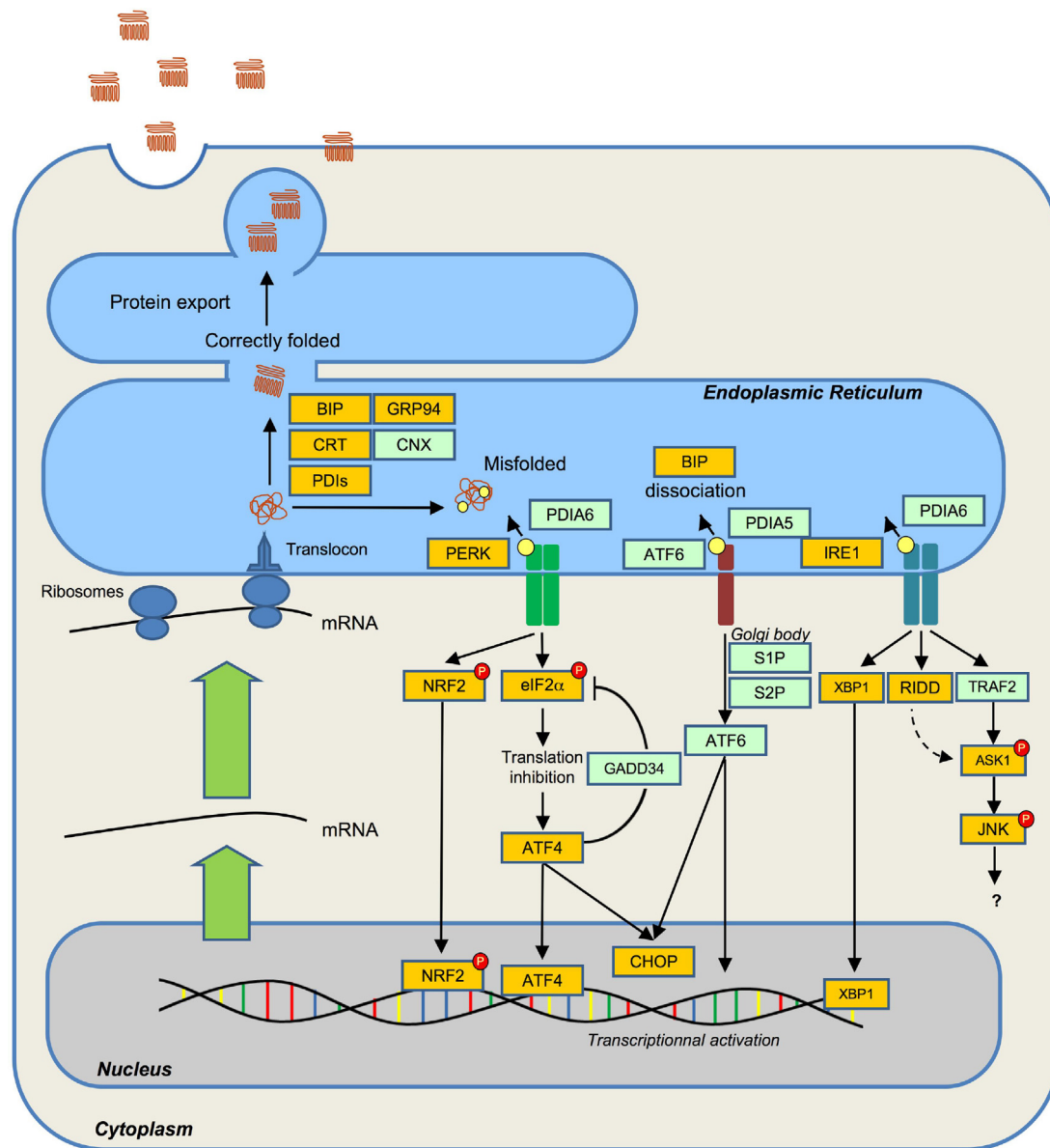


Fig. 1. Cancer relevant UPR signaling components. Major UPR signaling modules are drawn. Relevance to cancer is indicated as follows: orange – proteins directly implicated in the modulation of cancer cell features, including initiation, angiogenesis, inflammation, immunogenicity or resistance. Green – proteins whose modulation or activation is observed in cancer tissues or involved with cancer development or aggressiveness, but whose role in the control of cancer features is not clearly defined.

transcriptional activator. Finally, IRE1 (Inositol Requiring Enzyme 1), an ER resident type 1 transmembrane protein, has two enzymatic activities in its cytosolic domain: a serine/threonine kinase and an endoribonuclease activity. The endoribonuclease activity itself has two distinct molecular functions: (i) it participates in the unconventional splicing of the XBP1 transcription factor mRNA [4]; (ii) it degrades the mRNA of several secreted proteins, a process called RIDD (Regulated IRE1-Dependent Decay of RNA) [5]. The integration of signals from these three molecular pathways leads to a general transcription and translation reorientation, in favor of cell survival. Among the cellular processes regulated, the antioxidant capacity is increased, protein synthesis is decreased and the expression of ER chaperones/ER quality control proteins involved in protein folding (BiP, GRP94, CRT, PDIs) and in misfolded protein degradation is enhanced [6–8]. Finally, if ER homeostasis is not restored, ER stress can trigger apoptosis [9,10] (Fig. 1).

It is well established that differentiated cells such as neurons, blood cells, pancreatic β -cells, hepatocytes, all require a dedicated

secretory pathway with appropriate specialized regulations [11]. In accordance with this, an increasing number of studies have shown a dependency of specific UPR components for the differentiation of particular cell types. For instance, the IRE1-XBP1 branch is required for the differentiation of pancreatic β cells, plasma cells, or adipocytes [12–16] and disturbance of the PERK-ATF4 pathway triggers defects in oligodendrocytes, pancreatic and skeletal functions [17–21].

2. The “secretory switch” in transformed cells

Most cancers have to cope with increasing fluxes of proteins through their secretory pathway. This high secretory protein demand is caused by different hallmarks of cancer [2] and comprises all the processes that increase gene expression, in an unspecific manner, such as aneuploidy or the universal amplifier of transcription, MYC [22,23]. Hence, it is not surprising that

aneuploidy was found to be associated to hypersensitivity to conditions interfering with protein synthesis and protein folding in yeast [24] and in human cancer cells [25], and that MYC transformation requires a reliable secretory pathway to mediate its oncogenic potential [26]. Moreover, cell transformation can result in an increase in proliferation and metabolic demand, thereby leading to nutrient (i.e. glucose, amino acids) depletion and subsequent ER stress [27]. This means that during the cell transformation process, a “secretory switch” occurs and provides the transformed cells with novel secretory properties, which will in turn impact on cell homeostasis and interaction with the stroma.

What are the consequences of the “secretory switch” and associated ER stress on tumor–stroma interactions? First, it can lead to microenvironment architecture destabilization by remodeling of the extracellular matrix (ECM) through changes in ECM components abundance or matrix metalloproteinases (MMPs) expression, and consequently, to an increase in cancer dissemination and invasion [28,29]. Second, by modulating messenger (i.e. chemokines, cytokines) or contact/adhesion protein abundance (i.e. membrane receptors, integrins), the “secretory switch” and associated ER stress can trigger cancer cell proliferation, migration or tumor angiogenesis. Third, it can lead to remodeling of the immune response and ER stress transmission in the tumor microenvironment [30,31]. Fourth, it can modulate tumor immunogenicity by stimulating surface exposure of ER chaperones such as CRT [2,32,33] (Fig. 2).

2.1. UPR involvement in gastrointestinal cancer initiation

The UPR is a central pathway for intestinal functions and differentiation, and the human gastrointestinal tract represents an interesting example of UPR specialization. This is well illustrated by the immunostaining of UPR components in the normal intestine, which showed that UPR activation occurs in a heterogeneous manner in intestinal cell populations. Indeed, GRP78 abundance appeared high in transit amplifying cells (TA), low in intestinal stem cells (ISC) and heterogeneous in Paneth cells [34]. This suggests that the UPR could be induced with intestinal cell differentiation or could represent a pathway driving differentiation. One element supporting the second hypothesis is that activation of the PERK/eIF2 α axis in itself is sufficient to trigger the loss of ISC stemness [34]. Considering that ISCs are thought to represent the cells of origin for most colorectal cancers (CRCs) [35], this suggests that ER stress could have central implication for cancer initiation in the gastrointestinal tract.

Apart from the PERK/eIF2 α arm, specialization of the UPR in the intestine is partly due to the IRE1 branch. Indeed, the gastrointestinal tract is the main tissue where the two IRE1 paralogs, IRE1 α and IRE1 β are expressed (in the epithelial cells) [36]. Although the functions of IRE1 β are not entirely understood, IRE1 β –/– mice showed a role in the control of mucin production in goblet cells [37] and in resistance to chemically induced colitis [36]. Moreover, XBP1 deficiency or expression of XBP1 variants was associated with Crohn’s disease and ulcerative colitis [38]. Interestingly, IRE1 β and XBP1 deficiency were both associated with increased ER stress and inflammation of the intestine. A more recent report described the association between inflammatory bowel disease and tumorigenesis upon targeted deletion of XBP1 in the intestine [39]. In this model, XBP1 loss in epithelial cells results in an increase in colorectal cancer and colitis-associated cancer. This effect was attributed to an increase in ISC and TA cells, and a dysfunction of Paneth cells. ISC hyperproliferation was linked to increased WNT11 expression in Paneth cells and TA cells hyperproliferation to the activation of an ER stress-dependent interleukin/STAT3 pathway. Interestingly, in this study, a transgenic mouse model presenting a double deletion of XBP1 and IRE1 α was found to not present ISC hyperproliferation

observed in XBP1 deficient mice. Although one can ask the question of the roles of others components of the UPR, and especially IRE1 β in these processes, the authors proposed that IRE1 α is an important mediator of ER stress induced ISC expansion, in an XBP1-independent manner, which might leave room for an instrumental role of RIDD. In addition, XBP1 deletion in Paneth cells revealed that the IRE1 α –TNF α /NF- κ B pathway was central for ER stress-induced inflammation [40]. Parallel to these mechanisms, autophagy induced by the PERK/eIF2 α /ATF4 signaling axis partly restrained IRE1 activation and ER stress-induced intestinal inflammation. This was proposed as an explanation for the identification of mutations in autophagy components as risk factors in Crohn’s disease [40]. Finally, adding to the role of the UPR in intestinal epithelium, XBP1 was also identified as a susceptibility locus associated with esophagus squamous cell carcinoma [41] and ER stress was shown to induce epithelial differentiation in precursor cells in the esophagus [42] and also may be linked to Barrett’s syndrome [43].

These studies provide good examples of (i) how the UPR can fine-tune the entire functions and differentiation of the gastrointestinal epithelia by integrating information from the microenvironment and (ii) how deregulation of this molecular pathway (XBP1 deletion) can impact inflammatory bowel disease and gastrointestinal cancer initiation.

2.2. UPR in blood cancers: transformation, progression and drug resistance

Blood associated cancers represent interesting models in which UPR signals might determine tumor phenotypes. First of all, the expression and activity of UPR components including BiP, IRE1 α , BLIMP1, and XBP1 are required for terminal differentiation of B cells into plasma cells and are found overexpressed in plasma cell-derived multiple myeloma (MM). MM evolves from a highly prevalent premalignant condition termed monoclonal gammopathy of undetermined significance (MGUS). A MGUS/MM phenotype was recently reported in transgenic mice with Em-directed expression of the XBP1 spliced isoform (XBP1s). This was corroborated with the aberrant expression of known human MM dysregulated genes and thus implicates XBP1s dysregulation in MM pathogenesis [44]. Interestingly, Cre-mediated and inducible deletion of BiP, BLIMP1 or XBP1 consistently induces cellular stresses and cell death in normal pre-B cells and in pre-B-cell acute lymphoblastic leukemia (ALL) driven by BCR-ABL and NRAS [45]. Moreover, two ALL clinical trials revealed that high XBP1s levels at the time of diagnosis predicted poor outcome. As such, pharmacological inhibition of IRE1-induced selective cell death in patient-derived pre-B ALL cells and significantly prolonged survival of transplanted mice. As a consequence, pre-B ALL cells appeared uniquely vulnerable to ER stress [45]. In a recent study, the Hypoxia Inducible Factor (HIF)-2 α was implicated in the engraftment ability of human acute myeloid leukemia (AML) cells and in the maintenance of hematopoietic stem and progenitor cells (HSPCs). The mechanism controlling maintenance of HSPCs also involved ER stress signaling as HIF-2 α -deficient HSPCs displayed increased production of reactive oxygen species (ROS), which subsequently triggered apoptosis by activation of the UPR [46]. Hence these results might suggest an instrumental role of the UPR in HSPC differentiation program and in maintenance of the AML phenotype. In another model of blood-derived cancer, chronic myeloid leukemia (CML), apoptotic death triggered by the BCR-ABL inhibitor, imatinib, is activated downstream of ER stress [47]. Moreover, imatinib resistance in CML K562 cells was bypassed when preventing the activation of the ATF6 arm of the UPR, thus demonstrating the strong interconnection of these pathways in acquisition of tumor cell phenotypes [48].

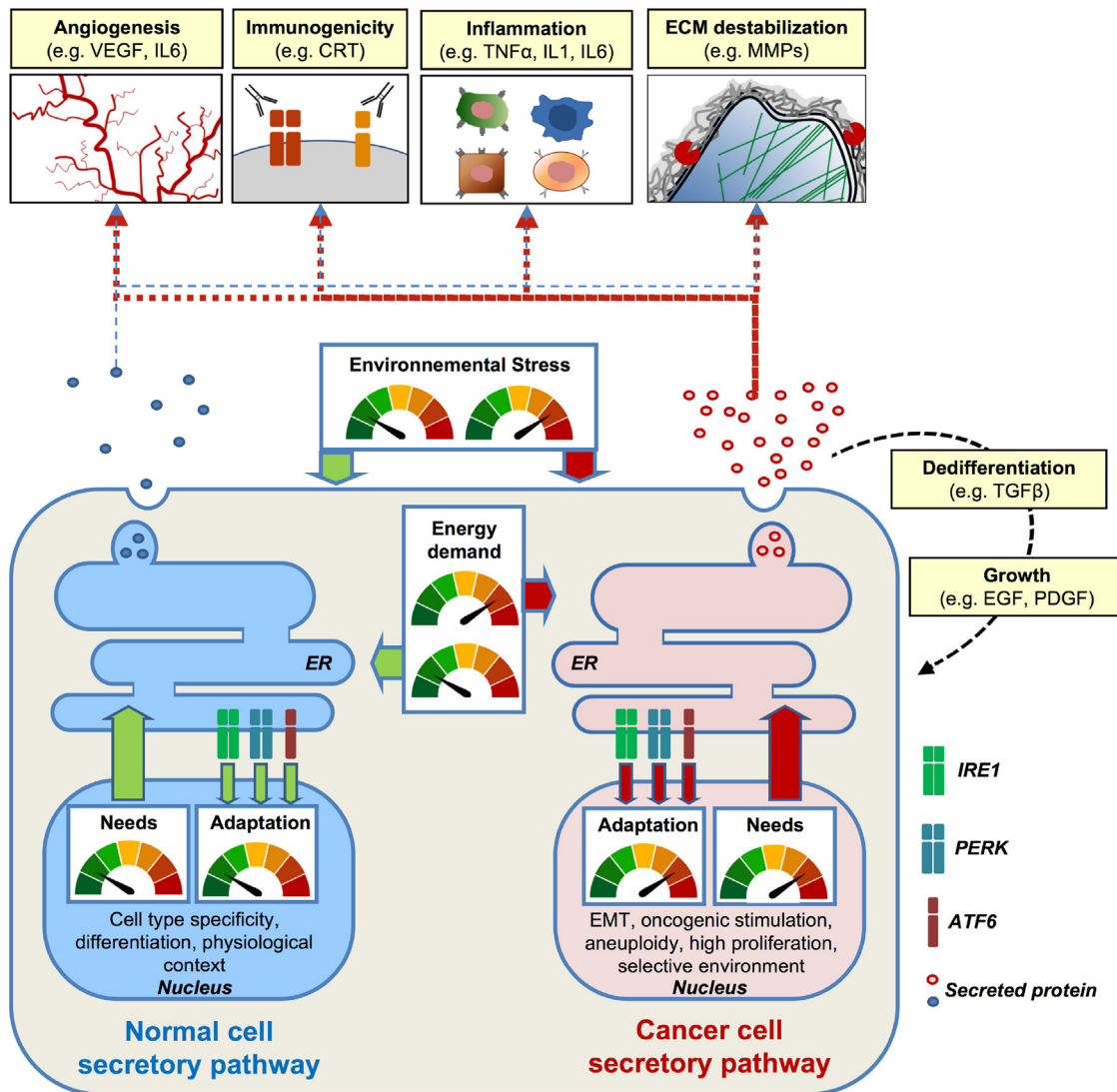


Fig. 2. Normal vs. cancer cell secretory pathway control and biological outcomes. In normal cells, the secretory pathway adapts to fluctuation of environmental stresses and intracellular needs, through a complex molecular signaling pathway: the unfolded protein response (UPR). This adaptation program is triggered by three ER transmembrane sensors, namely IRE1, PERK and ATF6. The cell secretory proteins needs vary depending on cell type, differentiation state or on the physiological context. In cancer cells, the secretory pathway is subjected to a strong environmental pressure due to environmental stress factors, such as hypoxia, oxidative stress or chemotherapies, and to oncogenic pressure (e.g. *Myc*/aneuploidy stimulation of transcription). Furthermore, in these cells, the increase in secretion demand is also dependent on a substantial requirement of energy and amino acid supply. The integration of both intrinsic and extrinsic challenges results in disturbance of the ER homeostasis causing the UPR to be constitutively active in these cells. Disturbance of the secretory pathway will, as a consequence, lead to the modulation of the secretion of proteins important for cancer features such as growth factors and their associated receptors, extracellular matrix proteins, matrix metalloproteinases, inflammatory factors, integrins, immunogenic factors or pro-angiogenic factors.

3. UPR and EMT: an intricate relationship

Epithelial to mesenchymal transition (EMT) enables carcinoma cells to acquire key malignant traits such as migratory and invasion properties, induces stem cell properties and drug resistance [49–51]. Hallmarks of this transition are repression of epithelial markers, up-regulation of mesenchymal markers and changes in morphology. During EMT, the phenotype of carcinoma cells is largely modified, for example the loss of epithelial polarity and *zonula adherens* mediated by the down-regulation of E-cadherin. E-cadherin is a cell adhesion protein and its cytosolic domain is associated at the membrane of the cell to β -catenin, which is a major player of the canonical WNT pathway [52]. Through EMT, diminution of E-cadherin leads to the release of β -catenin, resulting in its nuclear translocation. In the nucleus, β -catenin associates with transcription factors of the TCF/LEF family to regulate transcription of genes involved in EMT, migration and invasion.

Activation of the UPR has been involved in a growing number of cancers [53], but the link between UPR and EMT has been studied recently in breast cancers. Indeed, recent reports show an inter-relationship between UPR signals and EMT, in a context specific manner [54–56].

3.1. Activation of the UPR is instrumental for EMT induction

This phenomenon was first reported in thyroid cells, in which tunicamycin or thapsigargin triggered signaling by the proto-oncogene tyrosine kinase SRC, caused dedifferentiation through the down-regulation of thyroid specific genes and induced an EMT-like phenotype. This included the change in the organization of the polarized epithelial monolayer, the formation of actin stress fibers, the loss of trans-epithelial resistance, the down-regulation of E-cadherin and the up-regulation of mesenchymal markers such as vimentin, α -smooth actin, α 11 collagen and SNAI1/SIP1. Moreover

the use of PP2, a SRC kinase inhibitor, prevented dedifferentiation and EMT, thus confirming the involvement of the SRC pathway [54]. Moreover, the UPR (induced by tunicamycin or overexpression of a variant protein) in alveolar epithelial cells was shown to trigger the SRC and β -catenin pathways [57]. Again, the use of PP2 also blocked the EMT and maintained the epithelial phenotype. Notably no increase in TGF- β 1, an important mediator of EMT, was observed in this report. Interestingly, in renal proximal tubular epithelial cells, thapsigargin induced an EMT whereas tunicamycin did not [58]. ER stressors that alter calcium fluxes between the ER lumen and the cytosol such as thapsigargin lead to an increased expression of TDAG51 and TGF- β 1. TDAG51 interacts with the cytoskeleton and induces shape changes as well as the activation of WNT signaling thereby leading to EMT. As such, overexpression of TDAG51 alone was able to induce an EMT phenotype in HK-2 cells. The serine/cysteine protease inhibitor SCCA1 is deregulated in many cancers associated with poor differentiation and aggressiveness. In mammary epithelial cells (MCF10A), overexpression of SCCA1 induces chronic UPR. This non-lethal chronic UPR activates NF- κ B that leads to IL-6 production, resulting in EMT-like phenotypes [59]. It has been recently reported that IL-6 signaling plays a critical role in driving EMT through cell autonomous inflammation [60,61]. In light of these reports, activation of UPR can lead to EMT through several mechanisms including IL-6, SRC and/or WNT signaling, but how the UPR is triggered, either through alteration of calcium concentrations or increase of improperly folded proteins, might also determine the biological outcome. Thus, one might hypothesize that ER stressed cells enter dedifferentiation/EMT to change their phenotypes and consequently lower ER stress [57].

3.2. EMT induces activation of the UPR in colorectal and breast carcinoma

Cells subjected to EMT are also known to display an important secretory phenotype notably by changes in ECM protein secretion [62,63]. This could represent a cause for ER stress and UPR activation. In colorectal carcinoma cells (SW480, HCT116), stabilization of HIF1 α through CoCl₂-mediated inhibition of proline hydroxylase, or serum starvation, induces EMT and the subsequent activation of the UPR [64]. This mechanism is in part dependent upon ZEB-1, which is the main factor for EMT in colorectal carcinoma cells and a transcriptional repressor for E-cadherin [52,65,66]. In mammary epithelial cells, EMT induction by TWIST overexpression correlates with PERK constitutive activation [55]. Other branches of the UPR (i.e. IRE1 α and ATF6) were not involved in this process. Interestingly, inhibition of PERK activity attenuated cells' ability to migrate and to form tumor spheres, thereby indicating that PERK might be involved in EMT-dependent cell malignancy. In addition, PERK signaling in EMT dedifferentiated cells leads to constitutive activation of NRF2, a master regulator of cellular response to oxidative damage, causing these cells to become chemoresistant through expression of antioxidant enzymes and drug efflux pumps [67–69]. These results might therefore explain the correlation observed between PERK activation status and highly aggressive and poorly differentiated breast cancer tumors. It is noteworthy that in tubular epithelial cells both EMT and UPR are activated simultaneously through reactive oxygen species (ROS) and SRC kinase-dependent pathways [70].

4. Targeting UPR as a novel approach to treat EMT chemoresistant cells

As there is a hierarchical relationship between UPR and EMT it could allow the development of new treatment strategies. Indeed, in diseases where EMT is induced by UPR, e.g. lung fibrosis, chronic

kidney disease or breast cancer [57–59], targeting the UPR or the downstream activated pathways (e.g. SRC, WNT) with inhibitors might be an efficient way to prevent cells from undergoing EMT. In vitro, results were already observed with PP2 targeting the SRC kinase and preventing both PC C13 cells (thyroid cells) and alveolar epithelial cells from undergoing an EMT [54,57]. Currently, ER stress drugs are only used to treat multiple myeloma patients [71], however because EMT is associated with chemoresistance and invasiveness [49–51], there is a critical need to develop new approaches, therefore it might be very attractive to exploit the ER stress-sensitivity exhibited by cells subjected to EMT [64]. Indeed it was shown that in breast cancer cells several ER stressors such as tunicamycin, thapsigargin, DTT and A23187 render EMT undergoing cells more sensitive to cell death (up to 25 fold for thapsigargin). These treatments could even selectively eliminated EMT undergoing cells when co-cultivated with normal cells [55]. Also targeting the PERK pathway that is constitutively active in breast cancer [56,72] could also be a promising option. Indeed PERK is required for the cells to secrete new extracellular matrix and to enable EMT undergoing cells to invade, metastasize and form tumor spheres. PERK is also responsible for the constitutive activation of NRF2 in EMT undergoing human breast epithelial cells, causing cells to become MDR. Inhibition of PERK caused the decreased expression of 58 of the 142 NRF2 target genes and sensitized cells to chemotherapy. Together these observations suggest that targeting the UPR and its downstream effectors could be a key therapeutic strategy in the treatment of drug-resistant cancer cells.

4.1. UPR control of glioblastoma phenotypes

Glioblastoma (GBM) is the most common primary brain tumor in humans and remains incurable [73]. Despite the therapeutic efforts made in recent years, mortality is still close to 100% at 5 years. Different factors are involved in GBM aggressiveness, among which angiogenesis and tumor cell invasion/infiltration are critical [74,75]. Moreover, the mesenchymal phenotype is another hallmark of tumor aggressiveness in GBM [76,77]. Remarkably, a single UPR component, IRE1 α , appeared to regulate these three features of GBM aggressiveness. Indeed, it was shown that expression of a dominant negative form of IRE1 α triggered a mesenchymal drift in glioblastoma, characterized by modulation of the expression of extracellular matrix, angiogenesis, and inflammation proteins. This is in agreement with other studies reporting IRE1 α coding gene somatic mutations in GBM [78,79] and ranking IRE1 α as the fifth highest mutated kinase, carrying at least one driver mutation [78]. The mesenchymal drift accompanied by IRE1 α inactivation was due to a set of pathways acting in a synergistically manner. In this model, IRE1 α -driven modulation of angiogenesis was attributed to the positive regulation of pro-angiogenic factors such as VEGF-A, IL-1 β and IL-6 secretion [80,81] and the cleavage of the mRNA coding for the circadian gene PERIOD1 [82]. Adding to its role in the regulation of angiogenesis, PERIOD1 also regulated IRE1 α -dependent GBM infiltration [82]. Another IRE1 α endoribonuclease substrate, the mRNA coding for the extracellular matrix protein SPARC was also found to be involved in modulation of GBM invasion ability in an autocrine fashion [83]. Taken together, these data underline that IRE1 α in itself can send both intracrine and autocrine signals to control the phenotype, the physiology and the aggressiveness of GBM.

4.2. UPR in triple negative breast cancer

Triple negative breast cancers (TNBC) represent the most aggressive breast cancer subtype, with high rates of tumor recurrence and poor overall survival [84]. Although the lack of expression of the estrogen, progesterone and HER2 receptor clusters, these

tumors within the same group still comprise heterogeneous and poorly characterized breast cancers with no selective therapy [85]. Recently, a study by Chen and colleagues [86] revealed the contribution of the UPR to TNBC, in particular through the cross-talk with HIF-1 α , a transcription factor previously shown to be of particular importance in the hypoxic response in TNBC. Chen and colleagues characterize a new molecular mechanism, XBP1s-dependent HIF-1 α activation in TNBC, thereby indicating potential novel therapeutic strategies mediated through the inhibition of XBP1 in TNBC [87]. These observations could also be linked to the acquisition of a mesenchymal phenotype by breast tumor cells. Indeed, increased expression of XBP1 is associated with the progression of breast cancer and XBP1s is significantly over-expressed in matched metastatic tumors, which can act as a major regulator of EMT through SNAIL signaling [88]. Moreover, as autophagy and UPR signaling also appear to be interconnected, combined chloroquine (CQ), a pharmacological inhibitor of autophagy, with other drugs known to act as ER stress enhancers (nefnavir (an HIV protease inhibitor) and celecoxib (a cyclooxygenase-2 inhibitor) or its non-coxib analog 2,5-dimethyl-celecoxib (DMC)) were tested in TNBC. Addition of CQ resulted in synergistic enhancement of tumor cell killing by ER stress aggravators *in vitro* and *in vivo*, thus opening novel therapeutic avenues for TNBC [89].

5. Conclusions and future perspectives

Collectively, the aforementioned information sheds light on the intricate cancer signaling networks into which the UPR is involved. From this analysis it becomes evident that characterizing the UPR status in tumors might not only represent a good predictor of the disease outcome but also constitute an essential toolkit for better defining personalized treatments and following up treatment efficacy. As such, a thorough analysis of the UPR in tumors could be envisioned to firstly select the best and most relevant markers/predictors of tumor characteristics and then secondly to apply the most efficient targeted therapies to those tumors. Needless to say that in this context, therapies targeting the UPR itself could also be of interest either alone or as adjuvant therapies. In conclusion, the specificity of UPR signals and its impact on tumor phenotype represents an interesting avenue to better characterize carcinogenesis but also when documented in patients' tumors will constitute a novel basis for tumor typing and specialized treatments.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We apologize to colleagues whose work was not cited in this review due to space limitation. This work was funded by grants from Institut National du Cancer (INCa) and Ligue Contre le Cancer to EC. ND was supported by a post-doctoral fellowship from Fondation de France.

References

- [1] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
- [2] Dejeans N, Manie S, Hetz C, Bard F, Hupp T, Agostinis P, et al. Addicted to secrete – novel concepts and targets in cancer therapy. *Trends Mol Med* 2014;20:242–50.
- [3] Garg A, Maes H, van Vliet AR, Agostinis P. Targeting the hallmarks of cancer with therapy-induced endoplasmic reticulum (ER) stress. *Mol Cell Oncol* 2015 [in press].
- [4] Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 2001;107:881–91.
- [5] Maurel M, Chevet E, Tavernier J, Gerlo S. Getting RIDD of RNA: IRE1 in cell fate regulation. *Trends Biochem Sci* 2014;39:245–54.
- [6] Lee AH, Iwakoshi NN, Glimcher LH. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol* 2003;23:7448–59.
- [7] Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *National Institute of Environmental Health Sciences. Mol Cell* 2003;11:619–33.
- [8] Wu J, Rutkowski DT, Dubois M, Swathirajan J, Saunders T, Wang J, et al. ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. *Dev Cell* 2007;13:351–64.
- [9] Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 2011;334:1081–6.
- [10] Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* 2013;12:703–19.
- [11] Rutkowski DT, Hegde RS. Regulation of basal cellular physiology by the homeostatic unfolded protein response. *J Cell Biol* 2010;189:783–94.
- [12] Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravalles EM, et al. Plasma cell differentiation requires the transcription factor XBP-1. *Nature* 2001;412:300–7.
- [13] Zhang K, Wong HN, Song B, Miller CN, Scheuner D, Kaufman RJ. The unfolded protein response sensor IRE1alpha is required at 2 distinct steps in B cell lymphopoiesis. *J Clin Invest* 2005;115:268–81.
- [14] Tsang KY, Chan D, Bateman JF, Cheah KS. *In vivo* cellular adaptation to ER stress: survival strategies with double-edged consequences. *J Cell Sci* 2010;123:2145–54.
- [15] Sha H, He Y, Chen H, Wang C, Zenno A, Shi H, et al. The IRE1alpha-XBP1 pathway of the unfolded protein response is required for adipogenesis. *Cell Metab* 2009;9:556–64.
- [16] Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? *Nat Rev Cancer* 2004;4:966–77.
- [17] Delepine M, Nicolino M, Barrett T, Golamaully M, Lathrop GM, Julier C. EIF2AK3, encoding translation initiation factor 2-alpha kinase 3, is mutated in patients with Wolcott-Rallison syndrome. *Nat Genet* 2000;25:406–9.
- [18] Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, et al. Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. *Mol Cell* 2001;7:1153–63.
- [19] Lin W, Harding HP, Ron D, Popko B. Endoplasmic reticulum stress modulates the response of myelinating oligodendrocytes to the immune cytokine interferon-gamma. *J Cell Biol* 2005;169:603–12.
- [20] Wang W, Lian N, Li L, Moss HE, Wang W, Perrier DS, et al. Atf4 regulates chondrocyte proliferation and differentiation during endochondral ossification by activating *Ihh* transcription. *Development* 2009;136:4143–53.
- [21] Zhang P, McGrath B, Li S, Frank A, Zambito F, Reinert J, et al. The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol Cell Biol* 2002;22:3864–74.
- [22] Lin CY, Lovén J, Rahl PB, Paranal RM, Burge CB, Bradner JE, et al. Transcriptional amplification in tumor cells with elevated c-Myc. *Cell* 2012;151:56–67.
- [23] Nie Z, Hu G, Wei G, Cui K, Yamane A, Resch W, et al. c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell* 2012;151:68–79.
- [24] Torres EM, Sokolsky T, Tucker CM, Chan LY, Boselli M, Dunham MJ, et al. Effects of aneuploidy on cellular physiology and cell division in haploid yeast. *Science (New York, NY)* 2007;317:916–24.
- [25] Tang YC, Williams BR, Siegel JJ, Amon A. Identification of aneuploidy-selective antiproliferation compounds. *Cell* 2011;144:499–512.
- [26] Barna M, Pusic A, Zollo O, Costa M, Kondrashov N, Rego E, et al. Suppression of Myc oncogenic activity by ribosomal protein haploinsufficiency. *Nature* 2008;456:971–5.
- [27] Huber AL, Lebeau J, Guillaumot P, Petrilli V, Malek M, Chilloux J, et al. p58(IPK)-Mediated attenuation of the proapoptotic PERK-CHOP pathway allows malignant progression upon low glucose. *Mol Cell* 2013;49:1049–59.
- [28] Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med* 2011;17:320–9.
- [29] Leung CT, Brugge JS. Outgrowth of single oncogene-expressing cells from suppressive epithelial environments. *Nature* 2012;482:410–3.
- [30] Mahadevan NR, Rodvold J, Sepulveda H, Rossi S, Drew AF, Zanetti M. Transmission of endoplasmic reticulum stress and pro-inflammation from tumor cells to myeloid cells. *Proc Natl Acad Sci U S A* 2011;108:6561–6.
- [31] Mahadevan NR, Zanetti M. Tumor stress inside out: cell-extrinsic effects of the unfolded protein response in tumor cells modulate the immunological landscape of the tumor microenvironment. *J Immunol* 2011;187:4403–9.
- [32] Senovilla L, Vitale I, Martins I, Tailler M, Pailletet C, Michaud M, et al. An immunosurveillance mechanism controls cancer cell ploidy. *Science (New York, NY)* 2012;337:1678–84.
- [33] Luo B, Lee AS. The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. *Oncogene* 2013;32:805–18.
- [34] Heijmans J, van Lith de Jeude JF, Koo BK, Rosekrans SL, Wielenga MC, van de Wetering M, et al. ER stress causes rapid loss of intestinal epithelial stemness through activation of the unfolded protein response. *Cell Rep* 2013;3:1128–39.
- [35] Vermeulen L, Snippert HJ. Stem cell dynamics in homeostasis and cancer of the intestine. *Nat Rev Cancer* 2014;14:468–80.

- [36] Bertolotti A, Wang X, Novoa I, Jungreis R, Schlessinger K, Cho JH, et al. Increased sensitivity to dextran sodium sulfate colitis in IRE1beta-deficient mice. *J Clin Invest* 2001;107:585–93.
- [37] Tsuru A, Fujimoto N, Takahashi S, Saito M, Nakamura D, Iwano M, et al. Negative feedback by IRE1beta optimizes mucin production in goblet cells. *Proc Natl Acad Sci U S A* 2013;110:2864–9.
- [38] Kaser A, Lee A-H, Franke A, Glickman JN, Zeissig S, Tilg H, et al. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell* 2008;134:743–56.
- [39] Niederreiter L, Fritz TM, Adolph TE, Krismer AM, Offner FA, Tschurtschenthaler M, et al. ER stress transcription factor Xbp1 suppresses intestinal tumorigenesis and directs intestinal stem cells. *J Exp Med* 2013;210:2041–56.
- [40] Adolph TE, Tomczak MF, Niederreiter L, Ko HJ, Bock J, Martinez-Naves E, et al. Paneth cells as a site of origin for intestinal inflammation. *Nature* 2014;503:272–6.
- [41] Wu C, Kraft P, Zhai K, Chang J, Wang Z, Li Y, et al. Genome-wide association analyses of esophageal squamous cell carcinoma in Chinese identify multiple susceptibility loci and gene–environment interactions. *Nat Genet* 2012;44:1090–7.
- [42] Rosekrans SL, Heijmans J, Buller NV, Westerlund J, Lee AS, Muncan V, et al. ER stress induces epithelial differentiation in the mouse oesophagus. *Gut* 2014.
- [43] Pohler E, Craig AL, Cotton J, Lawrie L, Dillon JF, Ross P, et al. The Barrett's antigen anterior gradient-2 silences the p53 transcriptional response to DNA damage. *Mol Cell Proteomics* 2004;3:534–47.
- [44] Carrasco DR, Sukhdeo K, Protopopova M, Sinha R, Enos M, Carrasco DE, et al. The differentiation and stress response factor XBP-1 drives multiple myeloma pathogenesis. *Cancer Cell* 2007;11:349–60.
- [45] Kharabi Masouleh B, Geng H, Hurtz C, Chan LN, Logan AC, Chang MS, et al. Mechanistic rationale for targeting the unfolded protein response in pre-B acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A* 2014;111:E2219–28.
- [46] Rouault-Pierre K, Lopez-Onieva L, Foster K, Anjos-Afonso F, Lamrissi-Garcia I, Serrano-Sanchez M, et al. HIF-2alpha protects human hematopoietic stem/progenitors and acute myeloid leukemic cells from apoptosis induced by endoplasmic reticulum stress. *Cell Stem Cell* 2013;13:549–63.
- [47] Pattacini L, Mancini M, Mazzacurati L, Brusa G, Benvenuti M, Martinelli G, et al. Endoplasmic reticulum stress initiates apoptotic death induced by ST1571 inhibition of p210 bcr-abl tyrosine kinase. *Leuk Res* 2004;28:191–202.
- [48] Higa A, Taouji S, Lhomond S, Jensen D, Fernandez-Zapico ME, Simpson JC, et al. Endoplasmic reticulum stress-activated transcription factor ATF6alpha requires the disulfide isomerase PDIA5 to modulate chemoresistance. *Mol Cell Biol* 2014;34:1839–49.
- [49] Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial–mesenchymal transitions in development and disease. *Cell* 2009;139:871–90.
- [50] Nieto MA, Cano A. The epithelial–mesenchymal transition under control: global programs to regulate epithelial plasticity. *Semin Cancer Biol* 2012;22:361–8.
- [51] Baum B, Settleman J, Quinlan MP. Transitions between epithelial and mesenchymal states in development and disease. *Semin Cell Dev Biol* 2008;19:294–308.
- [52] Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. *Cancer Metastasis Rev* 2009;28:151–66.
- [53] Clarke HJ, Chambers JE, Liniker E, Marciniak SJ. Endoplasmic reticulum stress in malignancy. *Cancer Cell* 2014;25:563–73.
- [54] Ulianic L, Garbi C, Treglia AS, Punzi D, Miele C, Raciti GA, et al. ER stress is associated with dedifferentiation and an epithelial-to-mesenchymal transition-like phenotype in PC13 thyroid cells. *J Cell Sci* 2008;121:477–86.
- [55] Feng YX, Sokol ES, Del Vecchio CA, Sanduja S, Claessen JH, Proia TA, et al. Epithelial-to-mesenchymal transition activates PERK-eIF2alpha and sensitizes cells to endoplasmic reticulum stress. *Cancer Discov* 2014;4:702–15.
- [56] Del Vecchio CA, Feng Y, Sokol ES, Tillman EJ, Sanduja S, Reinhardt F, et al. De-differentiation confers multidrug resistance via noncanonical PERK-Nrf2 signaling. *PLoS Biol* 2014;12:e1001945.
- [57] Tanjore H, Cheng DS, Degryse AL, Zoz DF, Abdolrasulnia R, Lawson WE, et al. Alveolar epithelial cells undergo epithelial-to-mesenchymal transition in response to endoplasmic reticulum stress. *J Biol Chem* 2011;286:30972–80.
- [58] Carlisle RE, Heffernan A, Brimble E, Liu L, Jerome D, Collins CA, et al. TDAG51 mediates epithelial-to-mesenchymal transition in human proximal tubular epithelium. *Am J Physiol Renal Physiol* 2012;303:F467–81.
- [59] Sheshadri N, Catanzaro JM, Bott A, Sun Y, Ullman E, Chen E, et al. SCCA1/SerpinB3 promotes oncogenesis and epithelial–mesenchymal transition via the unfolded protein response and IL-6 signaling. *Cancer Res* 2014.
- [60] Gao SP, Mark KG, Leslie K, Pao W, Motoi N, Gerald WL, et al. Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. *J Clin Invest* 2007;117:3846–56.
- [61] Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M, et al. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Invest* 2007;117:3988–4002.
- [62] Kirk SJ, Cliff JM, Thomas JA, Ward TH. Biogenesis of secretory organelles during B cell differentiation. *J Leukoc Biol* 2009;87:245–55.
- [63] Fox RM, Hanlon CD, Andrew DJ. The Creba/Creb3-like transcription factors are major and direct regulators of secretory capacity. *J Cell Biol* 2010;191:479–92.
- [64] Zeindl-Eberhart E, Brandl L, Liebmann S, Ormanns S, Scheel SK, Brabletz T, et al. Epithelial–mesenchymal transition induces endoplasmic–reticulum-stress response in human colorectal tumor cells. *PLoS ONE* 2014;9:e87386.
- [65] Spaderna S, Schmalhofer O, Wahlbuhl M, Dimmler A, Bauer K, Sultan A, et al. The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. *Cancer Res* 2008;68:537–44.
- [66] Vandewalle C, Van Roy F, Bex G. The role of the ZEB family of transcription factors in development and disease. *Cell Mol Life Sci* 2009;66:773–87.
- [67] Alam J, Stewart D, Touchard C, Boinalpally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 1999;274:26071–8.
- [68] Alam J, Wicks C, Stewart D, Gong P, Touchard C, Otterbein S, et al. Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells, role of p38 kinase and Nrf2 transcription factor. *J Biol Chem* 2000;275:27694–702.
- [69] Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J* 1988;2:2557–68.
- [70] Lee JY, Chang JW, Yang WS, Kim SB, Park SK, Park JS, et al. Albumin-induced epithelial–mesenchymal transition and ER stress are regulated through a common ROS-c-Src kinase-mTOR pathway: effect of imatinib mesylate. *Am J Physiol Renal Physiol* 2011;300:F467–81.
- [71] Curran MP, McKeage K. Bortezomib: a review of its use in patients with multiple myeloma. *Drugs* 2009;69:859–88.
- [72] Hong M, Kim H, Kim I. Ribosomal protein L19 overexpression activates the unfolded protein response and sensitizes MCF7 breast cancer cells to endoplasmic reticulum stress-induced cell death. *Biochem Biophys Res Commun* 2014;450:673–8.
- [73] Adamson C, Kanu OO, Mehta AI, Di C, Lin N, Mattox AK, et al. Glioblastoma multiforme: a review of where we have been and where we are going. *Expert Opin Investig Drugs* 2009;18:1061–83.
- [74] Westphal M, Lamszus K. The neurobiology of gliomas: from cell biology to the development of therapeutic approaches. *Nat Rev Neurosci* 2011;12:495–508.
- [75] Zhong J, Paul A, Kellie SJ, O'Neill GM. Mesenchymal migration as a therapeutic target in glioblastoma. *J Oncol* 2010;2010:430142.
- [76] Phillips HS, Kharbanda S, Chen R, Forrester WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006;9:157–73.
- [77] Carro MS, Lim WK, Alvarez MJ, Bollo RJ, Zhao X, Snyder EY, et al. The transcriptional network for mesenchymal transformation of brain tumours. *Nature* 2010;463:318–25.
- [78] Greenman C, Stephens P, Smith R, Dalglish GL, Hunter C, Bignell G, et al. Patterns of somatic mutation in human cancer genomes. *Nature* 2007;446:153–8.
- [79] Parsons DW, Jones S, Zhang X, Lin JC-H, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science (New York, NY)* 2008;321:1807–12.
- [80] Auf G, Jabouille A, Guerit S, Pineau R, Delugin M, Boucheccareilh M, et al. Inositol-requiring enzyme 1alpha is a key regulator of angiogenesis and invasion in malignant glioma. *Proc Natl Acad Sci U S A* 2010;107:15553–8.
- [81] Drogat B, Auguste P, Nguyen DT, Boucheccareilh M, Pineau R, Nalbantoglu J, et al. IRE1 signaling is essential for ischemia-induced vascular endothelial growth factor-A expression and contributes to angiogenesis and tumor growth in vivo. *Cancer Res* 2007;67:6700–7.
- [82] Pluquet O, Dejeans N, Boucheccareilh M, Lhomond S, Pineau R, Higa A, et al. Post-transcriptional regulation of PER1 underlies the oncogenic function of IRE1alpha. *Cancer Res* 2013;73:4732–43.
- [83] Dejeans N, Pluquet O, Lhomond S, Grise F, Boucheccareilh M, Juin A, et al. Autocrine control of glioma cells adhesion and migration through IRE1alpha-mediated cleavage of SPARC mRNA. *J Cell Sci* 2012;125:4278–87.
- [84] Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med* 2010;363:1938–48.
- [85] Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* 2011;121:2750–67.
- [86] Chen X, Iliopoulos D, Zhang Q, Tang Q, Greenblatt MB, Hatziaepostolou M, et al. XBP1 promotes triple-negative breast cancer by controlling the HIF1alpha pathway. *Nature* 2014;508:103–7.
- [87] Lu Y, Liang FX, Wang X. A synthetic biology approach identifies the mammalian UPR RNA ligase RtcB. *Mol Cell* 2014;55:758–70.
- [88] Li H, Chen X, Gao Y, Wu J, Zeng F, Song F. XBP1 induces snail expression to promote epithelial-to-mesenchymal transition and invasion of breast cancer cells. *Cell Signal* 2014.
- [89] Thomas S, Sharma N, Golden EB, Cho H, Agarwal P, Gaffney KJ, et al. Preferential killing of triple-negative breast cancer cells in vitro and in vivo when pharmacological aggravators of endoplasmic reticulum stress are combined with autophagy inhibitors. *Cancer Lett* 2012;325:63–71.

We have observed that many reports have implicated the UPR in different tumors as playing a pro-survival, pro-adaptation role therefore great efforts to target this pathway in cancer cells are being undertaken (27a). However the contribution of the UPR to malignancy is complex and differs from a given tumor type to another due to the cell characteristics and the conditions to which they are exposed. More mechanistic and physiological studies are therefore needed for each tumor to ensure the efficacy of pharmacological small molecules. In cancer cells, the stress induced by the accumulation of unfolded proteins in the ER triggers the UPR to restore protein homeostasis. The UPR signalling and the transcriptional reprogramming that accompany it, not only help the cancer cells to cope with the unfolded proteins but has also been shown to participate in the acquisition of malignant traits such as growth, angiogenesis, invasion and metastasis or inflammation. Although the molecular events triggered by the UPR are well described, the transcriptional mechanism underlying the transcriptional reprogramming still remains poorly defined.

The genomic instability and the high proliferation rate of cancer cells cause them to be overloaded with proteins including mutated proteins that cannot find their correct conformation. As previously mentioned, such conditions trigger ER stress and the UPR, but another key player of maintenance of protein homeostasis is p97/VCP. Indeed, p97/VCP is not only part of the ERAD pathway but it is also involved in other degradation processes such as autophagy and works in concert with the Ubiquitin Proteasome System to degrade ubiquitinated proteins. Interestingly p97/VCP was found overexpressed in many cancers, suggesting that its role in the regulation of protein homeostasis might be key for cancer cell survival.

II) p97/VCP/CDC-48

The cdc-48 gene was discovered thirty years ago in a genetic screen conducted in *S. cerevisiae* and named Cell Division Cycle 48 among other genes that caused cell cycle arrest (27b). CDC-48 mammalian homolog was later reported as a 97 kDa protein precursor for the small peptide valosin, and therefore name as valosin-containing protein (VCP) or p97. Although the valosin was a purification artefact unrelated to p97, the VCP nomenclature is still being used. Studies on p97 are also conducted in other species like *D. melanogaster* where it is the name Transitional endoplasmic reticulum ATPase TER94 (TER94) that is often used, or *C. elegans*

which also use the name cdc-48 but interestingly have two copies of the gene (cdc-48.1 and cdc-48.2).

1) Structure of p97/VCP

The p97 protein belongs to the ATPase Associated with various cellular Activity (AAA⁺) family. AAA⁺ proteins are characterized by the presence of AAA domains that contain Walker A and B motifs. Proteins containing such Walker motifs are found throughout evolution (from archae bacteria to human) which suggest that they are fundamental in all life forms (28). Moreover, p97 is strongly and ubiquitously expressed in all multicellular organisms, for example in human p97/VCP proteins account for about 1% of the total cell proteins (29). The human p97/VCP is an 806 amino acid (AA) long protein and has four domains (30) (**Figure 6A**): the N domain starts at AA 1 to 187 and its role is to facilitate cofactors binding and substrate recognition

- after a short linker the D1 domain (AA 208 to 459) contains Walker A and B motifs and although it possesses ATP activity it is believed that its main role is to help p97/VCP to find its quaternary structure
- after a short linker the D2 domain (AA 481 to 761) also contains Walker A and B motifs and is the main site for ATP hydrolysis
- finally the C terminal tail (AA 762 to 806) serves as a PTM site and binding site for p97/VCP cofactors

In the cell p97/VCP is inactive as a monomer, but like other AAA⁺ proteins is active as a hexamer (**Figure 6B**)(28). Electron microscopy analyses revealed that p97/VCP/CDC-48 forms a hexameric ring which allows access for its cofactors to bind its N domain or C terminal tail.

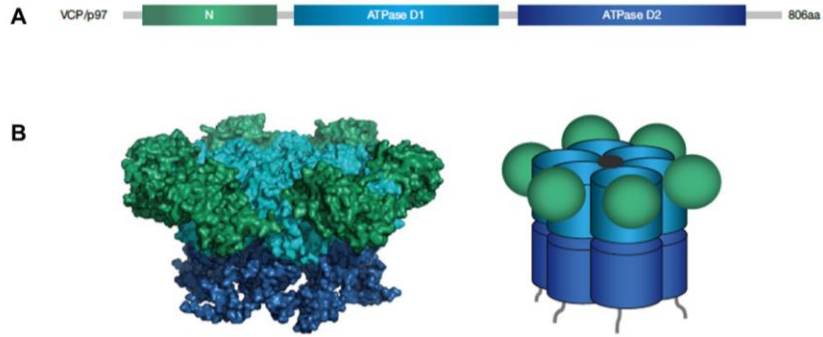


Figure 6: Schematic representation p97/VCP domains and quaternary structure - A) p97/VCP 4 domains: N domain for cofactors binding, D1 domain for ATPase and quaternary structure, D2 domain main ATPase domain and C terminal tail for PTM and cofactors binding. B) p97/VCP function as a hexamer in the cells, the domain colours are conserved between A) and B), its structure quaternary structure allows ATPase activity and cofactors binding at its N domains and C terminal tails (adapted from 30).

In the rest of the text p97/VCP will refer to the human protein/gene, CDC-48 to the yeast protein/gene and CDC-48.1 or CDC-48.2 to the worm protein/gene and p97 will refer to the gene/protein of multiple or other species.

2) Functions of p97/VCP/CDC-48

p97's main function is to segregate (extract) a given protein from protein complex, organelle membrane or chromatin thereby facilitating its recycling or degradation by the proteasome (**Figure 7**)(30). p97 associates with various cofactors/adaptors and these interactions are key for its activity and functional diversity (**Figure 8**)(31). Most of p97 partners possess ubiquitin-X (UBX) domain or UBX-like domain and can bind to ubiquitin (32). Among them are many E3 ubiquitin ligases which are important for specific substrate recognition, but also E4B/UFD2 an ubiquitin ligase E4 which can extend shorter ubiquitin chain to promote proteasome degradation (33). Interestingly, p97 can also interact with deubiquitinating enzymes (DUB) that can remove ubiquitin from protein thus promoting substrate recycling over proteasome degradation (30). p97 adaptors are also important to promote its recruitment to a specific subcellular compartment as they can interact with protein from this subcellular compartment. For example Ufd1/Npl4 can interact with the ER transmembrane protein VIMP (VCP interacting membrane protein) to recruit p97 at the ER membrane so it can participate in ERAD (34). To date p97 functions can be grouped in three major categories all mediated by its

segregase activity. These categories comprise regulation of protein homeostasis, membrane fusion and protein trafficking and chromatin-associated functions.

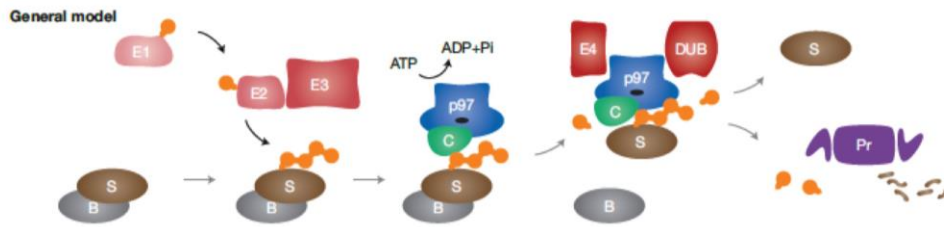


Figure 7: Schematic representation p97 activity - In this example p97 substrate: the “S” protein in brown is in complex with the protein B, once “S” is ubiquitinated by the UPS it is recognized by p97 and its ubiquitin binding cofactors. p97 can then separate the protein “S” from its partner with the force provide by ATP hydrolysis. From there if the ubiquitin chain is maintained or extend the protein is sent to the proteasome for degradation, however if the ubiquitin chain is removed by deubiquitinase which can associate with p97, the protein is recycled (adapted from 30).

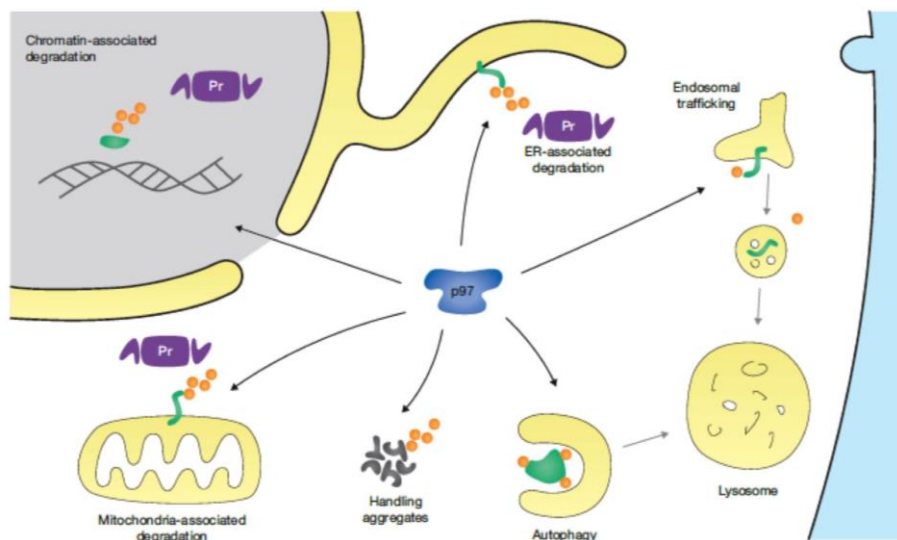


Figure 8: Schematic representation of p97 functions in cellular processes - A wide range of cellular processes required p97 mediated degradation such as: ERAD, mitochondria-associated degradation, autophagy, aggregates handling, chromatin-associated degradation and Endosomal trafficking (adapted from 30).

a) Regulation of Protein homeostasis

Short-lived, misfolded or damaged proteins are degraded by the UPS. Ubiquitination occurs on lysine residues through the sequential activity of ubiquitin activating (E1), ubiquitin conjugating (E2) and ubiquitin ligase (E3) enzymes. p97 thanks to its various cofactors/adaptors

including several E3 enzymes is involved in the regulation of several protein homeostasis processes (**Figure 8**).

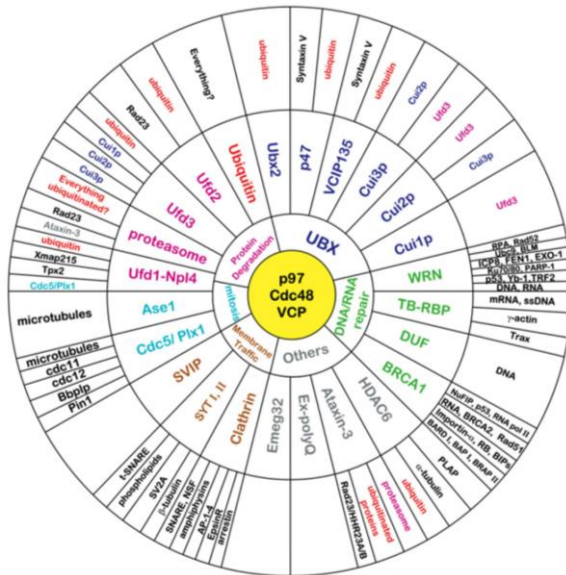


Figure 9: Representation of p97/VCP/CDC-48 cofactors and their functions – To date more than 40 cofactors are known for p97/VCP/CDC-48. This diagram shows in each pathway what cofactors are involved and their interacting partners (adapted from 32).

- **ERAD** - As indicated earlier, misfolded proteins in the ER have to be exported to the cytosol for proteasome mediated degradation. After a portion of the misfolded proteins emerge in the cytosol Derlin proteins which belong to the UPS and VIMP (VCP interacting membrane protein) recruit p97/VCP and its cofactors Ufd1/Npl4. Then p97/VCP provides the force to pull the protein through the retrotranslocon to the cytosol, as the protein is pulled out it is also processed by the UPS (**Figure 5**). It is also believed that p97/VCP might play a role in the shuttling of the misfolded protein to the proteasome but this process is poorly defined. Additionally, p97/VCP is capable of releasing membrane-bound transcription factors such as Nrf1 which are not degraded but instead translocate to the nucleus to activate transcription (35) Similarly, CDC-48 was shown to be required for the cleavage of SREBP from Golgi membrane in yeast (36).

- **Mitochondria-associated degradation** - It has also been showed in yeast that CDC-48 with its cofactor Vms1 (VCP/Cdc48-associated mitochondrial stress-responsive 1), Npl4, Ufd1 and the cooperation of the UPS are responsible for the quality control of proteins of the outer mitochondrial membrane (OOM) such as Fzo1 and Mcl1 (37). This observation is reinforced by the fact that CDC-48 cofactors Ufd1 and Npl4 are found highly enriched at the surface of the mitochondria.

- **Co-translational degradation** - p97/VCP/CDC-48 is also involved in a process called co-translational degradation, in fact during protein translation errors in the mRNAs or its degradation can cause the ribosome to stall and thus cannot complete the translation, the nascent peptide is then polyubiquitinated to be recognized by p97/VCP/CDC-48-Ufd1-Npl4 and directed to the proteasome for degradation (31).

- **Autophagy and aggregate handling** - p97/VCP/CDC-48 was also reported to be part of other degradation mechanisms: autophagy and aggregate handling. Indeed several reports have shown that CDC-48 is required for autophagosomes maturation into autolysosomes, as in Cdc-48 knock down cells immature autophagosomes accumulate (38a, 38b)). Aggregation of misfolded proteins in the cytosol can be caused by denatured protein, heat shock or overexpression of insoluble/mutant protein, to cope with these misfolded proteins the cell stores them into aggresome before their degradation by autophagy. HDAC6 is key player in this process as it was shown to be capable of shuttling ubiquitinated misfolded proteins to aggresome along the microtubules (39). Interestingly, in 2006 it was shown that the p97/VCP-HDAC6 protein ratio could control the fate and destination of ubiquitinated proteins: aggresome or proteasome (**Figure 8**). Indeed, HDAC6 can by interacting with its substrates hide their ubiquitin tags and promote aggregate formation which will be handled by autophagy. If the p97/VCP-HDAC6 ration is imbalanced p97/VCP can segregate HDAC6 from its ubiquitin substrates thus the ubiquitin tag is unmasked and the revealed protein is taken in charge by the UPS pathway. Later, another layer of complexity was added to the HDAC6-p97/VCP interaction as these proteins also control HSF1 a major transcription factor of the heat shock pathway. In fact, HSF1 was discovered to be part of an inactive complex in basal condition with p97/VCP, HDAC6 and HSPs (Heat Shock Proteins) (40) (**Figure 10**). However, upon accumulation of ubiquitinated proteins in the cytosol (caused by heat shock or impairment of the proteasome) the complex dissociates thus HDAC6 and p97/VCP can cope with ubiquitinated substrates and HSF1 activates transcription of its target genes which include chaperones, overall all the dissociated members work at restoring protein homeostasis.

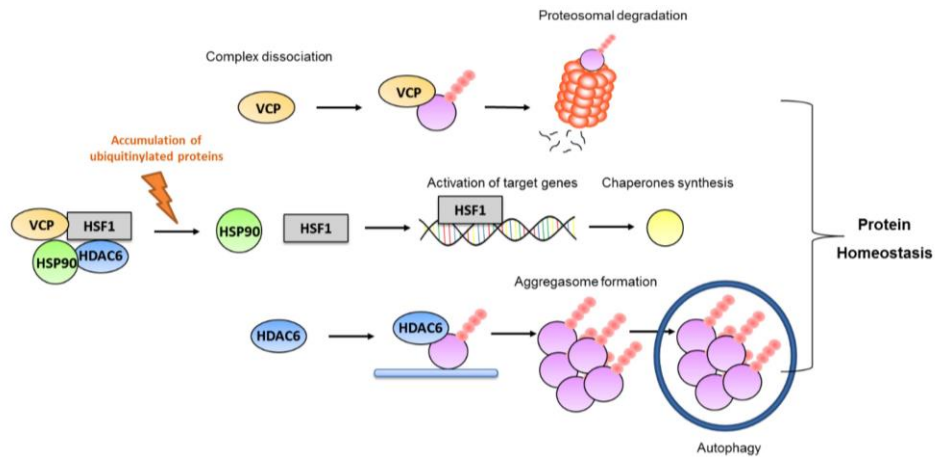


Figure 10: Schematic representation of the role of HDAC6, p97/VCP and HSF1 in response to accumulation of ubiquitinated proteins - In basal conditions p97/VCP, HDAC6, HSF1 and HSP90 form an inactive complex. However upon accumulation of ubiquitinated proteins the complex is dissociated. p97/VCP and its cofactors target ubiquitinated substrates for proteasomal degradation. HSF1 translocates to the nucleus to activate its target genes which include chaperones. HDAC6 shuttles ubiquitinated proteins along microtubules and promotes aggregates formation. All these mechanisms help the cell to restore its protein homeostasis

b) Membrane fusion and trafficking

During the cell cycle both the Golgi apparatus and the ER experience dramatic changes in shape and structure to the point where during mitosis the ER and Golgi are fragmented in vesicles and tubules that therefore need to be reassembled in the daughter cells. In this context, p97/VCP and its cofactors p47 and VCIP 135 are required for membrane fusion and reassembly of both the ER and Golgi apparatus (41). Moreover, p97/VCP was also shown to regulate the assembly of transitional ER, a process coordinated by p97/VCP phosphorylation: when not phosphorylated p97/VCP associates with p47 and syntaxin-5 an ER membrane protein involved in vesicle docking and fusion and promotes the assembly of transitional ER whereas phosphorylation of p97/VCP inhibits these interactions thus the formation of transitional ER (42). However, the precise role of p97/VCP in this process is not clear although it seems to involve binding and regulation of syntaxin-5 an ER membrane protein involved in vesicle docking and fusion. Recent studies have revealed a new role for p97/VCP in endocytic trafficking, indeed p97/VCP was found to interact with EEA1 (endosomal antigen-1) a protein responsible for the fusion of endocytic vesicles to form early endosomes (43). Upon p97/VCP silencing (pharmacological and siRNA mediated) the EEA1 oligomeric state was disturbed causing a defect

in early endosome maturation and size. Later another study linked p97/VCP to endocytic trafficking as p97/VCP and its cofactor UBXD1 were shown to interact with ubiquitinated-caveolin-1 (CAV1) a protein that is part of the endocytic machinery. In this study p97/VCP inhibition (pharmacological or siRNA mediated) caused an accumulation of enlarged endosomes and a defect of CAV1 sorting into intraluminal vesicles. Although, their results slightly differ those two reports provide strong evidence of the involvement of p97/VCP in the endosomal trafficking but its precise role remains to be defined.

c) Chromatin-associated functions

p97 protein can be found in the nucleus of all species, in yeast CDC-48 translocation relies on the phosphorylation of its last tyrosine, indeed after phosphorylation CDC-48 tridimensional structure is changed and reveals a nuclear import sequence (44)(**Figure 11**). However there is no tyrosine kinase in *S. cerevisiae*, only dual specificity protein kinases that phosphorylate serine/threonine or tyrosine residues and the enzyme responsible for this phosphorylation is still unknown.

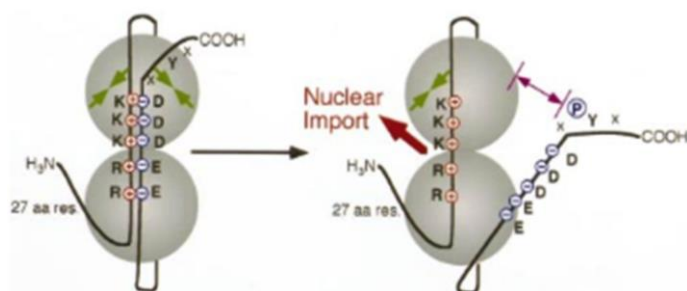


Figure 11: Schematic representation of CDC48 nuclear import mechanism - Phosphorylation of CDC-48 at the penultimate tyrosine changes its tridimensional structure to reveal a nuclear import sequence (adapted from 44).

In human, this tyrosine is conserved and can be phosphorylated by the protein kinase v-Src but this event regulates its activity during ERAD by preventing binding of p97/VCP partners: Ufd3 /or PNGase rather than inducing p97/VCP translocation. However, a recent report suggests that rather the N domain of the protein is required for p97/VCP translocation to the nucleus whereas the C terminal tail seems to control its export (45). In the nucleus, a wide range of cellular processes require p97/VCP dependent extraction of ubiquitin modified proteins such as: transcription, cell cycle progression, DNA damage response and DNA replication.

- **Cell cycle progression and DNA replication** - Aurora B kinase extraction by p97 at the end of mitosis from the chromatin of *Xenopus laevis* was the first case that reported extraction of

a nuclear protein by p97 (46). Indeed, extraction of the ubiquitin modified Aurora B kinase mediated by p97 and Ufd1-Npl4 is required for the formation of the nuclear envelope. As additional evidence that p97 and cofactors are required in this process, siRNA mediated silencing of Ufd1 or Npl4 caused defect in chromosome segregation and multi-lobed nuclei. DNA replication is a process tightly linked to the cell cycle progression as it ensures that both daughter cells will receive the correct amount of genetic material. p97 and its *C. elegans* orthologues CDC-48.1 and CDC-48.2 (duplicate gene) have been shown to participate to this process in an evolutionary conserved manner. Indeed in both human and worm CDT-1, a protein involved in the formation of the prereplication complex needs to be regulated by p97/CDC-48 to ensure that a single round of DNA amplification happens (47). This degradation of CDT-1 implicated p97/CDC-48 and its cofactor UFD-1/NPL-4, in fact worms lacking any of these proteins displayed increased level of CDT-1 are defective in S phase progression.

- **DNA damage response** - p97/VCP's role in DNA damage response is not simple as its substrates differ between different reparation mechanisms and different species. Historically, first evidence of p97/VCP implication in the DNA damage response came in 2000 when p97/VCP was found to interact with BRCA1 a well described tumour suppressor protein involved in double strand breaks (DSB) repair (48). In 2005 and 2007, the human p97 was identified as a novel substrate of PIKK (phosphatidylinositol 3-kinase-related kinase) kinases: ATM, ATR and DNA-PK, kinases that are known to orchestrate double strand break reparation (49-50). The authors also reported an accumulation of serine 784 phosphorylated p97/VCP at the site of DNA double-strand breaks. In 2007, a proteomic analysis of ATM substrates another well described kinase involved in DSB repair revealed p97/VCP and other proteins of the UPS as substrates Although these reports seem to point toward a role for p97/VCP in the DNA damage response it is only more recently that precise molecular event have been described.

- **Transcription coupled Nucleotide Excision Repair (NER)** - Ultraviolet light (UV) induces DNA lesions in the form of abnormal nucleotide dimers (pyrimidine-pyrimidone or cyclobutane pyrimidine dimers) which must be repaired to preserve the genome integrity and because they can stall the RNA pol II complex. Such photolesions are repaired by transcription-coupled Nucleotide Excision Repair (NER). In yeast, UV induces the ubiquitylation of RBP1 the largest subunit of the Pol II by CUL3 or RSP5 ubiquitin ligases (E3) to which the CDC-

48/UBX5 complex can bind (51). Then, extraction of RBP1 from the photolesion site by CDC-48/UBX5 allows the NER machinery to repair (**Figure 12**). Although, the ubiquitinylation of RBP1 in response to UV is conserved in human it is not established if p97/VCP is involved in its degradation.

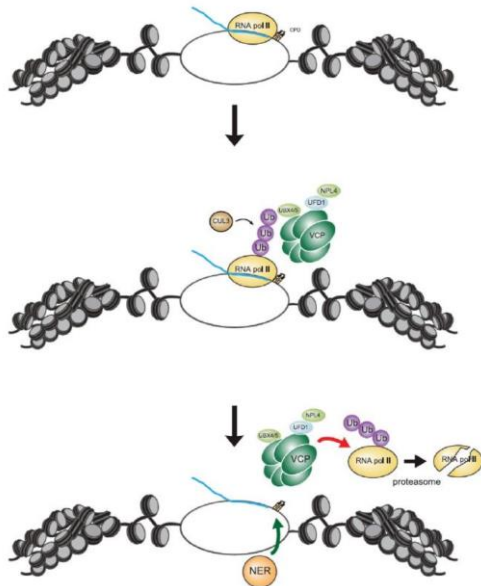


Figure 12: Schematic representation of the role of p97 during transcriptional coupled NER (REF) - UV light can cause DNA lesions such as abnormal DNA dimers which can stall the RNA pol II. The stalled RNA pol II is ubiquitinylated by CUL3 (E3) to which p97 and its cofactors can bind. CDC-48 then extracts the RNA pol II from the lesion site to the proteasome for degradation thus allowing access to the NER machinery for reparation (adapted from 51).

- **Global genome NER** - DDB2 and XPC are lesion-recognition proteins which can associate with photolesions independently of transcription to initiate a process called global genome NER. Upon recognition of a lesion DDB2 recruits an ubiquitin ligase CRL4 complex which ubiquitinylates XPC and DDB2 causing the recruitment of p97/VCP at the UV-induced lesion (52). The recruitment of p97/VCP depends on the UFD1/NPL4 and UBX5 cofactors. In the absence of functional p97/VCP cells accumulate supra-physiological levels of K48-ubiquitinylated DDB2 and XPC at UV lesions site suggesting that the extraction of these proteins is mediated by p97/VCP. In conclusion, it appears that p97/VCP/CDC-48 extraction of ubiquitinylated proteins is required in both NER pathways.

- **Translesion synthesis** - During replication if the DNA polymerases encounter an UV-induced lesion it can also cause them to stall. The stalling replication fork triggers ubiquitinylation of PCNA a processivity factor resulting in the replacement of the DNA polymerase by a translesion synthesis polymerase. Although the translesion polymerase is less processive and accurate it is able to incorporate a nucleotide at the opposite of the lesion and therefore continue the duplication process. Once the lesion is passed the translesion polymerase

should be removed and replaced by a more faithful and processive DNA polymerase. Although, the precise mechanism is unknown, p97/VCP seems to be required in extraction of the translesion polymerase. Indeed, DVC1 (DNA damage-targeting VCP adaptor C1orf124) a newly discovered p97/VCP adaptor is believed to recruit p97/VCP/UFD1/NPL4 thanks to its interaction with PCNA at the replication fork and facilitate polymerase switching (51). In fact, depletion of DVC1 results in persistent association of PCNA with the translesion polymerase causing an increased amount of mutations because the polymerase cannot be switched. This suggests a role for p97/VCP/DVC1 in the rapid extraction of the translesion polymerase.

- **DNA double strand breaks repair** - The two ubiquitin-ligases RNF8 and RNF168 (E3) are known to orchestrate the DNA damage response at either double strand breaks or lesions in human cells. First, RNF8 is recruited at the damaged site and induces ubiquitinylation of chromatin-associated proteins which leads to the recruitment of RNF168 as it possesses ubiquitin binding domains (53). Then RNF168 induces ubiquitinylation of H2A and H2AX histones to which 53BP1 can bind, in fact 53BP1 needs to recognize both ub-H2A and di-methyl H4K20 to bind (54). Two reports have implicated p97/VCP and its ubiquitin binding cofactors UFD1-NPL4 as key players in this process (51) (**Figure 13**).

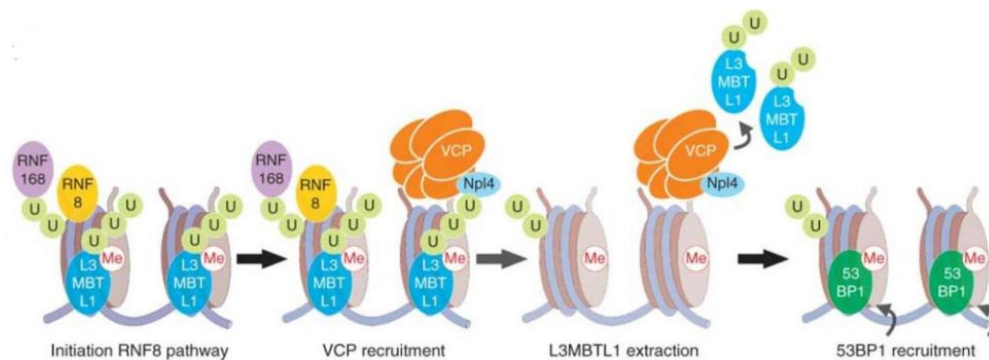


Figure 13: Schematic representation of the role of p97/VCP during Double Strand Breaks - RNF8 and RNF168 are recruited at the DSB site and ubiquitinate the chromatin associated proteins including L3MBTL1 which interact with the di-methyl H4K20. Then, p97/VCP and its cofactors UFD1/NPL4 which can bind to ubiquitin are recruited at the DSB site to extract L3MBTL1 thereby allowing binding at the same histone mark of the repair factor 53BP1 (adapted from 54).

However it is unclear how p97/VCP/UFD1/NPL4 is recruited as the first report suggests that this complex is recruited by K-48 ubiquitin modified proteins induced by RN8 whereas the second

suggests that both RNF8 and RNF168 which induce K63 chains are required. In this context, it was shown that p97/VCP is required to extract L3MBTL1 ubiquitinated by RNF8 from dimethyl H2K20 to allow binding of 53BP1 without which the DNA response would be interrupted. Additionally, JMJD2A/B a histone demethylase that can bind to the same mark is also ubiquitinated by RNF8 and then extracted during the DNA damage response, however the implication of p97/VCP has not yet been addressed. In conclusion, p97/VCP can promote the recruitment of the repair factors to the damaged site by extracting proteins that are occupying the same histone mark.

3) p97/VCP/CDC-48 role in transcription regulation

In 2012, a study based on a global gene microarrays on patients with Paget's disease of bone a disease caused by mutations in the p97/VCP gene revealed that more than 60 genes are significantly deregulated in those patients (55). Analysis of the deregulated genes showed that several pathway are affected by p97/VCP mutation including regulation of actin cytoskeleton, autophagy or lysosome. Moreover, several reports across human, yeast, worm or fruit fly have documented the molecular mechanism by which p97 can regulate gene expression. These reports suggest that there are two main mechanisms by which p97 can regulate gene expression: either by regulating the stability of transcription factors or by inducing change in histone marks.

p97 regulation of transcription factors occurs at different locations in the cell. At the ER membrane p97/VCP is required for the release of Nrf1 and the proteolytic activation of Mga2p and Spt23p (35). At the golgi membrane p97/VCP is required for the proteolytic activation of SREBP (36). As mentioned earlier, in the cytosol p97/VCP associates with HDAC6 and HSP90 to sequester HSF1 (40). Additionally, Chromatin Immunoprecipitation experiments, have revealed that p97/CDC-48 can associate directly at the chromatin with mono-ubiquitin transcription factors such as LexA-VP16, Met4 or Smad2/3 to prevent binding to their target promoter without degradation (57). Finally, other transcription factors often relevant for the study of cancer such as p53, H1F1 α , NFKB were shown to interact with p97/VCP although the localisation of the interaction was not assessed (58, 59, 60). Other reports have revealed that p97 can also regulate gene expression by affecting certain histone marks. Although the role of ubiquitinylation of histone H2B is not well understood, this mark undergoes major changes

during cell differentiation (61). Recently CDC-48 and its cofactor Ubx3 were shown to facilitate the transcription coupled recruitment of Lge1 a cofactor of the H2B ubiquitin ligase Bre1 (61). In another study, it was shown that accumulation of polyglutamine aggregates induces p97/VCP nuclear translocation. This translocation was associated with a global decrease of Histone H3 and H4 acetylation and as a result a diminution in transcription (62). Consistent with these observations, expression of mutant p97/VCP that cannot translocate to the nucleus were able to restore the histone H3 and H4 acetylation levels. These reports suggest an important role of p97/VCP/CDC-48 in the coordination of chromatin remodeling processes.

4) p97/VCP in diseases

a) **IBMPFD and ALS** - As p97/VCP is involved in a wide range of cellular processes and is a crucial keeper of the cell protein homeostasis it is not surprising that mutation in the gene *p97/VCP* that encode for the p97/VCP protein have been genetically linked to degenerative disorders (31,55). Indeed, autosomal dominant mutations in *p97/VCP* are responsible for multisystem proteinopathy (MSP) a degenerative disorder which can manifest clinically as Amyotrophic Lateral Sclerosis (ALS), Inclusion Body Myopathy (IBM), Paget's disease of the bone (PDB), Frontotemporal Dementia (FTD), or as a combination of these disorders (REF). The three later phenotypes are often referred to as IBMPFD (Inclusion Body Myopathy with Paget disease and Frontotemporal Dementia). Is it noteworthy, that more recently patients with MSP due to *p97/VCP* mutations have displayed additional symptoms such as Parkinsonism, ataxia, cataracts, dilated cardiomyopathy, hepatic fibrosis or hearing loss. More than 30 missense mutation in *p97/VCP* have been identified (31) and most of these mutations are clustered between the N and D1 domains of the protein (**Figure 6**), this region is thought to control the orientation of the N domain to which cofactors bind. As most mutations are in this region it suggests that it is important for the disease pathogenesis. Other mutations have been reported to affect p97/VCP basal ATP hydrolysis activity but not all (63), suggesting that it may not be the cause of the disease, moreover no mutation appear to impact the oligomerization of the protein (31). Additionally, two studies have reported that these disease-associated mutations affect p97/VCP interaction with cofactors suggesting that these mutation will not affect all p97/VCP function but only the function related to those cofactors (64). As p97/VCP knock-out

mice are not viable, this could explain how patients or mice with these mutations develop normally, suggesting that p97/VCP functions in cycle control or cellular division are not affected by these mutations. Overall, these observations suggest that it is the imbalanced cofactor association of p97/VCP that is most likely to be responsible for the MSP. Common cellular features of all IBMPFD or ALS, are impaired ERAD and accumulation of ubiquitinated protein aggregates indicate that the protein quality control system and protein aggregate clearance system are defective in these disorders. This is consistent with the fact that p97/VCP is required for autophagy and autophagosome biogenesis. Indeed depletion of p97/VCP or expression of dominant-negative p97/VCP mutant resulted in the accumulation of LC3II and immature autophagosomes (38). Additionally, accumulation of TDP-43 inclusions is also causal of FTD and ALS. TDP-43 is a RNA binding protein that can regulate processes such as transcription, pre-mRNA splicing, RNA transport and translation. Recently Ritso et al. showed a genetic interaction between p97 and TDP-43 and that p97 mutation caused accumulation of TDP-43 in the cytosol which had similar toxic effects (65). Moreover, TDP-43 mutations enhance the genetic interaction with p97 and also lead to cytotoxic accumulation of TDP-43. These results suggest that the degeneration caused by p97/VCP mutations is partly mediated by the toxic function of TDP-43 in the cytosol.

b) **Cancer** - p97/VCP was found overexpressed in different cancers e.g.: hepatocellular carcinoma, non-small cell lung carcinoma, osteosarcoma, oesophageal squamous cell carcinoma, gingival squamous cell carcinoma, prostate cancer, colorectal carcinomas, gastric carcinoma, pancreatic endocrine neoplasms, pancreatic cancer, and follicular thyroid cancer (66,67). Several studies have reported a correlation between the level of p97/VCP and adverse prognosis (67-68). However, due to the wide range of cellular functions that p97/VCP regulates it is difficult to anticipate the exact contribution(s) of this protein in oncogenesis. Most of the functions in which p97/VCP is involved are relevant to cancer development for instance the control of transcription, cell-cycle progression, autophagy, proteostasis, endocytic processes or DNA-damage mechanisms. Observation of cancer cells with elevated p97/VCP revealed a decreased ability for the cell to undergo apoptosis which suggest an anti-apoptotic role for p97/VCP (69). Such a role is coherent with the fact that in cancer cells the genomic abnormalities and the resulting synthesis disturb the protein homeostasis and lead to proteotoxic stress. As

p97/VCP is essential to restore protein homeostasis in the cell, elevated level of p97/VCP could prevent the proteotoxic stress from inducing apoptosis. Consistent with a pro-survival role of p97/VCP in cancer, another study have shown that p97/VCP is involved in the regulation of I κ B, an inhibitor of NF κ B (Nuclear Factor kappa B) a transcription factor which control genes involved in cell proliferation (70). In fact, Valla and colleagues have suggested that p97/CDC-48 and the UPS regulate NSCLC tumor genesis and metastasis via NF κ B and p53 (58). This may apply to other cancers as increased levels of p97/VCP may cause the degradation of NF κ B repressor and consequently promote cell proliferation. Numerous studies have also linked p97/VCP to the regulation of key cancer proteins such as p53, BRCA1, HIF1a, but it is still unclear how these regulations contribute to oncogenesis (48, 58, 59, 60). However, p97/VCP inhibitors have shown encouraging results. In hepatocellular cancer, one of the only treatment available Sorafenib a multikinase inhibitor which was shown to target p97/VCP (71). Indeed Sorafenib prevents the phosphorylation of p97/VCP which results in stabilizing its membrane association, causing ER stress and ultimately apoptosis. Upon treatment with a reversible inhibitor of p97/VCP: Dbeq the cells were further sensitized to Sorafenib strengthening the idea that that Sorafenib apoptosis is indeed mediated by p97/VCP impairment. NMS-873 is an allosteric inhibitor that impairs p97/VCP enzymatic activity and was shown to activate the UPR, interfere with autophagy and induce cancer cell death in U2OS and HCT166 (72). More recently, CB-5083 which binds to the p97/VCP D2 domain had an important apoptotic effect in HCT116 xenografted mice (73). In fact, CB-5083 treatment caused accumulation of ubiquitinated proteins, retention of ERAD substrates and irresolvable proteotoxic stress and as a consequence apoptosis mediated by the UPR. These studies suggest that targeting p97/VCP might be a viable strategy in certain cancers, however further tests are needed to ensure the safety of these drugs. Indeed, recent studies have shown that ER stress which can be caused by p97/VCP inhibition or p97/VCP inhibition itself can promote aggressiveness of cancer cells such as induction of EMT.

HYPOTHESIS AND OBJECTIVES

ER stress is a condition often met by tumor cells as they are exposed to challenging conditions that alter protein homeostasis (i.e. intrinsic and extrinsic stress). Recently it has become clear that ER stress signalling and the UPR contribute to cancer biology and the acquisition of malignant characteristics such as inflammation, invasion and metastasis, angiogenesis or resistance to chemotherapy (74,75). Although the key initiation mechanisms of the UPR are now well established and comprise activation of the three sensors (IRE1, PERK, ATF6) which leads to the production of transcription factors (XBP1s, ATF4, ATF6f), the resulting transcriptional outputs are far more complex than expected. Indeed, the exact nature of the genes activated under ER stress-mediated cellular reprogramming and how these genes switch from a repressed to an activated state remains unclear. As a consequence, identifying the components that regulate ER stress genes might help to characterize the gene sets regulated during the UPR and by extent to better understand the contribution of this pathway to cancer development.

The initial observation that CDC-48.2 knockout worms are impaired for the induction of ER stress genes upon exposure to tunicamycin led us to propose that beyond its role in ERAD, CDC-48 might have a role during ER stress in the regulation of ER stress genes (76). Moreover, although p97/VCP overexpression is observed in many cancers and sometimes associated with poor prognosis in patients, the mechanisms by which this protein contributes to malignancy is unknown. As a consequence we hypothesized that since ER stress is involved in cancer development and p97/VCP is a regulator of ER stress genes expression, overexpression of p97/VCP in cancers might play an important role in cellular reprogramming occurring in this context and therefore may provide some elements of explanation to its contribution to malignancy.

To address these points the objectives of my thesis are 1) to demonstrate that p97/VCP/CDC-48 is a genuine regulator of ER stress genes expression, and 2) to understand the molecular mechanism underlying this regulation, including the identification of the genes regulated, the cofactors of p97/VCP/CDC-48 in this process, the client proteins and the cellular localisation of these interactions.

EXPERIMENTAL APPROACH

Co-immunoprecipitation:

Huh7 or HeLa were cultured in 10 cm plates for co-immunoprecipitation. Two plates were used for each experimental conditions as one was used as a control to check the binding of our target proteins on the magnetic beads. The plates were placed on ice and the media was removed. The cells were washed twice with 5mL of cold PBS (PBS, 14190-094, Life Technology). 500 μ L of Lysis buffer (30 mM TrisHCl pH 7.5, 150 mM NaCl, 1.5% CHAPS) containing protease and phosphatase inhibitors (Complete EDTA, 04693159001, Roche and PhosphoSTOP, 04906837001, Roche) was then added to each plates and the cells were scrapped every 10 mins for 30 mins. The lysates were transferred to 1.5 mL tubes and briefly vortex before being centrifuge at 17 000g for 15 mins at 4°C. The supernatant were collected and the pellets discarded. The protein concentration of the supernatants were measured and adjusted to 2 μ g/ μ L (if not possible to 1 μ g/ μ L). For each immunoprecipitation 20 μ g was saved to be used as input and 1 μ g of specific antibody was added to 1000 μ g of the supernatants and incubated overnight on a rotating wheel at 4°C. The next day, 30 μ L magnetic beads (coupled with protein A (Dynabeads PG, 10003D, Thermo Fisher Scientific) for Rabbit antibody or with protein G (Dynabeads PA, 10001D, Thermo Fisher Scientific) for Mouse antibody) were washed 3 times in the lysis buffer and then incubated with the lysates for 15 mins at RT. Note that the magnetic bead manufacturer advise longer incubation time (45 to 60 mins) but it causes p97/VCP to bound to the beads. After 15 mins using the magnetic stand the magnetic beads were recuperated and the lysates discarded. The magnetic beads were then washed 3 times in the lysis buffer to remove aspecific bindings. Then 30 μ L of Laemmli buffer 1X was added to the beads, mixed by vortex and the mixtures were heated at 95°C for 5 mins. The supernatants were briefly centrifuge and separate from the magnetic beads using the magnetic stand and then loaded along with the input on a polyacrylamide gel of the appropriate concentration for immunoblotting.

Immunohistochemistry:

Huh7 cells were cultured on cover slips in 12 wells plates for immunohistochemistry. For each well/cover slip the media was removed and the cells were washed twice with cold PBS (PBS,

14190-094, Life Technology). After, the cells were incubated with a 4% Paraformaldehyde (PFA) in PBS solution for 10 mins and then were washed three times with cold PBS for 5 mins. The cell were permeabilise by incubation with a 0.1% Triton in PBS solution for 15 mins. After a quick wash the cells were incubated with H₂O₂ to inhibit the endogenous peroxidases. After a quick wash the cells were incubated with a 3% BSA in PBS for 1 hour to prevent aspecific binding of the primary antibody. After a quick wash the p97/VCP antibody (60316-1, Proteintech) was diluted at the 1:200 in the blocking solution (3%BSA in PBS) and incubated with the cells for 2 hours. After removing the antibody solution the cells were washed 3 times with PBS for 5 mins. The cells were then incubated with a secondary antibody: Flex-HRP (EnVision System, K406987-2, Dako) for 30 mins and then washed 3 times with PBS for 5 mins. Then in the dark (to protect the signal from light) the cells were incubated with 3,3'-diaminobenzidine (DAB, EnVision System, K406987-2, Dako) for 10 mins. The cells were then wash with distilled water before being incubated with Hemalum for 3 mins to color the nucleus. After 2 washings with distilled water, the cells are washed with Ammonium Peroxide and then dehydrated by a succession of baths in 95° Alcohol for 1 min, 100° Alcohol for 1 min twice and Toluene for 5 min twice. Finally the cover slips were fixed using mounting medium on a slide before microscopic analysis.

Silencing using siRNA:

Huh7, HeLa, U87, U251, Panc1 cells were cultured in 6 wells plates before protein or RNA extraction. The day before the transfection 200 000 cells were plate in 1mL of medium (DMEM GlutaMAX, 10566-016, Thermo Fisher Scientific) complement with 10% SVF. The day of the transfection the old medium was replaced by 1 mL of fresh medium and for each reaction two tubes were prepared: in the first tube 150µL of OptiMem (31985062, Thermo Fisher Scientific) was mixed with 4.5 µL of RNAimax by pipetting up and down (13778030, Thermo Fisher Scientific) and in the second tube 150µL of OptiMEM was mixed with 3.5 µL of siRNA (CTL, p97/VCP, HDAC1, RuvBL2 or USF2) at [20µM] by pipetting up and down. Note that the manufacturer recommend to use a final concentration from [10 nM] to [100 nM] and we found that the [40 nM] final concentration was the best for the efficiency of the silencing and the survivability of the cells. After 5 mins the two tubes were mixed and after 15 mins incubation at

RT, 250 μ L of the mixture was drop in droplets to the corresponding well before being mixed by gently rocking the plate back and forth. After 48h the cells were ready to be harvest for either protein or RNA extraction. Note that the efficiency of the silencing was tested by immunoblotting and qPCR.

List of siRNAs used in this study:

GENE	SEQUENCE 5' – 3'
p97/VCP	(GAAUAGAGUUGUUCGGAU)TT
HDAC1	(CAGCGACUGUUUGAGAACC)TT
RUVBL2	(GAGAUCCAGAUUGAUCGACCAGCAA)TT
USF2	(CCUCCACUUGGAAACGGUA)TT

Real Time-Quantitative PCR:

Huh7, HeLa, U87, U251, Panc1 cells were cultured in 6 wells plates for RNA extraction. Total RNAs were extracted using 1mL of Trizol reagent (15596018, Invitrogen) and the manufacturer recommendation. For the Reverse Transcription 2 μ g of mRNA were used with the Maxima Reverse Transcriptase (ER0741, Life technologies) following the manufacturer recommendation. For quantitative RT-PCR (RT-qPCR) all reactions were conducted using the SYBR qPCR Premix Ex Taq (TAKRR420W, Ozyme) in the QuantStudio 5 thermocycler (Applied Biosystem). Each PCR was performed in technical triplicates in 384-well plate. Each well contain 5 μ L of SYBR qPCR Premix Ex Taq, 0.4 μ L of Forward and Reverse primer at [10 μ M], 2.6 μ L au water and 2 μ L of cDNA diluted to the 1:200 for a total volume of 10 μ L. The cycle of amplification consists of the following steps: Pre-run at 95°C for 30 secs, 40 cycles of amplification (95°C for 5 secs, 60°C for 34 secs) and 95°C for 15 secs and 60° for 1 min for the dissociation step. For analysis each sample was normalized to Gapdh and/or Actin expression level.

List of the qPCR primers used in this study:

GENE	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'
------	----------------------	----------------------

p97/VCP	CCATCCGGAAAGGAGACATTT	GTCTGGAGCAACAATGCAATAAG
GAPDH	GACCTGACCTGCCGTCTAGAAAAA	ACCACCCTGTTGCTGTAGCCAAAT
GLI1	AGGGAGTGCAGCCAATACAG	ATTGGCCGGAGTTGATGTAG
USF2	TTCGGCGACCACAACATCCAG	CAGTCACCTGGACTACGCGGT
IRE1A	GCCACCCTGCAAGAGTATGT	ATGTTGAGGGAGTGGAGGTG
BIP	TGTTGGAAGATTCTGATTTGAAGA	TCACTCGAATACCATTACAT
EDEM	AGTCATCAACTCCAGCTCAA	AACCATCTGGTCAATCTGTCTG
ERDJ4	TGGTGGTTCCAGTAGACAAAGG	CTTCGTTGAGTGACAGTCTGC
HERPUD	TCCTCCTCCTGACGTTGTAAA	TGTTGCGXATCTAGTACATCC
ORP150	GAAGATGCAGAGCCCATTTC	TCTGCTCCAGGACCTCCTAA
RUVBL2	AAGTCCCGGAGATCCGTGAT	CGACCGGCAATCTTCCCTTC
CHOP	AAGGCACTGAGCGTATCATGT	TGAAGATACTTCCTTCTTGAACA

All the other methods used during my thesis are described in the following method article or in the materials and methods section of the science articles (Article 3 and 4).

ARTICLE 2

Adaptation of the Secretory Pathway in Cancer Through IRE1 Signaling

Stéphanie Lhomond, Nestor Pallares, Kim Barroso, Kathleen Schmit, Nicolas Dejeans, Hélène Fazli, Saïd Taouji, John B. Patterson, and Eric Chevet

Abstract

The unfolded protein response (UPR) was originally identified as a signaling network coordinating adaptive and apoptotic responses to accumulation of unfolded proteins in the endoplasmic reticulum (ER). More recent work has shown that UPR signaling can be triggered by a multitude of cellular events and that the UPR plays a critical role in the prevention of cell transformation but also in tumor development. This has been particularly well illustrated with studies on one of the three major ER stress sensors, IRE1. This ER resident type I transmembrane protein senses luminal ER stress and transduce signals through its cytosolic RNase activity. IRE1 signaling has been shown to contribute to the progression of solid tumors through pro-angiogenic mechanisms. Herein, we expose the methodologies for investigating IRE1 signaling in tumor cells and in tumors. Moreover, we show that selective pharmacological inhibition of IRE1 RNase activity sensitizes tumor cells to ER stress.

Key words Endoplasmic reticulum, Unfolded protein response, IRE1, ERN1, XBP1, IRE1 inhibitors

1 Introduction

Twenty-five years ago, the existence of a signaling pathway was identified in mammalian cells to control adaptation to protein folding defect. This occurs through the transcriptional upregulation of key ER chaperones [1] mediated by three classes of ER stress sensors, namely, Inositol-requiring enzyme-1 (IRE1, α and β isoforms), activating transcription factor 6 (ATF6) (α and β isoforms) and protein kinase RNA-like ER kinase (PERK) [2]. PERK activation also involves its dimerization and auto-transphosphorylation [3, 4]. Activated PERK phosphorylates the translation initiator factor eIF2 α , inhibiting protein synthesis, and nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor involved in redox metabolism [5]. This reduces the load of newly synthesized

proteins entering the ER, thus having an important pro-survival effect [6]. Phosphorylation of eIF2 α limits the amount of active ribosomes and allows the translation of mRNAs containing short open reading frames (micro-ORFs) in their 5'-untranslated regions, including Activating Transcription Factor-4 (ATF4). ATF4 controls the expression of genes involved in redox and amino acid metabolism, in addition to ER chaperones and foldases [7, 8]. ATF4 also regulates the expression of important genes involved in apoptosis including the transcription factor C/EBP-homologous protein (CHOP) and growth arrest and DNA damage-inducible-34 (GADD34) (*see above*). GADD34 participates on a feedback loop to dephosphorylate eIF2 α by interacting with protein phosphatase 1C (PP1C), restoring protein synthesis [9]. Finally, ATF6 α is a type-II ER located protein that contains a bZIP transcription on its cytosolic domain. Upon ER stress ATF6 α translocates to the Golgi apparatus where it is cleaved by S1P and S2P proteases to release a cytosolic fragment (ATF6c) [10, 11]. ATF6c is a transcription factor that regulates the expression of genes of the ERAD pathway among other target genes [12, 13]. Exclusive or combined action of ATF6c and XBPIs may also have a differential effect on gene expression [14].

Activation of IRE1 α involves its oligomerization, and auto-transphosphorylation, leading to a conformational change that activates the RNase domain. IRE1 α RNase excises a 26-nucleotide intron of the X-Box binding protein-1 (XBPI) encoding mRNA, which is then religated by a yet unknown RNA ligase. This changes the coding reading frame of the mRNA, leading to the expression of an active transcription factor, termed XBPIs, for the spliced form [12, 15, 16]. XBPIs trans-activates a subset of target genes involved in protein folding, endoplasmic reticulum-associated degradation (ERAD), protein translocation to the ER, and protein secretion [17, 18] (Fig. 1). IRE1 α also signals through the scaffolding of many adapter proteins and regulators, a dynamic protein platform referred to as the UPRosome [5] (Fig. 1). IRE1 α interacts with the adapter protein TRAF2, leading to the downstream activation of the kinase JNK [19]. IRE1 α RNase activity also degrades a subset of mRNA through a process known as regulated IRE1-dependent decay of mRNA (RIDD) [20–22] (Fig. 1). The pool of RNAs degraded by RIDD depends on the cell type affected and targets mRNAs encoding for proteins of the secretory pathway. The selectivity of IRE1 α to degrade particular RIDD substrates may depend on the presence of a conserved nucleotide sequence accompanied by a defined secondary structure [20–23]. Moreover, IRE1 α has also been shown to cleave premature microRNAs thereby impacting on the control of apoptosis [24]. Furthermore, the regulation of IRE1 α expression levels by microRNAs was shown to impact on its biological functions [25–27].

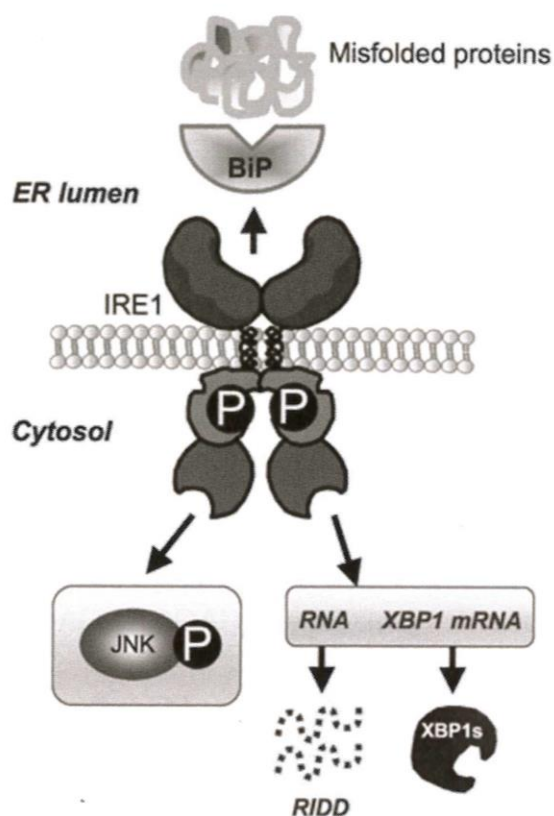


Fig. 1 Schematic representation of IRE1 signaling. Upon accumulation of misfolded proteins in the ER, BiP is titrated away from IRE1 leading to IRE1 oligomerization and downstream signaling. Three major signaling pathways are activated downstream of IRE1 including the activation of the JNK cascade, the unconventional splicing of XBP1 mRNA and the regulated IRE1 dependent decay of mRNA (RIDD)

The role of IRE1 in cancer has been well documented [28–30]. In particular, we have shown that in glioblastoma IRE1 activity contributes to tumor growth through the activation of pro-angiogenic and pro-inflammatory pathways [28, 31], thereby indicating that IRE1 could represent a potentially relevant therapeutic target in this disease. Herein, we list the methodologies used in our laboratory to investigate and pharmacologically perturb IRE1 [32] signaling in glioblastoma cells.

2 Materials

2.1 Cell Lines and Mouse Strains

1. Human glioblastoma derived cells U87MG were from ATCC.
2. RagGamma mice were produced in the Bordeaux 1 University animal house (Dir. R. Pineau).

2.2 Antibodies

1. Mouse monoclonal antibodies against XBP1s (clones 2G4 and 5E4) were produced in-house and respectively used for immunohistochemistry and immunoblotting.

2. Rabbit antisera to JNK1 (SantaCruz, CA, USA), anti phospho-JNK (Cell Signaling Technology, Danvers, MA, USA)
3. Rabbit antisera to IRE1 (SantaCruz, CA, USA), rabbit monoclonal antibodies to phospho-IRE1 (S724) (Abcam, Cambridge, MA, USA).
4. Vimentin (dil. 1/400) (Acris Antibodies, Herford, Germany)
5. CD31 (dil. 1/200) (BD Pharmingen, Franklin Lakes, NJ, USA)
6. Secondary antibodies: Alexa 547 (FluoProbes 547H Donkey Anti-Rat IGG FP-SB6110) 1/200, Alexa 488 (FluoProbes 488H Donkey Anti-Mouse IGG FP-5A4110) 1/200 or EnVision FLEX/HRP (Dako F8010/F8012/F8024).
7. Hoechst ((Molecular Probes 34580) 1/1,000).

2.3 PCR Primers and siRNAs

1. RT-PCR: hPer1 Fwd, 5'-GGGTCCTCCAGTGATAGCAA-3'; Rev, 5'-GAGGAGGAGGCACATTTACG-3' (amplicon length: 386 bp).
2. RT-PCR: hGapdh Fwd, 5'-ACCACCATGGAGAAGGCTGG-3'; Rev, 5'-CTCAGTGTAGCCCAGGATGC-3' (amplicon length: 528 bp).
3. RT-PCR: hPer2, Fwd, 5'-TACGCTGGCCACCTTGAAGTA-3'; Rev, 5'-CACATCGTGAGGCGCCAGGA-3' (amplicon length: 386 bp).
4. siRNA: GL2, 5'-CGUACGCGGAAUACUUCGA-3'; Ire1 α , 5'-UUACUGGCUUCUGAUAGGA-3'; Xbp1, 5'-CUCAU GGCCUUGUAGUUGA-3'.
5. qPCR: hPER2, Fwd, 5'-TACGCTGGCCACCTTGAAGTA-3'; Rev, 5'-CACATCGTGAGGCGCCAGGA-3'.
6. qPCR: hPER1, Fwd, 5'-TATACCCTGGAGGAGCTGGA-3'; Rev, 5'-AGGAAGGAGACAGCCACTGA-3'.
7. qPCR: 18S, Fwd, 5'-GGATCCATTGGAGGGCAAGT-3'; Rev, 5'-CCGCTCCCAAGATCCAATA-
8. qPCR: miR-17, Fwd, 5'-CAAAGUGCUUACAGUGCAGG UAG-3'; Rev, universal primer provided in the miScript SYBR Green PCR Kit (Qiagen, Ref: 218073).

2.4 Chemicals

1. IRE1 inhibitors: irestatin (Axon Medchem, Vienna, VA, USA), toyocamycin (Sigma-Aldrich, St Louis, MO, USA), and MKC8866 (labeled MKC; MannKind Corporation, WO 2011/127070 A2) [33].
2. Tunicamycin (Calbiochem, Merck KGaA, Darmstadt, Germany).
3. Acrylamide-Bis-acrylamide 30:1 (Bio-Rad, Hercules, CA, USA).
4. SDS (Thermo Fisher Scientific, Waltham, MA, USA).

2.5 SDS-PAGE and Phos-tag SDS- PAGE Gel Components

1. 1.5 M Tris-HCl solution, pH 8.8 (4× solution for resolving gel): add about 100 mL of distilled water to a 1 L graduated cylinder or a glass beaker. Weigh 181.7 g of Tris and transfer to the cylinder. Add distilled water to a volume of 900 mL. Mix and adjust pH with HCl. Make up to 1 L with distilled water. Filter the solution. Store at 4 °C in the dark.
2. 0.5 M Tris-HCl solution, pH 6.8 (4× solution for stacking gel): add about 100 mL of distilled water to a 1-L graduated cylinder or a glass beaker. Weigh 60.6 g of Tris and transfer to the cylinder. Add distilled water to a volume of 900 mL. Mix and adjust pH with HCl. Make up to 1 L with distilled water. Store at 4 °C.
3. 10 % (w/v) SDS solution: add about 100 mL of distilled water to a 1-L graduated cylinder or a glass beaker. Weigh 100 g of SDS and transfer to the cylinder. Add distilled water to a volume of 1 L. Store at room temperature.
4. 5.0 mM Phos-tag solution containing 3 % (v/v) methanol: add 0.1 mL of methanol to the oily product Phos-tag AAL-107 plastic tube (Wako Cat. No. 304-93525). Dilute the methanol solution with 3.2 mL of distilled water by pipetting. Wrap the tube with aluminum foil. Keep the solution in 2-mL microtubes at 4 °C in the dark.
5. 10 mM MnCl₂ solution: add about 50 mL of distilled water to a 500-mL graduated cylinder or a glass beaker. Weigh 0.10 g of MnCl₂•4H₂O (MW: 198) and transfer to the cylinder. Add distilled water to a volume of 500 mL. Mix and store at room temperature.
6. 10 % (w/v) ammonium persulfate solution: weigh 500 mg of (NH₄)₂S₂O₈ (MW: 228) and transfer to a 15 mL conical flask. Add distilled water to a volume of 5 mL. Mix and aliquot in 2-mL microtubes placed at -20 °C for long-term storage.
7. 30 % acrylamide-Bis solution (29.2:0.8 acrylamide-Bis) (Bio-Rad, Hercules, CA, USA). Store at 4 °C.
8. *N, N, N, N'*-tetramethyl-ethylenediamine (TEMED) (Sigma Chemical Company, St. Louis, MO, USA). Store at 4 °C.
9. Running buffer, pH 8.3 (10× solution): add about 100 mL distilled water to a 1-L graduated glass beaker. Weigh 30.2 g of Tris, 10.0 g of SDS, and 144 g of glycine and transfer to the glass beaker. Add distilled water to a volume of 500 mL. Vortex to pre-dissolve Tris, SDS, and glycine, then add distilled water to a volume of 900 mL. Mix and adjust pH to 8.3. Make up to 1 L with distilled water. Store at room temperature.
10. Sample buffer (3× solution): add about 1 mL of distilled water to a 10-mL graduated cylinder. Weigh 1.5 mg of bromophenol blue and 0.60 g of SDS and transfer to the cylinder. Add 3 mL

of glycerol and 3.9 mL of solution b (0.5 M Tris-HCl solution, pH 6.8). Add distilled water to a volume of 8.5 mL. Mix and aliquot in 2-mL microtubes placed at -20°C for long-term storage. Just before use, thaw the sample buffer and add 15 % of 2-mercaptoethanol.

2.6 Immunoblotting Components

1. PVDF membranes (Millipore, Darmstadt, Germany).
2. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine and 10 % methanol.
3. Phosphate-buffered saline (PBS; 10 \times): 1.5 M NaCl, 0.1 M Tris-HCl, pH 7.4.
4. PBST: TBS containing 0.1 % Tween 20.
5. Blocking solution: 3 % bovine serum albumin (BSA) in PBS. Store at 4°C .
6. Diluent solution: 5 % BSA in PBST. Store at 4°C .
7. Mini PROTEAN[®] 3 System glass plates (catalog number 1653311) (Bio-Rad); medium binder clips (1¼ in.); plastic container.
8. Wypall X-60 reinforced paper (Kimberly-Clark, Neenah, WI, USA).

2.7 RNA Extraction and RT-PCR

2.7.1 RNA Extraction

1. TRIzol[®] Reagent (life technologies, Ref: 15596026).
2. Chloroform (Sigma-Aldrich, Ref: C2432), Isopropanol (Carlo Erba, Ref: 415156).
3. Ethanol 75 %.

2.7.2 Reverse Transcription

1. Nuclease-free water.
2. Random Hexamer 100 pmol (Thermo Scientific, Ref: SO142).
3. dNTP Mix, 10 mM each (Thermo Scientific, Ref: R0191).
4. 5 \times RT Buffer (Thermo Scientific, Ref: EP074).
5. Ribolock[™] RNase Inhibitor (Thermo Scientific, Ref: EO0381).
6. Maxima[®] Reverse Transcriptase (200 U/ μL) (Thermo Scientific, Ref: EP0741).
7. miScript II RT Kit (Qiagen, Ref: 218160).

2.7.3 PCR Amplification

1. 10 \times PCR Buffer minus MgCl_2 (Life technologies, Ref: 18067-017).
2. 50 mM MgCl_2 (Life technologies, Ref: 18067-017).
3. 200 U/ μL *Taq* DNA Polymerase (Life technologies, Ref: 10342-053).
4. dNTP Mix, 10 mM each (Life technologies, Ref: 18427013).
5. miScript SYBR Green PCR Kit (Qiagen, Ref: 218073).

2.7.4 Agarose Gel Electrophoresis

1. Agarose (UltraPure™ Agarose, Ref: 16500-100).
2. Tris base, acetic acid and EDTA buffer (TAE) (Sigma-Aldrich, Ref: T9650).
3. UltraPure™ 10 mg/mL Ethidium Bromide used at 0.5 µg/mL (Life technologies, 15585-011).
4. Loading dye 5× (Qiagen, Ref: 1037649).

2.8 Plasmid Transduction

1. Lentiviral particles containing pCDH lentivector (System Biosciences).
2. U87-MG cells.
3. DMEM medium (Life technologies, Ref: 11570586).

2.9 Immunohistochemistry

2.9.1 CD31 and Vimentin Staining

1. CD31 Rat Anti-Mouse (dil. 1/200) (BD Pharmingen, 550274).
2. Vimentin Mouse (dil.1/400) (Acris BM5050P).
3. Hoechst (Invitrogen 34580).
4. Secondary antibody Alexa 488 (Interchim, FP-SA4110).
5. Secondary antibody Alexa 547 (Interchim).
6. PFA (Electron Microscopy Sciences 15710).
7. Albumin from bovine serum (SIGMA A2153).
8. Mounting medium (Interchim, FP-483331 FluoroMount-G).

2.9.2 Hand E

1. Harris Haematoxylin (RAL Diagnostics 361070-2500).
2. Eosin (Sigma-Aldrich E4009).
3. Mounting medium (HistoLab 00811).

2.9.3 XBP1s Staining

1. Mouse anti-XBP1s antibodies were made in-house.
2. EnVision FLEX/HRP (Dako DM822).
3. EnVision FLEX SUBSTRATE BUFFER (Dako DM823).
4. EnVision FLEX DAB + CHROMOGEN (Dako DM827).
5. Triton (SIGMA T5;830-0).
6. Albumin from bovine serum (SIGMA A2153).
7. Harris Haematoxylin (RAL Diagnostics 361070-2500).
8. Mounting medium (HistoLab 00811).

3 Methods

3.1 Immunoblot

1. Resolve samples by SDS-PAGE and transfer onto PVDF membranes (EMD Millipore, Billerica, MA, USA) using liquid transfer for 40 min at 30 V using and the transfer buffer: 25 mM Tris-HCl, 192 mM glycine pH 8.8.

2. Wash membranes with distilled water and incubate with Ponceau S (0.1 % (x/v) Ponceau S in 1 % (v/v) acetic acid) for 5 min prior to extensive washing with distilled water. Block membranes with PBS, 0.1 % Tween 20 (PBST), and 3 % (w/v) bovine serum albumin for 45 min at room temperature.
3. Dilute primary antibodies with PBST at the appropriate dilution (*see* Subheading 2.2) and incubate with the membrane overnight at 4 °C.
4. Wash membranes 5× 5–10 min with PBST prior to incubating with HRP-conjugated secondary antibodies (dil. 1/5,000) for 45 min at room temperature.
5. Wash membranes with PBST 5× 5–10 min (Figs. 2 and 3).
6. Incubate membranes with chemoluminescent reagent (KPL, Gaithersburg, MD, USA) as recommended by the manufacturer and expose to X-ray films. Quantify bands with ImageJ software (NIH).

3.2 Phos-tag Analysis

Carry out all procedures at room temperature unless otherwise specified (*see* Note 1).

1. Prepare the resolving gel by mixing 2.5 mL of resolving buffer, 3.33 mL of acrylamide mixture, 4 µL of Phos-tag solution, 100 µL of MnCl₂ solution, and 3.87 mL of distilled water in a 50 mL conical flask. Add 100 µL of SDS, 50 µL of ammonium persulfate, and 10 µL of TEMED, and cast gel within a 7.25 cm × 10 cm × 1.5 mm gel cassette. Allow space for stacking the gel and gently overlay with isobutanol or water.
2. Prepare the stacking gel by mixing 2.5 mL of resolving buffer, 1.5 mL of acrylamide mixture, and 5.84 mL water in a 50 mL conical flask. Add 100 µL of SDS, 50 µL of ammonium persulfate, and 10 µL of TEMED. Insert a 10-well gel comb immediately without introducing air bubbles.
3. Sample preparation and electrophoresis: Plate U87MG cells onto 6-well plates (200,000 cells/well). 24 h post seeding, treat cells with tunicamycin (5 µg/mL) or vehicle (DMSO) for 6 h. Lyse cells in RIPA buffer with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Mix 12 µL cell lysate samples (around 30 µg total proteins) with 6 µL of 2-mercaptoethanol containing sample buffer. Heat at 95 °C for 5 min and centrifuge the heated samples at 3,000 × *g* for 30 s to bring down the condensate. Load 18 µL of each sample or 5 µL of protein standard in the gel. Electrophoresis should be performed at 10–15 mA until the dye front (from the bromophenol blue dye in the samples) has reached the bottom of the gel.
4. Following electrophoresis, pry the gel plates open with the use of a spatula. The gel remains on one of the glass plates. Remove

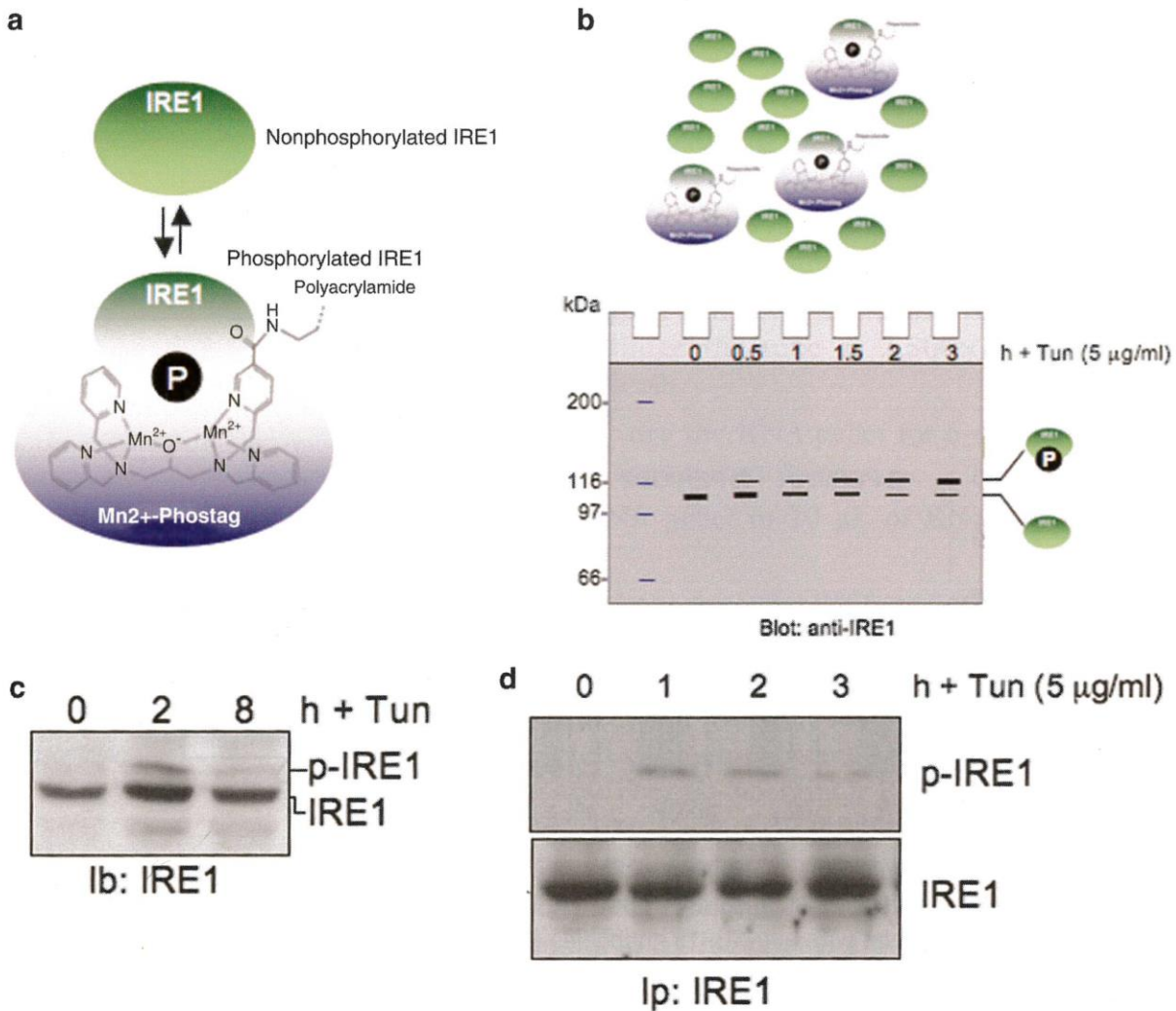


Fig. 2 IRE1 phosphorylation analysis. **(a)** Schematic representation of Phos-tag-p-IRE1 interaction. **(b)** Schematic representation of the Phos-tag analysis protocol. **(c)** IRE1 phosphorylation analysis using Phos-tag. U87 cells were lysed and protein samples were resolved by SDS-PAGE and Phos-tag. Following transfer onto PVDF membranes, IRE1 and p-IRE1 are visualized by immunoblot with anti-IRE1 antibodies. **(d)** IRE1 phosphorylation analysis by immunoblotting using anti p-IRE1 (S724). U87 cells were lysed and protein samples were immunoprecipitated with anti-IRE1 antibodies. Immunoprecipitates were resolved by SDS-PAGE, transferred onto PVDF and immunoblotted with anti-p-IRE1 or anti-IRE1 antibodies

the stacking gel. Rinse the gel twice with a general transfer buffer containing 10 mM EDTA for a minimum of 10 min with gentle agitation, to eliminate the manganese ions (Mn^{2+}) from the gel. Transfer carefully to a container with western blot transfer buffer without EDTA for 10 min.

5. Cut a PVDF membrane to the size of the gel and immerse in methanol. Rinse twice in distilled water and once with transfer buffer. Samples are transferred onto PVDF membranes using liquid transfer for 3 h at 10 V at 4 °C using the transfer buffer. Wash membranes with distilled water and incubate with Ponceau S (0.1 % (x/v) Ponceau S in 1 % (v/v) acetic acid) for

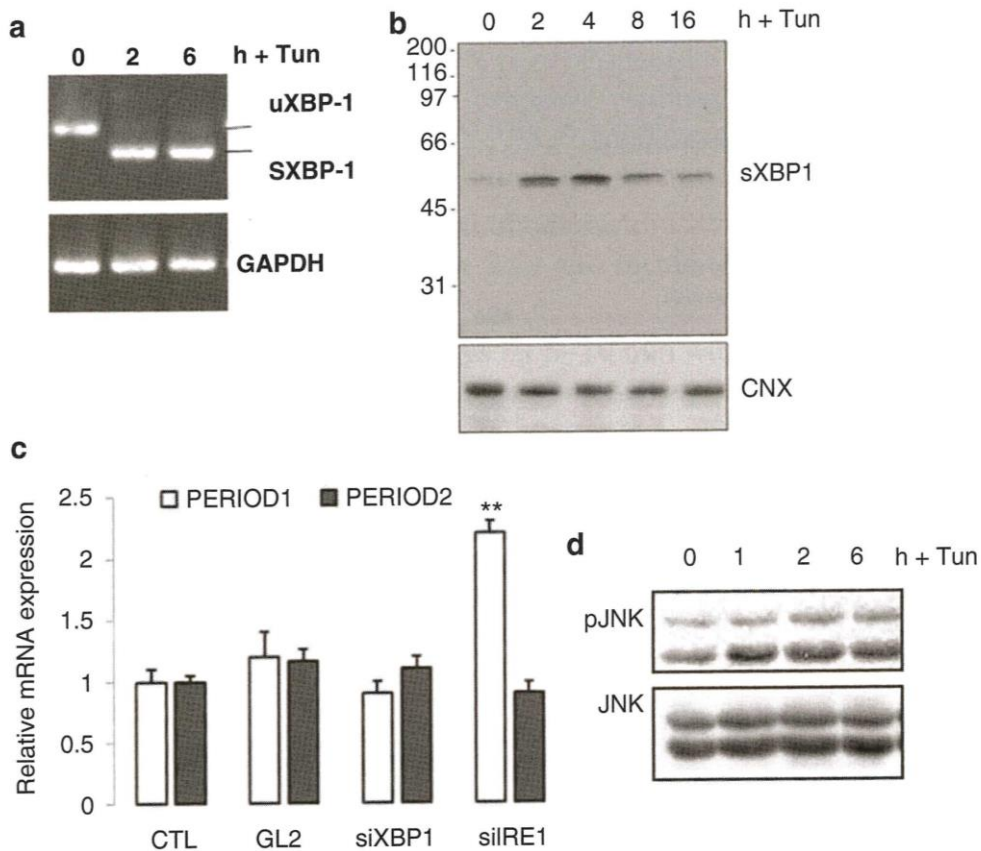


Fig. 3 Analysis of IRE1 downstream signaling. **(a)** XBP-1 mRNA splicing. **(b)** XBP1s protein expression. U87 cells were lysed and protein samples were resolved by SDS-PAGE. Following transfer onto PVDF membranes, XBP1s is visualized by immunoblot with anti-XBP1s antibodies **(c)** Analysis of RIDD activity towards PER1 mRNA. **(d)** JNK phosphorylation in response to tunicamycin-induced ER stress. U87 cells treated with tunicamycin were lysed and protein samples were resolved by SDS-PAGE and Phos-tag. Following transfer onto PVDF membranes, JNK1 and p-JNK1 are visualized by immunoblot with anti-JNK1 and anti-p-JNK1 antibodies (*see Note 2*)

5 min prior to extensive washing with distilled water. Block membranes using PBS, 0.1 % Tween 20, and 3 % (w/v) bovine serum albumin for 45 min at room temperature (Fig. 2).

3.3 RT-PCR for XBP1 mRNA Splicing

3.3.1 mRNA Extraction

mRNA extraction should be performed in a RNase-free environment.

1. After stress, remove medium from the wells and wash cells with PBS.
2. Add 1 mL of TRIzol® Reagent for 10 min in each well. Lyse the cells directly in the wells by pipetting the cells up and down several times.
3. Transfer each extract in a clean 1.5 mL tube and add 200 μ L of chloroform.
4. Vortex the tubes vigorously for 15 s. Incubate for 2–3 min at room temperature.
5. Centrifuge the samples at 12,000 $\times g$ for 15 min at 4 °C. Remove the aqueous phase of the sample by angling the tube at 45° and

pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase. Place the aqueous phase (about 0.4 mL) into a new tube.

6. Add 0.4 mL of isopropanol to the aqueous phase. Incubate at $-80\text{ }^{\circ}\text{C}$ for 1 h or at $-20\text{ }^{\circ}\text{C}$ overnight.
7. Centrifuge at $12,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. Remove the supernatant from the tube, leaving only the RNA pellet.
8. Wash the pellet, with 1 mL of 75 % ethanol. Vortex the sample briefly, then centrifuge the tube at $7,500\times g$ for 5 min at $4\text{ }^{\circ}\text{C}$. Discard the wash.
9. Vacuum-dry or air-dry the RNA pellet for 5–10 min. Do not dry the pellet by vacuum centrifuge.
10. Resuspend the RNA pellet in 20 μL of RNase-free water at $4\text{ }^{\circ}\text{C}$ for 20 min.
11. After homogenization, dose the RNA at 260 nm. Check the 260/230 and 260/280 ratios for protein contaminant.

3.3.2 Reverse Transcription

1. In a clean 200 μL tube, use 1 μg of RNA as template for the reaction, then add the following reaction components (manufacturer protocol, Thermo Scientific): 1 μL Random Hexamer, 1 μL dNTP Mix 10 mM, 4 μL 5 \times RT Buffer, 0.5 μL RibolockTM Rnase Inhibitor, 1 μL Maxima[®] Reverse Transcriptase Complete with RNase-free Water to 20 μL .
2. Start with 10 min at $25\text{ }^{\circ}\text{C}$ followed by 30 min at $50\text{ }^{\circ}\text{C}$ and terminate the reaction by heating at $85\text{ }^{\circ}\text{C}$ for 5 min.

3.3.3 XBP1 Splicing Polymerase Chain Reaction

PCR reaction should be performed in a DNA-free environment. Use of “clean” dedicated automatic pipettors and aerosol resistant barrier tips are recommended.

1. In a clean 200 μL tube, use 1–2 μL from the RT-PCR reaction mix as template for the reaction, then add the following reaction components (manufacturer protocol, life technologies): 0.3 μM Forward primer, 0.3 μM Reverse primer, 5 μL 10 \times Buffer minus MgCl_2 , 2 μL MgCl_2 (50 mM), 0.5 μL *Taq* DNA Polymerase, 1 μL dNTP Mix 10 mM, nuclease-free water to 50 μL .
2. PCR program: initial denaturation step start at $95\text{ }^{\circ}\text{C}$ for 10 min, followed by 40 cycles of: 30 s denaturation step at $95\text{ }^{\circ}\text{C}$, 45 s annealing step at $60\text{ }^{\circ}\text{C}$ and 45 s elongation step at $72\text{ }^{\circ}\text{C}$. The PCR reaction was finalized by 10 min elongation at $72\text{ }^{\circ}\text{C}$.

3.3.4 Agarose Gel Electrophoresis

1. Cast a 4 % agarose gel containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide in TAE buffer.
2. Mix 10 μL of PCR reaction with 2.5 μL of 5 \times loading dye.

3. Load the mix onto the gel and set the power supply at 100 V for 2 h.
4. Observe the result under UV light, prolong the migration time if the Xbp1 unspliced and Xbp1 spliced forms are not separated enough (Fig. 3).

3.4 Measuring RIDD Activation

3.4.1 IRE1 mRNA Decay

This protocol was designed to measure the RIDD activation of IRE1 in U87MG human cells and can be used to evaluate IRE1 mRNA decay activity regulators (*see Note 3*).

1. Incubate 300,000 cells by well in 4 wells of a 6-well plate, 48 h before siRNA transfection.
2. Transfect Cells by using the siRNAi Max Lipofectamine reagent (Invitrogen Corp.). Briefly, for each siRNA, dilute 9 μ L of RNAiMAX Reagent in 150 μ L of Opti-MEM[®] Medium (Life technologies) and 30 pmol of siRNA in 150 μ L of Opti-MEM[®] Medium. Add the diluted siRNA to diluted Lipofectamine[®] RNAiMAX and incubate for 5 min at room temperature. Add 250 μ L of this solution to the cells and incubate for 2–4 days.
3. RNA extraction, reverse transcription and PCR—Perform these steps as described in Subheading 3.3.1, except for the PCR program. Samples were denatured for 10 min at 95 °C, then cycled for 30 cycles (denaturation: 95 °C, 30 s; annealing: 62 °C, 30 s; elongation: 72 °C, 45 s) and then subjected to a final elongation of 10 min at 72 °C.
4. Resolve PCR products on 2 % agarose gels.
5. Quantify the bands using the ImageJ software (NIH). Normalize by dividing the PERIOD1 and PERIOD2 signal to the signal of the GAPDH. Normalization of each biological replicate can be performed by dividing the values by the mean of all values of the corresponding experiment or by dividing each value by the control (Fig. 3).

3.4.2 IRE1 miRNA Decay

1. Seed 200,000 cells by well in a 6-well plate, 48 h before RNA extraction. If a treatment is required, adjust the time according to the duration of treatment. Briefly, for each sample, dilute drugs in DMEM Medium (Life technologies) for the appropriate concentration and incubate until RNA extraction.
2. RNA extraction is performed as described in Subheading 3.3.1.
3. miRNA are specifically reverse transcribed using the miScript II RT kit (Qiagen). In a clean 200 μ L tube, use 500 ng of total RNA as template for the reaction, then add the following reaction components (manufacturer protocol, Qiagen): 4 μ L 5 \times miScript HiSpec Buffer, 2 μ L 10 \times miScript Nucleics Mix, 2 μ L miScript Reverse Transcriptase Mix and complete with

RNase-free water to 20 μ L. Incubate for 60 min at 37 °C followed by 5 min at 95 °C to inactivate miScript Reverse Transcriptase Mix.

4. SYBR-based Quantitative PCR: in a clean 200 μ L tube, use 1–2 μ L from the RT reaction mix as template for the reaction, then add the following reaction components (manufacturer protocol, Qiagen): 12.5 μ L 2 \times QuantiTect SYBR Green PCR Master Mix, 2.5 μ L 10 \times miScript Universal Primer, 2.5 μ L 10 \times miScript Primer assay and complete with nuclease-free water to 25 μ L. Samples were denatured for 15 min at 95 °C, then cycled for 40 cycles (denaturation: 94 °C, 15 s; annealing: 55 °C, 30 s; elongation: 70 °C, 30 s).
5. Quantification of miRNA by Delta Ct analysis and normalization of each biological replicate can be performed with RNA U6B.

3.5 Immunohistochemistry for Vimentin/CD31 and XBP1s

The entire process for Vimentin and CD31 staining is performed at room temperature in a moist chamber (*see Note 4*).

3.5.1 Tissue Preparation

1. Dry the sheets for 15 min.
2. Fix the tissue with PAF 4 %: 10 mL Formaldehyde 16 % (Electron Microscopy Sciences 15710) plus 30 mL of PBS 1 \times .
3. Wash with 1 \times PBS for 5 min. Do this process 3 times.
4. Permeabilize with PBS with Triton 0.1 % for 1 h (Triton 100 %—1 mL in 1 L of PBS 1 \times).
5. Wash with 1 \times PBS 3 times for 5 min.

3.5.2 Blocking and Antibody Reaction (Immunofluorescence)

1. Mark the area around the tissue with a Dako Pen (Dako 52002).
2. Saturate with PBS BSA 5 % for 1 h (1 L PBS 1 \times plus 50 mg of albumin from bovine serum (Sigma 96 %)). Wash with PBS for 5 min, 3 times.
3. Primary antibody: PBS BSA 1 % with Anti-Vimentin 1/400 (Mouse IgG1) (Acris BM5050P) for 1 h.
4. Wash with 1 \times PBS 3 times for 5 min.
5. Secondary antibody: PBS BSA 1 % with Alexa 488 (FluoProbes 488 Donkey Anti-Mouse IGG FP-5A4110), at a 1/200 dilution for 30 min.
6. Wash with 1 \times PBS for 5 min 3 times.
7. Primary antibody: PBS BSA 1 % with CD31 Purified Rat Anti-Mouse 1/200 (BD Pharmingen, 550274) for 1 h.
8. Wash with 1 \times PBS for 5 min 3 times.

9. Secondary antibody: PBS BSA 1 % with Alexa 547 (FluoProbes 547 Donkey Anti-Rat IGG FP-SB6110) (dilution 1/200) with Hoechst (PBS BSA 1 % + Hoechst (1/1,000)) for 30 min.
10. Wash with 1× PBS 3 times for 5 min.
11. Mount with 100 μ L of glue Interchim (FP-483331 FluoroMount-G Four immunofluorescent) and one coverglass (RS France Coverglass 24×60 mm 0.13–0.17 mm).

3.5.3 Antibody Reaction (HRP)

1. Primary antibody: PBS BSA 1 % with Monoclonal XBPIs IgG1 Mouse for 2 h.
2. Wash with 1× PBS 3 times for 5 min.
3. Secondary antibody: EnVision FLEX/HRP (Dako K8010/K8012/K8024) for 30 min.
4. Wash with PBS 1× 5 min. Do this 3 times.
5. Reveal with EnVision FLEX DAB+CHROMOGEN (Dako K8010/K8012/K8024).
6. Wash with distilled water for 5 min. Stain with hemalun for 3 min.
7. Wash with running water for 5 min.
8. Wash with distilled water and three drops of NH_3 .
9. Dehydrate by increasing battery graduation alcohol and toluene.
10. Mount with 100 μ L of mounting medium (PERTEX HistoLab F/00811) and a coverglass (RS France Coverglass 24×60 mm 0.13–0.17 mm) (Fig. 4).

4 Notes

1. For Phos-tag analysis gels must be run at rather low voltage (10–15 mA/gel) to allow better resolution and sharp bands (Fig. 2c). For the detection of IRE1 ser724 phosphorylation using the phosphospecific antibodies, it is better to immunoprecipitate IRE1 (following cell lysis with RIPA buffer containing protease and phosphatase inhibitors (Complete and PhosSTOP; Roche, Basel, Switzerland)) overnight at 4 °C. Immunoprecipitates are then resolved by SDS-PAGE and transferred onto PVDF membranes prior to immunoblotting using anti p-IRE1 antibodies (Fig. 2d).
2. For quantifying the increase in XBPIs expression by immunoblot (Fig. 3b), the amount of basal and ER stress-induced XBPIs should be investigated in preliminary experiments and depend on the cell lines/tissues to be analyzed. Several cell lines exhibit strong basal XBPIs such as HeLa cells or human hepatoma HuH7 cells.

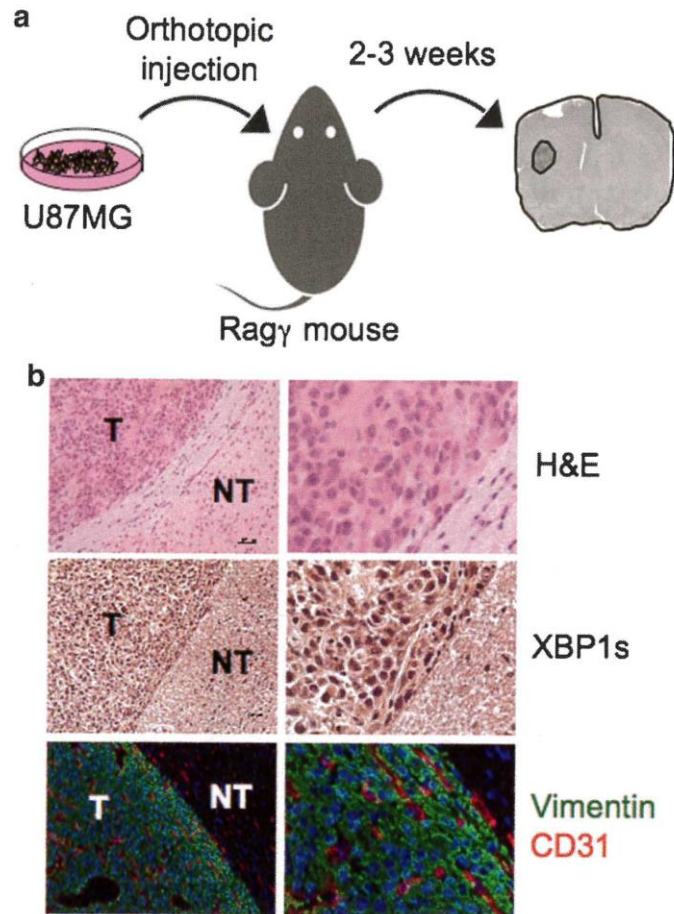


Fig. 4 Orthotopic glioblastoma model in the mouse. **(a)** Schematic representation of the orthotopic graft injection of U87MG cells into immunocompromised mice. **(b)** Following injection of U87 cells and 2–3 weeks, mouse brains were collected and preserved. Sections were performed and staining with H and E, anti-XBP1s antibodies (revealed using HRP-conjugated secondary antibodies), anti-Vimentin, anti-CD31 (revealed with fluorescently labeled secondary antibodies). *T* tumoral; *NT* nontumoral (see Note 5)

3. RIDD activity: Note that if you intend to validate the ability of IRE1 to cleave an mRNA upon stress, it is necessary to block the transcriptional regulation of potential substrates in order to validate their posttranscriptional regulation by IRE1. Furthermore, in this protocol the degradation of PERIOD1 mRNA was used as a marker of IRE1 endoribonuclease basal activation [31] (Fig. 3). Depending of the cell type, and the expression level of PERIOD1, it could be necessary to use another previously identified substrate of IRE1 mRNA decay activity, such as GPC3 [27] or SPARC [34]. The siIRE1 is used as a positive control of IRE1 modulation and PERIOD2 mRNA, a non-target of IRE1, as a negative control. The siGL2 represents a control unspecific siRNA. The siXBP1 is used to confirm that the regulation of the IRE1 mRNA target is not due to transcriptional regulation mediated by XBP1.

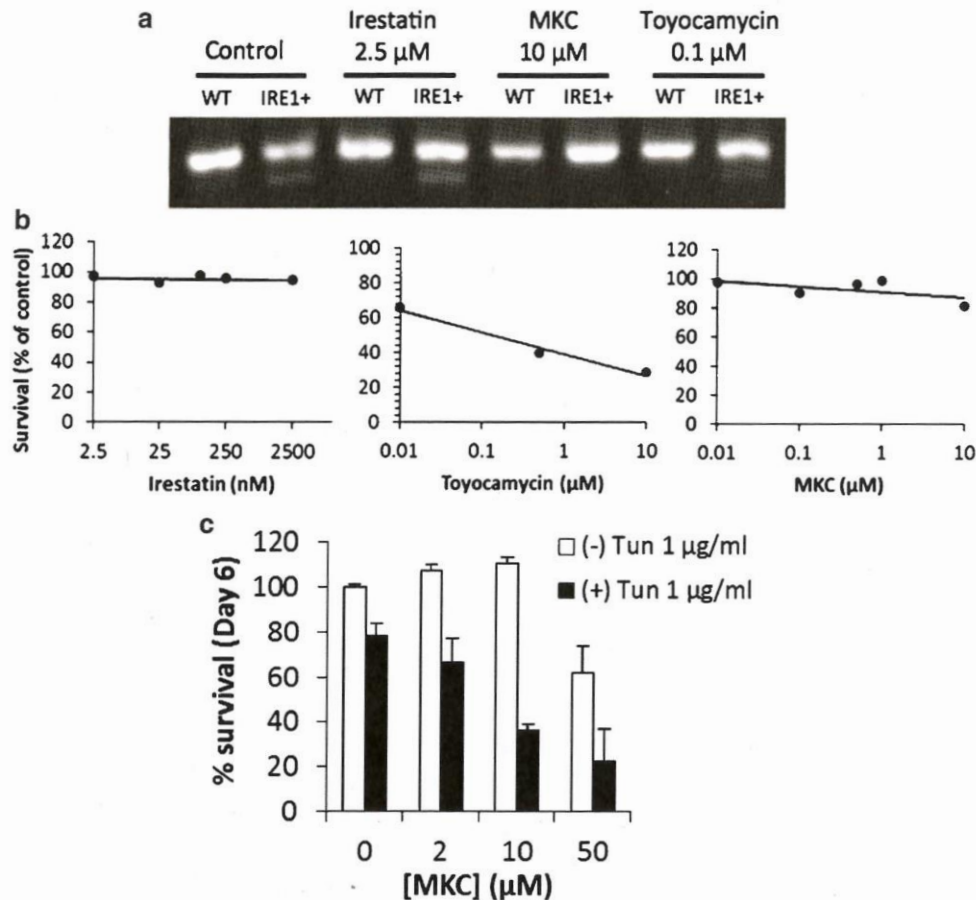


Fig. 5 Impact of IRE1 inhibition on U87 cells sensitivity to tunicamycin-induced ER stress. **(a)** Impact of toyocamycin, irestatin, and MKC8866 (labeled MKC) on XBP1 mRNA splicing activity. The concentrations used are indicated in the figure. XBP1 mRNA splicing activity was evaluated in control U87 cells and in U87 cells stably overexpressing wild-type IRE1, a situation sufficient for IRE1 activation. **(b)** Toxicity of toyocamycin, irestatin, and MKC8866 (labeled MKC) as assessed using sulforhodamine-B staining and increasing concentrations of the compounds. **(c)** Synergistic effects of MKC8866 and tunicamycin on toxicity in U87 cells (*see Note 6*)

- For immunohistochemistry experiments, all the solutions are prepared extemporaneously and conserved fresh. Hematoxylin can be used several times but the incubation time must increase with recycled solutions. Finally, the volume of each solution necessary for each slide (Dakopen-delimited area) is of about 300 μ L.
- For xenografts, cells must be resuspended in 100 μ L (1.5 mL tubes) or 50 μ L (round bottom tubes) in order for the syringe used for injection to homogenize properly the cell suspension and collect 1 μ L/brain for injection. Injecting more than 10,000 cells is not necessary.
- For inhibition of IRE1 activity, treatment with the inhibitors must be performed 48 h ahead of the treatment with tunicamycin at sub-toxic doses. In our experimental conditions and using our experimental model, only MKC was found to have an effect on cell sensitization to tunicamycin-induced death (Fig. 5).

Acknowledgements

This work was funded by grants from INSERM, Institut National du Cancer (INCa), La Ligue Contre le Cancer to EC. S.L. was supported by a PhD scholarship from the French government, and N.D. was supported by a postdoctoral fellowship from the Fondation de France.

References

1. Kozutsumi Y, Segal M, Normington K, Gething MJ, Sambrook J (1988) The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature* 332:462–464
2. Walter P, Ron D (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334:1081–1086
3. Kimata Y, Kohno K (2011) Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells. *Curr Opin Cell Biol* 23:135–142
4. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2:326–332
5. Hetz C (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 13:89–102
6. Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M et al (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* 6:1099–1108
7. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calton M et al (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* 11:619–633
8. Lerner AG, Upton JP, Praveen PV, Ghosh R, Nakagawa Y, Igarria A et al (2012) IRE1 α induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress. *Cell Metab* 16:250–264
9. Novoa I, Zeng H, Harding HP, Ron D (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2 α . *J Cell Biol* 153:1011–1022
10. Haze K, Yoshida H, Yanagi H, Yura T, Mori K (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* 10:3787–3799
11. Asada R, Kanemoto S, Kondo S, Saito A, Imaizumi K (2011) The signalling from endoplasmic reticulum-resident bZIP transcription factors involved in diverse cellular physiology. *J Biochem* 149:507–518
12. Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T et al (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev* 16:452–466
13. Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshida H et al (2007) Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6 α and XBP1. *Dev Cell* 13:365–376
14. Shoulders MD, Ryno LM, Genereux JC, Moresco JJ, Tu PG, Wu C et al (2013) Stress-independent activation of XBPs and/or ATF6 reveals three functionally diverse ER proteostasis environments. *Cell Rep* 3(4):1279–1292
15. Calton M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP et al (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 415:92–96
16. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107:881–891
17. Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH, Arias C et al (2007) XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. *Mol Cell* 27:53–66
18. Lee A-H, Iwakoshi NN, Glimcher LH (2003) XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol* 23:7448–7459

19. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP et al (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287:664–666
20. Han D, Lerner AG, Vande Walle L, Upton J-P, Xu W, Hagen A et al (2009) IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. *Cell* 138:562–575
21. Hollien J, Lin JH, Li H, Stevens N, Walter P, Weissman JS (2009) Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. *J Cell Biol* 186:323–331
22. Hollien J, Weissman JS (2006) Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* 313:104–107
23. Oikawa D, Tokuda M, Hosoda A, Iwawaki T (2010) Identification of a consensus element recognized and cleaved by IRE1 alpha. *Nucleic Acids Res* 38:6265–6273
24. Upton JP, Wang L, Han D, Wang ES, Huskey NE, Lim L et al (2012) IRE1alpha cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. *Science* 338:818–822
25. Dai BH, Geng L, Wang Y, Sui CJ, Xie F, Shen RX et al (2013) microRNA-199a-5p protects hepatocytes from bile acid-induced sustained endoplasmic reticulum stress. *Cell Death Dis* 4:e604
26. Maurel M, Chevet E (2013) Endoplasmic reticulum stress signaling: the microRNA connection. *Am J Physiol Cell Physiol* 304:C1117–C1126
27. Maurel M, Dejeans N, Taouji S, Chevet E, Grosset CF (2013) MicroRNA-1291-mediated silencing of IRE1alpha enhances Glypican-3 expression. *RNA* 19:778–788
28. Auf G, Jabouille A, Guerit S, Pineau R, Delugin M, Bouche-careilh M et al (2010) Inositol-requiring enzyme 1alpha is a key regulator of angiogenesis and invasion in malignant glioma. *Proc Natl Acad Sci U S A* 107:15553–15558
29. Drogat B, Auguste P, Nguyen DT, Bouche-careilh M, Pineau R, Nalbantoglu J et al (2007) IRE1 signaling is essential for ischemia-induced vascular endothelial growth factor-A expression and contributes to angiogenesis and tumor growth in vivo. *Cancer Res* 67:6700–6707
30. Moenner M, Pluquet O, Bouche-careilh M, Chevet E (2007) Integrated endoplasmic reticulum stress responses in cancer. *Cancer Res* 67:10631–10634
31. Pluquet O, Dejeans N, Bouche-careilh M, Lhomond S, Pineau R, Higa A et al (2013) Posttranscriptional regulation of PER1 underlies the oncogenic function of IRE1alpha. *Cancer Res* 73:4732–4743
32. Hetz C, Chevet E, Harding HP (2013) Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* 12:703–719
33. Volkmann K, Lucas JL, Vuga D, Wang X, Brumm D, Stiles C et al (2011) Potent and selective inhibitors of the inositol-requiring enzyme 1 endoribonuclease. *J Biol Chem* 286:12743–12755
34. Dejeans N, Pluquet O, Lhomond S, Grise F, Bouche-careilh M, Juin A et al (2012) Autocrine control of glioma cells adhesion and migration through IRE1alpha-mediated cleavage of SPARC mRNA. *J Cell Sci* 125:4278–4287

RESULTS

In 2008, in an attempt to identify new regulators of the UPR pathway, transgenic worms expressing a transcriptional reporter where the GFP is under the control of an ER stress promoter were subjected to a small RNA interference (RNAi) screening (76). The screening worked as follows: upon Tunicamycin treatment a pharmacological ER stressor (Tun) the worms become fluorescent, therefore if the fluorescence is modified by a RNAi it suggests that the silenced gene is an effector of the ER stress response: the UPR (**Figure 14**). Using this method we have identified CRP-1 a GTPase that belongs to the Rho family as a regulator of ER stress genes in *C. elegans* (76). In order to characterize the molecular mechanism behind this regulation CRP-1 partners were identified by mass spectrometry, among them several proteins involved in DNA remodelling and CDC-48 were found. Interestingly, CDC-48 and CRP-1 knock out worms displayed the same phenotype: impaired induction of ER stress genes induced by tunicamycin, suggesting a requirement of both CRP-1 and CDC-48 to induce these genes. These results suggested for the first time that CDC-48 role in ER may go beyond ERAD and ER formation, but that CDC-48 might also be involved in the regulation of ER stress genes.

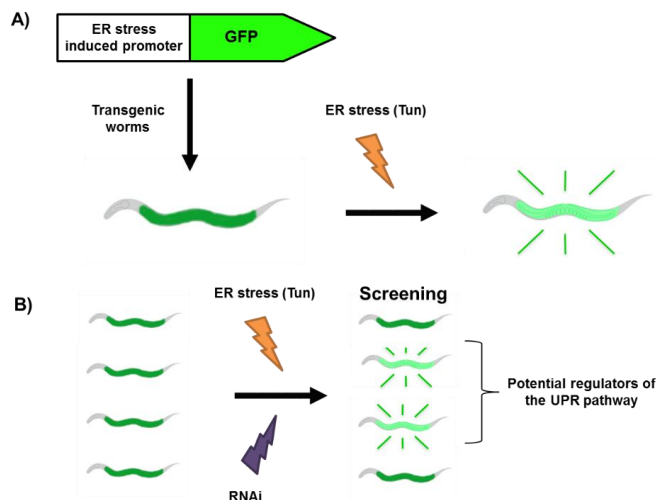


Figure 14: Schematic representation of the ER stress regulators screening in C. elegans - C. elegans worms expressed a transgenic GFP under the control of an ER stress induced promoter. In ER stress condition induced by Tun the transgene is activated resulting in GFP production and observable fluorescence. B) Using these transgenic C. elegans we were able to identify potential regulators of the UPR by measuring fluorescence of the different RNAi treated worms.

To better understand how p97/CDC-48 regulates ER stress genes, we came back to the observation that CDC-48.2 knock out worms are impaired in their capacity to induced *ckb-2p::gfp* (our ER stress reporter) in the presence of Tun and performed a new screening to identify proteins that could restore this phenotype. The following paper present our findings.

In this study, I helped with the characterization of ER stress gene expression under RuvBL2/Reptin silencing conditions in both *C. elegans* and human cells. I also contribute to characterize the interaction between p97/VCP and RuvBL2/Reptin. Finally, I have tested the effect of RuvBL2 silencing during ER stress on the activation of the IRE1 pathway.

ARTICLE 3

Genome-wide screen identifies a novel p97/CDC-48-dependent pathway regulating ER stress-induced gene transcription

Esther Marza^{1,2,4}, Saïd Taouji^{1,2*}, Kim Barroso^{1,2*}, Anne-Aurélie Raymond^{2,3*}, Léo Guignard^{2,4}, Marc Bonneau^{2,5}, Nestor Pallares^{1,2}, Jean-William Dupuy^{2,5}, Martin E. Fernandez-Zapico⁶, Jean Rosenbaum^{2,3}, Francesca Palladino⁷, Denis Dupuy^{2,4} and Eric Chevet^{1,2,8,9}

¹INSERM, UMR1053, Team «Endoplasmic Reticulum stress and cancer» F-33076 Bordeaux, France. ²Univ. Bordeaux, F-33000, Bordeaux, France. ³INSERM, UMR1053, «REPTeam» F-33076 Bordeaux, France. ⁴INSERM U869, ARNA laboratory, F-33000 Bordeaux, France. ⁵Plateforme Proteome, F-33000, Bordeaux, France. ⁶Schulze Center for Novel Therapeutics, Division of Oncology Research, Mayo Clinic, Rochester, MN, USA. ⁷Laboratory of Molecular and Cellular Biology, CNRS UMR5239, Université de Lyon, Ecole Normale Supérieure, 69364 Lyon Cedex 07, France. ⁸Centre Régional de Lutte Contre le Cancer Eugène Marquis, 35000 Rennes, France.

*equally contributed to this work

⁹Correspondance to EC - Email: eric.chevet@inserm.fr

Abstract

The accumulation of misfolded proteins in the endoplasmic reticulum (ER) activates the Unfolded Protein Response (UPR^{ER}) to restore ER homeostasis. The AAA⁺ ATPase p97/CDC-48 plays key roles in ER stress by promoting both ER protein degradation and transcription of UPR^{ER} genes. Although the mechanisms associated with protein degradation are now well established, the molecular events involved in the regulation of gene transcription by p97/CDC-48 remain unclear. Using a reporter-based genome-wide RNAi screen in combination with quantitative proteomic analysis in *C. elegans*, we have identified RUVB-2, a AAA⁺ ATPase, as a novel repressor of a subset of UPR^{ER} genes. We show that degradation of RUVB-2 by CDC-48 enhances expression of ER stress response genes through an XBP1-dependent mechanism. The functional interplay between CDC-48 and RUVB-2 in controlling transcription of select UPR^{ER} genes appears conserved in human cells. Together, these results describe a novel role for p97/CDC-48, whereby its role in protein degradation is integrated with its role in regulating expression of ER stress response genes.

Highlights

- 1) p97/CDC-48 induces retpin degradation upon ER stress
- 2) Reptin is a repressor of both ATF6 activation and XBP1 mRNA splicing
- 3) p97/cdc-48-mediated retpin degradation promotes ER adaptive response to stress

Two sentences summary

Upon Endoplasmic Reticulum stress, p97/CDC-48 mediates retpin degradation thereby enabling both ATF6 activation and XBP1 mRNA splicing. This work uncovers another layer in the regulation of canonical ER stress signaling.

Introduction

The endoplasmic reticulum (ER) protein quality control system ensures the correct folding of transmembrane and secretory proteins before their export from this organelle [1]. Accumulation of improperly folded proteins in the ER triggers the unfolded protein response (UPR^{ER}) to restore ER homeostasis. This is achieved by enhancing ER-Associated Degradation (ERAD), increasing ER protein folding capacity, decreasing protein translation and inducing a defined gene expression profile (UPR^{ER} genes) [2]. Although most of these molecular events are clearly established, the mechanism leading to the transcriptional regulation of specific genes under ER stress remains poorly understood.

Here, using as a model the nematode *C. elegans*, we identify a novel functional partner for p97/CDC-48, an AAA⁺ ATPase involved ER stress response, in the regulation of ER stress-associated UPR^{ER} gene transcription. *C. elegans* expresses two p97/CDC-48 homologs, *cdc-48.1* and *cdc-48.2*, which share similar functions in ERAD. While simultaneous silencing of both *cdc-48.1* and *cdc-48.2* leads to ER stress, UPR^{ER} gene activation and lethality [3]. Inactivation of either *cdc-48.1* or *cdc-48.2* is viable but abolishes the transcriptional activation of UPR^{ER} genes in response to ER stress [4]. Using a *C. elegans* strain mutant for the p97/CDC-48 homolog *cdc-48.2*^(-/-), we performed a genome-wide RNAi screen to identify proteins involved in the activation of UPR^{ER} genes during ER stress. We found that the AAA⁺ ATPase RUVB-2 is a regulator of the ER stress response by repressing the transcription of select UPR^{ER} genes in non-stressed conditions in both *C. elegans* and human cells. In response to ER stress, RUVB-2 is degraded in a CDC-48-dependent manner, thereby relieving repression of UPR^{ER} genes. Altogether, our results identify a novel mechanism controlling gene expression downstream of p97/CDC-48 and unveil a novel function for RUVB-2 and its human homolog Reptin as a key regulator of the transcriptional response to ER stress.

Results and Discussion

A genome-wide screen identifies *cdc-48* genetic interactors regulating ER stress-induced gene expression.

RNAi-mediated knockdown of *cdc-48.1* or *cdc-48.2* in *C. elegans* abolishes the ER stress-induced expression of a set of UPR^{ER} genes including *ckb-2* [4]. Using a transcriptional reporter

expressing GFP under the control of the *ckb-2* promoter, we confirmed the requirement for *cdc-48.1* and *cdc-48.2* in ER stress-induced gene transcription (Fig. 1A). Mutant *cdc-48.1*^(-/-) and *cdc-48.2*^(-/-) worms failed to respond to the ER stress inducer tunicamycin while *ckb-2p::gfp* fluorescence was increased more than 3-fold in wild-type (WT) worms (Fig. 1B). RNAi inactivation of *ire-1*, the main sensor of ER stress and mediator of UPR^{ER} signalling, resulted in a significant decrease in fluorescence intensity in both *ckb-2p::gfp* and *cdc-48.2*^{(-/-); ckb-2p::gfp} worms (Fig. 1A, Table S1). These results confirm that *ckb-2p::gfp* transcription is IRE1-dependent, as expected of a *bona fide* UPR^{ER} reporter.

Because p97/CDC-48 is involved in protein degradation [5], we reasoned that it might modulate ER stress-induced *ckb-2p* transcription by eliminating a transcriptional repressor. To address this hypothesis, we designed an RNAi suppressor screen to identify genes whose knockdown could restore tunicamycin *ckb-2p::gfp* activation following ER stress in a *cdc-48.2*^(-/-) mutant background ([4]; Fig. 1C). We performed the screen in liquid culture by feeding *cdc-48.2*^{(-/-); ckb-2p::gfp} synchronized L1 larvae with double stranded RNA (dsRNA)-expressing *E. coli* derived from the *C. elegans* ORFeome library that targets 11,698 open reading frames covering 62% of *C. elegans* genes [6]). We then exposed the worms to a concentration of tunicamycin (0.5 µg/ml for 16 hours) leading to maximal *ckb-2p::gfp* induction in WT worms grown in liquid culture, and analyzed them by flow cytometry [7] to measure their length, number and fluorescence intensity. Each RNAi clone was tested in duplicate and the mean Z-score was calculated. Two-hundred and forty one RNAi clones synergized with *cdc48.2*^(-/-) to decrease *ckb-2p::gfp* expression in our primary screen (mean Z-score value less than -1.5, or one of the two independent Z scores less than -3) (Fig. 1C). Of these, 59 clones significantly decreased GFP fluorescence below 0.75-fold ($P < 0.05$) (Fig. 1D, Table S2). One-hundred and seventy-seven RNAi clones instead reproducibly increased (average Z-score > 1.5 or one of the two individual Z-scores > 3) GFP fluorescence 1.5-fold above the fluorescence intensity measured with *cdc-48.2*^{(-/-); ckb-2p::gfp} worms fed with an empty vector and treated with tunicamycin ($P < 0.05$). These were classified as potential suppressors of the *cdc48.2*^(-/-) phenotype.

To discriminate between ER stress-dependent and independent activation of *ckb-2p::gfp* transcription, we measured fluorescence intensity in *cdc-48.2*^{(-/-); ckb-2p::gfp} worms fed with candidate RNAi clones and treated either with tunicamycin or vehicle (DMSO; Fig. 1E).

Seventy-seven RNAi clones showing a similar increase in the fluorescence ratios under both conditions were considered ER stress-independent and not further analysed (Fig. 1F, Table S3). By contrast, 100 RNAi clones which restored *ckb-2p::gfp* activation in the *cdc48.2^(-/-)* mutant background specifically under tunicamycin treatment were identified as ER-stress dependent suppressors of *cdc48.2^(-/-)* (Fig. 1E, Table S4). We next investigated if the genes targeted by these RNAi clones could activate gene transcription specifically under ER stress independently of *cdc-48.2*. If a targeted gene acts exclusively in the same genetic pathway as *cdc-48.2*, then its knockdown by RNAi should not increase *ckb-2p::gfp* transcription in a WT background, nor have an additive effect with the *cdc-48.2^(-/-)* mutation on *ckb-2p::gfp* transcription. We quantified and compared *ckb-2p::gfp* fluorescence intensities in both WT and *cdc-48.2^(-/-)* mutant worms fed with RNAi and exposed to tunicamycin. Twenty-seven RNAi clones increased fluorescence intensities in WT more than in *cdc-48.2^(-/-)* worms (fold change ≥ 1.4 , Fig. 1G). Nine other clones showed higher fluorescence in mutant worms compared to WT (fold change ≥ 1.4 , Fig. 1I), similar to *cdc-48.1* RNAi. The corresponding 36 genes (27+9) were therefore not considered as strict suppressor of *cdc-48.2* and were not further analyzed. We thus identified 64 suppressor RNAi clones that did not show any synthetic enhancement phenotype in *cdc-48.2^(-/-)* relative to WT (fold-change < 1.4 and $P < 0.05$, Fig. 1H). Taken together, these results identify genes controlling *ckb-2p::gfp* expression upon ER stress in a CDC-48 dependent fashion, and may provide mechanistic insight for the role of CDC-48 in ER stress induced gene expression (Fig. 2A). Among these candidates, the AAA+ ATPase *Ruvb2* was of particular interest.

To confirm the RNAi screen findings, we conducted a quantitative proteomic analysis to identify proteins whose levels are modified in *cdc-48.2^(-/-)*; *ckb-2p::gfp* worms exposed to tunicamycin. We selected proteins represented by at least two peptides and that had a peptide ratio above 2 or below 0.5 between WT and mutant worms exposed to tunicamycin. Ninety three proteins increased and 15 proteins decreased in abundance in *cdc-48.2^(-/-)* mutants compared to the WT (Fig. 2B, Table S5). RUVB-2 was the only suppressor identified in our RNAi screen for which an increase in protein abundance could be detected in *cdc-48.2^(-/-)*; *ckb-2p::gfp* compared to *ckb-2p::gfp* worms (2.5 ± 0.5 fold increase, Fig. 2C). Because the quantity of *ruvb-2* mRNA was not increased (Fig. S4A) under these conditions, the increased abundance of RUVB-2 in *cdc-*

cdc-48.2^(-/-) mutants is likely due to attenuation of protein degradation rather than to increased transcription.

Conserved RUVB-2 and CDC-48 dependent regulation of UPR^{ER} gene expression.

RNAi knockdown of *ruvb-2* restored *ckb-2p::gfp* activation both in *cdc-48.2^(-/-)* and *cdc-48.1^(-/-)* mutant worms exposed to tunicamycin (Fig. 3A-B). This suggests that, under ER stress, the repressor RUVB-2 is degraded through a CDC-48.1-dependent mechanism to allow full *ckb-2p::gfp* induction. Moreover, knockdown of *xbp-1* reduced *ckb-2p::gfp* expression in *cdc-48.2^(-/-)*; *ckb-2p::gfp* worms treated with tunicamycin compared to the DMSO-treated ones (Fig. 3C). Combined RNAi-mediated knock-down of *xbp-1* and *ruvb-2* decreased *ckb-2p::gfp* fluorescence to the same level observed using *xbp-1* RNAi alone. This suggests that RUVB-2 is degraded through a CDC-48-dependent mechanism in response to tunicamycin, thus allowing XBP-1s to activate *ckb-2* expression. *Ruvb-2* inactivation also restored the expression of ER homeostasis regulators (CKB-2, F22E5.6, Y71F9AL.17/COPA-1) observed upon ER stress in WT animals [8] in *cdc-48.2^(-/-)* tunicamycin-treated worms (Fig. 3D and S4B). These results suggest that RUVB-2 represses the expression of select UPR^{ER} target genes. We next tested whether this function was conserved in human cells. To this end, Huh7 cells transfected with the ER stress response element reporter gene (ERSE::tomato [9]) were knocked-down for *Reptin* using stable integration of a doxycycline-inducible short hairpin RNA (shRNA [10]) (Fig. 3E, left). Induction of *Reptin* shRNA synergized with tunicamycin treatment to activate ERSE::tomato transcription, demonstrating that Reptin can also repress ER stress-mediated transcription in human cells. Of note, the silencing of the Reptin homolog Pontin did not affect the transcription of the ERSE::tomato reporter under basal conditions or upon tunicamycin-induced ER stress (Fig. 3E, right). We next quantified the mRNA amounts of 4 genes whose products are involved in the control of ER homeostasis (BiP, CHOP, EDEM1, ORP150). This revealed that Reptin silencing significantly increased the expression of BiP, CHOP and EDEM1 while did not affect that of ORP150 (Fig. 3F). Moreover, Reptin overexpression led to the significant repression of select genes (CHOP, EDEM1, ORP150) under basal conditions when compared to control transfected cells (Fig. S5). Altogether, these results suggest the existence of a conserved role for Reptin in repressing expression of ER stress response genes.

Post-translational control of Reptin expression by p97/CDC-48 impacts on ER stress response in human cells.

Further, we examined whether p97/CDC-48 could also act by triggering the degradation of Reptin in response to ER stress as observed in *C. elegans* (Fig. 2). Reptin protein levels were significantly decreased upon tunicamycin treatment whereas p97/CDC-48, Pontin and Calnexin protein expression remained unaffected (Fig. 4A). Conversely, addition of the p97/CDC-48 inhibitor DBeQ, stabilized Reptin levels under ER stress (Fig. 4B). We then tested whether p97 and Reptin interacted physically using co-immunoprecipitation. These results were confirmed by determining Reptin's half-life upon stress (Table S6) and the values obtained under basal conditions were in the range of those determined in *S. cerevisiae* or *S. pombe* [11]. Reptin immunoprecipitates contained p97/CDC-48 and the interaction was modulated by tunicamycin-induced ER stress, DBeQ or both (Fig. 4C). Interestingly, when the reverse experiment was carried out, Reptin was found in the p97/CDC-48 immunoprecipitate as well as a slower migrating Reptin immunoreactive species (Fig. 4D, arrow). Sequential immunoprecipitation with p97/CDC-48 and Reptin antibodies suggested that the latter corresponds to an ubiquitylated form of Reptin (Fig. 4E). Hence, p97/CDC-48 might control Reptin levels through an ubiquitin-dependent mechanism.

XBP1 mRNA splicing and ATF6 activation are partly regulated by a p97/reptin signalling axis

To follow up on the role of Reptin degradation upon ER stress in the expression of ER stress genes, we sought to test whether artificial modulation of Reptin expression also impacted the activation of the three UPR signalling arms. Reptin silencing slightly increased the expression of the ER stress upregulated chaperones GRP78 and GRP94, which are canonical targets of ATF6 and XBP1s signaling, under basal conditions (Fig. 5A), but did not affect tunicamycin-induced phosphorylation of eIF2 α (Fig. 5B). ATF6 cleavage activation was increased after Reptin silencing in HuH7 cells (Fig. 5C). In accordance with this observation, reptin silencing also enhanced the expression of XBP1u mRNA under basal conditions, as could be expected since XBP1u is a target gene of ATF6 (Fig. 5D). Moreover, this occurred without affecting the

expression levels of the newly discovered XBP1 mRNA ligase RtcB [12] (Fig. 5D). XBP1 mRNA splicing was also increased when Reptin was silenced both in basal conditions and ER stress (Fig. 5E). Conversely, DBeQ-mediated p97/CDC-48 inhibition (Fig. 5F) or siRNA-mediated p97/CDC-48 silencing (Fig. S6) and the subsequent stabilization of Reptin led to reduced XBP1 mRNA splicing. Hence, partial stabilization of Reptin has a major impact on XBP1 mRNA splicing, which in turn impacts dramatically on the expression of various UPR^{ER} genes. However we could not detect an interaction between Reptin and XBP1s protein (Fig. 5G). Altogether, these results might indicate that Reptin is degraded through ubiquitin and p97/CDC-48-dependent mechanisms under ER stress and further support the role of Reptin in the control of select UPR^{ER} genes through repression of XBP1 mRNA splicing and of ATF6 activation.

In the present work, we have uncovered a novel regulatory mechanism of UPR^{ER} genes expression in response to ER stress conserved throughout metazoan evolution involving two AAA⁺ ATPases, RUVB-2 (or Reptin) and CDC-48 (or p97). In this model, RUVB-2, which mostly localizes to the cytoplasm and the nucleus, plays an important role in the regulation of XBP1 mRNA splicing by a yet unknown mechanism. Upon ER stress, RUVB-2 is degraded through an ubiquitin and p97/CDC-48-dependent mechanism, thereby allowing the ER stress specific transcription factors ATF6 and XBP-1 to activate the transcription of UPR^{ER} genes. Beyond unravelling a novel UPR^{ER} regulatory network, our data point towards the putative role of Reptin in non-conventional mRNA splicing. Our findings suggest that p97/CDC-48-induced degradation of target proteins plays an important role in the ER homeostasis control both, in the cytoplasm, to influence ERAD and to modulate UPR^{ER} gene transcription [13].

Materials and Methods

RNAi screen - The RNAi feeding screen was performed in liquid culture using EM2 animals and carried out as previously described with some modifications [4]. RNAi clones from the Worm ORFeome version 1.1 library [6] were grown overnight at 37°C in 96-well plates. Each RNAi plate included a positive control (Y37D8A.10 encoding for a signal peptidase identified in a preliminary screen or BC14636 worms fed with the L4440 empty vector) and a negative control (*gfp* RNAi). RNAi expression was induced with 1mM IPTG for 1h before bacteria were added to the L1 larvae. Adult worms were bleached and the obtained L1 larvae (200) were added to each

well of 96-well plates along with the induced bacteria and S-Medium, 50 µg/ml ampicillin, 1mM IPTG buffer with a final well volume of 150 µl. The 96-well plates were incubated at 20°C with shaking. Forty-eight hours later ER stress was induced by tunicamycin (0.5 µg/ml) for 16 hours and measurements taken using the COPAS Biosort flow cytometer (Union Biometrica, Holliston, MA, USA). Experiments for each 96-well plate from the RNAi library were performed in duplicate. Fluorescence average value for each plate was calculated and used to calculate the individual RNAi fold change. Plates showing no fluorescence induction in the positive control, no fluorescence decrease in negative control, or a high fluorescence mean were discarded and retested.

COPAS measurements – The COPAS biosort analyzer was purchased from Union Biometrica (Holliston, MA, USA). Photomultiplier tube control (PMT1) was set up at 600 so that the green fluorescence emission was not saturated in BC14636 worms exposed to tunicamycin (maximum signal) and still detectable in EM2 worms exposed to *gfp* RNAi (minimum signal). Plates were read through a ReFLx module. Raw data extracted from COPAS included worm axial length (time of flight), worm number (extinction), and fluorescence (green fluorescence emission). Raw data were processed as previously described [14] and used for quantitative analyses.

Acknowledgements - We thank Dr. R. Pedeux for help with the post-translational modifications of reptin, E Attebi and K Rebora for help with *C. elegans* screens, Drs E. Snapp and F. Schoenen for the ERSE::Tomato construct and DBeC, respectively, Dr. S. Mitani for the *cdc-48.1(tm544)* and *cdc-48.2(tm659)* strains.

Author contribution - E.M., A.A.R., S.T., K.B., E.C. and J.W.D. performed experiments. L.G. and D.D. developed the bioinformatics tools. M.B. supervised the proteomics analysis. J.R. and F.P. provided tools. E.M., D.D., E.C. coordinated the study. E.M., M.F.Z., D.D. and E.C. wrote the manuscript.

Conflict of interest - The authors declare no conflict of interest.

References

1. Ellgaard L, Helenius A (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* **4**: 181-191
2. Hetz C, Chevet E, Harding HP (2013) Targeting the unfolded protein response in disease. *Nat Rev Drug Discov* **12**: 703-719
3. Mouysset J, Křhler C, Hoppe T (2006) A conserved role of *Caenorhabditis elegans* CDC-48 in ER-associated protein degradation. *Journal of structural biology* **156**: 41-49
4. Caruso ME, Jenna S, Bouche-careilh M, Baillie DL, Boismenu D, Halawani D, Latterich M, Chevet E (2008) GTPase-mediated regulation of the unfolded protein response in *Caenorhabditis elegans* is dependent on the AAA+ ATPase CDC-48. *Mol Cell Biol* **28**: 4261-4274
5. Dantuma NP, Hoppe T (2012) Growing sphere of influence: Cdc48/p97 orchestrates ubiquitin-dependent extraction from chromatin. *Trends Cell Biol* **22**: 483-491
6. Reboul J *et al* (2003) *C. elegans* ORFeome version 1.1: experimental verification of the genome annotation and resource for proteome-scale protein expression. *Nat Genet* **34**: 35-41
7. Squiban B, Belougne J, Ewbank J, Zugasti O (2012) Quantitative and automated high-throughput genome-wide RNAi screens in *C. elegans*. *J Vis Exp* **in press**:
8. Shen X, Ellis RE, Sakaki K, Kaufman RJ (2005) Genetic interactions due to constitutive and inducible gene regulation mediated by the unfolded protein response in *C. elegans*. *PLoS Genet* **1**: e37
9. Lajoie P, Snapp EL (2011) Changes in BiP availability reveal hypersensitivity to acute endoplasmic reticulum stress in cells expressing mutant huntingtin. *J Cell Sci* **124**: 3332-3343
10. Haurie V *et al* (2009) Adenosine triphosphatase pontin is overexpressed in hepatocellular carcinoma and coregulated with reptin through a new posttranslational mechanism. *Hepatology* **50**: 1871-1883
11. Christiano R, Nagaraj N, Fröhlich F, Walther TC (2014) Global Proteome Turnover Analyses of the Yeasts *S. cerevisiae* and *S. pombe*. *Cell Rep* **9**: 1-7
12. Kosmaczewski SG, Edwards TJ, Han SM, Eckwahl MJ, Meyer BI, Peach S, Hesselberth JR, Wolin SL, Hammarlund M (2014) The RtcB RNA ligase is an essential component of the metazoan unfolded protein response. *EMBO Rep*
13. Fessart D, Marza E, Taouji S, Delom F, Chevet E (2013) P97/CDC-48: Proteostasis control in tumor cell biology. *Cancer Lett* **337**: 26-34
14. Dupuy D *et al* (2007) Genome-scale analysis of in vivo spatiotemporal promoter activity in *Caenorhabditis elegans*. *Nat Biotechnol* **25**: 663-668

Figure legends

Figure 1: RNAi screening identifies *cdc-48.2* genetics interactors in the *ckb-2* transcriptional response to ER stress. (A) *cdc-48.2* is required to activate *ckb-2p::gfp* transcription in response to tunicamycin. Images of adult worms (left) expressing *gfp* under the control of the *ckb-2* gene promoter in WT (upper panels) and in *cdc-48.2^(-/-)* mutants (lower panels) exposed to tunicamycin (5 μ g/ml) or DMSO for 16 hours. (Scale bar: 50 μ m, obj.: 10x). (B) Significant changes in fluorescence intensities were quantified using flow cytometry. L1 larvae (*ckb-2p::gfp* and *cdc-48.2^(-/-)*; *ckb-2::pgfp* larvae) were fed with bacteria expressing the L4440 empty vector or *ire-1* RNAi in liquid culture and exposed to tunicamycin (0.5 μ g/ml) or DMSO for 16 hours. F0 was defined as the fluorescence intensity obtained in *ckb-2p::gfp* worms fed with the empty vector and treated with DMSO. (Mean \pm s.e.m, N=8, 200 worms/experiment). *P* values were calculated using multiple t-test corrected using the Holm-Sidak method ***P*<0.001; **P*<0.01. (C) Genome-wide RNAi screen identifies suppressors and enhancers of *cdc-48.2^(-/-)* in *ckb-2p::gfp* transcription. Volcano plots present results obtained using *C. elegans* ORFeome library. (D) Re-testing of RNAi clones from first round. Classification of ER stress dependence of the 177 suppressor RNAi clones able to restore *ckb-2p::gfp* transcription in *cdc-48.2^(-/-)* mutant background. *cdc-48.2^(-/-)*; *ckb-2p::gfp* synchronized L1 larvae were fed with the dsRNA expressing bacteria in liquid culture, treated with tunicamycin (0.5 μ g/ml) or DMSO for 16 hours and fluorescence intensities were measured by flow cytometry. Tunicamycin dependent RNAi clones were defined as those that significantly increased fluorescence ratio following tunicamycin treatment ((E); Tunicamycin/DMSO F/F0 fold change>1.5). Tunicamycin independent RNAi clones were defined as those increasing *ckb-2p::gfp* fluorescence ratio in both conditions ((F); Tunicamycin/DMSO F/F0 fold change <1.5, *P*>0.05). Fluorescence ratios obtained with *ruvb-2* RNAi are shown in magenta. Fluorescence ratios obtained with *ckb-2p::gfp* worms fed with the empty vector and treated with tunicamycin (2.38 \pm 0.18) or DMSO (1.05 \pm 0.2) are shown in cyan. F0 was defined as the fluorescence intensity obtained in *cdc-48.2^(-/-)*; *ckb-2p::gfp* worms fed with the empty vector and treated with tunicamycin or DMSO, respectively. (Mean \pm s.e.m, N=5). Identification of ER stress dependent RNAi clones targeting genes involved in the same genetic pathway as *cdc-48.2* to increase *ckb-2p::gfp* transcription. Fluorescence ratio were determined on *cdc-48.2^(-/-)*; *ckb-2::gfp* and *ckb-2::gfp* worms fed with the suppressor RNAi clones and treated

with tunicamycin (0.5 $\mu\text{g/ml}$) for 16 hours. Graphs present the RNAi clones whose effect on *ckb-2p::gfp* fluorescence was higher ((**G**), (*ckb-2::gfp* F/F0/*cdc-48.2^(-/-)*; *ckb-2::gfp* F/F0) fold-change >1.4-fold), similar ((**H**), (*ckb-2::gfp* F/F0/*cdc-48.2^(-/-)*; *ckb-2::gfp* F/F0) fold-change <1.4, $P>0.05$) or lower ((**I**), (*ckb-2::gfp* F/F0/*cdc-48.2^(-/-)*; *ckb-2::gfp* F/F0) fold-change <0.75) in *ckb-2p::gfp* worms compared to *cdc-48.2^(-/-)*; *ckb-2p::gfp* worms. Fluorescence ratios obtained with *ruvb-2* RNAi and the two controls empty vector and *cdc-48.1* control RNAis are shown in magenta, cyan and brown, respectively. (Mean \pm s.e.m, N=5).

Figure 2: Identification of RUVB2 as a candidate CDC-48 target. (**A**) List of RNAi clones suppressing the *cdc-48.2^(-/-)* phenotype. (**B**) Graph representing identified peptide number identified in function of peptide quantity ratio. *cdc-48.2^(-/-)*; *ckb-2::gfp* and *ckb-2::gfp* synchronized L1 larvae were grown to the L4 stage and exposed to tunicamycin (5 $\mu\text{g/ml}$) for 16h on plates. Proteins (60 μg) were separated on a 10% SDS gel. A coomassie blue staining image representative of the SDS gel is shown on the left (1: *cdc-48.2^(-/-)*; *ckb-2::gfp*, 2: *ckb-2::gfp*). Gel lanes were cut into slices before proteins were in-gel digested. Peptides were then identified and quantified by label-free LC-MS/MS mass spectrometry. Peptides that were more (magenta) or less (cyan) abundant in the *cdc-48.2^(-/-)*; *ckb-2::gfp* than in *ckb-2::gfp* worms were defined as those having a ratio above 1.5 or below 0.5, respectively. N=3. (**C**) Graph representing peptide quantity ratio ($(\text{cdc-48.2^(-/-); ckb-2::gfp})/(\text{ckb-2::gfp})$) for the 93 proteins that are more abundant in *cdc-48.2^(-/-)* mutant background compared to WT background. (Mean \pm s.e.m, N=3).

Figure 3: RUVB-2 is a transcriptional repressor inactivated by CDC-48 upon ER stress. (**A**) Images of *cdc-48.2^(-/-)*; *ckb-2::gfp* adult worms fed with either the L4440 empty vector (upper panel) or *ruvb-2* RNAi (lower panel) and treated with tunicamycin (5 $\mu\text{g/ml}$) or DMSO for 16 hours on NGM agar plates. (Scale bar: 50 μm , obj: 10x). (**B**) Fluorescence was quantified by flow cytometry on *ckb-2::gfp*, *cdc-48.1^(-/-)*; *ckb-2::gfp* and *cdc-48.2^(-/-)*; *ckb-2::gfp* worms fed with *ruvb-2* RNAi or empty vector starting at the L1 stage in liquid culture and exposed to tunicamycin (0.5 $\mu\text{g/ml}$) or DMSO for 16 hours. Fluorescence (F) was normalized to the basal fluorescence obtained with empty vector and DMSO in the WT background (F0). (Mean \pm SD, N=5) *** $P < 0.001$. (**C**) Fluorescence (F) was quantified by flow cytometry on *ckb-2::gfp* and

cdc-48.2^(-/ -); *ckb-2::gfp* worms fed with either *ruvb-2* and empty vector (1:1), *xbp-1* and empty vector (1:1), *ruvb-2* and *xbp-1* RNAi (1:1), or empty vector alone and treated with tunicamycin (0.5 µg/ml) or DMSO for 16 hours. Fluorescence (F) was normalized to the basal fluorescence obtained with the empty vector and DMSO in the WT background (F0). (Mean ± s.e.m, N = 3). *P* values were calculated using multiple t-test corrected using the Holm-Sidak method. ***P*<0.01 ; ****P*<0.001. **(D)** RT-qPCR quantification of the relative expression levels of 3 endogenous ER homeostasis genes (ERp19, F22E5.6, Y71F9AL.17/COPA-1), *Ckb-2* and *Ruvb-2* following tunicamycin treatment in *cdc-48.2^(-/ -)* worms subjected or not to *ruvb-2* RNAi. Bars represent the mean of 3 biological replicates. (Mean ± s.e.m, N=3) ***P*<0.01; ****P*<0.001. **(E)** Fluorescence was quantified in HuH7 cells expressing the ERSE::Tomato construct and either the *Reptin* shRNA induced with doxycycline (left) or the the Pontin shRNA (transient, right). Cells were exposed to tunicamycin (5 µg/ml) for 4 hours prior to measurement. Data are presented as mean ± SD of 3 independent experiments. Note that Reptin levels were decreased upon Tunicamycin treatment (see also Fig. 4A) **(F)** RT-qPCR analysis of four ER homeostasis control genes under basal conditions or upon tunicamycin treatment (5 µg/ml, 16 hours) in HuH7 cells subjected or not to doxycycline-induced *Reptin* silencing. Data are presented as mean ± SD of 3 independent biological triplicates. (Mean ± s.e.m, N = 3) *P* value was calculated using multiple t-test corrected using the Holm-Sidak method. **P*<0.05; ***P*<0.01.

Figure 4: p97/CDC-48-mediated degradation of Reptin upon ER stress. **(A)** Reptin, Pontin, calnexin and quantification by immunoblot. Values are expressed as a percentage of the initial protein abundance in total HuH7 cell lysate before addition of tunicamycin (5 µg/ml), (Mean ± SD, N=5). **P*<0.05; ***P*<0.01. **(B)** Immunoblot analysis of Reptin in total protein extracts from HuH7 cells exposed to tunicamycin (5 µg/ml) for 0 to 2 hours. Protein levels were normalized to Calnexin (mean ± SD, N=3). **P*<0.05; ***P*<0.01. **(C)** HuH7 cells expressing FLAG tagged Reptin were treated either with the p97/CDC-48 inhibitor DBeQ (20 µM, D), the ER stress inducer tunicamycin (2 µg/ml; T) or both for 4 hours. FLAG tagged Reptin was immunoprecipitated from total protein extracts using anti-FLAG antibodies and p97/CDC-48 association was analyzed by immunoblot. **(D)** HuH7 cells were treated either with DBeQ (20 µM, D), (2 µg/ml; T) or both for 4 hours. P97/CDC-48 was immunoprecipitated from total protein

extracts using an antibody specific for p97/CDC-48 and repton association was analyzed by immunoblotting. (E) HuH7 cells were treated either with DBeQ (20 μ M, D), tunicamycin (2 μ g/ml; T) or both for 4 hours. P97/CDC-48 was immunoprecipitated from total protein extracts using anti-p97/CDC-48 antibodies. P97/CDC-48 immunoprecipitate was disrupted with 50 μ l of 1% SDS and heated at 95°C for 5 min. Beads were removed and the supernatant quenched with PBS containing 1% TX-100. Repton was then sequentially immunoprecipitated and the resulting immunoprecipitate immunoblotted with anti Ubiquitin or anti Repton antibodies.

Figure 5: Repton silencing enhances ATF6 and XBP1s activation. (A) GRP78 and GRP94 expression was detected using anti-KDEL antibodies (top blot) in HuH7 cells treated or not with tunicamycin and/or doxycycline (Dox) to induce repton silencing (bottom blot). Expression of p97 was also monitored (middle blot). (B) eIF2 α phosphorylation was monitored using specific antibodies (top blot) and reported to the total expression (bottom blot) in HuH7 cells treated or not with tunicamycin and/or doxycycline (Dox). (C) ATF6 activation was monitored in the same experimental conditions using antibodies against the N-terminal domain of ATF6. (D) Expression of unspliced XBP1 mRNA as determined by RT-PCR and expression of the XBP1s ligase RTCB as determined by immunoblot using anti RTCB antibodies in HuH7 cells treated or not with tunicamycin and/or doxycycline (Dox). (E) XBP-1 mRNA splicing as determined by RT-PCR under basal conditions or upon tunicamycin treatment (5 μ g/ml for 16 hours) in HuH7 cells subjected or not to doxycycline-induced *Repton* silencing. Three independent experiments were performed and a representative image is shown. (F) HuH7 cells were treated either with DBeQ (20 μ M, D), tunicamycin (2 μ g/ml; T) or both for 4 hours. XBP-1 mRNA splicing was determined by RT-PCR (Mean \pm SD, N = 3). *P* values were calculated using multiple t-test corrected using the Holm-Sidak method. **P* < 0.05; ***P* < 0.01. (G) HuH7 cells were treated with tunicamycin (2 μ g/ml; T) for 1 hour. XBP1s was immunoprecipitated and the complex was immunoblotted with anti Repton antibodies (top blot). Total cell lysate (TCL) was immunoblotted with anti Repton (middle blot) or anti XBP1s (bottom blot).

Figure 1

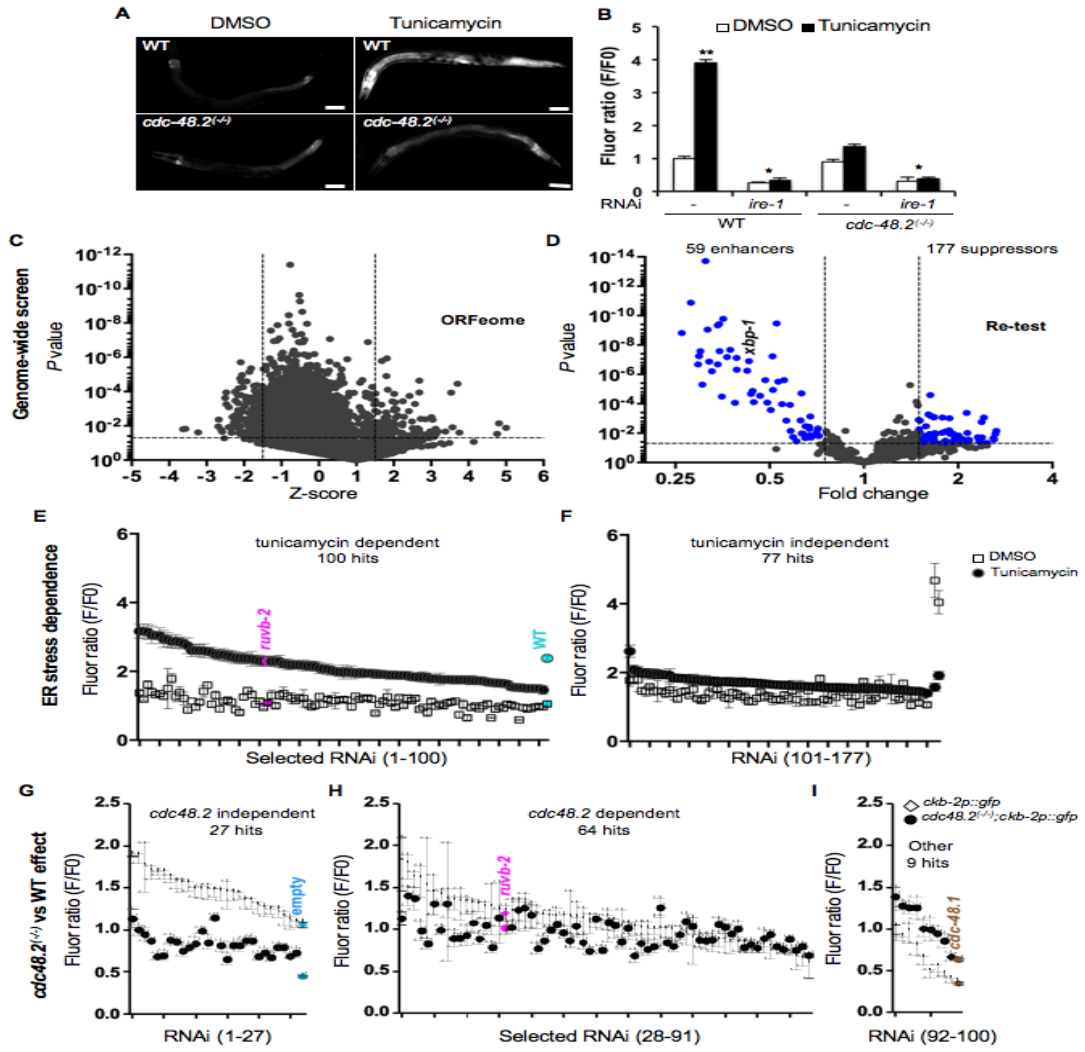


Figure 2

A

Process	Sequence Name	Gene Name	Brief description of gene product
Metabolism (lipid)	C24A11.9	<i>coq-1</i>	putative hexaprenyl pyrophosphate synthetase
Metabolism (lipid)	C49D10.4	<i>nac-10</i>	O-acyltransferase homolog
Metabolism (lipid)	Y42G9A.4	<i>mvk-1</i>	orthologous to the human gene MEVALONATE KINASE
Metabolism (lipid)	C52B9.1	<i>cka-2</i>	isoform of choline kinase
Metabolism (redox)	C29E4.7	<i>gsto-1</i>	thiol oxidoreductase and dehydroascorbate reductase
Metabolism (redox)	F56I10.3	<i>cao-1</i>	cysteine dioxygenase
Metabolism (RNA)	F25H5.6	<i>mvpl-54</i>	mitochondrial ribosomal protein, large
Metabolism (RNA)	R74.5	<i>asd-1</i>	alternative Splicing Defective
Metabolism (protein)	Y67D8C.5	<i>ccl-1</i>	Hect E3 ubiquitin ligase 1
Metabolism (protein)	F59A3.3	<i>mvpl-24</i>	Mitochondrial Ribosomal Protein, Large
Metabolism (protein)	K07C5.7	<i>tbl-15</i>	putative tubulin polyaminoacid ligase
Metabolism (protein)	M01F1.1	<i>gly-14</i>	N-acetylglucosaminyltransferase I (Gln1)
Metabolism (protein)	F26D10.10	<i>ghn-5</i>	glutamine synthetase (glutamate-ammonia ligase)
Signaling	Y4/D3B.2	<i>nlp-21</i>	neuropeptide-like proteins
Signaling	I0Z11.3	<i>srv-1b</i>	Serpentine receptor, class U
Signaling	T22B7.5	<i>srv-7</i>	serpentine receptor, class V
Signaling	ZC204.11	<i>btb-13</i>	BTB (Broad/complex/Tramtrack/Eric a brac) domain protein
Signaling	M01G12.1	<i>sri-14</i>	serpentine receptor, class I
Signaling	F45C12.10	<i>math-29</i>	MATH (meprin-associated Traf homology) domain containing
Signaling	F55A12.3	<i>ppk-1</i>	phosphatidylinositol-4-phosphate 5' kinase
Signaling	K07C5.8	<i>cash-1</i>	orthologous to Drosophila CKA and the human striatins
Signaling	C04F12.11	<i>arri-20</i>	ARRESTIN Domain protein
Signaling	F14I3.2	<i>gpl-1</i>	homologous to mammalian G protein-coupled receptor kinase in Ierac1or 1
Traffic/transport	Y48G1A.3	<i>daf-25</i>	C. elegans ortholog of mammalian Ankyr2
Traffic/transport	C06G3.2	<i>klp-18</i>	kinesin motor protein
Traffic/transport	C54C10.3	<i>pmp-3</i>	putative ABC transporter orthologous to human ABCD4
Traffic/transport	M03F8.2	<i>pst-1</i>	orthologous to the PAPS1 transporter
Transcription	K08A2.5	<i>nhr-88</i>	nuclear Hormone Receptor family
Transcription	T22D1.10	<i>ruvb-2</i>	RUVB (recombination protein) homolog

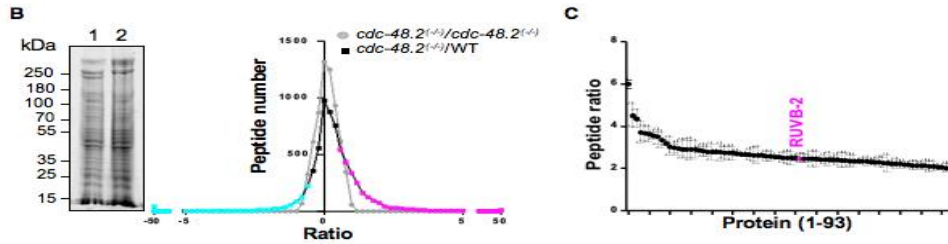


Figure 3

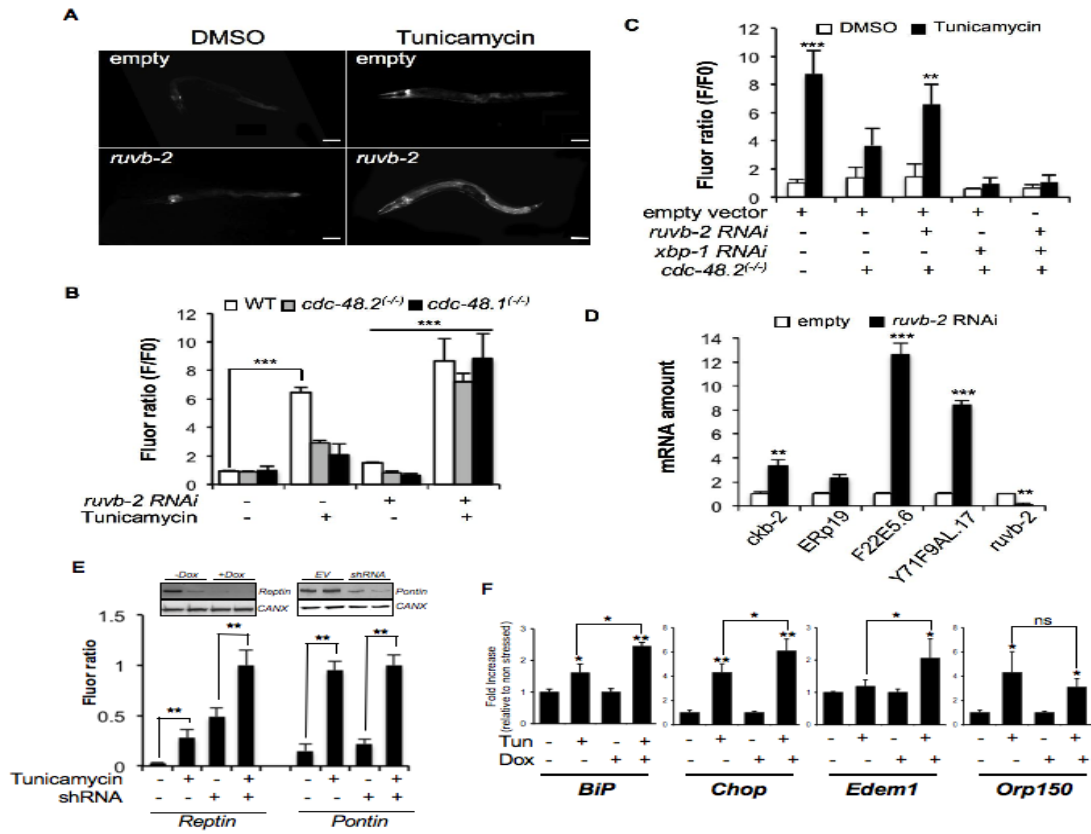


Figure 4

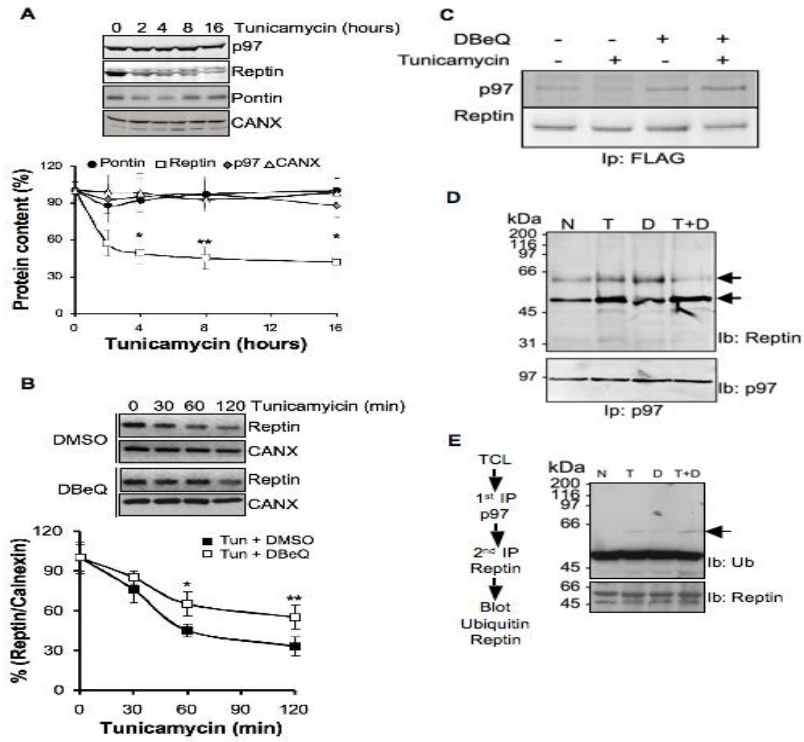
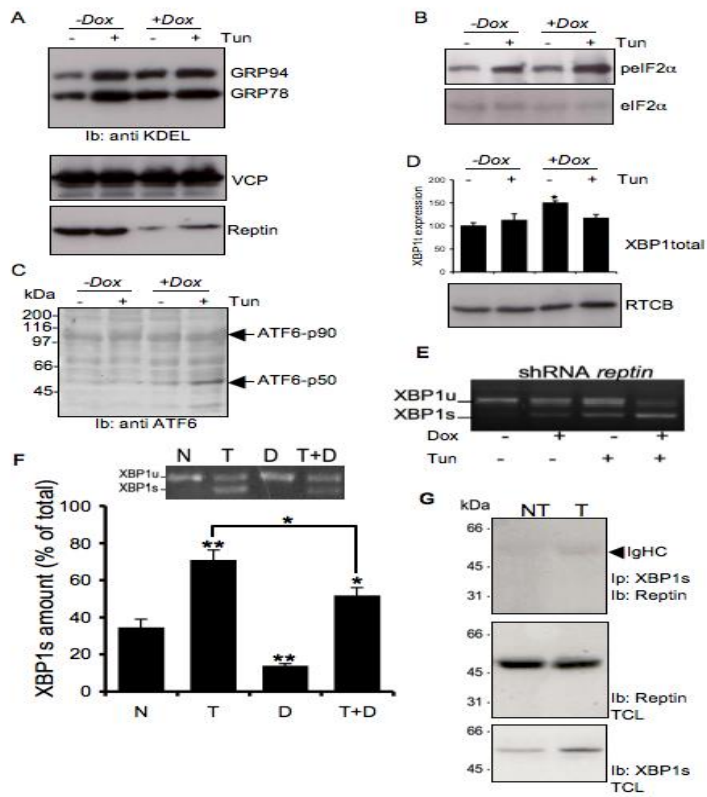


Figure 5



In the preceding paper, we have identified RUVBL2 as an evolutionary conserved repressor of certain ER stress genes including BiP, CHOP and EDEM in ER stress conditions. This repression relies at least in part on the inhibition of ATF6 activation and XBP1 mRNA splicing. Upon ER stress induction, the ubiquitin modified RUVBL2 is specifically degraded in a p97/VCP dependant manner causing an activation of the genes it normally represses. Although these results have showed that the two AAA+ ATPase (RUVBL2 and p97/VCP) are regulating ER stress genes in basal and stress conditions they raised several questions:

- What is the molecular mechanism by which p97/VCP-RuvBL2 regulates ER stress genes: do they affect histones, transcription factors, other repressors?
- How does p97/VCP-RUVBL2 affect ATF6 activation and XBP1 mRNA splicing?
- Where does this interaction take place: in the nucleus or in the cytosol?
- Could p97/VCP-RUVBL2 regulate the transcription of other genes, even beyond ER stress conditions?

To address these questions we have tried to identify other actors that are part of the regulation of ER stress genes by p97/VCP-RUVBL2. Reasoning that these actors might also be regulated by p97/VCP and ER stress like RuvBL2 is. Therefore we looked back at our screening data: the 93 proteins that accumulate under ER stress in CDC-48.2 knock out worms and check for proteins that are described to play a role in gene expression regulation. Among the proteins involve in gene expression regulation one candidate in HAD-1 the orthologue of Human HDAC1 and HDAC2 particularly caught our attention. Indeed, HDAC1 was interesting for two reasons: i) it was described to be a negative regulator of BiP in basal conditions which is removed from BiP gene upon ER stress (77) ii) HDAC1 was described to interact physically with RUVBL2 in chromatin remodelling complex (78). This suggest that HDAC1 might cooperate with RuvBL2 to regulate expression of BiP and possibly other ER stress genes. As a result, we hypothesize that a p97/VCP-RUVBL2-HDAC1 complex might exist in the cell and be responsible for the regulation of certain ER stress genes including BiP. To test this, we will first check if HDAC1 or HDAC2 are regulated by p97/VCP and under ER stress in human cells. We will then test the existence of a p97/VCP-RUVBL2-HDAC1 complex. And finally we will test the impact of this complex on the regulation of ER stress genes. The following article presents our findings.

In this study, I contribute to show that HDAC1/2 are regulated by p97/VCP and under ER stress. I showed that p97/VCP, RUVBL2 and HDAC1 are interacting together in repressor complexes. Moreover, these repressor complexes interact selectively with the major ER stress transcription factors to repress their target genes. Finally, I contribute to show that p97/VCP role of gene expression control extend to other genes including the oncogenes IGF2R and GLI1.

ARTICLE 4

A novel p97/VCP-mSin3A-HDAC1/2 complex antagonizes non-canonical activation of the Hedgehog-GLI1 pathway by Endoplasmic Reticulum stress

Kim Barroso^{1,2,5}, Luciana L. Almada³, Rachel L. O. Olson³, Holger W. Auner⁴, Rémy Pedeux^{2,5}, Martin Fernandez-Zapico^{4,*} and Eric Chevet^{2,5,*}

¹Université de Bordeaux, Bordeaux, France; ²INSERM U1242, “Oncogenesis, Stress, Signaling”, Université de Rennes 1, Rennes, France; ³Schulze Center for Novel Therapeutics, Division of Oncology Research, Mayo Clinic, Rochester, MN, USA; ⁴Imperial College London, London, UK; ⁵Centre de Lutte Contre le Cancer Eugène Marquis, Rennes, France.

Keywords: UPR, Proteasis, p97/VCP, Hedgehog, GLI1, HDACs, mSin3A

*equal contribution and correspondence to **MFZ** - Schulze Center for Novel Therapeutics, Division of Oncology Research, Mayo Clinic, Rochester, MN, USA. Email : FernandezZapico.Martin@mayo.edu and **EC** - INSERM U1242, “Oncogenesis, Stress, Signaling”, Centre de Lutte Contre le Cancer, Avenue de la bataille Flandres Dunkerque, 35042 Rennes, France. Email : eric.chevet@inserm.fr

Abstract

Upon accumulation of improperly folded proteins in the Endoplasmic Reticulum (ER), an adaptive pathway named the Unfolded Protein Response (UPR) is triggered to restore ER homeostasis. The UPR controls the expression of stress genes to increase ER folding and clearance capacities. Although the molecular events triggered by the UPR are well described, the transcriptional mechanism underlying the regulation of ER stress target genes cannot completely account for the transcriptional outputs observed. Here, we have found that p97/VCP, an AAA+ ATPase known to modulate ER stress target genes expression, dynamically interacts with RuvBL2 and the mSin3A-HDAC1/2 complex. These complexes selectively interact with the major ER stress transcription factors ATF4, ATF6, and XBP1s to control the transcription of their respective target genes. Furthermore we have identified USF2 a regulator of the GLI1 gene as a novel target of the p97/VCP-RuvBL2-mSin3A-HDAC1/2 complex. GLI1 is a known effector of the Hedgehog (Hh) signaling and is transcriptionally repressed by a RuvBL2-mSin3A-HDAC1/2 complex under basal conditions. Remarkably, upon ER stress GLI1 transcription is induced through a mechanism requiring p97/VCP-mediated extraction and degradation of RuvBL2 in an Hh-independent manner. Overall, our work demonstrates that p97/VCP controls the activation of Hh signaling upon ER stress in a ligand independent fashion and defines the interplay between the newly identified p97/VCP-mSin3A-HDAC1/2 complex and the transcription factor USF2 as an essential player in this phenomenon.

Introduction

The Endoplasmic Reticulum (ER) is the first compartment of the secretory pathway and is responsible for the folding, maturation, quality control and transport of secreted or transmembrane proteins. It therefore plays a key role in the maintenance of cellular homeostasis (1). In response to the accumulation of improperly folded proteins, a well-characterized signaling pathway, the unfolded protein response (UPR) is triggered to restore ER homeostasis by increasing protein folding and clearance capacities (2). Activation of the UPR promotes the activity of a subset of transcription factors to induce the expression of stress response genes that mainly encode for proteins involved in normal ER functions. We recently uncovered a novel role for the AAA⁺ ATPase (ATPases Associated with various cellular Activities) p97/VCP as a key player in the mediation of ER stress-induced transcriptional response (3), however the mechanisms underlying transcription regulation-mediated by this ATPase remains elusive. p97/VCP is an essential player in the control of protein homeostasis and its inactivation or loss of function have been implicated in numerous pathological states, including cancer (4). Most of p97/VCP functions were thus far linked to its segregase activity that is its capacity to disassemble and isolate a target substrate from membranes or large protein complexes, resulting in their further proteasomal degradation or recycling (5). Herein we show that p97/VCP interacts with RuvBL2-mSin3A-HDAC1/2 to regulate specific signaling complexes in a stress dependent manner. We show that p97/VCP-dependent complexes interact with specific transcription factors (ATF4, ATF6, XBP1s, USF2) and affect the expression of their respective target genes. Further analysis demonstrated that among the genes regulated by p97/VCP in an ER stress-dependent manner was GLI1, one of the main effector of Hedgehog pathway (Hh) which activation contributes to the development of several cancers (6,7). Interestingly, the regulation of GLI1 was non-canonical and independent of the Hh ligand. Our results highlight a novel signaling pathway contributing to the regulation of ER stress and leading to the non-canonical activation of the Hh pathway by p97/VCP containing complexes.

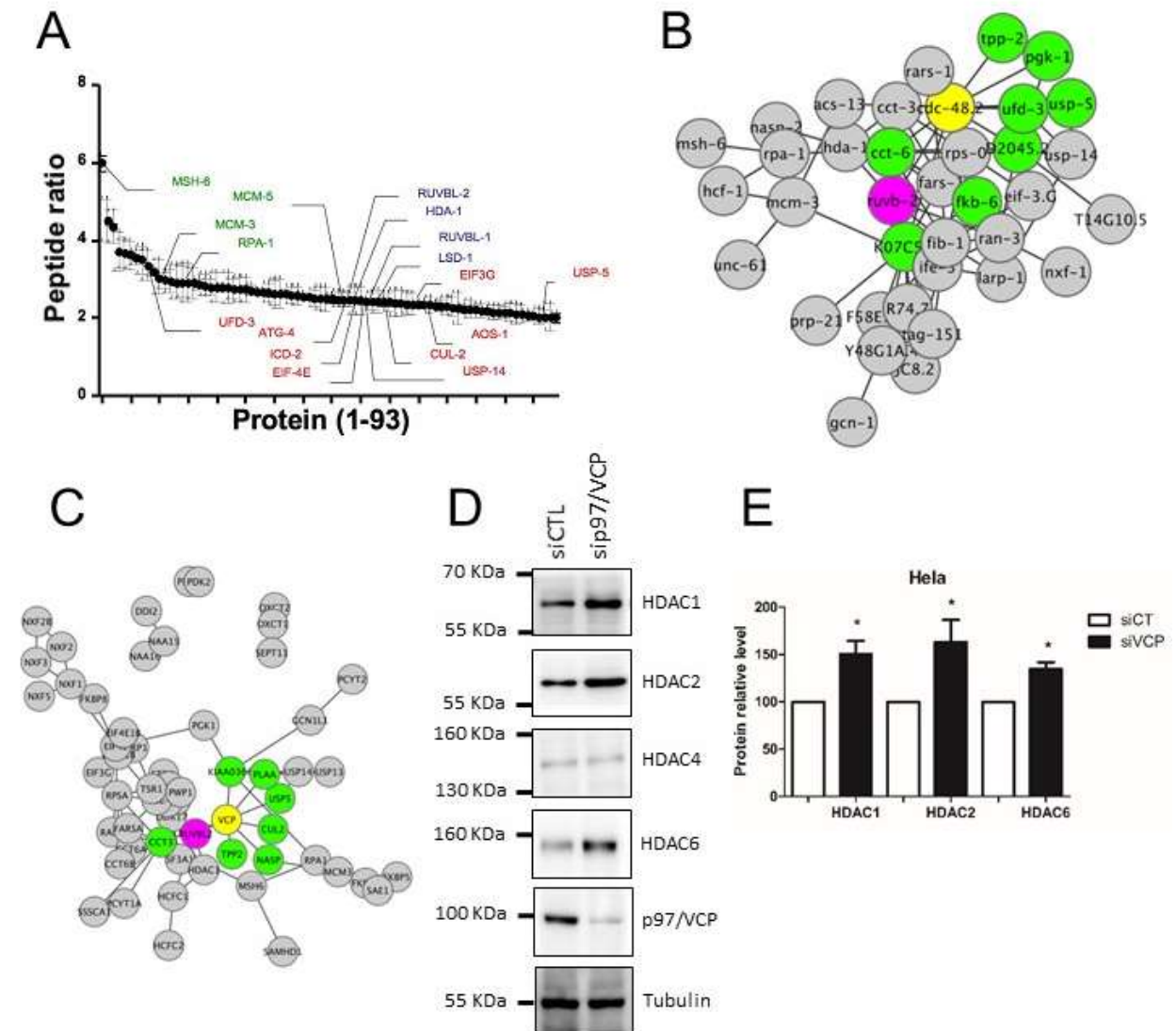
Results

p97/VCP regulates HDACs expression upon ER stress

We previously reported a repressor role of the AAA+ ATPase RuvBL2 of ER stress genes under basal non-stress conditions (3). Upon ER stress RuvBL2 is specifically degraded by a p97/VCP dependent mechanism that in turn allows the transcription of ER stress genes. We found that in *Cdc48.2* (*C. elegans* ortholog of p97/VCP) knock-out worms, key transcriptional regulators accumulated at the protein level including HAD-1 (the orthologue of HDAC1 and HDAC2) (**Fig. 1A**)(3). Further analysis of this interplay in *C. elegans* and in human cells using the STRING database, suggest that the interaction of p97/VCP with RuvBL2 might be at the center of a vast transcriptional network (**Fig. 1B-C**). To test if HDAC1 and HDAC2 were similarly regulated in human cells we transfected HeLa cells either with control (CTL) or p97/VCP siRNAs, and monitored the expression levels of several HDACs (HDAC1 to HDAC6) using immunoblotting (**Fig. 1D-E**). As observed in worms, both HDAC1 and HDAC2 accumulated in the p97/VCP knock-down cells. Similar, observations were made for HDAC6 which was not surprising as it was previously described to be regulated by p97/VCP (8), however no change in the expression of HDAC4 was observed, and HDAC3 and HDAC5 were expressed at undetectable levels in HeLa cells. Accumulation of HDAC1, HDAC2 and HDAC6 in p97/VCP-silenced cells was confirmed in other human cancer-derived cell lines including Huh7, U87, and U251 (**Fig. S1**, quantification not shown).

Figure 1: Evolutionary conserved regulation of HDACs by p97/VCP. **A.** Graph representing peptide quantity ratio ($((cdc-48.2^{(-/-)} ; ckb-2 ::gfp) / (ckb-2::gfp))$) for the 93 proteins that are more abundant in *cdc-48.2^{(-/-)}* mutant background compared to WT background (Mean s.e.m, see (3) for more details. **B.** RuvBL2 and Cdc-48 interacting network in *C. elegans*. **C.** RuvBL2 and p97/VCP interacting network in *H. sapiens*. **D.** HDAC1, HDAC2, HDAC4, HDAC6, p97/VCP (Knock Down control), Tubulin (loading control) by immunoblot in HeLa cells treated with siRNA CTL or siRNA VCP for 48h (N=3). **E.** Quantification of D, \pm SD, * = P value < 0.05.

Figure 1:

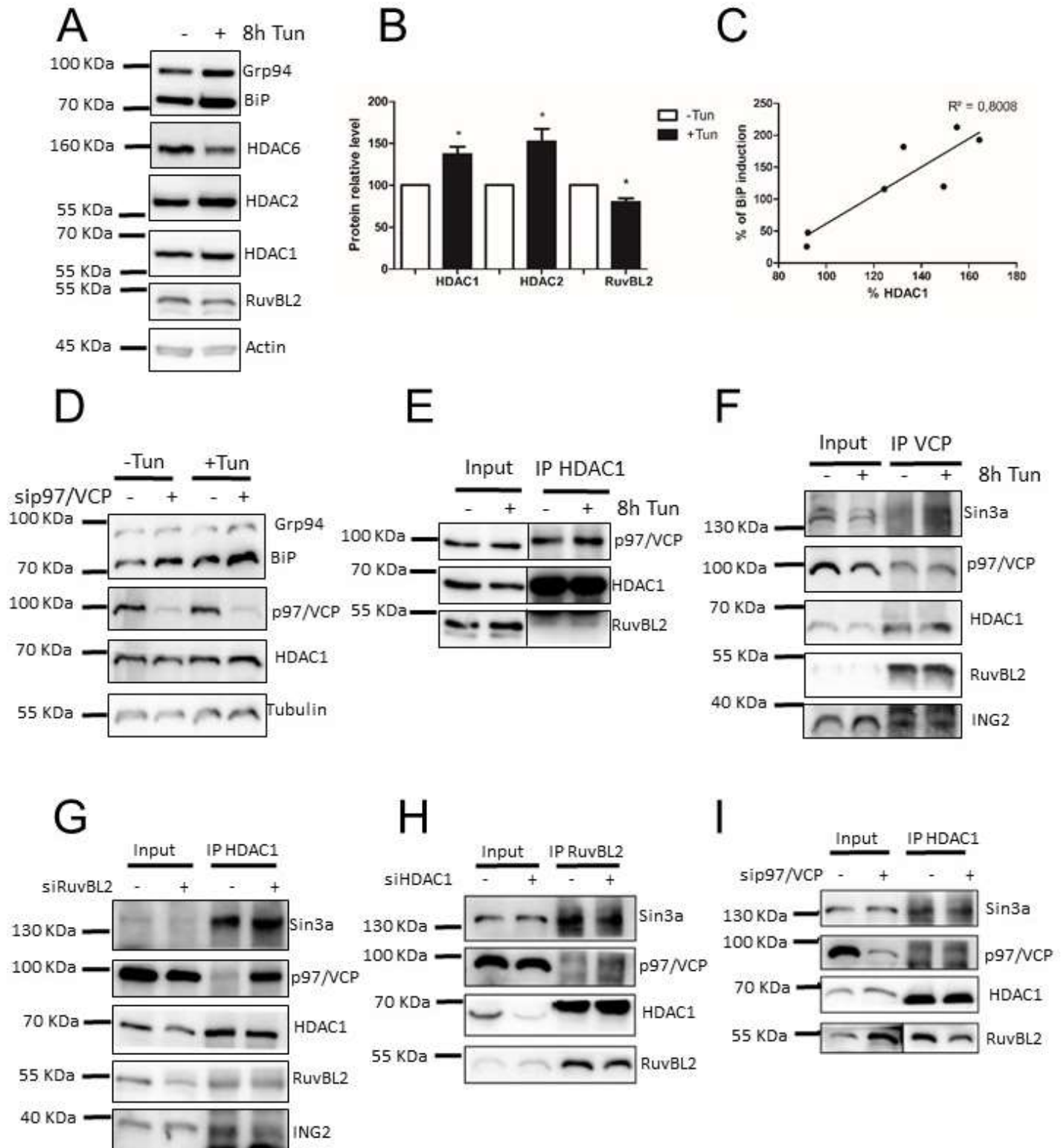


Given the role of p97/VCP in ER stress regulation we next examined if the expression of HDACs could also be regulated in a stress dependent manner by and possibly inside a complex that comprise RuvBL2. To prove existence of this signaling complex, we first tested whether the expression of proteins was regulated by p97/VCP upon ER stress. Upon treatment with the pharmacological ER stressor Tunicamycin (Tun), we found that RuvBL2 was destabilized, as previously shown, whereas HDAC1 and HDAC2 were stabilized when compared to untreated cells (**Fig. 2A-B**). Under the same experimental conditions HDAC6 expression remained unchanged (**Fig. 2A**, quantification not shown). Moreover we noticed that ER stress induction (as evaluated by monitoring BiP protein expression level) tend to correlated with HDAC1 protein level (**Fig.**

2C). We have previously shown that RuvBL2 expression was dependent on p97/VCP (3) and here using a siRNA mediated knock-down approach under basal or ER stress conditions, we confirmed that HDAC1 expression also depended on p97/VCP (**Figure 2D**). Indeed in cells subjected to ER stress and treated with sip97/VCP (**Fig 2D lane 4**), HDAC1 was further stabilized compared to cells subjected to stressed cells treated with siCTL (**Fig 2D lane 3**, quantification not shown). Together these results suggest that p97/VCP antagonizes ER stress-mediated regulation of HDAC1/2.

Figure 2: A p97/VCP-RuvBL2-HDAC1 complex is differentially regulated in basal and stress conditions. **A.** Grp94, BiP (ER stress control), HDAC6, HDAC2, HDAC1, RuvBL2, Actin (loading control) by immunoblot in untreated or exposed to tunicamycin (5µg/mL) for 8h HeLa cells (N=3). **B.** Quantification of A, ± SD, * = P value < 0.05. **C.** Correlation between BiP protein level after tunicamycin induction and HDAC1 protein level. **D.** Grp94, BiP (ER stress control), p97/VCP (Knock Down control), HDAC1, Tubulin (loading control) by immunoblot in HeLa cells treated with siRNA CTL or siRNA p97/VCP and exposed or not to tunicamycin (5µg/mL) for 8h (N=3). **E.** HDAC1 was immunoprecipitated from total protein extracts from untreated or exposed to tunicamycin (5µg/mL) for 8h HeLa cells using a specific antibody and p97/VCP, RuvBL2 association was analyzed by immunoblotting (N=3). **F.** p97/VCP was immunoprecipitated from total protein extracts from untreated or exposed to tunicamycin (5µg/mL) for 8h HeLa cells using a specific antibody and mSin3A, HDAC1, RuvBL2, ING2 association was analyzed by immunoblotting (N=3). **G.** HDAC1 was immunoprecipitated from total protein extracts from HeLa cells treated with siRNA CTL or siRNA RuvBL2 using a specific antibody and mSin3A, p97/VCP, RuvBL2, ING2 association was analyzed by immunoblotting (N=3). **H.** RuvBL2 was immunoprecipitated from total protein extracts from HeLa cells treated with siRNA CTL or siRNA HDAC1 using a specific antibody, and mSin3A, p97/VCP, HDAC1 association was analyzed by immunoblotting (N=3). **I.** HDAC1 was immunoprecipitated from total protein extracts from HeLa cells treated with siRNA CTL or siRNA p97/VCP using a specific antibody and mSin3A, HDAC1, RuvBL2 association was analyzed by immunoblotting (N=3).

Figure 2:



p97/VCP interacts with the mSin3-HDAC complex under ER stress conditions

Given the segregase role of p97/VCP, we then investigated whether this protein could interact directly with HDACs to control their expression. Using a co-immunoprecipitation approach, we found that p97/VCP co-immunoprecipitated with HDAC1, HDAC2 and HDAC6 (**Fig. S2**). The interaction between HDAC1 and p97/VCP was much stronger than that of HDAC2 or HDAC6 and as HDAC1 and HDAC2 protein levels were similar, this suggested a preferential binding of p97/VCP to HDAC1 in HeLa cells. These evidences lead us to hypothesize that a set ER stress genes could be regulated by a p97/VCP-RuvBL2-HDAC1 complex. As such we found that HDAC1 co-immunoprecipitated with both RuvBL2 and p97/VCP in HeLa cells and the interactions between p97/VCP and HDAC1 was stabilized upon ER stress (**Fig 2E**, quantification not shown). Moreover, as p97/VCP interacted with both HDAC1 and HDAC2, we also tested whether it could co-immunoprecipitate with the mSin3A chromatin remodeler complex that contain both of these HDACs (9). p97/VCP co-immunoprecipitated with mSin3A, HDAC1 and ING2 another member of the mSin3a complex (10) (**Fig. 2F**). To verify if HDAC1, RuvBL2 and p97/VCP can be part of the same complex we used a siRNA-mediated knock-down approach, reasoning that if they are part of the same complex, silencing of one member should affect the stability of the whole complex. Upon RuvBL2 knock down we found that HDAC1 and p97/VCP interaction was highly increased (**Fig. 2G**, quantification not shown). Upon HDAC1 silencing, p97/VCP and RuvBL2 interaction was enhanced and interestingly RuvBL2 appeared to form a complex with mSin3A thus suggesting that RuvBL2 not only interacted with HDAC1 in the β -Catenin repressor complex (11) but also interacted with the mSin3A complex (**Fig 2H**). Silencing p97/VCP also affected HDAC1/RuvBL2 interaction as less RuvBL2 was bound to HDAC1 in p97/VCP silenced cells (**Fig 2I**, quantification not shown). Together, these results suggested that HDAC1, RuvBL2 and p97/VCP can be found in the same complex, and that modulation of the expression level of one member of the complex affects the interaction of the two others. Moreover, p97/VCP and RuvBL2 were found associated with the mSin3A complex, a major chromatin remodeler further documenting the key role of the AAA+ ATPase in gene expression regulation. Given that p97/VCP have a plethora of cofactors, it is very likely that this protein can interact with signaling complexes other than mSin3A like RuvBL2 does to regulate gene expression.

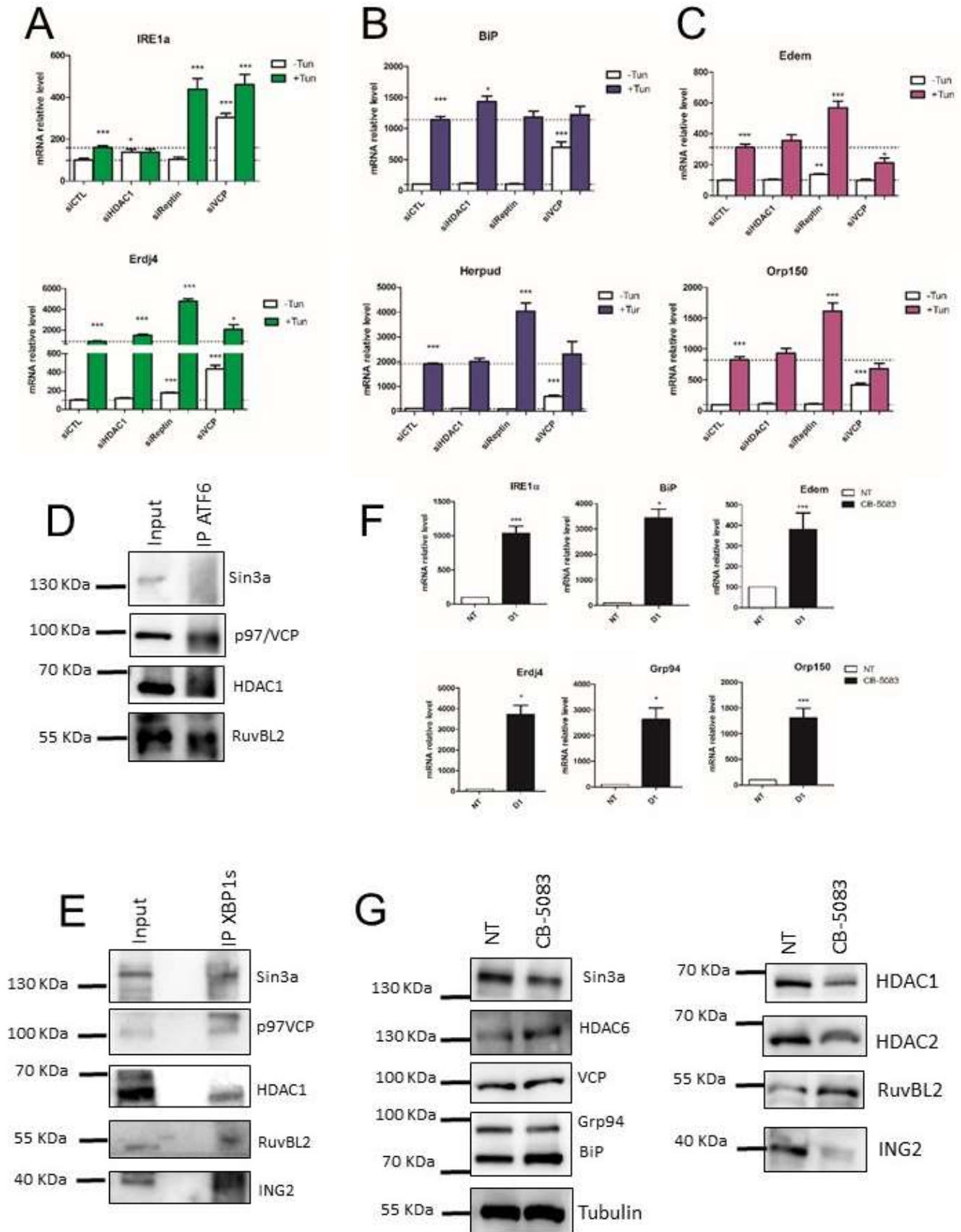
The p97/VCP-RuvBL2-HDAC signaling complex controls ER stress target genes

To further characterize the role of p97/VCP-RuvBL2-HDAC1 in the regulation of ER stress gene expression, we measured the expression of IRE1a, Erdj4, BiP, Herpud, Edem, Orp150, Grp94 and Chop mRNA using real-time PCR in HeLa cells transfected with siRNA CTL, HDAC1, RuvBL2 or p97/VCP under basal or ER stress conditions (**Fig. 3A-C and Fig. S3**). Knock-down of the members of the complex did not affect the expression of the genes tested in a similar fashion. Indeed HDAC1 knock-down had mild effect on IRE1, Erdj4 BiP and p97/VCP knock-down, described to lead to ER stress induction (19), exerted a stimulatory effect on the expression of most of the gene tested (IRE1, BiP, Erdj4, Herpud, Orp150, Grp94 and CHOP) mostly under basal conditions. Finally, RuvBL2 knock-down enhanced the induction of all genes tested but Grp94 under ER stress conditions. Although we demonstrated the existence of the p97/VCP-RuvBL2-HDAC1, these results indicate that the ER stress genes analyzed herein may not be regulated by the exact same mechanism. Interestingly, the ER stress genes whose expression was tested here are regulated by ATF4, ATF6 or XBP1s the 3 main ER stress transcription factors. We therefore hypothesized that the p97/VCP-HDAC1-RuvBL2 complex may directly control those transcription factors to regulate the transcription of their target genes. To test this, we evaluated the presence ATF4, ATF6, XBP1s in immune complexes containing p97/VCP-HDAC1-RuvBL2. We found that ATF6 and ATF4 co-immunoprecipitated with p97/VCP-HDAC1-RuvBL2 (**Fig. 3D and Fig. S4**) but not with mSin3A whereas XBP1s co-immunoprecipitated with p97/VCP-HDAC1-RuvBL2 and mSin3A (**Fig 3E**). This result was confirmed by the fact that only XBP1s co-immunoprecipitated with ING2, another member of the mSin3A complex. These data suggested that ER stress-induced transcription factors are differentially incorporated in select transcription complexes: ATF6 and ATF4 are regulated a by p97/VCP-RuvBL2-HDAC1 but not mSin3A whereas XBP1s is regulated by the mSin3a-p97/VCP-RuvBL2 complex. Moreover, this result also indicated that RuvBL2 and HDAC1 interact in different complexes in the cell as suggested in Kim et al., 2006 (12). To further prove the role of p97/VCP in the regulation of ER stress-induced gene transcription, we used the pharmacological p97/VCP inhibitor (CB-5083, 13) and monitored the induction of the above referenced ER stress genes using real time PCR (**Fig. 3F**). As expected, a strong induction of all the ER stress target genes tested was observed that was even stronger than that caused by p97/VCP siRNA silencing (**Fig. 3 A-C**). We then evaluated the impact of p97/VCP pharmacological inhibition on the protein levels of the different members of the complexes using immunoblot (**Fig.**

3G, quantification not shown). Interestingly, all member of the mSin3A complex: mSin3A, HDAC1, HDAC2, ING2 showed a decreased of protein level whereas HDAC6 and RuvBL2 were stabilized upon CB-5083 treatment. Overall, these results indicate that genetic or pharmacological inhibition of p97/VCP, which cause a diminution in protein level or inhibition of ATPase activity respectively have different effects on the stability of the signaling complex and as a result on ER stress target gene expression.

Figure 3: p97/VCP-RuvBL2-HDAC1 regulates ER stress genes through regulation of their transcription factors **A.** RT-qPCR analysis of two ATF6 targets (IRE1, Erdj4) in siRNA CTL, siRNA HDAC1, siRNA RuvBL2 or siRNA p97/VCP conditions with or without tunicamycin treatment (5µg/mL for 8h); ± SD; *, **, *** = P value < 0.05, 0.005, 0.001 respectively (N=3). **B.** Same experiment as in A for two XBP1s targets (BiP, Herpud1) **C.** Same experiment as in A for two ATF6 and XBP1s cooperative targets (Edem1, Orp150). **D.** ATF6 was immunoprecipitated from total protein extracts from HeLa cells exposed to tunicamycin (5µg/mL) for 8h using a specific antibody and mSin3A, p97/VCP, HDAC1, RuvBL2 association was analyzed by immunoblotting (N=3). **E.** XBP1s was immunoprecipitated from total protein extracts from HeLa cells exposed to tunicamycin (5µg/mL) for 8h using specific a antibody and mSin3A, p97/VCP, HDAC1, RuvBL2, ING2 association was analyzed by immunoblotting (N=3). **F.** RT-qPCR analysis of six ER stress genes (IRE1, Erdj4, BiP, Herpud, Edem, Orp150) target of ATF6 and/or XBP1s in HeLa cells treated with 1 µM of CB-5083 for 16h; ± SD; *, **, *** = P value < 0.05, 0.005, 0.001 respectively (N=3). **G.** mSin3a, HDAC6, p97/VCP, Grp94, BiP, HDAC1, HDAC2, RuvBL2, ING2, Tubulin (loading control) by immunoblot in untreated or exposed to 1 µM of CB-5083 for 16h HeLa cells (N=3).

Figure 3:



p97/VCP and USF2 regulate GLI1 mRNA expression

To identify additional genes that could be regulated by p97/VCP-dependent complexes, we tested the expression of 8 cancer relevant genes that were also deregulated at the transcriptional level in the p97/VCP-associated disease, inclusion body myopathy (14). To this end, the expression of BRCA1, EGFR, FGF2, FGF7, FGF12, GLI1, IGF2R and TGF α mRNA was investigated in HeLa cells silenced for p97/VCP or not using real time PCR (**Fig. 4A and Fig. S5**). This showed that IGF2R and GLI1 were upregulated in p97/VCP silenced HeLa cells while other genes were not significantly altered (**Fig. 4A**). Mutation or loss of heterozygosity of IGF2R has been associated with risk of hepatocellular carcinoma whereas GLI1 is one of the main transcription factors of the Hedgehog (Hh) pathway which activation has been implicated in the development of various cancers (6, 7). The p97/VCP-dependent regulation of IGF2R and GLI1 expression was conserved in various cancer derived cell lines including Huh7 and U87 thus suggesting a common mechanism of regulation (**Fig. S6**). We then hypothesized that this transcriptional upregulation could be the result of the stabilization of a transcription factor and therefore we tested transcription factors described in the literature to regulate IGF2R and/or GLI1 (15). We found that in p97/VCP silenced cells USF2 expression was enhanced whereas that of HSF1 remained unchanged (**Fig. 4B-C**). To test if USF2 controls the expression of GLI1 and IGF2R in HeLa cells, we silenced USF2 and measured the expression of GLI1 and IGF2R mRNA using real time PCR (**Fig. 4D and Fig. S6**). In USF2 knock-down cells the expression of GLI1 was decreased whereas that of IGF2R was unexpectedly increased (**Fig. 4D and Fig. S7**). These results suggest that USF2 stabilization in p97/VCP knock-down cells can explain GLI1 upregulation but not that of IGF2R. We then tested if, like other ER stress transcription factors (ATF4, ATF6, XBP1s), USF2 interacted with p97/VCP-dependent complexes (**Fig. 4E-F**). Interestingly, USF2, as for XBP1s, was found to interact with p97/VCP, HDAC1, RuvBL2 and mSin3A (**Fig 4F**). Together these results suggest that the GLI1 gene is regulated by USF2, which is itself regulated by the p97/VCP-RuvBL2-HDAC1-mSin3A complex.

Figure 4:

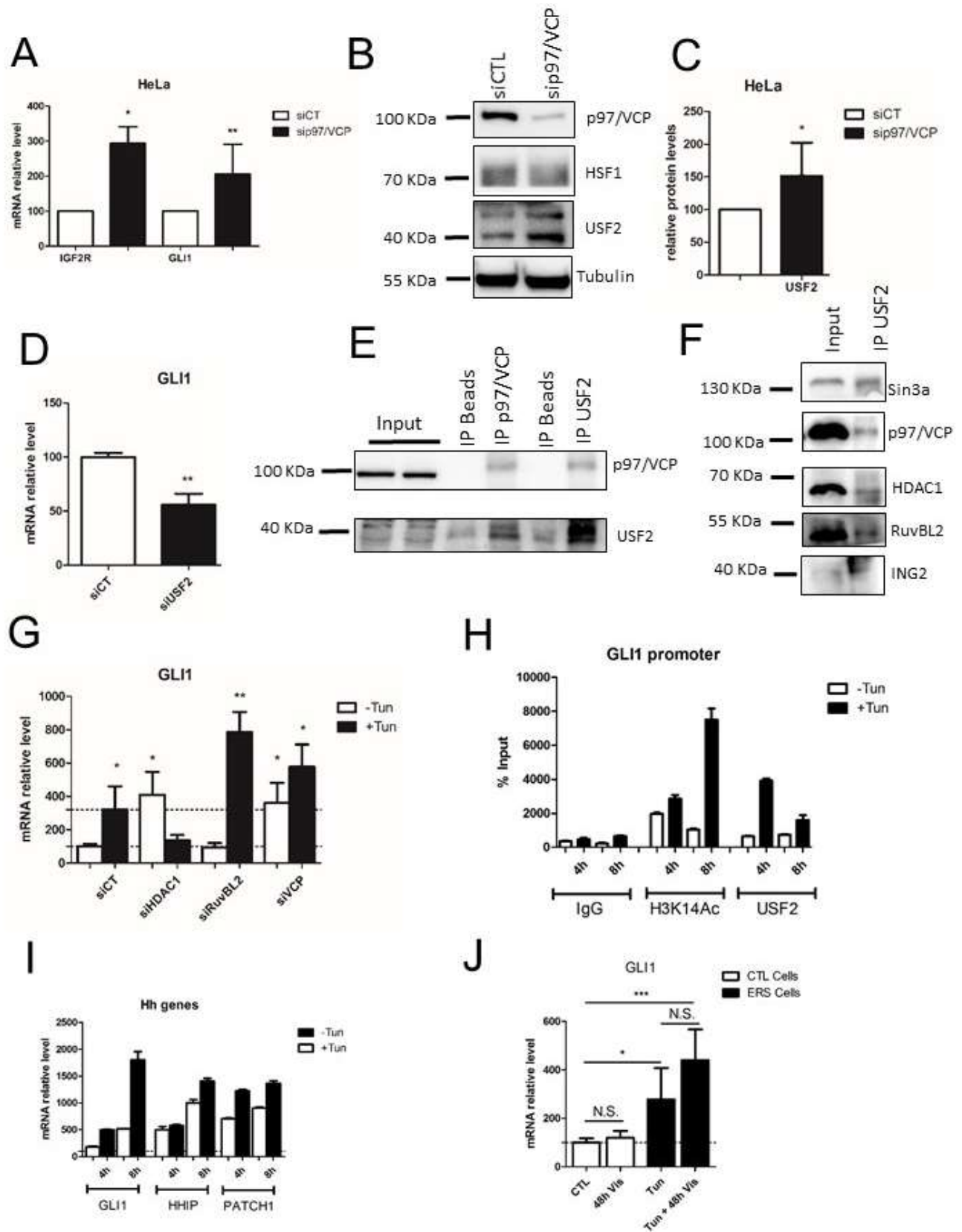


Figure 4: GLI1 is regulated by p97/VCP and ER stress. **A.** RT-qPCR analysis of IGF2R and GLI1 in HeLa cells treated with siRNA CTL or siRNA p97/VCP; \pm SD; *, ** = P value < 0.05, 0.005 respectively (N=3). **B.** p97/VCP (Knock Down control), HSF1, USF2, Tubulin (loading control) by immunoblot in HeLa cells treated with siRNA CTL or siRNA p97/VCP (N=3). **C.** Quantification of B; \pm SD; * = P value < 0.05. **D.** RT-qPCR analysis of GLI1 in HeLa cells treated with siRNA CTL or siRNA USF2; \pm SD; ** = P value < 0.005 (N=3). **E.** p97/VCP or USF2 were immunoprecipitated from total protein extracts from HeLa using specific antibodies and p97/VCP, USF2 association was analyzed by immunoblotting (N=3). **F.** USF2 was immunoprecipitated from total protein extracts from HeLa using a specific antibody and mSin3a, p97/VCP, HDAC1, RuvBL2, ING2 association was analyzed by immunoblotting (N=3). **G.** RT-qPCR analysis of GLI1 in siRNA CTL, siRNA HDAC1, siRNA RuvBL2 or siRNA p97/VCP conditions with or without tunicamycin treatment (5 μ g/mL for 8h); \pm SD; *, ** = P value < 0.05, 0.005 respectively (N=3). **H.** Binding of USF2 to the GLI1 promoter (site 1) and change of Acetylated H3K14 (H3K14 ac) was tested by ChIP under basal conditions, or after 4h or 8h of tunicamycin (5 μ g/mL); \pm SD; (N=2). **I.** RT-qPCR analysis of three Hh genes (GLI1, Hhip, Patch1) in HeLa cells under basal conditions, or after 4h or 8h of tunicamycin (5 μ g/mL) \pm SD; (N=3). **J.** RT-qPCR analysis of GLI1 in HeLa cells treated with or without tunicamycin (5 μ g/mL) for 8h and with or without Visomegib (1 μ M) for 48h; \pm SD; *, *** = P value < 0.05, 0.001 respectively (N=3).

ER stress induces the expression of GLI1 through a non-canonical mechanism

We showed that the p97/VCP-HDAC1-RuvBL2-mSin3a signaling complex regulates USF2 but because members of the complex are regulated upon ER stress, we then hypothesized that ER stress might affect USF2 stability or binding to the GLI1 promoter and therefore control GLI1 expression most likely in a Hh-independent manner. We thus tested this hypothesis using real time PCR and our result showed that beyond p97/VCP knock-down, tunicamycin-induced ER stress, HDAC1 knock-down under basal conditions or RuvBL2 upon ER stress also affected the expression GLI1 mRNA (**Fig. 4G**). These results suggest that all the members of the p97/VCP-HDAC1-RuvBL2 complex are important for the regulation of the GLI1 mRNA. To explain how ER stress induces GLI1 mRNA expression we performed Chromatin ImmunoPrecipitation (ChIP) on the GLI1 promoter where *in silico* analysis predicted a potential USF2 binding site. Our results showed that upon ER stress, USF2 was recruited to the GLI1 promoter (**Fig 4H**). Moreover, ER stress-mediated

USF2 recruitment to the GLI1 promoter was accompanied by an increase in acetylation of histone 3 lysine 14 (acetyl H3K14) a histone mark generally associated with transcriptional activation (16, **Fig 4H**). These results suggest that under basal conditions the removal or lack of acetylation at the GLI1 gene cause its repression, however upon ER stress the GLI1 promoter is acetylated and USF2 recruited and as a consequence the gene is activated. Therefore our next step was to evaluate whether GLI1 target genes in the Hh pathway were also induced by ER stress (**Fig 4I**). Our result showed all the Hh tested genes, namely Hhip, Patch, (and Notch1 not shown) were induced upon ER stress (**Fig 4I**, quantification not shown). To confirm that this activation was independent of the Hedgehog ligand we used Vismodegib a pharmacological inhibitor that prevent activation of the Hh pathway by binding to the smoothed receptor (SMO)(**Fig 4J**). After 48h of treatment with Vismodegib GLI1 mRNA expression was not significantly altered compared to control (Bar1 vs Bar2 **Fig 4J**) or ER stressed cells, respectively (Bar3 vs Bar 4 **Fig 4J**). This suggests that GLI1 induction during ER stress is independent of the Hh ligand. Together these results suggest GLI1 is regulated at least in part by acetylation and that ER stress or change in protein level of the member of the complex p97/VCP-RuvBL2-HDAC1 are sufficient to induce its expression independently of the ligand. As a possible consequence of GLI1 overexpression during ER stress we found that its target genes in the Hh pathway (Hhip, Patch1) are also induce.

Discussion

In this study we have shown that p97/VCP can interact in a stress dependent manner with signaling complexes that include HDAC1, RuvBL2 and transcription factors to control gene expression of ER stress genes and GLI1. These results have allowed us to propose a model (**Fig. 5**) in which under basal conditions, GLI1 and ER stress genes are repressed by deacetylation mediated by chromatin remodeler complexes that contain RuvBL2-HDAC1 with or without mSin3A. Deacetylation of the promoter renders it inaccessible for the transcription machinery and therefore prevents the transcription of the target genes. The fact that mSin3A can interact with transcription factors to be recruited at specific chromatin site is well established (17). This suggest that under basal conditions these repressor complexes could be recruited at GLI1 or ER stress gene promoters by interacting with the corresponding transcription factors: USF2, ATF4, ATF6 or XBP1s. Moreover, under basal conditions p97/VCP is mainly located in the cytosol, however like described by Wang et al. (18) upon ER stress it translocate to the nucleus, an observation that we also found

in our experimental system (**Fig. S8**). In the nucleus, p97/VCP is able to bind to the ubiquitinated RuvBL2 and to induce its extraction from the repressor complexes thereby leading to their inactivation and the subsequent increase in acetylation. This mechanism would then allow the chromatin to be in an open state, a favorable condition for transcription activation. As GLI1 is one of the main effector of the Hh pathway, its activation upon ER stress might therefore lead to the non-canonical activation of the Hh pathway, which could play a role in adaptation to stress. Overall, in our model GLI1 and ER stress genes are turned off by deacetylation under basal conditions, but upon ER stress they are turned on through a p97/VCP-dependent mechanism that acts as a molecular switch by inactivating repressor complexes.

Although p97/VCP silencing lead to the stabilization of RuvBL2 and HDACs, it is also known to cause ER stress and to induce the expression of ER stress genes (19). In the context of our model, this suggests that either the remaining pool of p97/VCP is sufficient to induce ER stress genes and/or the cell can induce these genes by other mechanisms independent of p97/VCP-mediated extraction of ubiquitinated-RuvBL2. As siRNA mediated knockdown (or pharmacological inhibition) are never fully effective it is conceivable that the remaining pool of p97/VCP is preferentially translocated to the nucleus where it could exert its functions towards RuvBL2 and thus induce transcription of ER stress genes. Additionally, in this scenario the lack of cytosolic p97/VCP could impair the degradation of RuvBL2 by the proteasome and explain its stabilization (**Fig. S8**). Example of such an alternative mechanism has been described in yeast as the degradation of most nuclear ubiquitinated proteins is mediated by the ubiquitin protein ligase San1. Moreover, Gallagher et al. showed that although San1 and p97/VCP orthologue CDC-48 have common substrates, CDC-48 is not universally required for the degradation of ubiquitinated nuclear proteins. Similarly, GLI1 is also induced upon p97/VCP silencing, although the mechanism described above might explain this phenotype, it is also possible that the accumulation of activators is responsible for GLI1 induction. Indeed, Smad2/3 are other transcription factors known to regulate GLI1 and they were recently demonstrated to interact with p97/VCP on chromatin regions corresponding to other genes (20). As a result it is probable that accumulation of USF2 and Smad2/3 is sufficient to induce GLI1 expression in p97/VCP-silenced cells.

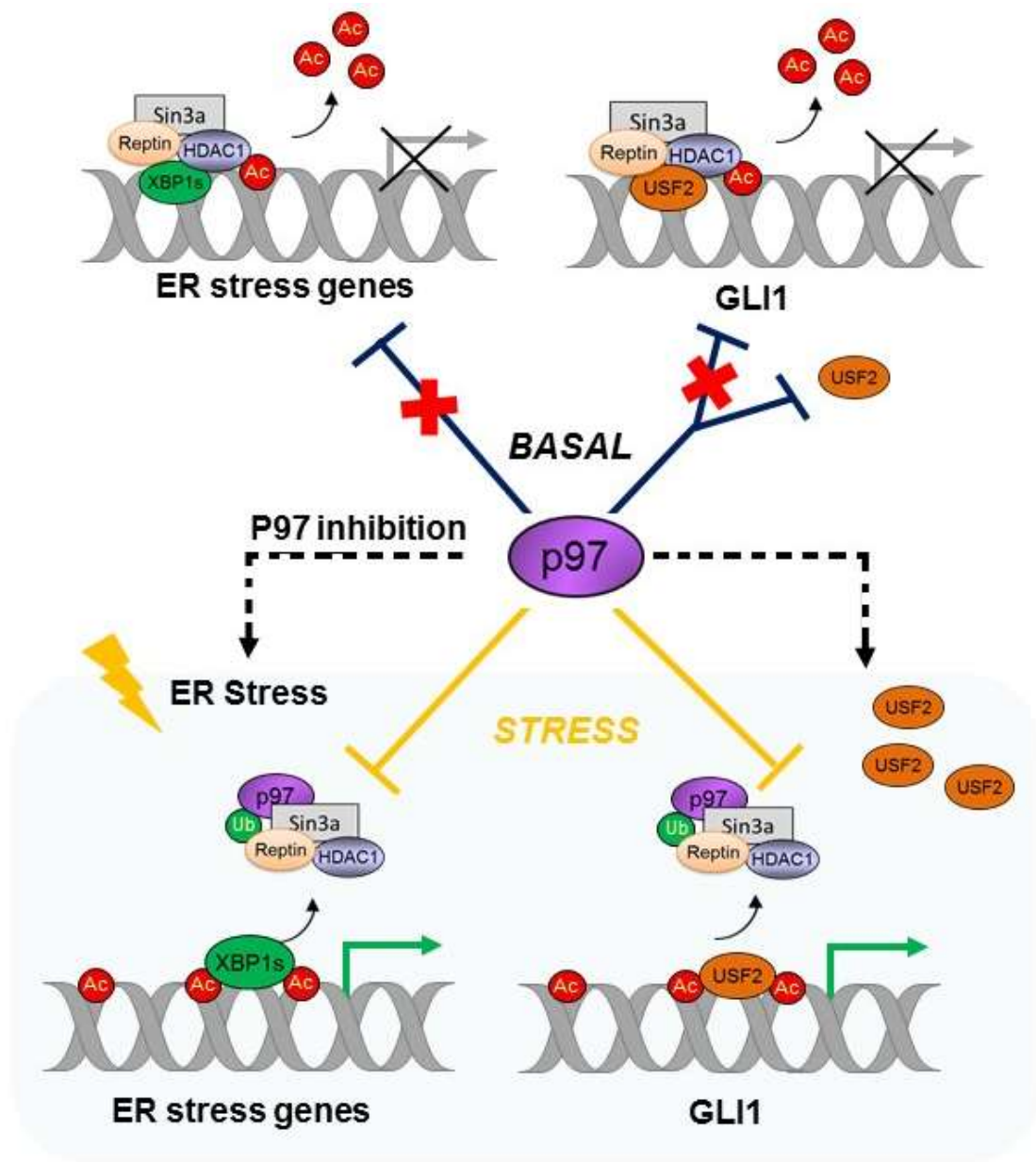


Figure 5: Model of GLI1 and ER stress genes regulation mediated by p97/VCP. Under basal conditions, RuvBL2-mSin3A-HDAC1 repress the expression of GLI1 and ER stress genes. Upon ER stress p97/VCP act as a molecular switch and inhibits the repressor complexes by promoting degradation of ubiquitinated RuvBL2 thus activating GLI1 and ER stress genes. Non-canonical activation of GLI1 leads to the expression of its target Hedgehog genes. Upon ER stress, p97/VCP can bound to ubiquitinated RuvBL2 to extract repressor complexes (RuvBL2-HDAC1 or RuvBL2-mSin3A), the target genes are then accessible for the transcription machinery.

Overall our work shows that p97/VCP is a molecular switch able to induce ER stress gene expression by inhibiting repressor complex upon ER stress. We have discovered that this mechanism is not exclusive to ER stress genes. Indeed, ER stress also leads to the non-canonical activation GLI1 in a p97/VCP and the RuvBL2-mSin3a-HDAC1/2 complex-dependent manner and as a consequence activation of Hh genes. As p97/VCP inhibition was described to induce Epithelial to Mesenchymal Transition (EMT)-like phenotypes (21) and also to contribute to the activation of pro-oncogenic genes (GLI1, the present study), we have to take into careful consideration the use of p97/VCP pharmacological inhibitors in neoplastic diseases.

References:

- 1 Hetz C, Chevet E, Oakes SA. Proteostasis control by the unfolded protein response. *Nat Cell Biol.* 2015;17(7):829-38.
- 2 Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science.* 2011;334(6059):1081-6.
- 3 Marza E, Taouji S, Barroso K, Raymond AA, Guignard L, Bonneu M, et al. Genome-wide screen identifies a novel p97/CDC-48-dependent pathway regulating ER-stress-induced gene transcription. *EMBO Rep.* 2015.
- 4 Fessart D, Marza E, Taouji S, Delom F, Chevet E. P97/CDC-48: Proteostasis control in tumor cell biology. *Cancer Lett.* 2013;337(1):26-34.
- 5 Meyer H, Wehl CC. The VCP/p97 system at a glance: connecting cellular function to disease pathogenesis. *J Cell Sci.* 2014;127(Pt 18):3877-83.
- 6 Oka Y, Waterland RA, Killian JK, Nolan CM, Jang HS, Tohara K, et al. M6P/IGF2R tumor suppressor gene mutated in hepatocellular carcinomas in Japan. *Hepatology.* 2002;35(5):1153-63.
- 7 Carpenter RL, Lo HW. Hedgehog pathway and GLI1 isoforms in human cancer. *Discov Med.* 2012;13(69):105-13.
- 8 Boyault C, Gilquin B, Zhang Y, Rybin V, Garman E, Meyer-Klaucke W, et al. HDAC6-p97/VCP controlled polyubiquitin chain turnover. *EMBO J.* 2006;25(14):3357-66.
- 9 Fleischer TC, Yun UJ, Ayer DE. Identification and characterization of three new components of the mSin3A corepressor complex. *Mol Cell Biol.* 2003;23(10):3456-67.
- 10 Shi X, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, et al. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature.* 2006;442(7098):96-9.
- 11 Rashid S, Pilecka I, Torun A, Olchowik M, Bielinska B, Miaczynska M. Endosomal adaptor proteins APPL1 and APPL2 are novel activators of beta-catenin/TCF-mediated transcription. *J Biol Chem.* 2009;284(27):18115-28.
- 12 Kim JH, Choi HJ, Kim B, Kim MH, Lee JM, Kim IS, et al. Roles of sumoylation of a reptin chromatin-remodelling complex in cancer metastasis. *Nat Cell Biol.* 2006;8(6):631-9.
- 13 Anderson DJ, Le Moigne R, Djakovic S, Kumar B, Rice J, Wong S, et al. Targeting the AAA ATPase p97 as an Approach to Treat Cancer through Disruption of Protein Homeostasis. *Cancer Cell.* 2015;28(5):653-65.
- 14 Nalbandian A, Ghimbovschi S, Radom-Aizik S, Dec E, Vesa J, Martin B, et al. Global gene profiling of VCP-associated inclusion body myopathy. *Clin Transl Sci.* 2012;5(3):226-34.

15 Villavicencio EH, Yoon JW, Frank DJ, Fuchtbauer EM, Walterhouse DO, Iannaccone PM. Cooperative E-box regulation of human GLI1 by TWIST and USF. *Genesis*. 2002;32(4):247-58.

16 Koch CM, Andrews RM, Flicek P, Dillon SC, Karaoz U, Clelland GK, et al. The landscape of histone modifications across 1% of the human genome in five human cell lines. *Genome Res*. 2007;17(6):691-707.

17 Dannenberg JH, David G, Zhong S, van der Torre J, Wong WH, Depinho RA. mSin3A corepressor regulates diverse transcriptional networks governing normal and neoplastic growth and survival. *Genes Dev*. 2005;19(13):1581-95.

18 Wang T, Xu W, Qin M, Yang Y, Bao P, Shen F, et al. Pathogenic Mutations in the Valosin-containing Protein/p97(VCP) N-domain Inhibit the SUMOylation of VCP and Lead to Impaired Stress Response. *J Biol Chem*. 2016;291(27):14373-84.

19 Chou TF, Brown SJ, Minond D, Nordin BE, Li K, Jones AC, et al. Reversible inhibitor of p97, DBE1, impairs both ubiquitin-dependent and autophagic protein clearance pathways. *Proc Natl Acad Sci U S A*. 2011;108(12):4834-9.

20 Ndoja A, Cohen RE, Yao T. Ubiquitin signals proteolysis-independent stripping of transcription factors. *Mol Cell*. 2014;53(6):893-903.

21 Shah PP, Beverly LJ. Regulation of VCP/p97 demonstrates the critical balance between cell death and epithelial-mesenchymal transition (EMT) downstream of ER stress. *Oncotarget*. 2015;6(19):17725-37.

22 Lo Re, A. E., Fernandez-Barrena, M. G., Almada, L. L., Mills, L., Elswa, S. F., Lund, G., Ropolo, A., Molejon, M. I., Vaccaro, M. I., and Fernandez-Zapico, M. E. A novel AKT1-GLI3-VMP1 pathway mediates Kras-induced autophagy in cancer cells. *J. Biol. Chem*. 2012; 287:25325–25334.

Materials and Methods

Antibodies, Cell lines and Reagent - Antibody against B-Actin and Tubulin were from Sigma (A2228, T5168), antibody against p97/VCP were from Proteintech (60316-1) and Progen (65278), antibody against RuvBL2 and ATF6 were from Abcam (ab137834, ab37149), antibody against HDAC1, HDAC2, HDAC3, HDAC4, HDAC5 and HDAC6 were from cell signaling (9928), antibody against USF2, mSin3A and ATF4 were from Santa Cruz (sc-862, sc-5299,sc-200), antibody against HSF1 and KDEL were from Enzo life science (ADI SPA 901 F, ADI SPA 827), antibody against ING2 and XBP1s were homemade. Tunicamycin (Tun) was from Calbiochem (EMD Biosciences, Inc., Darmstadt, Germany). Vismodegib (Vis) was from Selleckchem (GDC-0449). CB-5083 was a kind gift of Dr Holger Auner. All cell lines used in this study (HeLa, U87, U251, Panc1, HuH7) were cultured in DMEM from life technologies (41965) supplemented with 10% FBS from Sigma-Aldrich (12003C). All cell cultures were maintained in a 37°C incubator containing 5% CO₂.

Real Time-Quantitative PCR - Total RNA was extracted using Trizol reagent (Invitrogen). For the Reverse Transcription 2µg of mRNA were used with the Maxima Reverse Transcriptase from Life technologies (ER0741). For quantitative RT-PCR (RT-qPCR) all reactions were conducted using the SYBR qPCR Premix Ex Taq from Ozyme (TAKRR420W) and the QuantStudio 5 thermocycler (Life technologies). For analysis each sample was normalized to Gapdh and/or Actin mRNA. All primers used are listed in the Table S1

Small interfering RNA (siRNA) - All siRNA used in this study are listed in the Table S2. siRNAs were transfected into the cells by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols. Cells were used for the experiments 48h post-transfection.

Co-immunoprecipitation assay - After a cold PBS wash cell were lysed with lysis buffer containing 30 mM Tris-HCL, pH 7,5, 150 mM NaCl and 1,5% CHAPS (Calbiochem) and Protease and Phosphatase inhibitors were from Roche (05892 791 001, 04 906 837 001). Supernatant were recovered following centrifugation at 13,000 rpm for 15 mins at 4°C and incubated overnight with the adequate antibody. Magnetic beads (Life technologies 100006D, 100007D) after 3 washes in the lysis buffer were added to the immune complexes for 20 mins at RT with gentle rotation

followed by washing 3 times in the lysis buffer. Labeled proteins were resolved on 10% polyacrylamide gels.

Statistical analysis - All data were analyzed by Student's t-test. The GraphPad Prism 5 software was used for statistical analysis.

Interacting Network – p97/VCP and RuvBL2/Reptin network were established using the STRING database and represent using the Cytoscape software.

Chromatin Immunoprecipitation - ChIP was conducted as previously described (22). Briefly, 4 and 8 hours after Tunicamycin treatment, HeLa cells (15×10^6) were cross-linked with 1% formaldehyde, followed by cell lysis. DNA was sheared using a Bioruptor 300 (Diagenode, Denville, NJ) to fragment DNA to ~600 bp. Aliquots of the sheared chromatin were then immunoprecipitated using magnetic beads and a H3K14Ac antibody (Millipore) or USF2 (Santa Cruz) or a normal rabbit IgG (Abcam). Following immunoprecipitation, cross-links were removed, and immunoprecipitated DNA was purified using spin columns and subsequently amplified by quantitative PCR. PCR primers were designed to amplify regions of the GLI1 promoter containing potential USF2 binding sites. The sequences of the primers are as follows: sense, TGAGGGAGGATGCTTAGGGG; antisense, GGTCAAGAGATTGAGACCATCC. Samples for quantitative SYBR PCR were performed in triplicate using the C1000 Thermal Cycler (Bio-Rad, Hercules, CA). Results are represented as Percentage of Input.

Acknowledgements

This work was supported by grants from the Institut National du Cancer (INCa, PLBIO 2012, 2014) and la Ligue Contre le Cancer to EC. KB was funded by a grant from La Ligue Contre le Cancer.

Supplementary Material

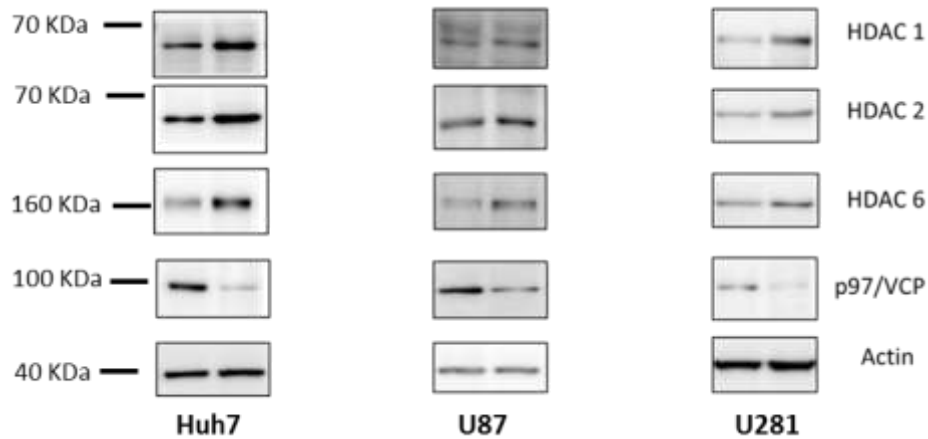


Figure S1: Analysis of the expression of HDAC1, HDAC2, HDAC6, p97/VCP (Knock Down control), Actin (loading control) by immunoblot in Huh7, U87 and U251 cells treated with siRNA CTL or siRNA p97/VCP for 48h (N=3).

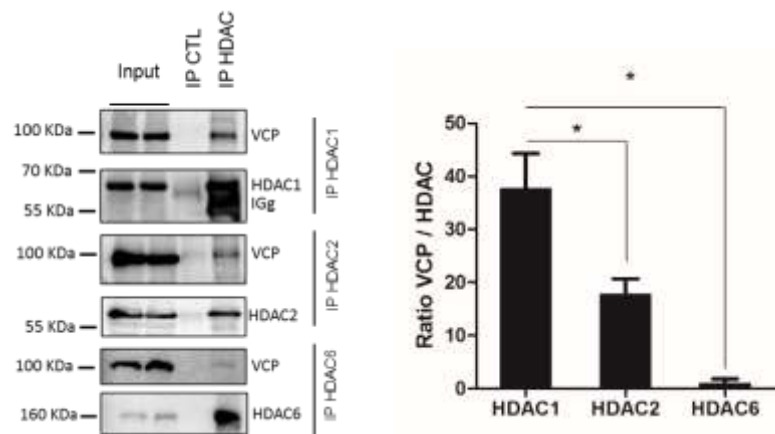


Figure S2: HDAC1, HDAC2 or HDAC6 were immunoprecipitated from total protein extracts using specific antibodies and p97/VCP association was analyzed by immunoblotting and quantification of the ration between p97/VCP and the corresponding HDAC, \pm SD; * = P value < 0.05 (N=3).

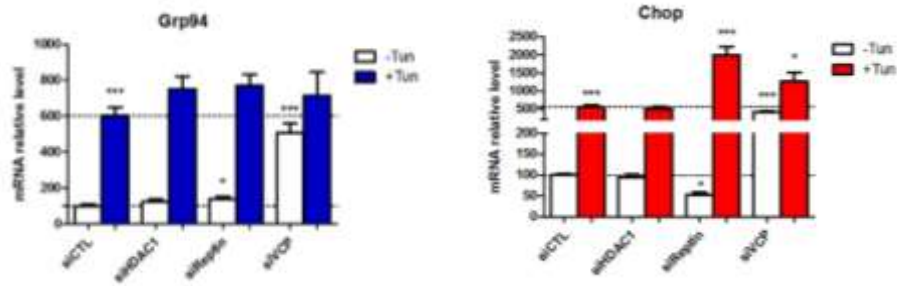


Figure S3: RT-qPCR analysis of Grp94 target of XBP1s and CHOP target of ATF4 in siRNA CTL, siRNA HDAC1, siRNA RuvBL2 or siRNA p97/VCP conditions with or without tunicamycin treatment (5µg/mL for 8h), *, *** = P value < 0.05, 0.001 respectively (N=3).

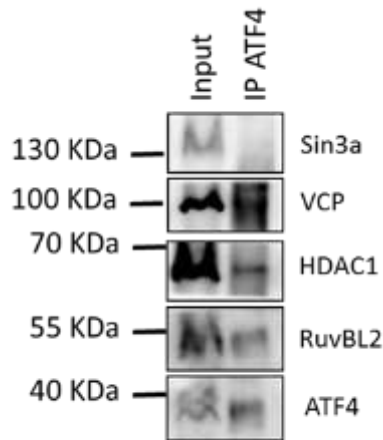


Figure S4: ATF4 was immunoprecipitated from total protein extracts from HeLa cells HeLa cells exposed to tunicamycin (5µg/mL) for 8h using a specific antibody and mSin3A, p97/VCP, HDAC1, RuvBL2 association was analyzed by immunoblotting (N=3).

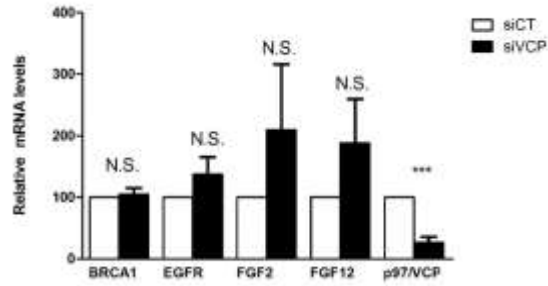


Figure S5: RT-qPCR analysis of BRCA1, EGFR, FGF2, FGF12 and p97/VCP in Huh7 cells treated siRNA CTL or siRNA p97/VCP (Knock Down control); \pm SD; *** = P value < 0.001 (N=3).

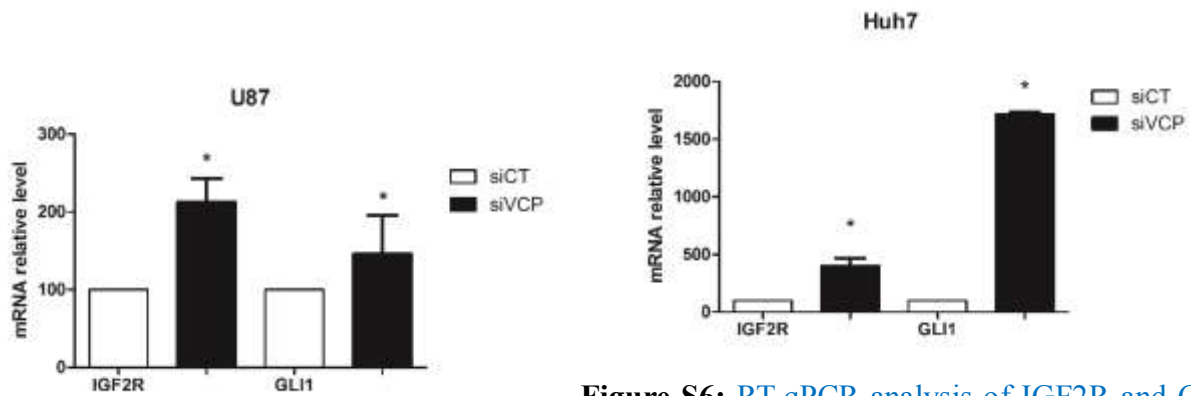


Figure S6: RT-qPCR analysis of IGF2R and GLI1 in U87 and Huh7 cells treated siRNA CTL or siRNA p97/VCP; \pm SD; * = P value < 0.05 (N=3).

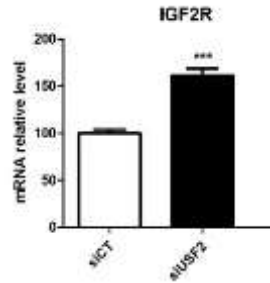


Figure S7: RT-qPCR analysis of IGF2R in Hela cells treated siRNA CTL or siRNA USF2; \pm SD; *** = P value < 0.001 (N=3).

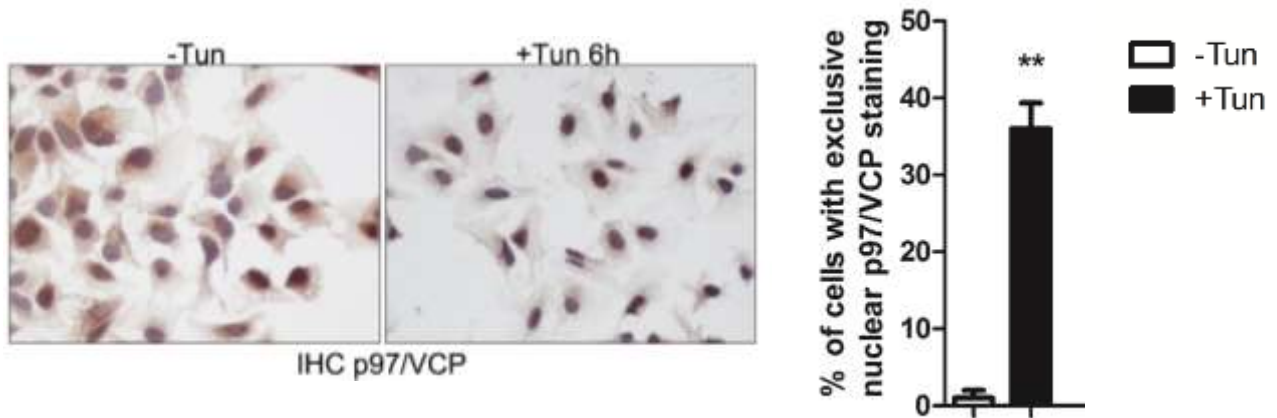


Figure S8: Immunohistochemistry staining of endogenous p97/VCP in Huh7 cells under basal conditions or after 6h of tunicamycin (5 μ g/mL) and quantification of cells with nuclear staining exclusively; \pm SD; ** = P value < 0.005 (N=3).

Table S1: qPCR primer list

GENE	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'
P97/VCP	CCATCCGGAAAGGAGACATTT	GTCTGGAGCAACAATGCAATAAG
GAPDH	GACCTGACCTGCCGTCTAGAAAAA	ACCACCCTGTTGCTGTAGCCAAAT
GLI1	AGGGAGTGCAGCCAATACAG	ATTGGCCGGAGTTGATGTAG
USF2	TTCGGCGACCACAACATCCAG	CAGTCACCTGGACTACGCGGT
IRE1A	GCCACCCTGCAAGAGTATGT	ATGTTGAGGGAGTGGAGGTG
BIP	TGTTGGAAGATTCTGATTTGAAGA	TCACTCGAATACCATTACAT
EDEM	AGTCATCAACTCCAGCTCCAA	AACCATCTGGTCAATCTGTCG
ERDJ4	TGGTGGTTCCAGTAGACAAAGG	CTTCGTTGAGTGACAGTCCTGC
HERPUD	TCCTCCTCCTGACGTTGAAA	TGTTGCGXATCTAGTACATCC
ORP150	GAAGATGCAGAGCCCATTTT	TCTGCTCCAGGACCTCCTAA
RUVBL2	AAGTCCCGGAGATCCGTGAT	CGACCGCAATCTTCCCTTC
CHOP	AAGGCACTGAGCGTATCATGT	TGAAGATACACTTCCTTCTGAACA

Table S2: siRNA list

GENE	SEQUENCE 5' – 3'
P97/VCP	(GAAUAGAGUUGUUCGGAAU)TT
HDAC1	(CAGCGACUGUUUGAGAACC)TT
RUVBL2	(GAGAUCCAGAUUGAUCGACCAGCAA)TT
USF2	(CCUCCACUUGGAAACGGUA)TT

DISCUSSION

p97/VCP is a molecular switch that activates transcription of ER stress genes during ER stress

It is well established that p97/VCP is an important player in the regulation of protein homeostasis and particularly during ER stress as it contributes to the degradation of terminally misfolded proteins from the ER in the ERAD pathway (23). However, our work has highlighted a novel role for the AAA+ ATPase p97/VCP during ER stress in the regulation of ER stress gene expression. We have demonstrated that p97/VCP/CDC-48 is required in *C. elegans* and Human for the induction of certain ER stress genes during ER stress. This induction is induced by the degradation of ubiquitylated RuvBL2 another AAA+ ATPase that acts as a repressor of ER stress genes under basal conditions. RuvBL2's repressor role relies on its interaction with a chromatin remodeler complex that contains HDAC1 and/or mSin3A. Our result suggest that these chromatin remodeler complexes might be recruited at their target sites by interacting with ER stress transcription factors (XBP1s, ATF6, ATF4) that are expressed at low levels in basal conditions. Interestingly, XBP1s interacts with RuvBL2-HDAC1-mSin3A whereas ATF6 and ATF4 interact with RuvBL2-HDAC1 only. In coherence, with Kim and colleagues findings this result suggests that RuvBL2 can interact with HDAC1 in different complexes (78). Overall these results have allowed us to propose a model (**Figure 15**).

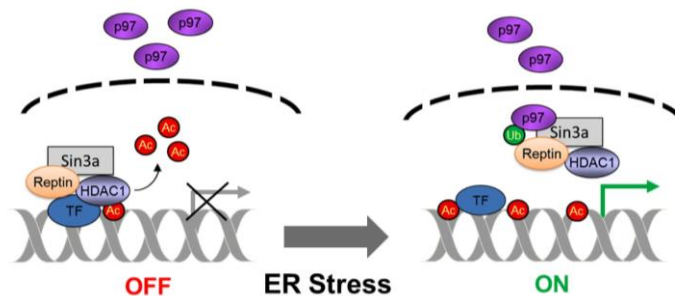


Figure 15: Model of the regulation of ER stress genes mediated by p97/VCP-RuvBL2-HDAC1 - In basal conditions, RuvBL2 and chromatin remodeler complexes are recruited at ER stress genes mediated by their association with ER stress transcription factors. RuvBL2 and HDAC1 and/or mSin3A prevent acetylation of ER stress genes thereby the chromatin is in a condensed “OFF” state. Upon ER stress induction, p97/VCP act as a molecular switch to turn “ON” ER stress by removing ubiquitylated RuvBL2. The removal of RuvBL2 causes the inactivation or dissociation of the repressor complex and allows the fixation of transcription factors and co-factors required for the activation of these ER stress genes.

This model is supported by Baumeister and colleagues findings (77a, 77b) which have shown that under basal conditions, BiP one of the main ER stress gene and a target of ATF6, is repressed by HDAC1 which is bound to the ERSE1 sequence. Upon ER stress HDAC1 is removed and as a result transcription of BiP is activated (77a, 77b). Interestingly, HDAC1 binds to the same sequence as XBP1s and ATF6, strengthening the idea that these repressor complex are recruited by their interaction with ER stress transcription factors. Moreover, HDAC1 but not HDAC2 knock down induces BiP expression suggesting that this gene is not regulated by mSin3A complex which contains both HDACs. This result is consistent with our finding that ATF6 interact with HDAC1 but not mSin3A and strengthens the idea that certain ER stress genes and transcription factors are regulated by different RuvBL2-HDAC1 complexes (78).

ER stress activate the Hedgehog pathway mediated by the p97/VCP induction of GLI1

Another key finding of our work is the fact that GLI1, a major transcription factor of the Hedgehog pathway which has been involved in cancer progression is regulated by p97/VCP and ER stress (**Figure 16**)(80). We showed that USF2 a transcription factor responsible for the regulation of the GLI1 gene (81) is also regulated by a RuvBL2-mSin3A-HDAC complex. In basal conditions, the deacetylation repressor complex is probably recruited to the Gli1 gene by association with USF2. Upon ER stress removal of ubiquitinated RuvBL2, inhibits the repressor complex, resulting in an increase in acetylation and ultimately activation of Gli1.

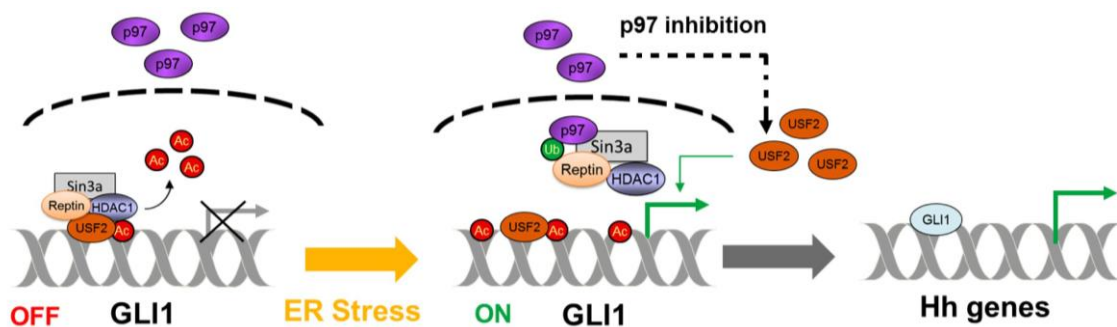


Figure 16: Model of the regulation of Hedgehog genes mediated by p97/VCP-RuvBL2-HDAC1 during ER stress - Under basal conditions, GLI1 is repressed by a repressor complex: RuvBL2-HDAC1-mSin3A that is recruited by USF2. Upon ER stress p97/VCP promotes the degradation of ubiquitinated RuvBL2 thereby inactivating this complex and thus activating transcription of GLI1. GLI1 then promotes the transcription of its target Hh genes.

We then tested the impact this induction of Gli1 and we found that all the Hh target genes tested are indeed induced upon ER stress. Overall our data indicates that ER stress leads to a non-canonical activation of the Hh pathway mediated by p97/VCP-RuvBL2-mSin3a-HDAC. Interestingly, p97/VCP silencing induces the expression of GLI1 a possible explanation is that in p97/VCP knockdown cell several activators of GLI1 accumulate. Indeed, Smad2/3 two transcription factors that are described to regulate GLI1 were also shown to be interacting with p97/VCP directly at the chromatin (57). Therefore in p97/VCP knockdown cells Smad2/3 and USF2 accumulation might be sufficient to cause induce GLI1 expression.

p97/VCP to regulate gene expression of distant pathways ?

We showed that p97/VCP is key to regulate GLI1 and ER stress genes in ER stress conditions. This regulation relies on the disruption of chromatin remodeler complex (RuvBL2-HDAC1 and RuvBL2-mSin3A-HDAC1) that target acetylated histones to repress their target genes. In fact this mechanism does not seem to be exclusive to ER stress as other reports have linked p97/VCP to the regulation of histone marks: Koike et al. showed that PolyQ cytosolic accumulation caused p97/VCP translocation to the nucleus and change in global acetylation of histones (62). Additionally, a report in yeast suggests the requirement of CDC-48 to regulate ubiquitinylation of H2B during replication (61). Moreover, p97/VCP gene regulation does not seem limited to ER stress and Hh genes as p97/VCP was shown to regulate Heat Shock genes by forming an inactive complex with HSF1 and HDAC6 in basal conditions (40). In other pathways, p97/VCP regulates IKB the repressor of NFkB in the nucleus to regulate genes involve in cell survival and CDC-48 and its cofactor UBX7 were shown to regulate HIF1 α and thereby genes involved in the response to hypoxia (59,60). Overall our results and these reports suggest that p97/VCP does not control transcription in one fashion, it seems to affect different histone marks but also regulates a variety of transcription factors involved in different pathways. As the result the extent of the genes it regulate in normal and cancer cells in a given condition is very hard to assess.

p97/VCP as a therapeutic target to treat cancer ?

p97/VCP is found overexpressed in many cancers and it is suggested that it contributes to oncogenesis by helping to cope with the proteotoxic stress that exist in fast dividing cancer cells (66). In our study, we have showed that p97/VCP induces gene expression by targeting chromatin modifier complexes. We have also shown that these complexes repressed their target genes by interacting with transcription factors including ATF6, ATF4, XBP1s and USF2. Given that the mSin3A complex regulated many genes by interacting with transcription factors it is likely that some of these genes are regulated by p97/VCP during ER stress. This mechanism could in part explain the complex transcriptional reprogramming that was observed during the UPR. And such transcriptional reprogramming events have been linked to the acquisition of malignant traits such as inflammation, immunogenicity or angiogenesis in cancers (26). Moreover, p97/VCP overexpression in cancer cells might have an important impact on gene expression as it may affect the stability of the complexes it control and thereby the expression of their target genes.

Although the impact of pharmacological inhibition of p97/VCP in cancer cells is hard to assess as it is involved in various cellular processes including cancer relevant processes (transcription, autophagy, protein homeostasis, DNA damage) it has shown some encouraging results. Indeed, in hepatocellular carcinoma, Sorafenib a kinase inhibitor the only treatment available was shown to prevent phosphorylation, cause ER stress and ultimately cancer cell death (71). When combined with Dbeq a specific p97/VCP inhibitor the effects were even stronger. More recently, NMS-783 and CB-5083 two p97/VCP inhibitors were also shown to induce apoptosis mediated by prolonged ER stress in cancer cell lines and xenografted mice, suggesting that p97/VCP is valid target for cancer treatment (72,73). However a recent report suggest that p97/VCP inhibition mediated by pharmacological inhibitor or siRNA induce Epithelial-Mesenchymal Transition like phenotype in cancer cell lines (81). Moreover, in our study p97/VCP protein level were reduced to 20-30% after siRNA treatment and caused prolonged ER stress but was not sufficient to induce apoptosis. It is also noteworthy that in p97/VCP silenced cell we noticed induction two oncogenes: GLI1 and IGF2R. In conclusion, although p97/VCP pharmacological inhibition has shown some encouraging results, it may also promote EMT or induction of oncogenes and therefore be an unsafe therapeutic strategy.

Perspectives

Although our work has unraveled some of the molecular mechanisms that occur before and after the induction of ER stress such as the recruitment of USF2 at the Gli1 promoter, the change in acetylation at the Gli1 promoter, the stronger interaction between p97/VCP and RuvBL2 we are still failing to have a dynamic vision of the events that occur and in particular at the chromatin. For instance, how and when p97/VCP shuttles to the nucleus is still unclear, in yeast it is thought that phosphorylation of the penultimate tyrosine is responsible for the nuclear import of CDC-48 (44) whereas in human the first study reported that residues 612 to 614 and their modifications are crucial for p97/VCP nuclear translocation (62) and a second study has shown that the N domain (the first 200 AA) are sufficient to be translocated to the nucleus (45). We are currently investigating the timeframe by which p97/VCP translocates to the nucleus and the relevance of its tyrosine phosphorylation in this process. Moreover, which cofactors are required if any for p97/VCP translocation to nucleus and which are involved in the recognition of ubiquitinated RuvBL2 remain to be examined. However, it is noteworthy that most but not all p97/VCP chromatin associated processes seem to involve Ufd1-Npl4 (31). Another limit of our model is the fact that we do not know yet how p97/VCP extraction/degradation of RuvBL2 affects the other members of the complex. Is the removal of RuvBL2 causing the dissociation of the complex from the chromatin or if p97/VCP also mediates their extraction? In fact, cullin3 a cofactor of p97/VCP was shown to interact with HDAC1 (83) therefore there is a possibility that p97/VCP mediates extraction of both RuvBL2 and HDAC1 from the chromatin to inactivate the complex. Finally, as p97/VCP is mostly found overexpressed in cancers (67), it would be interesting overexpressing it in our cells lines and tested the impact on gene expression, histone marks, protein stability as this could help us understand the physiology of cancer cells.

REFERENCES

- 1 Palade, G., Intracellular aspects of the process of protein synthesis. *Science*, 1975. 189(4206): p. 867.
- 2 Lavoie, C. and J. Paiement, Topology of molecular machines of the endoplasmic reticulum: a compilation of proteomics and cytological data. *Histochem Cell Biol*, 2008. 129(2): p. 117-28.
- 3 Berridge, M.J., M.D. Bootman, and H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol*, 2003. 4(7): p. 517-29.
- 4 Berridge, M.J., The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium*, 2002. 32(5-6): p. 235-49.
- 5 McPherson, P.S. and K.P. Campbell, The ryanodine receptor/Ca²⁺ release channel. *J Biol Chem*, 1993. 268(19): p. 13765-8.
- 6 Lytton, J., et al., Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J Biol Chem*, 1992. 267(20): p.14483-9.
- 7 Coe, H. and M. Michalak, Calcium binding chaperones of the endoplasmic reticulum. *Gen Physiol Biophys*, 2009. 28 Spec No Focus: p. F96-F103.
- 8 Fagone, P. and S. Jackowski, Membrane phospholipid synthesis and endoplasmic reticulum function. *J Lipid Res*, 2009. 50 Suppl: p. S311-6.
- 9 Helenius, J., et al., Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein. *Nature*, 2002. 415(6870): p. 447-50.
- 10 Underwood, K.W., et al., Evidence for a cholesterol transport pathway from lysosomes to endoplasmic reticulum that is independent of the plasma membrane. *J Biol Chem*, 1998. 273(7): p. 4266-74.
- 11 Wirtz, K.W., Phospholipid transfer proteins: from lipid monolayers to cells. *Klin Wochenschr*, 1991. 69(3): p. 105-11.
- 12 Radhakrishnan, A., et al., Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance. *Cell Metab*, 2008. 8(6): p. 512-21.
- 13 Wang, X., et al., SREBP-1, a membrane-bound transcription factor released by sterolregulated proteolysis. *Cell*, 1994. 77(1): p. 53-62.
- 14a Neuhof A, Rolls MM, Jungnickel B, Kalies KU, Rapoport TA. Binding of signal recognition particle gives ribosome/nascent chain complexes a competitive advantage in endoplasmic reticulum membrane interaction. *Mol Biol Cell*. 1998;9(1):103-15.
- 14b Kalies, K.U., D. Gorlich, and T.A. Rapoport, Binding of ribosomes to the rough endoplasmic reticulum mediated by the Sec61p-complex. *J Cell Biol*, 1994. 126(4): p.925-34.
- 15 Braakman I, Hebert DN. Protein folding in the endoplasmic reticulum. *Cold Spring Harb Perspect Biol*. 2013;5(5):a013201.
- 16 Araki K, Nagata K. Protein folding and quality control in the ER. *Cold Spring Harb Perspect Biol*. 2011;3(11):a007526.

- 17 Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N, et al. Global analysis of protein expression in yeast. *Nature*. 2003;425:737–741
- 18 Stephens, D.J., De novo formation, fusion and fission of mammalian COPII-coated endoplasmic reticulum exit sites. *EMBO Rep*, 2003. 4(2): p. 210-7.
- 19 Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). *Molecular biology of the cell*, Forth edn (New York, Garland Science).
- 20 McCracken, A.A. and J.L. Brodsky, Recognition and delivery of ERAD substrates to the proteasome and alternative paths for cell survival. *Curr Top Microbiol Immunol*, 2005. 300: p. 17-40.
- 21 Okuda-Shimizu, Y. and L.M. Hendershot, Characterization of an ERAD pathway for nonglycosylated BiP substrates, which require Herp. *Mol Cell*, 2007. 28(4): p. 544-54.
- 22 Hargrave PA 2001. Rhodopsin structure, function, and topography—the Friedenwald lecture. *Invest Ophthalmol Vis Sci* 42: 3–9
- 23 Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol*. 2008;9(12):944-57.
- 24 Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science*. 2011;334(6059):1081-6.
- 25 Hetz C, Chevet E, Oakes SA. Proteostasis control by the unfolded protein response. *Nat Cell Biol*. 2015;17(7):829-38.
- 26 Chevet E, Hetz C, Samali A. Endoplasmic reticulum stress-activated cell reprogramming in oncogenesis. *Cancer Discov*. 2015;5(6):586-97.
- 27a Backer MV, Backer JM, Chinnaiyan P. Targeting the unfolded protein response in cancer therapy. *Methods Enzymol*. 2011;491:37-56.
- 27b Moir, D., Stewart, S.E., Osmond, B.C., Botstein, D., 1982. Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics* 100, 547–563.
- 28 Zhang X, Shaw A, Bates PA, Newman RH, Gowen B, Orlova E, et al. Structure of the AAA ATPase p97. *Mol Cell*. 2000;6(6):1473-84.
- 29 Xia D, Tang WK, Ye Y. Structure and function of the AAA+ ATPase p97/Cdc48p. *Gene*. 2016;583(1):64-77.
- 30 Meyer, H., Bug, M., Bremer, S., 2012. Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. *Nat. Cell Biol.* 14, 117–123
- 31 Meyer H, Wehl CC. The VCP/p97 system at a glance: connecting cellular function to disease pathogenesis. *J Cell Sci*. 2014;127(Pt 18):3877-83.
- 32 Dreveny I, Pye VE, Beuron F, Briggs LC, Isaacson RL, Matthews SJ, et al. p97 and close encounters of every kind: a brief review. *Biochem Soc Trans*. 2004;32(Pt 5):715-20. 27b Moir, D., Stewart, S.E., Osmond, B.C., Botstein, D., 1982. Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. *Genetics* 100, 547–563.

- 33 Bohm S, Lamberti G, Fernandez-Saiz V, Stapf C, Buchberger A. Cellular functions of Ufd2 and Ufd3 in proteasomal protein degradation depend on Cdc48 binding. *Mol Cell Biol.* 2011;31(7):1528-39.
- 34 Nery FC, Armata IA, Farley JE, Cho JA, Yaqub U, Chen P, et al. TorsinA participates in endoplasmic reticulum-associated degradation. *Nat Commun.* 2011;2:393.
- 35 Radhakrishnan SK, den Besten W, Deshaies RJ. p97-dependent retrotranslocation and proteolytic processing govern formation of active Nrf1 upon proteasome inhibition. *Elife.* 2014;3:e01856.
- 36 Stewart EV, Lloyd SJ, Burg JS, Nwosu CC, Lintner RE, Daza R, et al. Yeast sterol regulatory element-binding protein (SREBP) cleavage requires Cdc48 and Dsc5, a ubiquitin regulatory X domain-containing subunit of the Golgi Dsc E3 ligase. *J Biol Chem.* 2012;287(1):672-81.
- 37 S. Xu, G. Peng, Y. Wang, S. Fang, M. Karbowski The AAA-ATPase p97 is essential for outermitochondrial membrane protein turnover *Mol. Biol. Cell*, 22 (2011), pp. 291–300
- 38a Ju JS, Weihl CC. p97/VCP at the intersection of the autophagy and the ubiquitin proteasome system. *Autophagy.* 2010;6(2):283-5.
- 38b Ju JS, Fuentealba RA, Miller SE, Jackson E, Piwnicka-Worms D, Baloh RH, et al. Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. *J Cell Biol.* 2009;187(6):875-88.
- 39 Boyault C, Gilquin B, Zhang Y, Rybin V, Garman E, Meyer-Klaucke W, et al. HDAC6-p97/VCP controlled polyubiquitin chain turnover. *EMBO J.* 2006;25(14):3357-66.
- 40 Pernet L, Faure V, Gilquin B, Dufour-Guerin S, Khochbin S, Vourc'h C. HDAC6-ubiquitin interaction controls the duration of HSF1 activation after heat shock. *Mol Biol Cell.* 2014;25(25):4187-94.
- 41 Uchiyama K, Jokitalo E, Kano F, Murata M, Zhang X, Canas B, et al. VCIP135, a novel essential factor for p97/p47-mediated membrane fusion, is required for Golgi and ER assembly in vivo. *J Cell Biol.* 2002;159(5):855-66.
- 42 Lavoie C, Chevet E, Roy L, Tonks NK, Fazel A, Posner BI, et al. Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly in vitro. *Proc Natl Acad Sci U S A.* 2000;97(25):13637-42.
- 43 Ramanathan HN, Ye Y. The p97 ATPase associates with EEA1 to regulate the size of early endosomes. *Cell Res.* 2012;22(2):346-59.
- 44 Madeo, F., Schlauer, J., Zischka, H., Mecke, D., Frohlich, K.U., 1998. Tyrosine phosphorylation regulates cell cycle-dependent nuclear localization of Cdc48p. *Mol. Biol. Cell* 9, 131–141.
- 45 Song C, Wang Q, Song C, Lockett SJ, Colburn NH, Li CC, et al. Nucleocytoplasmic shuttling of valosin-containing protein (VCP/p97) regulated by its N domain and C-terminal region. *Biochim Biophys Acta.* 2015;1853(1):222-32.

- 46 Dobrynin G, Popp O, Romer T, Bremer S, Schmitz MH, Gerlich DW, et al. Cdc48/p97-Ufd1-Npl4 antagonizes Aurora B during chromosome segregation in HeLa cells. *J Cell Sci.* 2011;124(Pt 9):1571-80.
- 47 Raman M, Havens CG, Walter JC, Harper JW. A genome-wide screen identifies p97 as an essential regulator of DNA damage-dependent CDT1 destruction. *Mol Cell.* 2011;44(1):72-84.
- 48 Zhang H, Wang Q, Kajino K, Greene MI. VCP, a weak ATPase involved in multiple cellular events, interacts physically with BRCA1 in the nucleus of living cells. *DNA Cell Biol.* 2000;19(5):253-63.
- 49 Mu JJ, Wang Y, Luo H, Leng M, Zhang J, Yang T, et al. A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. *J Biol Chem.* 2007;282(24):17330-4.
- 50 Livingstone M, Ruan H, Weiner J, Clouser KR, Strack P, Jin S, et al. Valosin-containing protein phosphorylation at Ser784 in response to DNA damage. *Cancer Res.* 2005;65(17):7533-40.
- 51 Dantuma NP, Acs K, Luijsterburg MS. Should I stay or should I go: VCP/p97-mediated chromatin extraction in the DNA damage response. *Exp Cell Res.* 2014;329(1):9-17.
- 52 Puumalainen MR, Lessel D, Ruthemann P, Kaczmarek N, Bachmann K, Ramadan K, et al. Chromatin retention of DNA damage sensors DDB2 and XPC through loss of p97 segregase causes genotoxicity. *Nat Commun.* 2014;5:3695.
- 53 Acs K, Luijsterburg MS, Ackermann L, Salomons FA, Hoppe T, Dantuma NP. The AAA-ATPase VCP/p97 promotes 53BP1 recruitment by removing L3MBTL1 from DNA double-strand breaks. *Nat Struct Mol Biol.* 2011;18(12):1345-50.
- 54 Ramadan K. p97/VCP- and Lys48-linked polyubiquitination form a new signaling pathway in DNA damage response. *Cell Cycle.* 2012;11(6):1062-9.
- 55 Nalbandian A, Ghimbovski S, Radom-Aizik S, Dec E, Vesa J, Martin B, et al. Global gene profiling of VCP-associated inclusion body myopathy. *Clin Transl Sci.* 2012;5(3):226-34.
- 56 Stewart EV, Lloyd SJ, Burg JS, Nwosu CC, Lintner RE, Daza R, et al. Yeast sterol regulatory element-binding protein (SREBP) cleavage requires Cdc48 and Dsc5, a ubiquitin regulatory X domain-containing subunit of the Golgi Dsc E3 ligase. *J Biol Chem.* 2012;287(1):672-81.
- 57 Ndoja A, Cohen RE, Yao T. Ubiquitin signals proteolysis-independent stripping of transcription factors. *Mol Cell.* 2014;53(6):893-903.
- 58 Valle CW, Min T, Bodas M, Mazur S, Begum S, Tang D, et al. Critical role of VCP/p97 in the pathogenesis and progression of non-small cell lung carcinoma. *PLoS One.* 2011;6(12):e29073.
- 59 Asai T, Tomita Y, Nakatsuka S, Hoshida Y, Myoui A, Yoshikawa H, et al. VCP (p97) regulates NFkappaB signaling pathway, which is important for metastasis of osteosarcoma cell line. *Jpn J Cancer Res.* 2002;93(3):296-304.
- 60 Alexandru G, Graumann J, Smith GT, Kolawa NJ, Fang R, Deshaies RJ. UBXD7 binds multiple ubiquitin ligases and implicates p97 in HIF1alpha turnover. *Cell.* 2008;134(5):804-16.

- 61 Bonizec M, Herissant L, Pokrzywa W, Geng F, Wenzel S, Howard GC, et al. The ubiquitin-selective chaperone Cdc48/p97 associates with Ubx3 to modulate monoubiquitylation of histone H2B. *Nucleic Acids Res.* 2014;42(17):10975-86.
- 62 Koike M, Fukushi J, Ichinohe Y, Higashimae N, Fujishiro M, Sasaki C, et al. Valosin-containing protein (VCP) in novel feedback machinery between abnormal protein accumulation and transcriptional suppression. *J Biol Chem.* 2010;285(28):21736-49.
- 63 Weihl, C. C., Dalal, S., Pestronk, A. and Hanson, P. I. (2006). Inclusion body myopathy-associated mutations in p97/VCP impair endoplasmic reticulum-associated degradation. *Hum. Mol. Genet.* 15, 189–199. doi:10.1093/hmg/ddi426
- 64 Kim, N. C., Tresse, E., Kolaitis, R. M., Molliex, A., Thomas, R. E., Alami, N. H., Wang, B., Joshi, A., Smith, R. B. and Ritson, G. P. et al. (2013b). VCP is essential for mitochondrial quality control by PINK1/Parkin and this function is impaired by VCP mutations. *Neuron* 78, 65–80. doi:10.1016/j.neuron.2013.02.029
- 65 Ritson GP, Custer SK, Freibaum BD, Guinto JB, Geffel D, Moore J, et al. TDP-43 mediates degeneration in a novel *Drosophila* model of disease caused by mutations in VCP/p97. *J Neurosci.* 2010;30(22):7729-39.
- 66 Fessart D, Marza E, Taouji S, Delom F, Chevet E. P97/CDC-48: Proteostasis control in tumor cell biology. *Cancer Lett.* 2013;337(1):26-34.
- 67 Liu Y, Hei Y, Shu Q, Dong J, Gao Y, Fu H, et al. VCP/p97, down-regulated by microRNA-129-5p, could regulate the progression of hepatocellular carcinoma. *PLoS One.* 2012;7(4):e35800.
- 68 S. Yamamoto, Y. Tomita, T. Uruno, Y. Hoshida, Y. Qiu, N. Iizuka, I. Nakamichi, A. Miyauchi, K. Aozasa, Increased expression of valosin-containing protein (p97) is correlated with disease recurrence in follicular thyroid cancer, *Ann.Surg. Oncol.* 12 (2005) 925–934.
- 69 Y. Tsujimoto, Y. Tomita, Y. Hoshida, T. Kono, T. Oka, S. Yamamoto, N. Nonomura, A. Okuyama, K. Aozasa, Elevated expression of valosin-containing protein (p97) is associated with poor prognosis of prostate cancer, *Clin. Cancer Res.* 10 (2004) 3007–3012.
- 70 Wang YB, Tan B, Mu R, Chang Y, Wu M, Tu HQ, et al. Ubiquitin-associated domain-containing ubiquitin regulatory X (UBX) protein UBXN1 is a negative regulator of nuclear factor kappaB (NF-kappaB) signaling. *J Biol Chem.* 2015;290(16):10395-405.
- 71 Yi P, Higa A, Taouji S, Bexiga MG, Marza E, Arma D, et al. Sorafenib-mediated targeting of the AAA(+) ATPase p97/VCP leads to disruption of the secretory pathway, endoplasmic reticulum stress, and hepatocellular cancer cell death. *Mol Cancer Ther.* 2012;11(12):2610-20.
- 72 Magnaghi P, D'Alessio R, Valsasina B, Avanzi N, Rizzi S, Asa D, et al. Covalent and allosteric inhibitors of the ATPase VCP/p97 induce cancer cell death. *Nat Chem Biol.* 2013;9(9):548-56.
- 73 Anderson DJ, Le Moigne R, Djakovic S, Kumar B, Rice J, Wong S, et al. Targeting the AAA ATPase p97 as an Approach to Treat Cancer through Disruption of Protein Homeostasis. *Cancer Cell.* 2015;28(5):653-65.
- 74 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000;100(1):57-70.

- 75 Urra H., Dufey E., Avril T., Chevet E. and Hetz C. (2016). ER stress and the hallmarks of cancer. *Trends in Cancer*. 5:252-262. *#
- 76 Caruso ME, Jenna S, Bouchecareilh M, Baillie DL, Boismenu D, Halawani D, et al. GTPase-mediated regulation of the unfolded protein response in *Caenorhabditis elegans* is dependent on the AAA+ ATPase CDC-48. *Mol Cell Biol*. 2008;28(13):4261-74.
- 77a Baumeister P, Luo S, Skarnes WC, Sui G, Seto E, Shi Y, et al. Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. *Mol Cell Biol*. 2005;25(11):4529-40.
- 77b Baumeister P, Dong D, Fu Y, Lee AS. Transcriptional induction of GRP78/BiP by histone deacetylase inhibitors and resistance to histone deacetylase inhibitor-induced apoptosis. *Mol Cancer Ther*. 2009;8(5):1086-94.
- 78 Kim JH, Choi HJ, Kim B, Kim MH, Lee JM, Kim IS, et al. Roles of sumoylation of a reptin chromatin-remodelling complex in cancer metastasis. *Nat Cell Biol*. 2006;8(6):631-9.
- 79 Wang T, Xu W, Qin M, Yang Y, Bao P, Shen F, et al. Pathogenic Mutations in the Valosin-containing Protein/p97(VCP) N-domain Inhibit the SUMOylation of VCP and Lead to Impaired Stress Response. *J Biol Chem*. 2016;291(27):14373-84.
- 80 Carpenter RL, Lo HW. Hedgehog pathway and GLI1 isoforms in human cancer. *Discov Med*. 2012;13(69):105-13.
- 81 Villavicencio EH, Yoon JW, Frank DJ, Fuchtbauer EM, Walterhouse DO, Iannaccone PM. Cooperative E-box regulation of human GLI1 by TWIST and USF. *Genesis*. 2002;32(4):247-58
- 82 Shah PP, Beverly LJ. Regulation of VCP/p97 demonstrates the critical balance between cell death and epithelial-mesenchymal transition (EMT) downstream of ER stress. *Oncotarget*. 2015;6(19):17725-37.

APENDIX

Beyond my study of the role of p97/VCP in the regulation of ER stress genes, I have also participated to another project of our group: the characterization of the functional impact of IRE1 α mutations found in Glioblastoma tumours.

In order to characterize the impact of the different IRE1 α mutants, we thought to test if these mutations were affecting p53 (**Figure 17**). Using western blot, we have compared the protein levels of p53 in U87 WT cells or U87 expressing an IRE1 α mutants (e.g: S769F, Q780*, P336L, A414T). No change was noticed in the expression of p53 in all mutants except in P336L expressing cells where p53 was increased by 15 fold. (**Figure 17A**). We confirmed by PCR that this was not due to an increase of mRNA level (**Figure 17B**). We also confirmed that p53 was not mutated by sequencing (Data not shown). Overall these results suggest that p53 elevated expression in IRE1a P336L expressing cells is not due to a mutation or a transcriptional event but most likely to a post translational event.

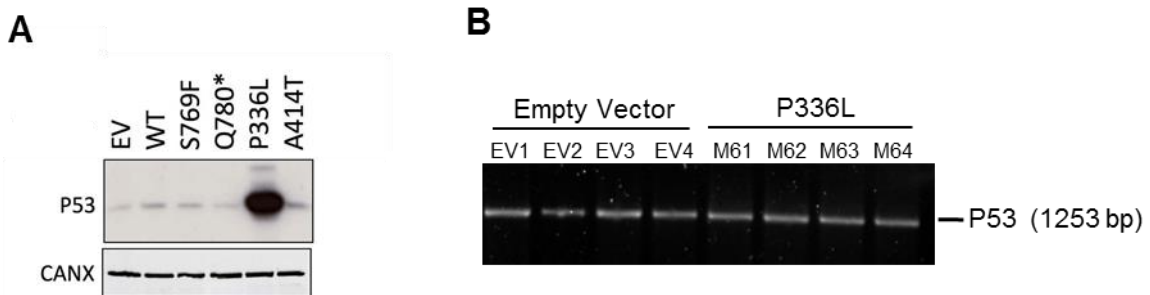


Figure 17: Analyse of p53 expression and mutation in U87 expressing IRE1 α mutants – A) Analysis of p53 protein level in U87 WT, U87 control (Empty vector – EV) or U87 expressing IRE1a mutants, CANX use as a loading control. **B)** Analysis of p53 mRNA expression in U87 control (EV) or in cells expressing the IRE1 P336L variant.

Antagonistic IRE1 RNase functions dictate glioblastoma tumor development

Stéphanie Lhomond^{1,17}, Tony Avril^{2,3,¶}, Nicolas Dejeans^{1,17,¶}, Mari McMahon^{2,3,4}, Raphaël Pineau^{1,2,3}, Olga Papadodima⁵, Konstantinos Voutetakis^{5,6}, Marianthi Logotheti^{5,7}, Néstor Pallares-Lupon¹, Kathleen Schmit¹, Pierre-Jean Le Reste^{2,8}, Amandine Etchevery⁹, Jean Mosser⁹, **Kim Barroso**^{1,2,3}, Elodie Vauléon^{2,3}, Marion Maurel^{2,3,4}, Gwénaële Jégou^{2,3}, Afshin Samali⁴, John B. Patterson¹⁰, Olivier Pluquet¹¹, Claudio Hetz^{12,13,14,15,16}, Véronique Quillien^{2,3}, Arisotelis Chatziioannou^{5,6} and Eric Chevet^{1,2,3*}

ABSTRACT

Abnormal proteostasis control is emerging as a major hallmark of cancer, driving tumor aggressiveness. Genetic and pharmacological evidence suggest that the endoplasmic reticulum (ER), a major site for protein folding and quality control, plays a critical role in cancer development. This has been applied to glioblastoma multiform (GBM), the most lethal primary brain cancer with an overall survival of 15 months and no effective treatment. The ER stress sensor IRE1alpha contributes to GBM progression, impacting tissue invasion and tumor vascularization. IRE1alpha is an RNase that signals by catalyzing the splicing of the mRNA encoding the transcription factor XBP1, in addition to regulate the stability of certain miRNAs and mRNAs through a process known as Regulated IRE1 Dependent Decay (RIDD). Somatic mutations in the IRE1alpha gene have been identified in GBM and other forms of cancer. Here we investigated the contribution of IRE1alpha signaling to GBM based on the systematic comparison of mutant forms identified in cancer, and demonstrated a relevant role to the disease. We also identified a novel mutation associated with GBM with functional consequences to tumor formation. Taking advantage of the specific signaling outputs of the RNase domain of IRE1alpha engaged by distinct GBM-related mutations, we defined specific signaling signatures that were confronted to human GBM transcriptomes. This approach allowed us to demonstrate the antagonistic roles of XBP1 mRNA splicing and RIDD on tumor outcomes. This study provides the first demonstration of a dual role of IRE1alpha downstream signaling in cancer and opens a new therapeutic window to impair tumor progression.