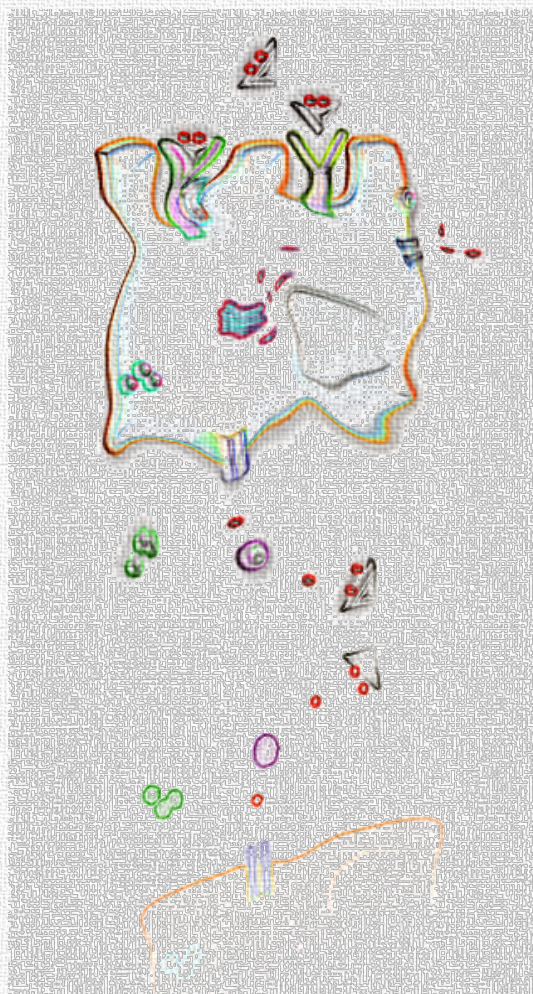




Impact of the UPR on the expression of relevant genes for cellular iron metabolism

Susana João Cunha Oliveira

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Impact of the UPR on the expression of relevant genes for cellular iron metabolism

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Orientador – Doutora Maria Ângela Brito de Sousa

Categoria – Professora Catedrática Jubilada/Emérita

Afiliação – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto

Co-orientador – Doutor Idílio Jorge Matias Pereira Pinto

Categoria – Investigador Auxiliar

Afiliação – Instituto de Biologia Molecular e Celular, Universidade do Porto

Co-orientador – Doutor Jorge Eduardo da Silva Azevedo

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto

Aos meus pais

“Procrastination is the thief of time”

Edward Young

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ABSTRACT

Abstract

The Unfolded Protein Response (UPR) is a highly specialized signaling program aimed at relieving the stress induced by folding-defective proteins accumulated in the endoplasmic reticulum (ER). Mounting evidence, however, has revealed the multifaceted nature of the UPR through its involvement in functions as diverse as cell differentiation, metabolic processes and inflammation. The connection with iron homeostasis, although suggested in earlier studies, remained largely unexplored. To examine whether activation of the UPR influences the expression of iron metabolism-related genes was therefore defined as the central aim of the current thesis.

The C282Y point transition of HFE disrupts systemic iron balance, underlying the majority of cases of the iron overload disorder Hereditary Hemochromatosis (HH). Apart from causing loss of protein function, the C282Y mutation was recently shown to trigger an UPR. The possibility of a C282Y-mediated interplay between the UPR pathways and cellular iron metabolism was first addressed in a stable-transfected model of HepG2 cells, with no signs of UPR activity being detected. Following a transient version of HFE expression in the same cells, canonical ER stress markers were partially stimulated by the C282Y mutant, thereby uncovering intrinsic peculiarities of the UPR signaling cascades. Transcript levels of hepcidin, a major regulator of iron homeostasis, were not influenced upon overexpression of any of the HFE forms, thus conflicting with the previously described C282Y-associated hepcidin down-modulation.

Considering the difficulty in ascertaining whether the results conveyed by the aforementioned model arose from the C282Y-imposed loss of protein function or UPR activation, our further strategy focused on chemically-induced ER stress. Dithiothreitol (DTT) and homocysteine (Hcys) treatments significantly reshaped the expression profiles of hepcidin, ferritin H and ferroportin genes in HepG2 cells. Moreover, the ER stress-associated regulation of hepcidin herein described was found to involve the concerted action of C/EBP α and CHOP transcription factors.

In order to consolidate our achievements with other than thiol-containing compounds, accumulation of unglycosylated clients in the ER was favored by tunicamycin (Tm) supplementation of cells. Under this stress condition, mRNA transcript levels of hepcidin, ferritin H and ceruloplasmin were substantially altered. Despite reinforcing the intermeshing of UPR and iron homeostasis, such approach

Abstract

also supported the conclusion that consequences of UPR activation might be dependent on the type of insult faced by the ER.

In summary, the work presented as part of this thesis clearly established the existence of a crosstalk between iron metabolism and the UPR signaling network. The repercussions of the current findings are discussed in the wider context of inflammation, neurodegeneration and viral infection, just to mention a few, and will certainly inspire promising research directions.

RESUMO

A *Unfolded Protein Response* (UPR) é um programa de sinalização altamente especializado empenhado em atenuar o *stress* induzido pela acumulação de proteínas incapazes de adquirir a conformação nativa no retículo endoplasmático (RE). A natureza multifacetada da UPR tem sido, no entanto, revelada através da crescente implicação em funções tão distintas quanto diferenciação celular, processos metabólicos e inflamação. Apesar de sugerida em estudos anteriores, a sua associação com a homeostasia do ferro permanecia largamente inexplorada. Avaliar se a activação da UPR influencia a expressão de genes envolvidos no metabolismo do ferro foi, desta forma, definido como objectivo central da presente tese.

A mutação pontual C282Y do HFE desregula o equilíbrio sistémico do ferro, sendo responsável pela maioria dos casos de Hemocromatose Hereditária (HH), doença de sobrecarga de ferro. Para além de determinar a perda de função da proteína, foi recentemente demonstrado que a mutação C282Y activa uma UPR. A possibilidade de uma correlação entre as cascatas de sinalização da UPR e o metabolismo celular do ferro mediada pela mutação C282Y foi primeiramente abordada num modelo estavelmente transfectado de células HepG2, sem que quaisquer sinais de uma UPR activa fossem detectados. Recorrendo a uma versão transiente de expressão do HFE nas mesmas células, marcadores canónicos de *stress* no RE foram parcialmente estimulados pelo mutante C282Y, expondo assim peculiaridades intrínsecas das vias de transdução da UPR. Os níveis de transcritos de hepcidina, principal regulador da homeostasia do ferro, permaneceram inalterados após sobre-expressão de todas as formas do HFE, o que contrasta com a diminuição associada à mutação C282Y previamente documentada.

Atendendo à dificuldade em discriminar se os resultados veiculados pelo modelo anterior resultavam da perda de função da proteína ou da activação da UPR impostas pela mutação C282Y, a estratégia seguinte residiu na indução química de *stress* no RE. Os tratamentos com ditioneitol (DTT) e homocisteína (Hcys) remodelaram significativamente os perfis de expressão dos genes da hepcidina, ferritina H e ferroportina em células HepG2. Ficou ainda demonstrado que a regulação da hepcidina associada ao *stress* no RE aqui descrita envolve a acção concertada dos factores de transcrição C/EBP α e CHOP.

Com o intuito de consolidar os nossos resultados com a acção de compostos não tiolados, a acumulação de proteínas não glicosiladas no RE foi favorecida pela adição de tunicamicina (Tm) às células. Nesta condição de *stress*, os níveis de RNAm de hepcidina, ferritina H e ceruloplasmina foram substancialmente alterados. Embora reforçando a interligação entre a UPR e a homeostasia do ferro, tal abordagem reiterou também que as consequências da activação da UPR podem ser dependentes do tipo de insulto enfrentado pelo RE.

Em síntese, o trabalho apresentado como parte desta tese estabeleceu claramente a existência de uma interface entre o metabolismo do ferro e as cascatas de sinalização definidas pelas UPR. As repercussões desta descoberta são discutidas no contexto mais abrangente da inflamação, neurodegeneração e infecções virais, só para citar alguns, e irão certamente inspirar promissoras linhas de investigação futuras.

LIST OF ABBREVIATIONS

List of Abbreviations

ADP	Adenosine diphosphate
ASK1	Apoptosis-signaling kinase 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
β_2 m	β_2 -microglobulin
BAP	BiP-associated protein
BBF2H7	BBF2 human homolog on chromosome 7
BiP	Immunoglobulin heavy chain-binding protein
BMP	Bone morphogenetic protein
BMPR	BMP receptor
C/EBP α	CCAAT/enhancer-binding protein α
CHO	Chinese hamster ovary cell line
CHOP	C/EBP homologous protein
CNX	Calnexin
COPII	Coat protein complex II
CREBH	cAMP response element-binding protein H
CRT	Calreticulin
Dcytb	Cytochrome b-like ferrireductase
DFO	Desferrioxamine
DMT1	Divalent metal transporter 1
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EDEM	ER degradation enhancing α -mannosidase-like protein
eIF2 α	α subunit of eukaryotic initiation factor 2
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
Erdj	J-domain containing proteins
ERGIC-53	ER-Golgi intermediate compartment of 53 kDa
ERO1	ER oxidoreductin 1
Erp57	Protein disulfide isomerase-related protein 57
ERSE	ER stress response element
GADD34	Growth arrest and DNA damage protein 34

List of Abbreviations

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF15	Growth and differentiation factor 15
GPI	Glycosylphosphatidylinositol
GRP78	Glucose-regulated protein of 78 kDa
GRP94	Glucose-regulated protein of 94 kDa
GSH	Glutathione
GTPase	Guanosine triphosphatase
HCP1	Heme carrier protein 1
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
Hcys	Homocysteine
HEK293T	Human embryonic kidney cell line
HepG2	Hepatocellular carcinoma cell line
HFE	Hemochromatosis gene
HH	Hereditary hemochromatosis
HIF-1 α	Hypoxia-inducible factor 1 α
HIV-1	Human immunodeficiency virus 1
HJV	Hemojuvelin
Hsp	Heat-shock protein
IFN γ	Interferon γ
IL	Interleukin
IRE	Iron responsive element
IRE1	Inositol-requiring enzyme 1
IRP	Iron regulatory protein
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KDEL	Lys - Asp - Glu - Leu
L1	Deferiprone
LPS	Lipopolysaccharide
MAPK/ERK	Mitogen-activated protein kinase/extracellular signal-regulated kinases
MHC	Major histocompatibility complex
nATF6	Nuclear ATF6
NF-Y	Nuclear factor Y
NIH3T3	Mouse embryonic fibroblast cell line
Nrf2	NF-E2-related factor
NTBI	Non-Tf-bound iron

OASIS	Old astrocyte specifically induced substance
OS9	Osteosarcoma 9
OST	Oligosaccharyltransferase
PBMCs	Peripheral blood mononuclear cells
PDI	Protein disulphide isomerase
PERK	Double-stranded RNA-dependent protein kinase-like ER kinase
PPIases	Peptidyl-prolyl isomerases
RIP	Regulated intramembrane proteolysis
RNC	Ribosome-nascent chain
S1P and S2P	Site-1 and -2 proteases
sHJV	Soluble HJV
SMAD	Mothers against decapentaplegic homologue
SR	SRP receptor
SRP	Signal recognition particle
STAT	Signal transduction and activator of transcription
STEAP3	Six-transmembrane epithelial antigen of the prostate 3
sXBP1	Spliced XBP1
TBI	Tf-bound iron
Tf	Transferrin
TfR	Tf receptor
Tisp40	Transcript induced in spermiogenesis 40
TNF α	Tumor necrosis factor α
Tm	Tunicamycin
UGGT	UDP-glucose:glycoprotein glucosyltransferase
uORF	Upstream open reading frame
UPR	Unfolded protein response
UPRE	UPR element
Usf2	Upstream stimulatory factor 2
UTR	Untranslated region
uXBP1	Unspliced XBP1
VIP36	Vesicular integral protein of 36 kDa
XBP1	X-box-binding protein 1
XTP3B	XTP3-transactivated gene B
ZIP14	Zrt-Irt-like protein 14

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CHAPTER I

General Introduction

Section I

ER stress & the Unfolded Protein Response

1. The Endoplasmic Reticulum protein factory

The endoplasmic reticulum (ER) is a multifunctional organelle with major roles in the secretory pathway. More than a mere transit compartment for secretory and membrane-targeted proteins, the ER is responsible for their biosynthesis, folding, assembly and modifications [Helenius et al. 1992]. The accomplishment of such variety of functions closely depends upon the specialized luminal conditions found in the ER, namely: abundance of resident molecular chaperones and folding enzymes [Meunier et al. 2002]; high Ca^{2+} stores necessary for optimal function of the former [Meldolesi and Pozzan 1998] and oxidizing milieu compatible with disulphide bond formation [Tu and Weissman 2004]. The accuracy of the process is ensured by stringent quality control mechanisms [Ellgaard and Helenius 2003], coupled to ER-associated degradation (ERAD) of aberrant proteins [Brodsky and McCracken 1999]. Operating together, both systems guarantee that only proteins whose native conformation was met are delivered to the Golgi apparatus towards their final destinations (Fig. 1).

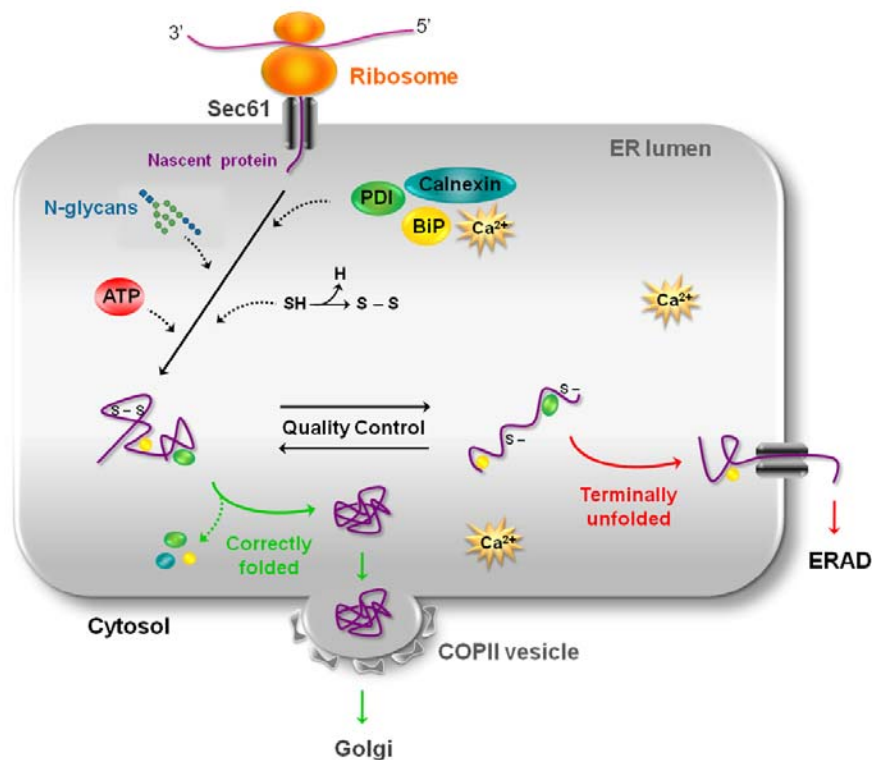


Figure 1. The endoplasmic reticulum (ER): a folding factory with two execution pathways. Proteins destined for the secretory route are targeted to the ER, where highly specialized maturation conditions

are provided. While entering the ER through the Sec61 translocon, nascent chains undergo a series of modifications, such as N-linked glycosylation and disulphide bond formation. In addition, growing polypeptides are engaged by the ER-resident chaperones (e.g. BiP and calnexin) and foldases (e.g. PDI) that assist their folding under strict quality control scrutiny. Properly folded clients are delivered to the Golgi via COPII-coated vesicles, towards the final destinations. Conversely, proteins that fail to reach the native state are retained and sent to ER-associated degradation (ERAD), a disposal pathway that prevents their toxic accumulation within the ER lumen.

1.1. Targeting proteins to the ER

A hydrophobic signal sequence typically localized at the N-terminus of secretory proteins targets them to the ER membrane [Blobel and Dobberstein 1975; Martoglio and Dobberstein 1998]. The process is mediated by the signal recognition particle (SRP), a cytosolic ribonucleoprotein that binds the address tag as it egresses from the ribosome, transiently arresting chain elongation [Walter and Johnson 1994]. The complex ribosome-nascent chain (RNC)-SRP is guided to the ER surface via interaction with the SRP receptor (SR) [Gilmore et al. 1982]. There, coordinated GTPase cycles elicit both SRP recycling and RNC docking into the multimeric Sec61 translocon, also allowing translation to resume [Connolly et al. 1991; Johnson and van Waas 1999]. As the growing polypeptide is vectorially delivered into the ER luminal space, the signal sequence is proteolitically cleaved [Mothes et al. 1994; Blobel and Dobberstein 1975] and the chain engaged by the local folding machinery, whose residency within the ER is ensured by a C-terminal KDEL-like sequence [Munro and Pelham 1987]. Although to a minor extent in mammalian cells, proteins can be post-translationally transferred across the ER membrane through SRP-independent pathways [Rapoport et al. 1999].

Regardless of the translocation mechanism, ER clients are folded to adopt the biologically active three-dimensional structure. Amongst the menu of possible folds, the preferred is the one that minimizes the global free energy and is referred to as the native state [Anfinsen 1973]. The burial of non-polar regions and electrostatic interactions in the protein's core, along with the exposure of polar side groups to the aqueous surroundings generally favors achievement of such state [Stevens and Argon 1999].

As postulated by Anfinsen and others in the early 1960's, instructions for the native structure are entirely codified in the linear amino acid sequence of a protein [Anfinsen et al. 1961; Epstein et al. 1963]. *In vivo*, however, this spontaneous self-

folding capacity is challenged by the intracellular crowding environment, making assistance from molecular chaperones and catalysts vital for the folding efficiency. A particularly thorough assistance is provided within the ER, since proteins exiting the organelle are no longer subject to chaperone surveillance and must, nevertheless, preserve their stability under demanding intra/extracellular conditions.

1.2. Assisted protein folding and Quality control in the ER

i. “Classical” molecular chaperones

By definition, molecular chaperones neither convey structural information dictating the protein's tertiary conformation nor accelerate the folding steps. Their inestimable role rather consists in preserving maturing polypeptides in a soluble, folding-competent state, thereby counteracting misfolding and aggregation [Ellis 1997]. The ER-lodged chaperones are not exception, being governed by the same functional tenets.

Prominent among the plethora of chaperones constitutively expressed in the ER is glucose-regulated protein (GRP)78, also known as immunoglobulin heavy chain-binding protein (BiP) [Haas and Wabl 1983; Munro and Pelham 1986]. While belonging to the 70 kDa heat-shock protein (Hsp) family, its regulation occurs independently of the cytosolic partners [Lee 1992]. BiP chaperoning activity relies on the promiscuous, low-affinity (1-100 mM) binding to hydrophobic segments exposed by protein folding intermediates [Blond-Elguindi 1993]. Such interaction, reflecting the coordinated regulation between the C- (peptide-binding) and N- (ATPase) terminal domains of BiP, involves repetitive ATP/ADP cycles through which maturing clients are transiently bound and released, progressing towards the native state [Gething 1999; Awad et al. 2008]. The functional cycle of BiP requires two additional classes of co-chaperones: J-domain containing proteins (ERdj1-7) that accelerate ATP-hydrolysis [Cheetham and Caplan 1998; Dudek et al. 2009] and nucleotide exchange factors (BAP and Hsp170) catalyzing the ADP/ATP switch reaction [Chung et al. 2002; Weitzmann et al. 2006]. Besides shielding immature substrates, several studies have highlighted the multitasking nature of BiP, including: i) maintenance of a competent permeability barrier in the ER membrane [Alder et al. 2005]; ii) driving force for

polypeptide translocation across the Sec61 pore [Matlack et al. 1999]; iii) preservation of ER Ca^{2+} homeostasis [Lièvrement et al. 1997]; iv) assistance of irreversibly unfolded proteins *en route* for degradation [Nishikawa et al. 2001] and v) regulation of the Unfolded Protein Response (UPR) signal transduction pathways (see below) [Hendershot 2004].

The most abundant, vertebrate restricted, glycoprotein operating in the ER is the Hsp90 homolog GRP94 [Koch et al. 1986; Argon and Simen 1999]. The molecule appears to function downstream of BiP, thus stabilizing partially folded intermediates [Melnick et al. 1994] through yet poorly characterized mechanisms. Importantly, the subset of known GRP94-assisted clients is relatively narrow, possibly a consequence of the binding selectivity/specificity imposed by the chaperone [Argon and Simen 1999]. Cumulatively to its role as chaperone, GRP94 has been linked to T-cell immune responses [Suto and Srivastava 1995; Berwin et al. 2002].

ii. **Lectin chaperones**

Originally named for their Ca^{2+} -buffering capacity [Michalak et al. 2002], calnexin (CNX) and calreticulin (CRT) are major components of the ubiquitous ER-localized lectin chaperones. Sharing high degree of sequence and structural identity, the proteins are distinguished by the topological environment: CNX is anchored to the membrane, whereas CRT is a soluble luminal version [Schrag et al. 2001]. Despite the extensive structural similarity, differences in the substrate preferences of the two homologues likely arose from the latter feature [Molinari et al. 2004].

The CNX/CRT chaperone system is specifically devoted to the folding of glycoproteins, a large fraction of clients traversing the secretory route. The N-glycosylation process involves the attachment of preassembled mannose-rich oligosaccharides ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) to target asparagine residues of proteins [Kornfeld and Kornfeld 1985]. The reaction is catalyzed by oligosaccharyltransferase (OST) and occurs as soon as the nascent chain emerges into the ER lumen [Nilsson and von Heijne 1993]. Aside from maximizing the global hydrophilicity and conformational stability of polypeptide backbones, oligosaccharide moieties support the recruitment of CNX/CRT [Helenius and Aebi 2004]. Their association with maturing clients, however, is unable to proceed until glucosidases I and II convert

the original N-glycan into the monoglucosylated form [Herscovics 1999], the unique recognized by the lectins [Hammond et al. 1994; Ware et al. 1995]. Exploiting such substrate constraint, glucosidase II excises the remaining glucose of the core oligosaccharide, while UDP-glucose:glycoprotein glucosyltransferase (UGGT) renders the opposite effect [Hebert et al. 1995]. This concerted action abolishes and restores the lectin engagement with client glycoproteins, respectively, representing the keystone of the so-called CNX/CRT cycle. Owing to the sentinel activity of UGGT [Ritter and Helenius 2000], the cycle continues until the native conformation is achieved or until degradative processes take place, thus constituting a crucial ER quality control checkpoint [Ellgaard and Helenius 2001; Trombetta and Parodi 2003].

Additional lectin-like proteins accumulate in the ER, namely those participating in the sorting of glycoproteins to the Golgi (ERGIC-53 and VIP36) [Hauri et al. 2000] or to the ERAD pathway (ER degradation enhancing α -mannosidase-like proteins – EDEMs) [Hosokawa et al. 2001].

iii. **Redox enzymes**

The oxidizing environment, one of the most remarkable features of the ER, is both a precondition and a consequence of the oxidative folding of exportable proteins [Hwang et al. 1992; Mezghrani et al. 2001]. In fact, maturation of ER clients involves formation of inter- and intramolecular disulphide bonds between cysteine residues, tailored to: i) determine functional properties and enhance the structural integrity of proteins; ii) restrict folding possibilities, thereby guiding the process; iii) support oligomerization and iv) provide additional folding supervision points [Tu and Weissman 2004; van Anken and Braakman 2005]. The importance of redox-regulated events in the secretory pathway is witnessed by the impressive collection of related catalysts housed in the ER, the oxidoreductases [Sevier and Kaiser 2006; Appenzeller-Herzog and Ellgaard 2008]. These enzymes not only catalyze the oxidation of thiols to generate disulphide bonds, but also the opposite reaction, conducive to reduction and rearrangement of disulphides. Such versatility underlies the isomerase activity of oxidoreductases, allowing the unscrambling of erroneous disulphide pairings towards the native ones [Fassio and Sitia 2002; Ellgaard 2004].

The classical ER oxidoreductase is protein disulphide isomerase (PDI) [Goldberger et al. 1963; Freedman et al. 1994]. The oxidation state of the cysteines found in the active-site Cys-Xaa-Xaa-Cys motif dictates its function as thiol-disulphide reductase, oxidase or isomerase [Schwaller et al. 2003; Appenzeller-Herzog and Ellgaard 2008]. The efficient completion of PDI redox cycling is ensured by the balanced action of ER oxidoreductin (ERO)1 and glutathione (GSH) [Frard and Kaiser 1999; Jessop and Bulleid 2004]. By transferring oxidizing equivalents, the former promotes PDI oxidation, whereas GSH drives the reverse effect. Along with the catalytic role, PDI displays chaperone-like behavior, recognizing and transiently binding aggregation-prone substrates [Wang and Tsou 1993].

Another well-known ER-resident oxidoreductase is ERp57. Although analogous to PDI in terms of domain organization and function, ERp57 forms one-to-one complexes with CRT and CNX, specifically assisting the oxidative folding of glycoproteins [Oliver et al. 1999]. This model nicely illustrates the cooperation established between enzymatic and chaperone systems during the folding pathways.

iv. **Peptidyl-prolyl isomerases**

The repertoire of folding catalysts working in the ER lumen also includes peptidyl-prolyl isomerases (PPIases). Members of this family facilitate the *cis-trans* isomerisation of peptide bonds preceding prolyl residues within polypeptide chains [Bose and Freedman 1994]. Given the ability to accelerate this rate-limiting step and to render the backbone of maturing clients flexible, PPIases are key adjuvant components of the folding process [Schmid et al. 1993].

In the last decades, a growing list of ER folding factors has emerged into the limelight. Although here described separately for the sake of clarity, these chaperone and foldase systems are not mutually exclusive, rather functioning *in vivo* in a network-like manner [Kleizen and Braakman 2004].

1.3. ER-Associated Degradation

The ER to Golgi transport via COPII-coated vesicles is, by principle, confined to properly folded and assembled proteins [Lee et al. 2004a]. Those that, in spite of all efforts, fail to attain the native conformation are diverted to an alternative execution pathway: degradation. The toxicity inherent to the accumulation of such aberrant clients is circumvented by a specialized disposal mechanism termed ERAD [Brodsky and McCracken 1999]. The process involves retrotranslocation/dislocation of faulty proteins to the cytosol where degradation by the ubiquitin-proteasome system takes place [Tsai et al. 2002; Kostova and Wolf 2003]. How the ERAD folding machinery discriminates on-pathway unstructured intermediates from dead-end misfolded proteins remains intriguing, however. The exact nature of the ERAD-fate determinants and the decision timing are elusive as well.

Recognition of ERAD substrates is the first step of this route, which likely relies on folding defects and/or prolonged ER retention. ER chaperones, such as BiP, have been proposed to contribute to ERAD client selection [Kabani et al. 2003]. Moreover, by maintaining aggregation-prone polypeptides in solution, BiP ensures their efficient retrotranslocation [Nishikawa et al. 2001]. PDI is another relevant mediator of the recognition process, partly due to its chaperone activity. Further, PDI directs ERAD-destined proteins to the dislocation machinery where, functioning as thiol-reductase, promotes useful unfolding events prior to their delivery to the cytosol [Gillece et al. 1999; Tsai et al. 2001]. Glycoproteins misfolded beyond rescue are distinguished by the presence of mannose-trimmed oligosaccharides [Fagioli and Sitia 2001]. Demannosylation prevents futile CNX/CRT cycles, also generating degradation tags recognized by EDEM family members [Molinari et al. 2003]. Besides participating in the mannose trimming [Olivari et al. 2006], these lectin-type molecules act as acceptors of terminally non-native conformers, thereby mediating the crosstalk between folding and ERAD routes [Oda et al. 2003]. Working in close proximity with the membrane-anchored ubiquitination complex, osteosarcoma (OS)9 and XTP3-transactivated gene B (XTP3B) are additional ER lectins that assist the targeting process of ill-fated glycoproteins [Christianson et al. 2008; Buschhorn et al. 2004].

Clients committed to ERAD traverse the ER membrane towards the cytosol. The identity of the dislocation channel is still a contentious issue. Although the Sec61 translocon pore has been suggested to accommodate such function [Wiertz et al. 1996; Schäfer and Wolf 2009], alternative candidates have emerged and include the membrane-embedded Derlin-1 protein [Ye et al. 2004]. According to other models, the retrotranslocon might be transiently created from one or many components [Vembar and Brodsky 2008].

Once in the cytosolic face of the ER, folding-defective proteins are polyubiquitinated through the sequential activity of the ubiquitin-activating (E1), -conjugating (E2) and -protein ligase (E3) enzymes [Hiller et al. 1996]. By virtue of this modification, ERAD substrates gain access to the 26S proteasome to be eventually degraded. The list of E3 ubiquitin variants partaking in ERAD is expanding, probably reflecting client-dependent specificity [Imai et al. 2001; Yoshida et al. 2002; Carvalho et al. 2006]. If the ERAD efficiency is hampered or inefficient, a backup clearance system consisting in the autophagic engulfment of ER portions is triggered [Klionsky 2007].

2. The Unfolded Protein Response

Notwithstanding the sophistication of the quality control mechanisms normally provided by the ER, certain physiological states and exogenous stimuli can compromise the above-described folding environment, unbalancing the load/capacity ratio of the ER. Such condition, collectively termed ER stress, is instigated by numerous acute and chronic factors. Disruption of Ca^{2+} stores, alteration of redox status, energy/nutrient deprivation and hypoxia fall into the first category [Rutkowski and Kaufman 2004; Bernales et al. 2006], whereas expression of mutant substrates or ER folding components [Kozutsumi et al. 1988], viral infection and even the potent secretory activity of certain cell types [Dimcheff et al. 2003; Calton et al. 2002] are examples of chronic stress insults. To cope with these deleterious scenarios and counteract the accumulation of misfolded clients, cells have evolved specialized signaling circuits referred to as the Unfolded Protein Response (UPR). Tailored to restore ER homeostasis, the UPR combines multiple synergistic strategies that encompass global suppression of protein synthesis and

translocation into the ER, transcriptional induction of ER chaperones and foldases to face the increased folding demands and improvement of ERAD machinery to bolster the clearance of irreparably unfolded proteins [Schröder and Kaufman 2005; Bernales et al. 2006]. If the pro-survival attempts are exhausted and the ER damage prevails, UPR-induced pro-apoptotic programs are executed [Xu et al. 2005].

In mammalian cells, three ER-resident transmembrane proteins operate as proximal sensors and define the major UPR signaling pathways – double-stranded RNA-dependent protein kinase-like ER kinase (PERK), inositol-requiring enzyme (IRE)1 and activating transcription factor (ATF)6 [Schröder and Kaufman 2005; Bernales et al. 2006]. Despite this diversity, association with BiP is proposed as a common regulator of the ER transducers. Under unstressed conditions, BiP binds the luminal domains of all sensors, rendering them inactive. As unfolded proteins clog the ER, BiP is competitively titrated away, allowing the PERK-, IRE1- and ATF6-dependent cascades to proceed [Bertolotti et al. 2000; Hendershot 2004] (Fig. 2). Over the past few years, significant progress has been made in understanding the UPR signaling network and a brief overview will be provided here.

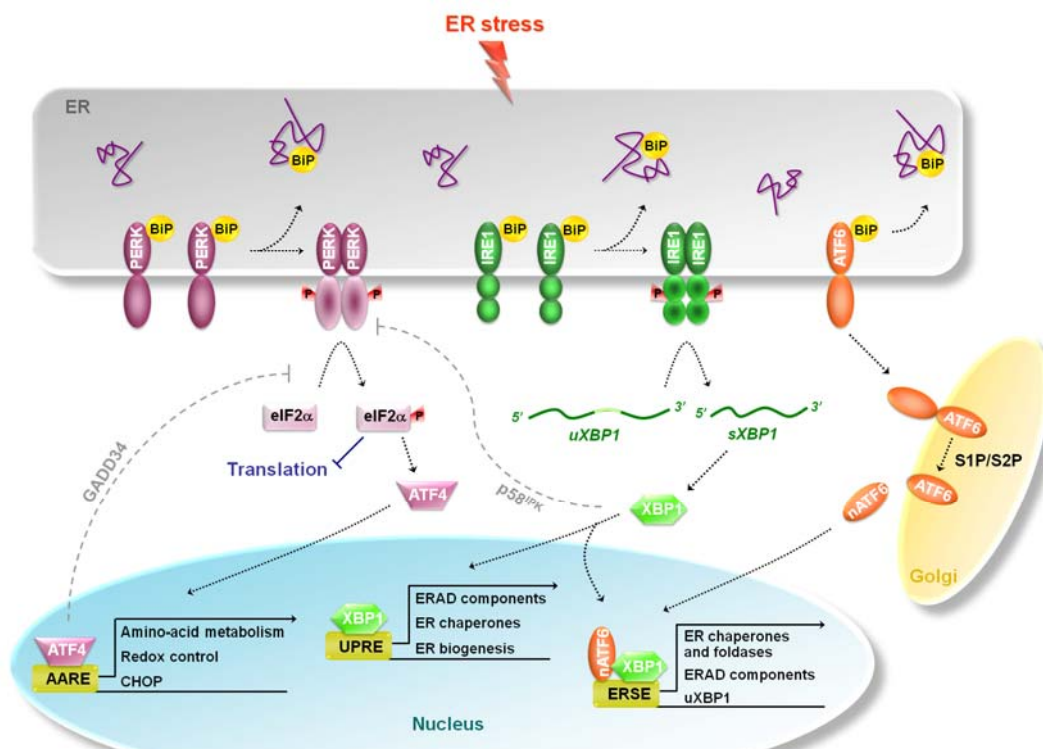


Figure 2. The unfolded protein response (UPR) network in mammalian cells. The endoplasmic reticulum (ER)-transmembrane proteins PERK, IRE1 and ATF6 act as proximal sensors of ER stress, initiating distinct downstream signaling cascades. Accumulated misfolded proteins titrate BiP away from

the luminal domains of each sensor, thereby enabling their activation. PERK, activated through homodimerization and *trans*-autophosphorylation, triggers phosphorylation of eIF2 α . In consequence, global protein synthesis is attenuated, while translation of selected transcription factors is enhanced. One such example is ATF4, whose target genes code for proteins involved in amino acid metabolism, antioxidative response and apoptosis (e.g. CHOP). ATF4 also induces the production of GADD34 that, promoting dephosphorylation of eIF2 α , allow translation to resume and define a negative feedback loop. Once dimerized and phosphorylated, IRE1 promotes splicing of uXBP1 mRNA. The spliced version (sXBP1) encodes a potent transcription factor that up-regulates the expression of folding and ERAD components. The IRE1/XBP1 axis provides a second feedback mechanism via p58^{IPK} which, interacting with PERK, prevents its phosphorylation and downstream cascade. Upon BiP dissociation, ATF6 migrates to the Golgi apparatus and undergoes a S1P/S2P-dependent proteolytic cleavage. The cytosolic domain (nATF6) is released and further translocated into the nucleus, activating the transcription of UPR target genes. Among these is uXBP1, the substrate of the IRE1 branch.

PERK, double-stranded RNA-dependent protein kinase-like ER kinase; IRE1, inositol-requiring enzyme 1; ATF6, activating transcription factor 6; BiP, immunoglobulin heavy chain-binding protein; eIF2 α , α subunit of eukaryotic initiation factor 2; ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; GADD34, growth arrest and DNA damage protein 34; XBP1, X-box-binding protein 1; ERAD, ER-associated degradation; S1P, site-1 protease; S2P, site-2 protease; AARE, amino acid response element; UPRE, unfolded protein response element; ERSE, ER stress response element. Grey dashed lines: negative feedback loops.

i. The PERK pathway

Massive accumulation of unfolded clients in the ER lumen sequesters BiP from PERK, unmasking the dimerization motifs of this type-I transmembrane protein [Bertolotti et al. 2000]. As a consequence, PERK homodimerizes and autophosphorylates, thereby activating its cytosolic kinase function toward the α subunit of eukaryotic initiation factor 2 (eIF2 α). By inhibiting the formation of ribosomal preinitiation complexes, phosphorylated eIF2 α transiently halts protein synthesis [Harding et al. 1999; Harding et al. 2000b] (Fig. 2). Such reaction is the primary line of defense against ER stress, since it promptly limits the load of ER clients while the downstream transcriptional response is set in motion. Another direct repercussion is cell cycle arrest, reflecting the rapid depletion of cyclin D1 protein pool [Brewer and Diehl 2000]. PERK concomitantly phosphorylates NF-E2-related factor (Nrf2), a transcription factor involved in oxidative stress protection [Cullinan et al. 2003].

Paradoxically to the general repression of protein synthesis, selective translation of a subset of mRNAs containing 5' upstream open reading frames (uORF) is triggered by the PERK-eIF2 α axis [Lu et al. 2004]. A prominent example is ATF4 that programs the expression of UPR-target genes encoding amino acid transporters, redox control and cell death-inducing molecules [Harding et al. 2000a;

Harding et al. 2003]. Integrating the last group is the transcription factor CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), whose pro-apoptotic action is apparently linked to growth arrest and DNA damage protein (GADD)34 up-regulation [Marciniak et al. 2004]. This protein mediates dephosphorylation of eIF2 α as part of a negative feedback loop to reverse the translational block [Novoa et al. 2001]. However, if ER homeostasis remains disrupted the recovery of protein synthesis might exacerbate ER stress and lead to cell-lethal outcomes.

In physiological contexts, signaling through PERK/eIF2 α exerts fundamental translational control in pancreatic β -cells, maintaining adequate levels of insulin production and contributing to their normal proliferation and survival [Zhang et al. 2006b]. Equally essential is the ATF4 function during osteoblast differentiation and collagen synthesis [Yang et al. 2004].

ii. **The IRE1 pathway**

ER stress signals are also integrated and transmitted through the ancient IRE1, a bifunctional transmembrane protein with both kinase and endoribonuclease activities at the cytosolic face [Cox et al. 1993; Tirasophon et al. 1998]. The structural homology shared by the luminal domains of PERK and IRE1 renders identical activation mechanisms. Accordingly, IRE1 activation relies on BiP dissociation which, in turn, determines its homodimerization and *trans*-autophosphorylation [Bertolotti et al. 2000]. An alternative model of sensing claims that the major histocompatibility complex (MHC)-like groove displayed by IRE1 directly detects and binds misfolded clients in the ER lumen [Credle et al. 2005]. The likelihood of this hypothesis, however, is weakened by the narrow dimension of the groove [Zhou et al. 2006].

Activated IRE1 drives the spliceosome-independent cleavage of a 26-base intron from X-box-binding protein (XBP)1 pre-mRNA [Yoshida et al. 2001; Calton et al. 2002]. Spliced XBP1 (sXBP1) encodes a potent bZIP transcription factor that up-regulates a battery of UPR-responsive genes harboring either ER stress response element (ERSE) or UPR element (UPRE) binding motifs [Yoshida et al. 2000; Yamamoto et al. 2004]. Its transcriptional output includes ERAD components, ER

chaperones, as well as modulators of lipid synthesis and ER expansion [Lee et al. 2003; Sriburi et al. 2004] (Fig. 2). Apart from the conventional cascade, it was recently uncovered that IRE1 mediates the selective clearance of ER-associated mRNAs encoding secreted proteins [Hollien and Weissman 2006]. Such pathway anticipates and rapidly prevents further ER crowding. An UPR termination signal emanates from the IRE1/XBP1 axis, having as executor p58^{IPK}. This co-chaperone interacts with PERK, impairing its phosphorylation and the corresponding downstream events [Lee et al. 2003; Yan et al. 2002].

Beyond the classical UPR targets, the IRE1/XBP1 sub-pathway is critically required for some cellular metabolic processes, including insulin production by pancreatic β -cells and hepatic lipogenesis [Lipson et al. 2006; Lee et al. 2008]. Consistent with the UPR's purpose of bolstering ER capacity, the IRE1 signaling branch is essential for B-cell differentiation into secretory plasma cells [Reimold et al. 2001; Zhang et al. 2005]. Recently, IRE1 activation was found in differentiating T-lymphocytes [Brunsing et al. 2008], likewise illustrating its physiological role.

iii. **The ATF6 pathway**

ATF6 is a type II ER membrane-anchored protein defining the third UPR arm [Haze et al. 1999]. In resting conditions, ER localization of ATF6 is guaranteed through chaperone tethering. Furthermore, the redox status of the ATF6 luminal portion was recently coupled to its activation state [Nadanaka et al. 2007]. Along with the accumulation of misfolded proteins, BiP dissociates from ATF6, allowing its mobilization to the Golgi compartment [Shen et al. 2002]. By releasing this transducer from CRT, underglycosylation of ATF6 has equivalent effects [Hong et al. 2004]. In the Golgi, site-1 and -2 proteases (S1P and S2P) promote regulated intramembrane proteolysis (RIP) of ATF6, liberating a soluble, active transcription factor (nATF6) [Ye et al. 2000]. Upon trafficking to the nucleus, nATF6 binds ERSE-containing promoters in concert with the constitutive NF-Y transcription factor [Yoshida et al. 2000], thereby inducing the expression of numerous ER folding assistants [Okada et al. 2002] (Fig. 2). Of note, nATF6 drives up-regulation of the unspliced XBP1 (uXBP1) mRNA, supplying the IRE1 pathway with its substrate [Yoshida et al. 2001]. Reciprocally, uXBP1 triggers nATF6 degradation, acting as

negative regulator of the ATF6 signaling cascade [Yoshida et al. 2009]. Convergent ATF6 and PERK signals were also reported, since both transducers lead to CHOP transcriptional activation [Ma et al. 2002]. Such findings illustrate the interactive and overlapping nature of the UPR branches.

Evidence for a broader physiological role of ATF6 stems from its ability to enhance acute inflammatory responses in liver cells undergoing ER stress [Zhang et al. 2006a].

Novel ATF6-related transducers, likely processed through ER stress-induced RIP, but exhibiting tissue-specific distributions have been identified. The list includes cAMP response element-binding protein (CREB)H [Zhang et al. 2006a], old astrocyte specifically induced substance (OASIS) [Kondo et al. 2005], BBF2 human homolog on chromosome 7 (BBF2H7) [Kondo et al. 2007] and transcript induced in spermiogenesis (Tisp)40 [Nagamori et al. 2005], whose expression is restricted to liver, astrocytes, neurons and spermatids, respectively. This diversity may reflect exclusive local strategies to tackle ER stress, also raising the possibility that each sensor regulates unique subsets of genes.

2.1. ER-stress induced apoptosis

From the preceding discussion, one can envision the UPR as a pro-survival program aimed at restoring ER homeostasis. However, persistent proteotoxic insults might render insurmountable levels of ER stress, eliciting a second execution plan: apoptosis. Although the mechanisms underlying this life/death decision are still barely understood, multiple ER-derived apoptotic signals have been described. One of the most studied relies on CHOP. Besides the above-mentioned induction of GADD34, the pro-death role of CHOP is likely mediated through both ERO1 α up-regulation [Marciniak et al. 2004] and repression of the antiapoptotic Bcl-2 [McCullough et al. 2001]. Furthermore, the intrinsic instability of CHOP mRNA and protein was recently shown to regulate the transition to apoptosis [Rutkowski et al. 2006]. Release of Ca²⁺ from the ER into the cytoplasm might also commit cells to apoptosis, either by mitochondrial-dependent or -independent induction of caspase cascades [Boya et al. 2002; Nakagawa and Yuan 2000]. A major ER stress-related death signal emanates from the IRE1 axis and involves activation of the apoptosis-

signaling kinase 1/c-Jun N-terminal kinase (ASK1/JNK) pathway [Urano et al. 2000; Nishitoh et al. 2002].

This topic was broadly addressed here, as a thorough review is beyond the scope of this thesis. Nevertheless, crucial questions remain open and should be mentioned, namely: how is the dichotomy adaptation vs apoptosis integrated by the same regulatory program? Which are the criteria governing the cell death commitment during the UPR? Is there an “ER stress threshold” dictating the switch from pro-survival efforts to apoptotic signals?

Section II

Iron genes & Iron metabolism

3. The outlines of iron metabolism

With the exception of some bacteria, all living organisms have an absolute requirement for iron. This commonality across species largely reflects the properties of iron as transition metal, namely the ability to rapidly transfer electrons and the versatility in binding ligands [Hentze et al. 2004]. Either as co-factor or biocatalyst, iron is critical for a variety of metabolic processes and enzymatic systems, ranging from oxygen transport and bioenergetics to DNA synthesis and repair [Drakesmith and Prentice 2008]. However, the avid redox activity of iron is potentially detrimental for lipids, proteins and nucleic acids. These biomolecules, particularly vulnerable to oxidative attack, are damaged by free radicals generated via the iron-catalyzed Fenton reaction [Papanikolaou and Pantopoulos 2005]. It is therefore mandatory to strictly regulate body iron stores, restricting the toxic accumulation of this metal without compromising its availability for cellular demand. A myriad of proteins and regulatory systems contributing to this goal has evolved in humans [Hentze et al. 2004]. Although still incompletely understood, components and principles of iron balancing have been unraveled in the last decades and will be summarized here.

3.1. Absorption, traffic and storage of iron

Even though all cells have daily iron needs for essential metabolic processes, they are quantitatively negligible comparing to the major body iron consumers – erythroid precursors. The iron necessary to sustain hemoglobinization of newly produced erythrocytes (approximately 20-25 mg/day) is mostly derived from the macrophage-mediated recycling of senescent red blood cells. Complementing this reutilization circuit, intestinal absorption contributes with 1-2 mg of iron per day, only compensating for losses through epithelial sloughing and bleeding episodes [Andrews 1999]. This description outlines two dominant players of systemic iron homeostasis – reticuloendothelial macrophages and duodenal enterocytes [Knutson and Wessling-Resnick 2003; Frazer and Anderson 2005]. Hepatocytes represent a third piece in this complex puzzle as the major site for iron storage and producers of a complex range of iron-related molecules [Graham et al. 2007] (Fig. 3).

The absence of an active physiological system underlying iron excretion in mammals renders duodenal absorption the primary regulated and regulating mechanism to control iron homeostasis [McCance and Widdowson 1937]. Regardless of the source, both heme and non-heme dietary iron are absorbed at the apical membrane of duodenal enterocytes. Import of the former proceeds via heme carrier protein (HCP)1 [Shayeghi et al. 2005], while acquisition of inorganic iron involves the combined action of the cytochrome b-like ferriredutase Dcytb and divalent metal transporter (DMT)1 [McKie et al. 2001; Gunshin et al. 1997]. Once inside enterocytes, iron is transferred across their basolateral membranes into the bloodstream. Ferroportin, the iron exporter [Donovan et al. 2000; McKie et al. 2000], and hephaestin, a ferroxidase that converts ferrous ions (Fe^{2+}) to the ferric state (Fe^{3+}) [Vulpe et al. 1999], are engaged in this step (Fig. 3). Of note, expression of these molecules is influenced by the iron content of enterocytes, thereby responding to the body requirements.

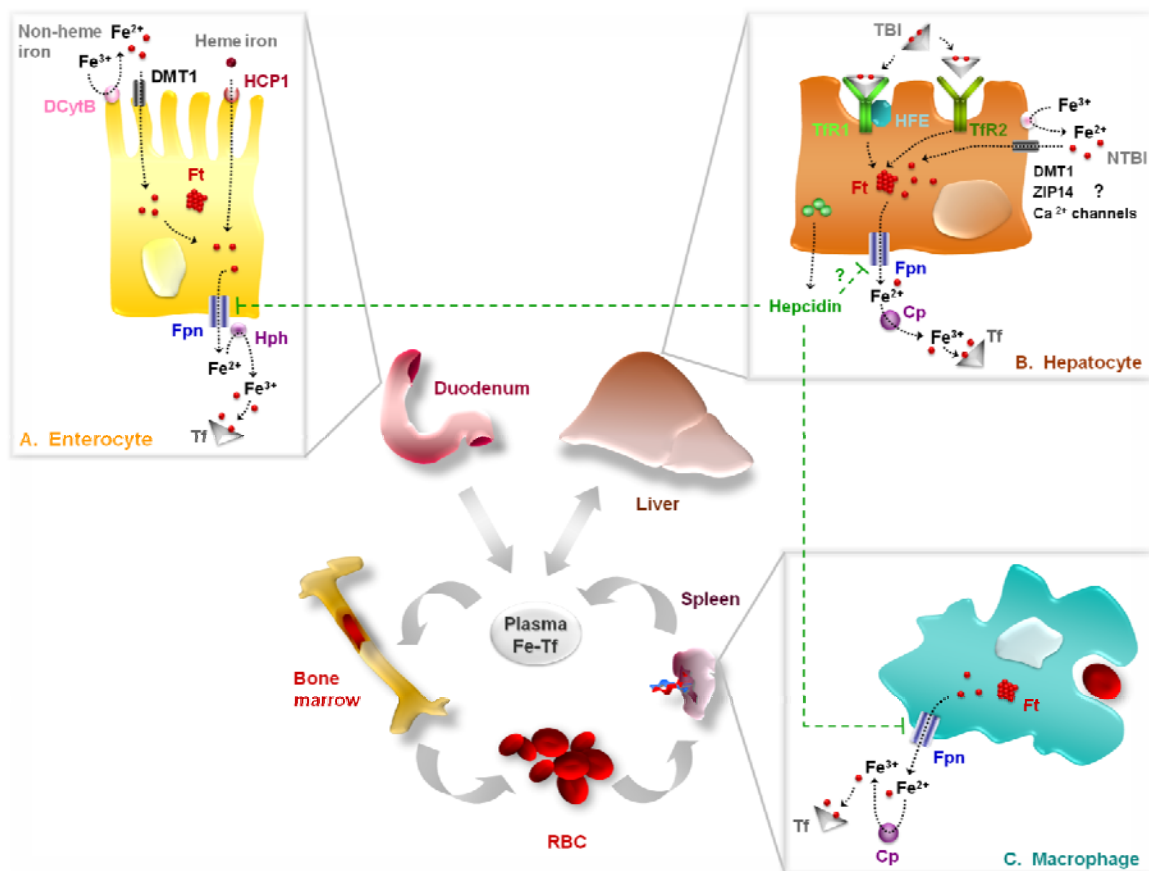


Figure 3. Overview of systemic and cellular iron metabolism. The central part of the scheme represents the close circuit of iron traffic. Dedicated to the production of red blood cells (RBC), erythroid bone marrow is the primary consumer of circulating transferrin (Tf)-bound iron (TfBI).

Senescent RBC are phagocytosed by macrophages of the reticuloendothelial system (spleen), process that guarantees the efficient recycling of the metal from hemoglobin and its return back to plasma. Dietary iron absorption in the duodenum only compensates for daily loss. Excess iron is deposited in the liver, mainly in hepatocytes, being mobilized from stores according to the body needs. The edged panels (A-C) depict iron transport in the major cellular players of iron homeostasis. **A**, Duodenal enterocytes absorb iron from diet at the apical brush border via divalent metal transporter (DMT)1, which is preceded by the duodenal cytochrome b (Dcytb)-mediated reduction of ferric ion (Fe^{3+}) to the ferrous state (Fe^{2+}). Heme is another source of dietary iron imported by heme carrier protein (HCP)1. Once in the cytoplasm, iron can be stored as ferritin (Ft) or released into the bloodstream through the basolateral membrane-localized ferroportin (Fpn). After oxidation by hephaestin (Hph), ferric iron circulates in the plasma bound to the shuttle-protein Tf that ensures its distribution to the various tissues. **B**, As specialized iron storage cells, hepatocytes display several modalities of iron acquisition. The best characterized is the uptake of TBI through a receptor-mediated endocytosis process involving either Tf receptor 1 or 2 (TfR1 or TfR2). Hepatocytes can also import non-Tf bound iron (NTBI) via carrier-mediated mechanisms, likely using DMT1, Zrt-Irt-like protein (ZIP)14 and Ca^{2+} channels as transporters. Imported iron is mostly sequestered by Ft for storage. When mobilization from stores is triggered, iron is released into the plasma by Fpn, which is coupled to the ferroxidase activity of ceruloplasmin (Cp). In addition, hepatocytes produce the major iron regulatory hormone hepcidin. This peptide targets Fpn, leading to its internalization and degradation, thus inhibiting iron release from duodenal enterocytes and reticuloendothelial macrophages. Hepcidin expression is modulated by iron levels, inflammation, hypoxia and erythropoietic demand. **C**, Erythrophagocytosis mediated by tissue macrophages serves the purpose of recycling iron. The metal can be stored within these cells as Ft or reloaded into plasma Tf upon Fpn-dependent export.

In the plasma, ferric ion generated by ceruloplasmin-mediated oxidation [Osaki et al. 1966; Harris et al. 1995] is loaded onto transferrin (Tf), yielding both mono- and diferric isoforms. Such high affinity complexes support the systemic delivery of iron, ensuring its solubility and circumventing free metal toxicity. Cellular uptake of Tf-bound iron (TBI) occurs through a specific endocytosis mechanism dependent on Tf receptor (TfR)1 [Dautry-Varsat et al. 1983; Enns et al. 1996]. During this pathway, holoTf/TfR1 complexes clustered in clathrin-coated pits are internalized into endosomes, where the acidic milieu promotes iron disassembly from Tf. Endosomal iron export towards the cytosolic labile pool is accomplished by DMT1 [Fleming et al. 1998], a process preceded by the STEAP3 (six-transmembrane epithelial antigen of the prostate 3)-triggered reduction of Fe^{3+} to Fe^{2+} [Ohgami et al. 2005]. Working as a recycling system, the vesicles carrying apoTf and TfR1 return to the cell surface and leave both elements available for further uptake cycles. A second, low affinity modality of TBI acquisition has been described. The mechanism relies on TfR2, a homolog of TfR1 that drives endocytic internalization of diferric Tf in restricted cell types, namely hepatocytes [Kawabata et al. 1999; Deaglio et al. 2002].

Although Tf prevails as the major iron shuttle system, non-Tf-bound iron (NTBI) also circulates in the plasma [Sarkar 1970]. Such heterogeneous pool,

consisting of low molecular iron aggregates mostly formed by citrate as chelator [Grootveld et al. 1989], expands in iron burden states as Tf binding capacity is surpassed. In these conditions, NTBI is rapidly cleared by hepatocytes [Brissot et al. 1985] through pathways involving DMT1, Ca^{2+} channels and Zrt-Irt-like protein (ZIP)14 as strong candidate carriers [Shindo et al. 2006; Oudit et al. 2003; Liuzzi et al. 2006] (Fig. 3).

Irrespective of the import variant, cytosolic iron is distributed to the requiring moieties (e.g. heme and Fe-S clusters) and metabolic activities. The excess is sequestered by ferritin, a multimeric protein composed by two subunit isoforms (heavy, H and light, L) that shields iron as ferrihydrite into a mineral core [Harrison and Arosio 1996]. Responding to the physiological needs, iron is mobilized from ferritin by still unclear mechanisms, likely based on lysosomal degradation [Kidane et al. 2006] or exit through the protein nanocage pores [Jin et al. 2001]. The proteasomal clearance of iron-poor ferritin was also reported [De Domenico et al. 2006]. The process here described is particularly prominent in hepatocytes, dedicated iron storage cells.

3.2. Regulation of iron metabolism

Maintenance of iron balance heavily depends on the dynamic regulation of molecules partaking in uptake, transport, storage and export of this biometal. Such molecular machinery reflects the demand for iron imposed by erythropoietic activity (*erythroid regulator*) and total body iron content (*stores regulator*) [Finch 1994], with preponderance of the former. The mechanisms controlling this modulation could be transcriptional, post-transcriptional or post-translational.

At a cellular level, post-transcriptional regulation is one of the best characterized. The system relies on cytoplasmic iron regulatory proteins (IRP1 and IRP2) that interact with conserved iron responsive elements (IREs) present in untranslated regions (UTRs) of several transcripts [Casey et al. 1988; Hentze and Kühn 1996]. The 5' or 3' location of IREs dictates the fate of the corresponding mRNAs upon IRP binding. At the 5' UTR, formation of IRE/IRP complexes blocks the translation initiation process, as occurs in ferritin and ferroportin transcripts [Gray and Hentze 1994; Lymboussaki et al. 2003]. Conversely, mRNAs harboring IREs on

the 3' UTR are stabilized following IRP recruitment, thus increasing their expression. DMT1 and TfR1 fall into the latter category [Gunshin et al. 2001; Casey et al. 1989]. The binding affinity of both IRP1 and IRP2 is primarily tuned by the cellular labile iron content, also responding to hypoxia [Hanson et al. 1999], reactive oxygen species and nitric oxide [Pantopoulos et al. 1996]. Accordingly, iron starvation stimulates the IRE/IRP interaction, whereas the opposite is favored under metal-replete conditions, thereby coordinating expression of key iron-related molecules [Hentze and Kühn 1996; Recalcati et al. 2010].

Transcriptional modulation of target genes appears as another regulatory strategy of iron metabolism. This system is essentially governed by iron status, cytokine stimuli and growth factors. Proinflammatory cytokines, such as tumor necrosis factor (TNF) α and interferon (IFN) γ , positively regulate ferritin H and DMT1, simultaneously decreasing ferroportin mRNA expression [Torti and Torti 2002; Ludwiczek et al. 2003]. These changes are collectively envisaged as a host iron-withholding program against invading pathogens. Acting as oxygen sensor, hypoxia-inducible factor (HIF)-1 α was shown to influence the transcription of DMT1, Dcytb, Tf, TfR1, ceruloplasmin and ferroportin [Li et al. 2008; Peyssonnaud et al. 2008], thus building-up the erythropoietic capacity and reconciling iron homeostasis with hypoxic states.

Notwithstanding the above-mentioned pathways, the seminal mechanism orchestrating systemic iron balance is post-translationally executed. The 25-residue peptide hormone hepcidin is the long-sought soluble regulator and ferroportin the cognate receptor [Nemeth and Ganz 2006]. Mainly secreted by hepatocytes [Park et al. 2001], hepcidin binds to the membrane-anchored ferroportin, triggering its internalization and lysosomal degradation [Nemeth et al. 2004]. Duodenal absorption and iron egress from macrophages are therefore inhibited, ultimately restricting the availability of the biometal in circulation.

3.2.1. **Hepcidin, the iron shepherd**

Hepcidin was first described in the context of inflammation as an antimicrobial molecule [Park et al. 2001]. Its connection to iron metabolism was uncovered when accidental down-regulation of hepcidin, derived from disruption of an adjacent locus (*Usf2*), was accompanied by severe iron overload [Nicolas et al. 2001]. Although fortuitous, such discovery would open a new field of intense research, largely focused on the molecular mechanisms driving hepcidin expression.

The multitasking trait of hepcidin arises from its responsiveness to iron stores, inflammation, anemia and hypoxia [Pigeon et al. 2001; Nicolas et al. 2002]. Hepcidin is physiologically stimulated in the first two conditions, with the converse occurring in the remaining ones. This versatility is also mirrored by the array of signaling pathways coordinating hepcidin transcription as-yet identified (Fig. 4).

i. **Regulation by iron**

The absence of IRE motifs within hepcidin mRNA excludes regulation through the IRE/IRP system. Its modulation is rather transcriptional, but the mechanism turned out more intricate than anticipated. Genetic disorders of iron metabolism have provided important clues. Affecting either the hemochromatosis gene HFE [Bridle et al. 2003], TfR2 [Nemeth et al. 2005] or hemojuvelin (HJV) [Papanikolaou et al. 2004], all such pathologies converge on inadequate production of hepcidin, suggesting an upstream role of the former molecules in the regulatory network.

Bone morphogenetic proteins (BMPs)-mediated cascade was recently coupled to iron-sensing and hepcidin activation [Truksa et al. 2006]. By signaling through specific receptors (BMPR) on the hepatocyte surface, these factors trigger phosphorylation of mothers against decapentaplegic homologue (SMAD) proteins (SMADs 1/5/8), dictating their interaction with a common co-SMAD, SMAD4. The complex then migrates to the nucleus and binds to DNA response elements in the hepcidin gene promoter, stimulating its transcription. In line with this, liver-restricted ablation of *Smad4* suppressed hepcidin synthesis [Wang et al. 2005], whilst supplementation with BMPs 2, 4, 6 and 9 conveyed stimulatory effects [Babitt et al. 2007]. HJV, a glycosylphosphatidylinositol (GPI)-linked membrane protein

predominantly found in hepatocytes, emerged as a focal piece of this pathway. Acting as a BMP co-receptor, HJV enhances both the BMPs/SMAD4 signaling cascade and the downstream impact on hepcidin expression [Babitt et al. 2006] (Fig. 4). This picture nicely accommodates the dramatic reduction of hepcidin levels and severe iron accumulation elicited by mutant forms of HJV, the leading cause of juvenile hemochromatosis [Papanikolaou et al. 2004]. The interplay between HJV and the BMPs cascade was further supported by the antagonizing effects produced by its soluble form (sHJV), likely due to competition for the same BMPRs [Lin et al. 2005; Babitt et al. 2007].

One question remains, however: how is the systemic iron status transmitted to the liver? The answer may, at least in part, lie in HFE, TfR1 and TfR2. Mutant forms of HFE, an atypical MHC-class I protein, accounts for the majority of “classical” hereditary hemochromatosis (HH) cases [Feder et al. 1996]. Competing with holoTf, HFE binds to TfR1 [Lebrón et al. 1999], which *per se* could define a pathway that lowers the amount of iron entering the cells, accordingly modulating hepcidin production. Nevertheless, fueled by the finding that HFE can also interact with TfR2 [Goswami and Andrews 2006], the mainstream view defends an alternative regulatory model. When the iron stores are replete, the abundant circulatory holoTf binds to TfR1 with avidity. Consequently, the released HFE would become available to associate with TfR2, initiating a signaling program aimed at inducing hepcidin expression [Schmidt et al. 2008; Gao et al. 2009]. The signaling would proceed through the mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) pathway, although a crosstalk with the BMPs cascade is likewise envisaged [Ramey et al. 2009] (Fig. 4).

In parallel, the liver-enriched C/EBP α nuclear factor has been correlated with both basal and iron-stimulated transcription of hepcidin in hepatocytes [Courselaud et al. 2002].

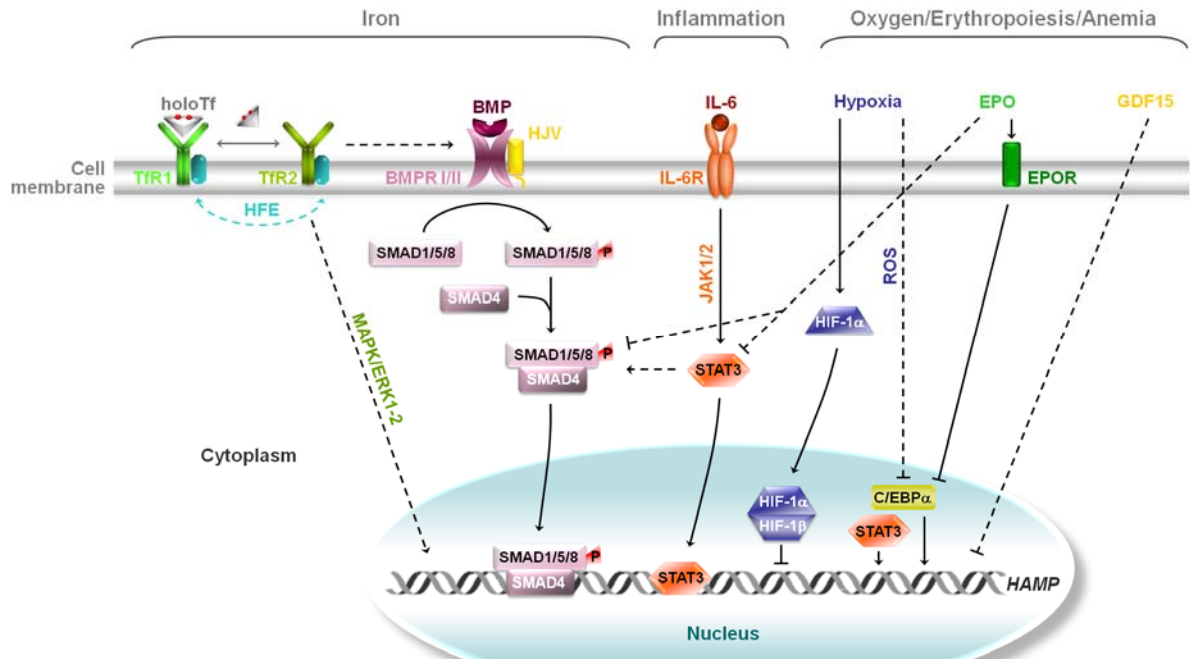


Figure 4. Major signaling pathways governing hepcidin expression. Hepcidin synthesis is primarily regulated at the level of transcription, responding to a variety of stimuli that ranges from iron content and inflammation to hypoxia and erythropoietic activity. At the cell surface, depending on the circulating concentration of holo-transferrin (holoTf), the hereditary hemochromatosis protein HFE associates either with transferrin receptor (TfR1) or TfR2. Under conditions of serum iron saturation, HFE is displaced from TfR1, becoming available to interact with TfR2. Such interaction triggers a signaling cascade, likely involving MAPK/ERK1-2 activation, aimed at up-regulate hepcidin (*HAMP*) transcription. Alternatively, it has been suggested that the HFE-TfR2 complex might signal through the bone morphogenetic proteins (BMPs)/mothers against decapentaplegic homologue (SMAD) route. This pathway is mediated by the binding of specific BMPs to the cognate multimeric receptors (BMPRI/II), with subsequent phosphorylation of the cytosolic SMADs1/5/8. Upon interaction with the co-SMAD4, the complex migrates to the nucleus, thereby stimulating *HAMP* gene expression. Acting as BMP co-receptor, the membrane-anchored form of hemojuvelin (HJV) potentiates this regulatory cascade. The responsiveness of *HAMP* to inflammatory states relies on the JAK1/2-dependent activation of signal transduction and activator of transcription (STAT)3, mostly triggered by the association of interleukin (IL)-6 to its cell surface receptor. A crosstalk between this classical route and the BMPs/SMAD signaling has been proposed as well. Hypoxic states were found to stabilize hypoxia-inducible factor (HIF)-1 α which, together with the constitutively expressed HIF-1 β , binds to the *HAMP* promoter, inhibiting its transcription. Increased levels of reactive oxygen species (ROS) generated during hypoxia, by impairing the CCAAT/enhancer binding protein (C/EBP) α and STAT3 binding to the *HAMP* promoter, were also implicated in such down-modulation. The regulatory model of the erythroid demand appears to be multifactorial. The hormone erythropoietin (EPO), signaling through its receptor (EPOR), was shown to directly decrease *HAMP* expression by antagonizing the C/EBP α binding activity. An indirect effect of EPO, rather involving suppression of the SMAD4 and STAT3 cascades, was recently suggested. The growth and differentiation factor (GDF)15, whose production reflects expansion of the erythroid compartment, was reported to straightly suppress *HAMP* transcription, via as-yet unknown mechanism. Solid lines: known/demonstrated pathways. Dashed lines: suggested/uncertain pathways.

ii. **Regulation by inflammation**

The antimicrobial activity of hepcidin stems from its enhanced synthesis under inflammatory conditions, with interleukin (IL)-6 as the principal mediator [Wrighting and Andrews 2006]. Converging on IL-6 production, IL-1 and bacterial lipopolysaccharide (LPS) stimuli render identical outcome [Lee et al. 2004b]. The effector pathway, triggered by IL-6 binding to its receptor, engages Janus kinase (JAK)1/2 and leads to activation of signal transduction and activator of transcription (STAT)3. Interacting with proximal elements of the hepcidin promoter, STAT3 induces its transcription [Verga Falzacappa et al. 2007]. Mounting evidence, however, highlights the essential role of the BMP/SMAD cascade in the IL-6-dependent stimulation of hepcidin, suggesting an interconnection between both pathways [Babitt et al. 2007; Verga Falzacappa et al. 2008] (Fig. 4).

Such a mechanism is thought to underlie the anemia of chronic disease typical of inflammatory episodes, strategically raised to deprive invasive microorganisms of iron [Nemeth et al. 2003].

iii. **Regulation by oxygen, erythropoiesis and anemia**

To meet the extraordinary erythropoietic demand, hypoxia and anemia down-modulate hepcidin levels, thereby allowing iron mobilization from stores and diet [Nicolas et al. 2002]. As referred above, hypoxia-stabilized HIF-1 α is translocated to the nucleus, directly targeting the hepcidin promoter and halting the transcription process [Peyssonnaud et al. 2007]. According to others, reactive oxygen species generated under hypoxic states might prevent the interaction of both C/EBP α and STAT3 with the hepcidin promoter, further suppressing its expression [Choi et al. 2007] (Fig. 4). Inhibition of 2-oxoglutarate-dependent oxygenases was also proposed to govern the HIF-unrelated repression of hepcidin under low oxygen tension [Braliou et al. 2008].

The nature of the erythropoietic regulator of hepcidin is debatable as well. One candidate is erythropoietin (EPO), an erythroid-stimulating hormone shown to directly decrease hepcidin expression through EPO receptor and C/EBP α [Pinto et al. 2008b]. An alternative via was recently presented, correlating the inhibitory action of EPO with impairment of the STAT3- and SMAD4-defined cascades [Huang et al.

2009]. Another plausible intermediary is the growth and differentiation factor (GDF)15, an oxygen-sensitive cytokine secreted by erythroid progenitors that likely defines a novel negative regulatory axis controlling hepcidin expression [Tanno et al. 2007] (Fig. 4).

How exactly the varied pathways controlling hepcidin expression are coordinated to produce a coherent response *in vivo* is still elusive but actively pursued. So far, hepcidin is consensually considered the focal point of iron balance, unifying the regulators classically classified as “erythroid” and “stores”. Moreover, ineffective modulation of hepcidin is currently viewed as the common pattern of all forms of hereditary iron-overload disorders, excepting those arising from ferroportin gene mutations.

Section III

Background, Research question & Outline of the thesis

Background

HFE – the Hereditary Hemochromatosis gene

Arising from defective regulation of iron absorption, HH is an iron overload disorder intrinsically related to *HFE* [reviewed in Beutler 1996]. The product of this gene shares structural homology to a MHC-class I protein, requiring association with β_2 -microglobulin (β_2m) for cell surface expression [Feder et al. 1996; Lebrón et al. 1998]. By contrast to conventional MHC-class I molecules, *HFE* is unable to bind peptides and has not been implicated in antigen presentation functions [Lebrón et al. 1998]. The demonstration that the *HFE*- β_2m heterodimer binds to TfR1 and inhibits competitively its interaction with diferric transferrin was the primary evidence coupling *HFE* to cellular iron homeostasis [Lébron et al. 1999; West et al. 2001]. C282Y and H63D are the two point mutations of *HFE* commonly linked to HH [Feder et al. 1996]. The former, resulting from a G-A transition that replaces the amino acid cysteine by tyrosine at position 282, is carried by the majority of HH patients, while the H63D, consisting in a C-G transition that determines a histidine to aspartate substitution at position 63, acquires clinical significance in C282Y/H63D compound heterozygotes [Waheed et al. 1997; reviewed in Lyon and Frank 2001].

The C282Y mutation and the Unfolded Protein Response

Blocking the formation of a disulphide bond in the α_3 domain of *HFE*, the C282Y mutation prevents the assembly with β_2m [Feder et al. 1997; Waheed et al. 1997]. As a consequence, the mutant protein fails to progress through the secretory pathway and remains in the ER as high molecular weight aggregates [Waheed et al. 1997; Pinto et al. 2007] that undergo proteasome-dependent degradation [Waheed et al. 1997]. Besides the early recognized loss of protein function [Drakesmith et al. 2002], it was recently shown that expression of the *HFE* C282Y mutant triggers activation of an UPR [de Almeida et al. 2007a; Lawless et al. 2007]. The finding that CRT, an ER-chaperone with anti-oxidative role, correlates with the clinical phenotype of HH-individuals further reinforced the interconnection between ER stress and the *HFE* C282Y mutant [Pinto et al. 2008a].

Looking beyond the classical UPR targets

As previously mentioned (Section I), the UPR is a highly evolved signaling network aimed at relieving the stress induced by the accumulation of improperly folded proteins in the ER lumen [Schröder and Kaufman 2005]. The classical actions driven by the UPR pathways comprise the bolstering of ER-folding capacity and chaperone machinery, as well as the clearance of irreparable proteins [Schröder and Kaufman 2005; Bernales et al. 2006]. Although our comprehension of how cells are reprogrammed during ER stress is still sparsely elucidated, rising evidence points to a broader spectrum of action for the UPR-derived transcriptional signals. Indeed, a set of experiments document the involvement of the UPR in either normal or pathophysiological processes, like B cell differentiation [Gass et al. 2002] and type II diabetes [Ladiges et al. 2005], respectively. By down-regulating both adiponectin and resistin [Hosogai et al. 2007; Lefterova et al. 2009], ER stress has also been implicated in the setting of obesity. Moreover, the UPR-inducible molecule CREBH was shown to modulate the expression of inflammatory-related genes [Zhang et al. 2006a]. In the context of MHC-class I, a causal relation between its expression and UPR induction was also reported. Importantly, it was demonstrated that MHC-class I expression is hampered throughout the UPR activated by either HFE C282Y mutant [de Almeida et al. 2005; de Almeida et al. 2007a], palmitate or glucose starvation [Granados et al. 2009].

Research question & Outline of the thesis

Based on the key concepts reviewed in **Sections I** and **II** and on the above background information (**Section III**), a putative crosstalk between ER stress events and cellular iron homeostasis was envisaged. The core question of the present thesis was therefore raised as follows: *Does the activation of an UPR influence the expression of iron metabolism-associated genes?*

Intending to shed light on this issue, distinct, although complementary, approaches were pursued. A detailed description of the experimental procedures is provided in **Chapter II**, while specific aims are further summarized at the beginning of each chapter.

Chapter III starts by evaluating the impact of the C282Y-associated UPR in both stable and transient HFE expressing models of human hepatoma HepG2 cells. To overcome the mildness of the UPR mounted under such conditions, our strategy focused on chemically-induced ER stress, with **Chapter IV** exploring the misfolding consequences elicited by dithiothreitol (DTT) and homocysteine (Hcys). This chapter comprises the published results. Since both are thiol-containing compounds and the net effect of the UPR has been suggested to depend on the type of insult used to perturb the ER environment [DuRose et al. 2006; Labunskyy et al. 2009], **Chapter V** covers the influence of tunicamycin (Tm) as stressor agent.

Finally, **Chapter VI** integrates insights from the preceding chapters and discusses promising avenues of future research.

CHAPTER II

Materials & Methods

Cell lines and Culture conditions. Human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX (Gibco, Grand Island, NY), containing 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin/amphotericin (Sigma, St. Louis, MO), unless otherwise stated. HepG2 cells stably overexpressing pcDNA3 (mock), HFE wild-type (WT), H63D and C282Y, previously isolated by limiting dilution [Pinto et al. 2007], were maintained in growth medium supplemented with 200 μ g/mL Geneticin (G418; Gibco). HFE WT- and H63D-positive HepG2 populations obtained by flow cytometric sorting with the 8C10 antibody (see below) were cultured under the same conditions. Human embryonic kidney (HEK) 293T cells were grown in Iscove's modified Dulbecco's medium (IMDM) with GlutaMAX (Gibco), containing 10% heat-inactivated fetal bovine serum (HyClone, Waltham, MA). This will be referred to as complete medium. All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Antibodies. Mouse anti-KDEL and rabbit anti- β -actin antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-GADD153 (R-20), goat anti-C/EBP α (C-18), goat anti-C/EBP α (C-18X), rabbit anti-C/EBP α (14AA) and rabbit anti-IgG antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-HFE CT-16 was a kind gift of Dr. Abdul Waheed (Saint Louis University School of Medicine, St. Louis, USA), whereas mouse anti-human HFE 8C10 antibody was kindly provided by Dr. R. Ehrlich (Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv, Israel).

Drugs and Chemicals. Dithiothreitol (DTT), homocysteine (Hcys) and tunicamycin (Tm) were purchase from Sigma-Aldrich (St. Louis, MO). Stock solutions of DTT and Hcys were prepared and frozen as single-use aliquots to maintain consistency among experiments. Desferrioxamine (DFO) was supplied by Novartis Pharma (Basel, Switzerland) and deferiprone (L1; Sigma-Aldrich) was a kind gift of Dr. Conceição Rangel.

Construction of lentiviral transfer vectors. The pCCL.PGK.TetLinker.WPRE (a generous gift of Dr. Stefano Rivella, Cornell University, NY, USA) is a lentiviral transfer vector that allows the expression of a gene of interest under control of the

phosphoglycerate kinase (PGK) promoter. Full-length human HFE and β_2m cDNAs were obtained by PCR amplification using as templates the pcDNA3.HFE-WT (a gift of Dr. Luísa Salter-Cid) or pcDNA3. β_2m plasmids (kindly provided by Dr. Hal Drakesmith, Oxford University, UK), respectively. Primers, whose sequences are listed in Table 1, were designed to contain the *Bam*HI (at 5' end) and *Sall* (at 3' end) unique restriction sites. The amplified fragments were cloned into pGEM-T Easy vector (Promega, Madison, WI) to facilitate digestion with *Bam*HI and *Sall* (Fermentas, Burlington, Canada). After digestion with the referred enzymes, HFE and β_2m fragments were inserted into the polylinker region of pCCL.PGK.TetLinker.WPRE vector at *Bam*HI and *Sall* sites, yielding the pCCL.HFE-WT and pCCL. β_2m constructs, respectively (Fig. 1). Plasmids containing the H63D and C282Y mutant forms of HFE were generated from pCCL.HFE-WT using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Fidelity of the cDNA inserts was confirmed by DNA sequencing.

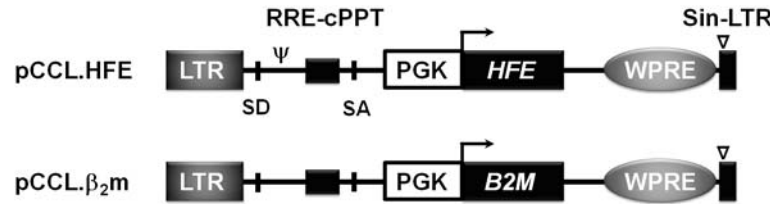


Figure 1. Schematic representation of the lentiviral vectors used in this study. LTR, long terminal repeat; Sin-LTR, self inactivating-LTR; SD, splice donor; SA, splice acceptor; RRE-cPPT, HIV rev-response and central polypurine tract elements; ψ , packaging signal; PGK, human phosphoglycerate kinase promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; *HFE*, cDNA encoding human HFE; *B2M*, cDNA encoding human β_2 -microglobulin.

Viral vector production. Lentiviral vector particles were produced essentially as described by Dull et al. [Dull et al. 1998]. Briefly, HEK293T cells were seeded at a density of 4×10^6 cells in 10 cm-culture plates with complete medium. The following day, cells were co-transfected with 3 μ g of VSV-G envelope plasmid, 5 μ g of pMDLg/pRRE packaging plasmid, 2.5 μ g of pRSV-REV expressing plasmid (kind gifts of Dr. Stefano Rivella) and 10 μ g of each gene transfer construct previously generated, using the calcium phosphate precipitation method. Conditioned supernatants containing the recombinant lentiviruses were collected 40 and 64 h

post-transfection, cleared by low-speed centrifugation, aliquoted and stored at -80°C. Virus titers were indirectly measured using a micro-reverse transcriptase assay, conducted as described by Lichtenstein et al. [Lichtenstein et al. 1995].

HepG2 cells transduction. Transduction of HepG2 cells with the harvested lentiviruses was carried out in 6-well plates, using a seeding density of 1.5×10^5 cells/well. One day after plating, conditioned supernatants were added to each well containing complete IMDM medium supplemented with 8 µg/mL polybrene (Chemicon, Billerica, MA). The final viral dilutions were 1:3 for pCCL.HFE-WT and pCCL.HFE-H63D and 1:2 for pCCL.HFE-C282Y. All cells were co-infected with the pCCL.β₂m expression construct. To mock-transduce HepG2 cells the pCCL empty transfer vector was employed. Cells were harvested 48 h post-infection and the transgene expression assessed by western blot.

Induction of ER stress and iron chelation. HepG2 cells were seeded at a density of 1.5×10^6 cells in 60-mm diameter culture dishes. One day after plating, cells were exposed to DTT, Hcys and Tm as follows: 1) for dose-response assays, cells were incubated for 6 h with increasing concentrations of the ER-stressors (ranging from 0.5-10 mM, 1-25 mM or 1-50 µg/mL for DTT, Hcys and Tm, respectively); 2) for time-course experiments, cells were cultured in the presence of 2 mM DTT or 5 µg/mL Tm from 2-24 h and 10 mM Hcys from 2-48 h. Control cells were treated with vehicle alone. In the iron chelation experiments, DTT-stimulated ER stress was induced in the presence of either DFO or L1 at 20, 50 or 100 µM per culture plate. mRNA levels of target genes were assessed in cells exposed to both DTT and iron chelators following 24 h of incubation.

RNA isolation and real-time RT-PCR. Total RNA was isolated from HepG2 cells using the RNeasy Midi Kit (QIAGEN, Hilden, Germany), followed by genomic DNA digestion with TURBO DNA-free (Ambion, Austin, TX). Reverse transcription was obtained from 1 µg of DNase-treated RNA employing the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA), according to the manufacturer's guidelines. For real-time quantification of mRNA levels, the synthesized cDNAs were amplified in duplicate by PCR in an iCycler iQ5 (Bio-Rad, Hercules, CA) using iQ SYBR

Green Supermix (Bio-Rad). At the end of the PCR cycling, melting curves were generated to ascertain the amplification of a single product and the absence of primer dimers. The primers used are listed in Table 2. Results were normalized to GAPDH as endogenous control. Relative expression levels were calculated as follows:

$$2^{(\text{Ct GAPDH gene} - \text{Ct gene of interest})} \times 1000.$$

Western blot. HepG2 cells were harvested and the pellets lysed in ice-cold lysis buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% Triton X-100) supplemented with 1x Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Total protein content of lysates was measured using the *RC/DC* Protein Assay (Bio-Rad) and 30 µg resolved by electrophoresis on 12% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose Hybond-C Extra membranes (Amersham Biosciences, Little Chalfont, UK). After blocking with 5% dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h, primary antibody incubations were performed overnight at 4°C. Blots were then washed three times with TBS-T and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature. After washing with TBS-T, signal was developed with the SuperSignal West Pico (Pierce, Rockford, IL) chemiluminescence kit and the blots exposed to CL-XPosure films (Pierce). For normalization of protein loading, blots were stripped and reprobed with an antibody against β-actin.

Preparation of nuclear extracts and Electrophoretic Mobility Shift Assay (EMSA).

Nuclear extracts from DTT- and control-treated cells were prepared essentially as described by Schreiber et al. [Schreiber et al. 1989]. Briefly, after harvesting and washing with ice-cold PBS, cells were suspended in hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM EDTA, 0.1 mM EGTA, 0.1% IGEPAL, 1 mM DTT, 1x Complete EDTA-free protease inhibitor cocktail [Roche Diagnostics]). Upon 15 min of incubation on ice, the nuclei were pelleted by centrifugation at 2000 *g* for 10 min and resuspended in high-salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 20% glycerol, 0.5 mM EDTA, 420 mM NaCl, 1 mM DTT, 1x Complete EDTA-free protease inhibitor cocktail). Following incubation on ice for 30 min with gentle agitation, nuclear debris were removed by centrifugation at 16000 *g* for 25 min and

the nuclear extract present on supernatants stored at -80°C . To investigate specific DNA-protein interactions, a fluorescence based EMSA was employed according to previously reported procedures [Ruscher et al. 2000]. The following single-stranded oligonucleotides encompassing the C/EBP α consensus sequence element [Ryden and Beemon 1989] were purchased from Thermo Scientific (Waltham, MA): 5'-Cy5-CTAGGGCTTGCGCAATCTATATTCG-3' (Cy5-labeled sense specific probe), 5'-CTAGGGCTTGCGCAATCTATATTCG-3' (sense specific competitor) and 5'-CGAATATAGATTGCGCAAGCCCTAG-3' as antisense complementary oligonucleotide. To generate double-stranded probes for EMSA, single stranded oligonucleotides were annealed by heating to 95°C for 1 min, followed by 30 min at 20°C . For binding reactions, 20 μg of nuclear extract was incubated with 1 pmol of double-stranded Cy5-labeled probe in binding buffer containing 20 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol and 250 ng poly[dI.dC] (Sigma). Binding reactions were carried out overnight at 4°C and then subjected to electrophoresis through 5% nondenaturing polyacrylamide gels using an ALF-Express DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). The temperature was maintained at 10°C by an ALFexpress II Cooler external thermostat (Amersham Pharmacia Biotech). Signals were analyzed using ALFwin 1.03 software (Amersham Pharmacia Biotech). For the competition assays, 50-fold excess unlabeled probe was added to the reaction 1 h prior to incorporation of the labeled duplex. For supershift experiments, nuclear extracts were pre-incubated overnight at 4°C with 2 μL of antibody against C/EBP α (C-18X) and the labeled probe added 1 h before the loading onto the gel.

Chromatin Immunoprecipitation (ChIP). HepG2 cells were grown in 100-mm diameter culture dishes to ~70% of confluence and subjected to time-course assays with 2 mM DTT as depicted above. ChIP analysis was performed as previously described by Kuo et al. [Kuo and Allis 1999] with some modifications. Cells were cross-linked in 1% formaldehyde (directly added to the culture medium) for 10 min at 37°C . After washing twice with cold PBS, cells were scraped and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) supplemented with 1x Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics) for 30 min on ice. Chromatin was sheared into 500-1000 bp size fragments by sonicating the cell

lysates with a Branson Sonifier 250 (10 cycles of 20 sec at 30% amplitude). After removing cell debris by centrifugation at 14000 *g* for 10 min, an aliquot of the supernatant was saved as input DNA. The remainder was 10-fold diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) and pre-cleared with Protein A Sepharose beads (Sigma-Aldrich) during 2 h at 4°C. Immunoprecipitation of chromatin was carried out overnight at 4°C using rabbit anti-C/EBP α antibody (14AA) or rabbit anti-IgG as serum control. Immune complexes were recovered with Protein A Sepharose beads for 2 h at 4°C. The beads were then washed sequentially with: a) Low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl); b) High-salt buffer (as previous but with 500 mM NaCl); c) LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1mM EDTA, 10 mM Tris-HCl pH 8.1) and d) TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.1). The immune complexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO₃) and protein-DNA crosslinks reversed for 6 h at 65°C in the presence of 0.2 M NaCl. Following Proteinase K (Sigma-Aldrich) digestion for 1 h at 45°C, DNA was extracted with phenol/chloroform, ethanol precipitated and dissolved in TE buffer containing RNase H. The chromatin fragments obtained upon immunoprecipitation were analyzed by quantitative real-time PCR, using 5 μ L of DNA template, iQ SYBR Green Supermix (Bio-Rad) and the primers as follows: 5'-TGTCGCTCTGTTCCCGCTTATC-3' (forward) and 5'-TCTGGTGTCTGGGACCGAGTGA-3' (reverse). This primer set was designed to amplify the -136/+9 region of human *HAMP* promoter that contains a literature-annotated C/EBP α binding site [Courselaud et al. 2002]. The relative enrichment was calculated by the $\Delta\Delta C_t$ method: upon normalization to the corresponding ChIP input, values were corrected for non-immune background according to the equation as follows:

$$2^{(C_{t \text{ neg IP}} - C_{t \text{ input}}) - (C_{t \text{ C/EBP}\alpha \text{ IP}} - C_{t \text{ input}})}$$

siRNA transfection. Small interfering RNAs (siRNAs) targeting human C/EBP α and CHOP were designed and synthesized by Eurogentec (Ougrée, Belgium). The siRNA sequences were as follows: C/EBP α (5'-CGCACCUGCAGUCCAGAU-3' and 5'-GAGACGUCCAUCGACAUC-3'); CHOP (5'- GCGCAUGAAGGAGAAAGAA-3' and 5'-GCUGAGUCAUUGCCUUUCU-3'). A scrambled siRNA (Eurogentec) was used as negative control. To improve the transfection efficiency, a cell-suspension variant of

the standard protocol was applied to HepG2 cells. Briefly, after trypsinization and dilution in antibiotic-free medium, 3×10^5 cells were transferred to 6-well plates containing the siRNA duplexes complexed with Lipofetamine 2000 (Invitrogen), previously diluted in Opti-MEM (Gibco). Following 48 or 24 h of transfection, for C/EBP α or CHOP respectively, the knock-down effectiveness was evaluated by immunoblot analysis.

Statistics. Results are expressed as average + 1 standard deviation (SD) of three independent experiments. Significant differences among sample means were detected using one-way ANOVA followed by Tukey test. Statistical significance was considered at $p < 0.05$. Statistical analysis was performed using SigmaStat software.

Table 1. Oligonucleotide primers used to amplify full-length cDNAs encoding HFE and β_2m . *Bam*HI and *Sal*I restriction sites are underlined.

Gene	Accession number	Forward primer (5' - 3')	Reverse Primer (5' - 3')
HFE	U60319	CCGGAGG <u>GATCC</u> GCCACCATGGGCCCCGAGCCAGGCC	CCGGAGG <u>TCGACTCA</u> CTCACGTTAGCTAAGACGTAGTGC
B2M	NM_004048	CCGGAGG <u>GATCC</u> GCCACCATGTCTCGCTCCGTGGCCTTAGCT	CCGGAGG <u>TCGACTT</u> ACATGTCTCGATCCCACTTAACATATCTTGG

Table 2. Sequences of the oligonucleotide primers used for quantitative real-time RT-PCR analysis.

Gene	Accession number	Protein Coded	Forward primer (5' - 3')	Reverse Primer (5' - 3')
GRP78	M19645	BiP	CCTGGGTGGCGGAACCTTCGATGTG	CTGGACGGGCTTCATAGTAGACCGG
GADD153	S40706	CHOP	GCCTTTCTCCTTTGGGACACTGTCCAGC	CTCGGCGAGTCGCCTCTACTTCCC
HAMP	NM_021175	Hepcidin	ATGGCACTGAGCTCCAGAT	TTCTACGTCTTGACGACATCC
CEBPA	NM_004364	C/EBP α	CTAGAGATCTGGCTGTGGGG	TCATAACTCCGGTCCCTCTG
SLC40A1	NM_014585	Ferroportin	CCCGGAGACAAGTCCTGAATC	TGGCCCATTTGCCACAAGGAG
FTH1	AF088851	Ferritin H	CAGAACTACCACCAGACTCAGA	TAGCCCCAGGCTTAGCTTTCA
ALB	NM_000477	Albumin	CAAAAACATGTGTTGCTGATGA	CTTGTTTTGCACAGCAGTCAG
GAPDH	NM_002046	GADPH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTTC
HFE	U60319	HFE	CTCCTTTGGTGAAGGTGACACATC	ATCACAATGAGGGGCTGATCCA
CP	NM_000096	Ceruloplasmin	TTTACATGCAAATGGGAGACA	TTCATCTTCCTTATCCACCTGA
TFR2	NM_003227	TfR2	CCATGTTCTGCAGTTCCTG	AATGTCCTGAGTCAGAGCGG

CHAPTER III

Does the HFE C282Y-triggered UPR have downstream effects on cellular iron metabolism?

Specific aims

As described in Chapter I, Section III, besides disrupting normal iron balance, the C282Y mutation of HFE causes ER stress. The current piece of work is an attempt to reconcile the double facet of this protein with the growing list of unraveled UPR-responsive targets. Specifically, the main objectives are:

- a. to examine whether the UPR induced by the C282Y mutant form of HFE affects cellular iron metabolism, namely by evaluating its impact on the expression of the major regulator hepcidin;
- b. to set-up an experimental model that enables the proper accomplishment of the foregoing task.

Results and Discussion

Stable expression of HFE C282Y mutant protein in HepG2 cells does not activate an UPR

Preceding studies reporting the correlation between expression of the HFE C282Y protein and the activation of an UPR [de Almeida et al. 2007a; Lawless et al. 2007] used HEK293T cells as model. Although valuable for their purposes, this cell line is unsuitable for the present issue. While major producers of iron-related proteins [reviewed in Graham et al. 2007], hepatocytes play a pivotal role in the orchestration of iron metabolism. Furthermore, hepatocytes naturally express HFE [Parkkila et al. 1997], thus harboring the cellular machinery to properly assemble and express the protein. In light of these observations, the hepatocytic model emerged as the appropriate platform for our study, which we mimicked using the human hepatoma HepG2 cell line as model.

We started by taking advantage of stably transfected HepG2 cells, previously generated by Pinto et al. [Pinto et al. 2007]. Regarding the stable transfectants of HFE WT and H63D, both subclones isolated by limiting dilutions (referred to as C) and populations purified by cell sorting (indicated by S) were assessed. On the other hand, the typical ER retention of the C282Y mutant impaired non-permeabilizing labeling of the protein, making cell sorting isolation technically unfeasible. To circumvent this constraint, five distinct subclones expressing the HFE C282Y mutant protein were analyzed. To verify the stability of the transfection in the cells employed, *HFE* transgene expression was monitored by quantitative real-time RT-PCR. As Fig. 1 depicts, all forms of *HFE* still greatly overexpressed in HepG2 cells.

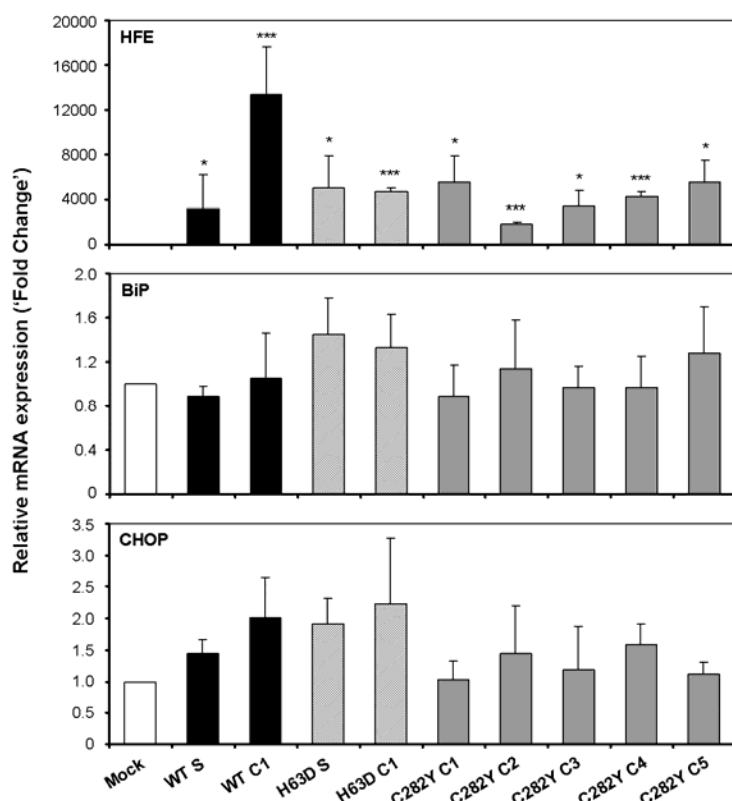


Figure 1. Stable-expressing model of HepG2 cells. HepG2 cells stably transfected with the pcDNA empty vector (mock), HFE WT, H63D or C282Y were used. The subclones isolated by limiting dilution are indicated by C, whereas the populations purified by flow cytometric sorting are specified by S. mRNA levels of HFE (upper panel) were analyzed by quantitative real-time RT-PCR to confirm its stable overexpression. BiP (middle panel) and CHOP (lower panel) mRNA levels were assessed as markers of UPR activation. Results were normalized to GAPDH and are displayed as fold change over mock-transfected cells. Each bar represents the average + SD of three independent experiments.

* $p < 0.05$, *** $p < 0.001$ vs mock.

Seeking for a signature of UPR activation, mRNA expression of the ER-resident chaperone BiP and the stress-inducible transcription factor CHOP was examined. Interestingly, BiP and CHOP transcript levels were not significantly altered in HepG2 cells overexpressing HFE C282Y mutant as compared with the remaining stable transfectants (Fig. 1). In terms of protein, the steady-state pool of BiP remained unaltered among the cell populations under scrutiny, whereas CHOP was undetectable (data not shown). In apparent contradiction to previous reports documenting the HFE C282Y ability to evoke an UPR [de Almeida et al. 2007a; Lawless et al. 2007], the results here achieved might be the corollary of a recently introduced concept: adaptation [Rutkowski et al. 2006]. According to this viewpoint, cells exposed to long-term stresses can permanently adjust their machinery to the noxious condition, thus maintaining cellular functions and evading to apoptosis. We therefore hypothesized that stable transfected HepG2 cells might face a chronic stress process, coping with the C282Y mutation without continuously recruiting the classical UPR partakers.

Transient HFE C282Y expression partially up-regulates markers of ER stress in HepG2 cells

Utilization of HepG2 stable-transfectants was hampered by the absence of measurable UPR activation markers. Hence, an alternative approach relying on transient expression of HFE was engineered. According to the literature [Liao and Ning 2006] and our own experience, HepG2 cells are relatively resistant to conventional lipid-based transfection methods, requiring selection and expansion of high expressing transfectants. To overcome this drawback, following generation of proper transfer constructs, a lentiviral vector strategy was used. The faulty assembly of HFE due to limiting levels of β_2m was prevented by co-expressing this protein in all experiments.

Infection of HepG2 cells with the viral particles resulted in high overexpression of HFE, detectable in terms of mRNA and protein levels (Fig. 2A and B, respectively). To compensate for the accelerated degradation of the HFE C282Y mutant [Waheed et al. 1997] and to equalize the final protein content amongst HFE forms, increased titers of the C282Y lentiviruses were intentionally transferred into cells (Fig. 2A, upper panel). Consistent with previous reports [Waheed et al. 1997; Wang et al. 2003], both WT and H63D forms of HFE migrated in two bands of ~48 and 45 KDa, likely reflecting different degrees of glycosylation. The higher molecular weight product was barely visible in the C282Y mutant, a consequence of its defective ER processing [Waheed et al. 1997] (Fig. 2B).

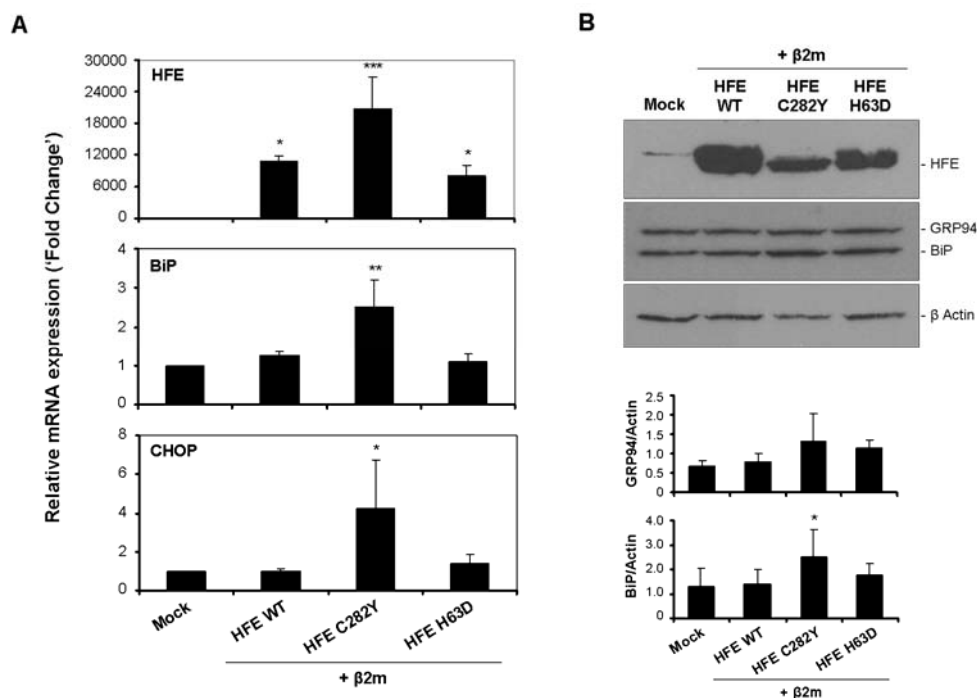


Figure 2. Transient-expressing model of HepG2 cells. HepG2 cells were infected with lentiviruses encoding HFE WT, C282Y or H63D. A transfer vector containing β_2m was co-expressed in these cells. Mock cells were infected with pCCL empty vector alone. **A**, 48 h post-infection, mRNA expression levels of HFE and the UPR markers BiP and CHOP were quantified by real-time RT-PCR. Results were normalized to GAPDH as endogenous control. Fold change over mock-infected cells was calculated and is shown as average + SD of three independent experiments. **B**, Whole cell lysates from mock- and HFE-infected HepG2 were assessed by western blot for HFE (using anti-HFE CT16), GRP94 and BiP (using anti-KDEL) expression. As loading control, membranes were stripped and reprobed for β -actin. Representative blots are displayed. Band intensity was quantified by densitometry, normalized to β -actin and plotted as average + SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs mock.

To evaluate whether an ER-stress response was elicited in the C282Y-transduced cells, expression of the canonical targets BiP and CHOP was measured. A significant up-regulation of both transcripts was found in cells overexpressing the C282Y variant of HFE (Fig. 2A). These results were further confirmed for BiP, whose protein levels in cells carrying the C282Y mutant were approximately twice the mock-values (Fig. 2B). On the contrary, CHOP was not detected by western blotting (data not shown). The other ER chaperone recognized by the anti-KDEL antibody, GRP94, was not altered as well (Fig. 2B).

Collectively, these results suggest that when expressed in HepG2 cells, the HFE C282Y mutant represents a mild ER insult, only partially activating an UPR.

Displaying potent secretory activity [Ji and Kaplowitz 2006], hepatocytes are intrinsically better equipped to deal with proteotoxic challenges, which might reasonably justify our observations. If compared to previous studies in HEK293T [de Almeida et al. 2007a; de Almeida et al. 2007b], these findings also highlight that the magnitude and nature of the ER stress response are heavily dependent on the cell type, even if evoked by a common stimulus. In line with this, the kinetic discrepancy between PERK activation and eIF2 α phosphorylation observed in CHO cells exposed to pharmacological stresses was lost in NIH3T3 fibroblasts subjected to identical treatments [DuRose et al. 2006].

Moreover, the different outcomes achieved with the two modalities of expression, stable or transient, should not be underestimated since they may reflect intrinsic characteristics of the UPR pathways, possibly correlated with the chronic vs acute duality.

Hepcidin levels are not affected by the transient expression of HFE

Albeit subtle, signs of a stressed ER were identified in the transient version of HFE expression, prompting us to further examine hepcidin levels in this model. As Fig. 3 depicts, hepcidin transcripts remained unchanged upon delivery of all forms of HFE into cells.

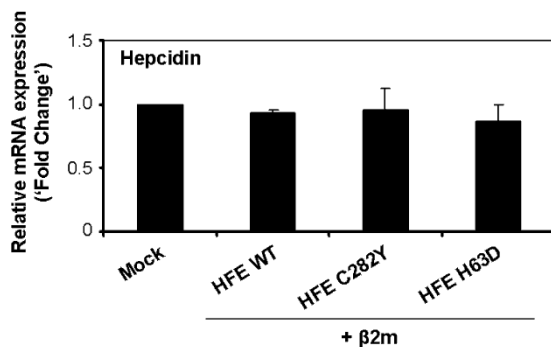


Figure 3. Hepcidin mRNA levels are not altered upon transient expression of all forms of HFE. HepG2 cells were transduced with lentiviruses encoding HFE WT, C282Y or H63D, with β_2m being co-expressed in all cases. Mock cells were infected with pCCL empty vector. 48 h post-infection, mRNA expression levels of hepcidin were measured by real-time RT-PCR. Results were normalized to GAPDH as endogenous control. Fold change over mock-infected cells was calculated and displayed as average + SD of three independent experiments.

Using stable transfectants, Pinto et al. reported down-regulated mRNA levels of hepcidin in HepG2 overexpressing the HFE C282Y mutant. Since endogenous HFE of those cells was preserved, the effects on hepcidin were not correlated with loss of the functional gene (as occurs in HH patients and *Hfe*^{-/-} mice), but rather to a direct influence of the C282Y mutation itself [Pinto et al. 2008a]. Such results were not recapitulated in our conditions, however. Explanation for this inconsistency likely rests on the stable *versus* transient dichotomy, involving the time-frame of HFE transgene expression – 48 h in our system, in contrast to the long-term expression of stable transfectants. This hypothesis is supported by similar discrepancies described elsewhere [Yanagi et al. 1989; Hamilton et al. 2000]. Conceivably, the lentiviral-based system may favor certain conditions under which the previously found hepcidin down-modulation is counteracted. One can speculate that, even though partial, the ER stress response elicited by the transient expression of the C282Y mutant is one of those conditions, playing a role in the hepcidin outcome.

Nevertheless, while conceptually interesting, the experimental model we set-up renders ambiguous data interpretation. Since HFE has been identified as an upstream regulator of hepcidin synthesis (see Chapter I, Section II) [Schmidt et al. 2008; Gao et al. 2009], this working system is unsuited to *per se* determine whether the observations arise from the C282Y loss of protein function or from the C282Y evoked UPR. A strategy for solving such issue would require the manipulation of the ER stress response with chemical chaperones, for example. However, for the sake of clarity, we decided to address each variable separately. Our initial question was therefore re-centered on the effects that generic, HFE-independent, UPR activation might impinge on the expression of iron metabolism-relevant molecules.

CHAPTER IV

ER Stress-inducible Factor CHOP Affects the Expression of Hepcidin by Modulating C/EBPalpha Activity

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Abstract

Endoplasmic reticulum (ER) stress induces a complex network of pathways collectively termed the Unfolded Protein Response (UPR). The clarification of these pathways has linked the UPR to the regulation of several physiological processes. However, its crosstalk with cellular iron metabolism remains unclear, which prompted us to examine whether an UPR affects the expression of relevant iron-related genes. For that purpose, the HepG2 cell line was used as model and the UPR was activated by dithiothreitol (DTT) and homocysteine (Hcys). Here, we report that hepcidin, a liver secreted hormone that shepherds iron homeostasis, exhibits a biphasic pattern of expression following UPR activation: its levels decreased in an early stage and increased with the maintenance of the stress response. Furthermore, we show that immediately after stressing the ER, the stress-inducible transcription factor CHOP depletes C/EBP α protein pool, which may in turn impact on the activation of hepcidin transcription. In the later period of the UPR, CHOP levels decreased progressively, enhancing C/EBP α -binding to the hepcidin promoter. In addition, analysis of ferroportin and ferritin H revealed that the transcript levels of these iron-genes are increased by the UPR signaling pathways. Taken together, our findings suggest that the UPR can have a broad impact on the maintenance of cellular iron homeostasis.

Introduction

The endoplasmic reticulum (ER) has evolved a high degree of plasticity, allowing the adjustment of its environment according to the transit of client proteins. The organelle homeostasis, however, can be threatened by numerous stimuli which overall contribute to the luminal accumulation of improperly folded proteins [reviewed in Schröder and Kaufman 2005]. Aiming at relieving such stressful condition, a finely coordinated signaling program known as Unfolded Protein Response (UPR) is elicited [Schröder and Kaufman 2005]. Its mechanisms of action can be summarized as follows: global repression of protein synthesis; induction of ER chaperones and foldases to meet the increased folding demands and enhancement of ER-associated degradation (ERAD) of irreversibly unfolded proteins [Schröder and Kaufman 2005; Bernales et al. 2006]. The UPR employs three ER-resident transmembrane proteins that operate as proximal sensors and define independent signaling pathways towards the cytosol/nucleus: PERK (double-stranded RNA-dependent protein kinase-like ER kinase), IRE1 (inositol-requiring enzyme 1) and ATF6 (activating transcription factor 6). The output of these cascades entails the selective activation of transcription factors whose main gene targets code for components of the ER protein-processing machinery [Rutkowski and Kaufman 2004]. Prominent among this category is immunoglobulin heavy chain-binding protein (BiP), an ER chaperone with key sentinel activity [Hendershot 2004].

The scope of the UPR-derived transcriptional signals goes beyond the classical targets. A paradigmatic example is cAMP response element-binding protein H (CREBH) which, albeit activated along the UPR, executes its transcriptional activity over genes encoding inflammatory proteins [Zhang et al. 2006a]. Likewise, the circulating iron-transport protein transferrin (Tf) was identified as a downstream target of CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) [You et al. 2003], a stress-inducible transcription factor. CHOP belongs to the C/EBP family and can heterodimerize with other members of the same class [Ron and Habener 1992]. Acting as dominant negative inhibitor of other C/EBP isoforms, namely C/EBP α , CHOP was reported to down-modulate *Tf* gene expression [You et al. 2003]. Interestingly, C/EBP α has also been described as transcriptional activator of hepcidin [Courselaud et al. 2002]. Although not formally tested, an identical

mechanism to that depicted for *Tf* was proposed to justify the impaired hepcidin transcription observed in two models of hepatic iron overload, induced by either hepatitis C virus [Nishina et al. 2008] or alcohol [Harrison-Findik et al. 2007].

As a major orchestrator of iron homeostasis [Ganz 2003], hepcidin binds to the iron exporter ferroportin and negatively regulates cellular iron release into circulation [Nemeth et al. 2004]. A poor induction of hepcidin despite the systemic iron overload has been found in Hereditary Hemochromatosis (HH) [Bridle et al. 2003]. The leading cause of this disorder – the C282Y mutation of HFE protein [Feder et al. 1996] – was recently coupled to the activation of an UPR [de Almeida et al. 2007; Lawless et al. 2007], which reinforces the interest of exploring the UPR signaling/iron metabolism interplay.

To clarify this putative interconnection, we examined whether activation of an UPR affects the expression of relevant iron-related genes. Being the cellular “factory of iron-proteins” [reviewed in Graham et al. 2007], hepatocytes emerged as the most relevant platform for our study, herein recapitulated by the well-characterized human hepatoma HepG2 cell line. Dithiothreitol (DTT) and homocysteine (Hcys) were used as UPR inducers. Both agents interfere with disulphide bond formation, thereby burdening the ER lumen with misfolded proteins [Braakman et al. 1992; Outinen et al. 1998; Zhang et al. 2001]. Using this approach, we show that the gene profiles of hepcidin, ferroportin and ferritin H are modulated throughout an active UPR. In addition, evidence supporting the involvement of C/EBP α and CHOP on the expression pattern exhibited by hepcidin is also provided.

Results

Experimental model of ER stress

Dose-response assays. The first part of the work was assigned to establish the minimum concentration of stressor agent able to robustly trigger an UPR. For this purpose, HepG2 cells were exposed for 6 h to increasing doses of DTT and Hcys, ranging from 0.5 to 10 mM and from 1 to 25 mM, respectively. The mRNA and/or protein levels of the ER-resident chaperones glucose regulated protein 94 (GRP94) and BiP, and of the stress-inducible transcription factor CHOP were examined as markers of UPR activation. Albeit with different magnitudes, both DTT and Hcys elicited a dose-dependent up-regulation of GRP94, BiP and CHOP, noticeable in terms of gene and/or protein expression (Fig. 1A and B). Of note, CHOP transcript levels were very low in basal conditions (Fig. 1A) and the respective protein was only detectable after treatment with the highest concentrations tested (DTT $\geq$ 2 mM and Hcys $\geq$ 5 mM; Fig. 1B). Since 2 mM DTT and 10 mM Hcys were enough to ensure a fully-activated UPR, distinguished by the simultaneous induction of BiP and CHOP, they were chosen as working concentrations for the time-course assays.

Time-course assays. In response to DTT, BiP and CHOP exhibited markedly different expression profiles within the 24 h time-frame analyzed. BiP transcripts increased consistently throughout the experiment, only declining in the final period of treatment, which is likely a consequence of the DTT labile properties (Fig. 1C). At the protein level, however, a persistent up-regulation of BiP was found at 24 h (Fig. 1D). Conversely, CHOP transcripts manifested a strong induction after 2 h of DTT incubation, rapidly abolished over time (Fig. 1C). This pattern of CHOP expression was further confirmed by the immunoblot results showing a protein peak at 5 h (Fig. 1D). Using Hcys as ER-stressor, BiP and CHOP mRNA levels were both gradually up-regulated over the 24 h treatment of HepG2 cells (Fig. 1C). Extending the temporal window of the assay, we observed a decline in the transcript abundance of these UPR targets until 48 h (Fig. 1C). The similarity in terms of mRNA expression was not mirrored by the protein profiles, however. As Fig. 1D illustrates, whereas BiP increased from 12 to 48 h of Hcys exposure, induction of CHOP protein became detectable at 5 h and persisted until 36 h of incubation.

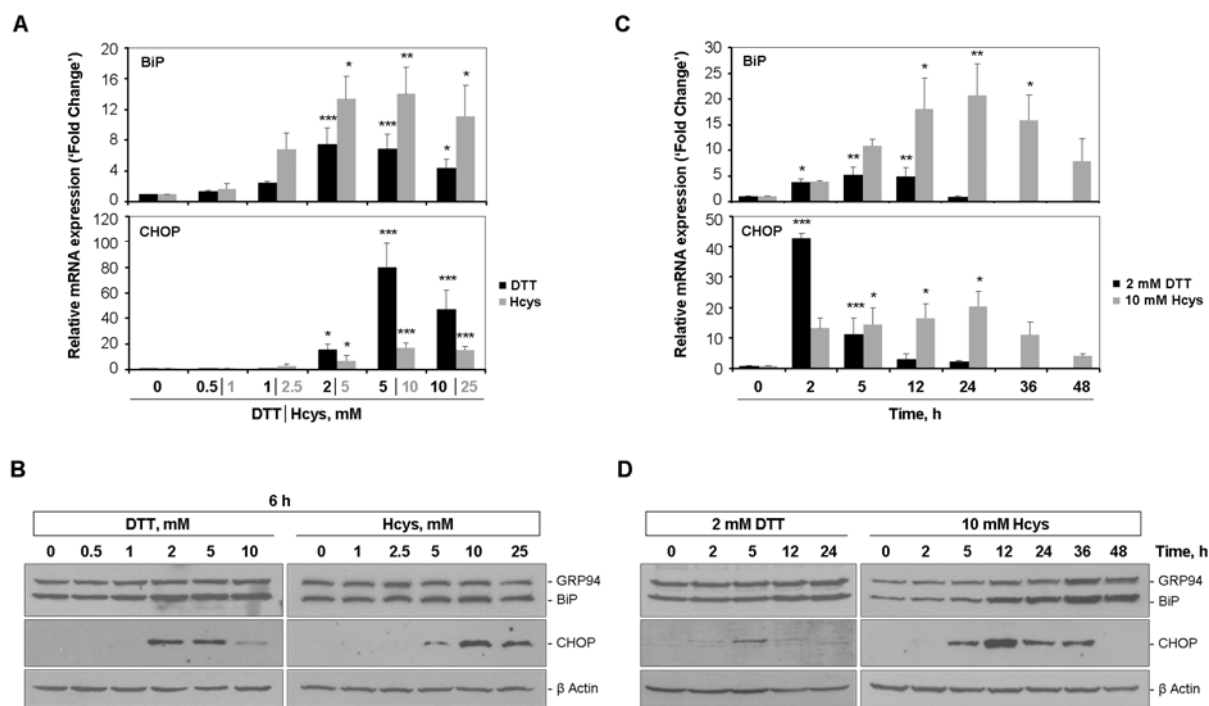


Figure 1. Monitoring of UPR activation markers in HepG2 cells during the DTT- and Hcys-induced ER-stress. Dose-response assays (**A** and **B**): HepG2 cells were incubated for 6 h with increasing concentrations of DTT or Hcys, as indicated. Control cells received vehicle alone. Time-course assays (**C** and **D**): HepG2 cells were exposed to 2 mM DTT or 10 mM Hcys for the indicated times. Control cells were left untreated. **A** and **C**, BiP and CHOP mRNA levels were assessed by real-time RT-PCR and normalized to *GAPDH* expression. Results are expressed as fold change over control-treated cells and represent the average + SD of three independent experiments. **B** and **D**, GRP94, BiP (using an anti-KDEL antibody) and CHOP were measured by western blot in whole cell lysates of HepG2 cells. As loading control, membranes were stripped and reprobed for β -actin. Representative blots of three independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Expression of iron-related genes is modulated in the context of an active UPR

To ascertain whether triggering of an UPR had an impact on cellular iron metabolism, gene expression profiling of hepcidin, ferritin H and ferroportin was performed on HepG2 cells. The choice of these genes was based on their well documented roles in iron homeostasis [reviewed in Chua et al. 2007].

Upon 5 h of DTT treatment, the expression levels of hepcidin in HepG2 cells were significantly reduced to approximately one-half the control values, which was followed by a 10-fold up-regulation at 24 h (Fig. 2A). Nearly identical effects were produced when Hcys was used as stressor agent, although with less pronounced increase (2-fold) of hepcidin transcripts in the late stage of treatment (Fig. 2B).

Ferroportin and ferritin H displayed analogous expression patterns in the presence of 2 mM DTT and 10 mM Hcys, with both mRNAs being induced in a time-dependent manner (Fig. 2A and B).

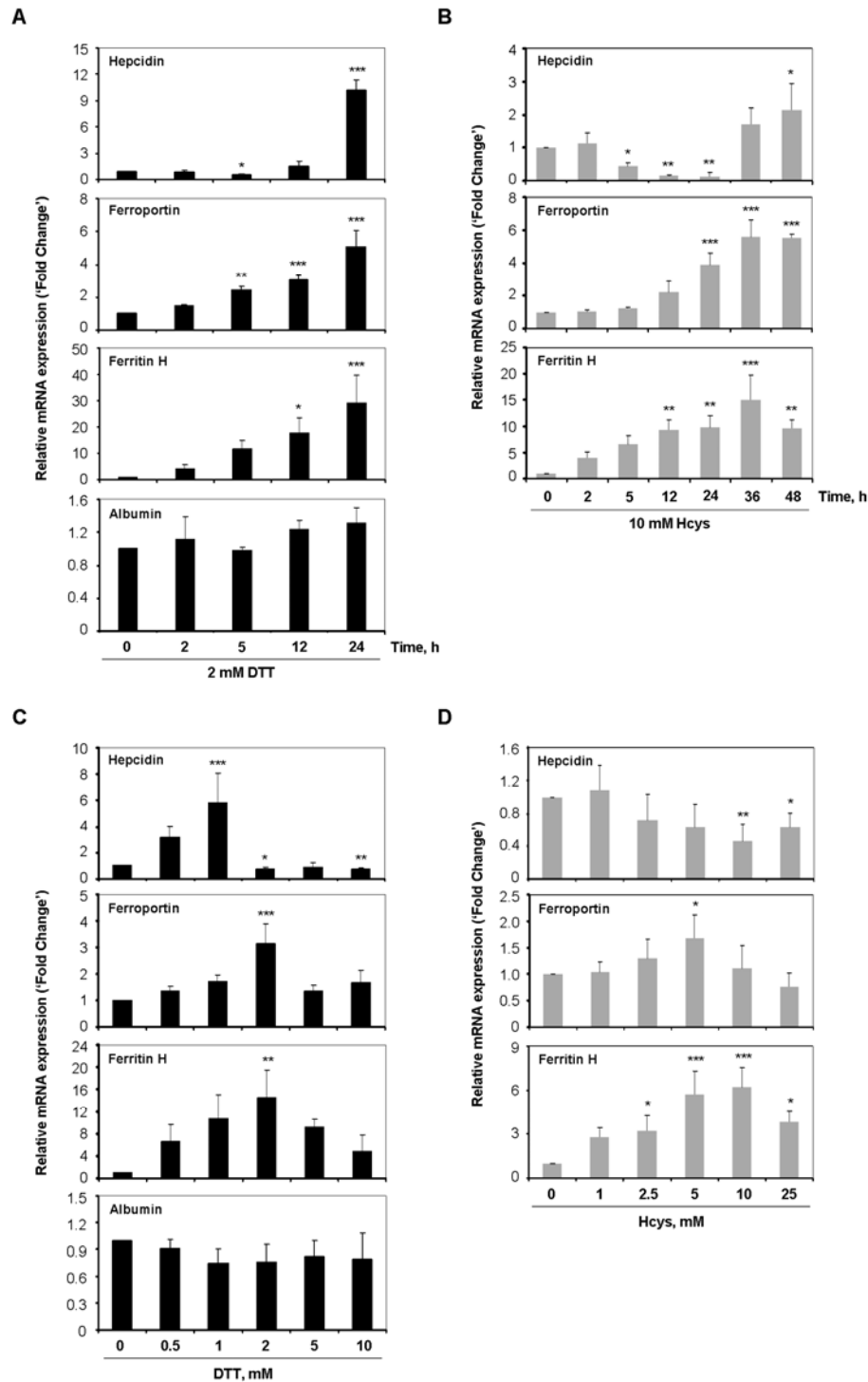


Figure 2. Expression of iron-related genes is modulated following UPR activation. HepG2 cells were cultured in the presence of 2 mM DTT (A) or 10 mM Hcys (B) for the indicated intervals. Untreated cells were used as control. In dose-response assays, HepG2 cells were treated for 6 h with DTT (C) or Hcys (D) at concentrations ranging from 0.5 to 10 mM and from 1 to 25 mM, respectively. Control cells were incubated with vehicle. After treatments, total RNA was isolated and mRNA levels of the “iron-genes” hepcidin, ferroportin and ferritin H assessed by real-time RT-PCR. Expression of albumin, a non-iron-related gene, was evaluated as control in cells subjected to the DTT-induced ER stress. Data were normalized to *GAPDH* and are expressed as fold change over control-treated cells. Each bar displays the average + SD of three independent experiments.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

To gain additional insight into the modulation of the iron-genes under scrutiny, we assessed the influence of varying doses of each UPR inducer on their transcript levels. As Fig. 2C depicts, hepcidin mRNA showed a biphasic pattern of expression in response to DTT. Indeed, low concentrations of this stressor (0.5 and 1 mM) significantly increased hepcidin transcript levels, whereas higher doses counteracted this effect. Unlike DTT, the up-regulation of hepcidin mRNA was not evident when cells were cultured in the presence of low concentrations of Hcys. However, the down-modulation associated with higher doses of stress was maintained (Fig. 2D). Concerning the other iron-related genes, both ferroportin and ferritin H mRNAs reached a peak with 2 mM DTT, decreasing thereafter. Equivalent outcome was found in Hcys treatments, with ferroportin and ferritin H increasing in a dose-dependent fashion until 5 and 10 mM of stressor agent, respectively.

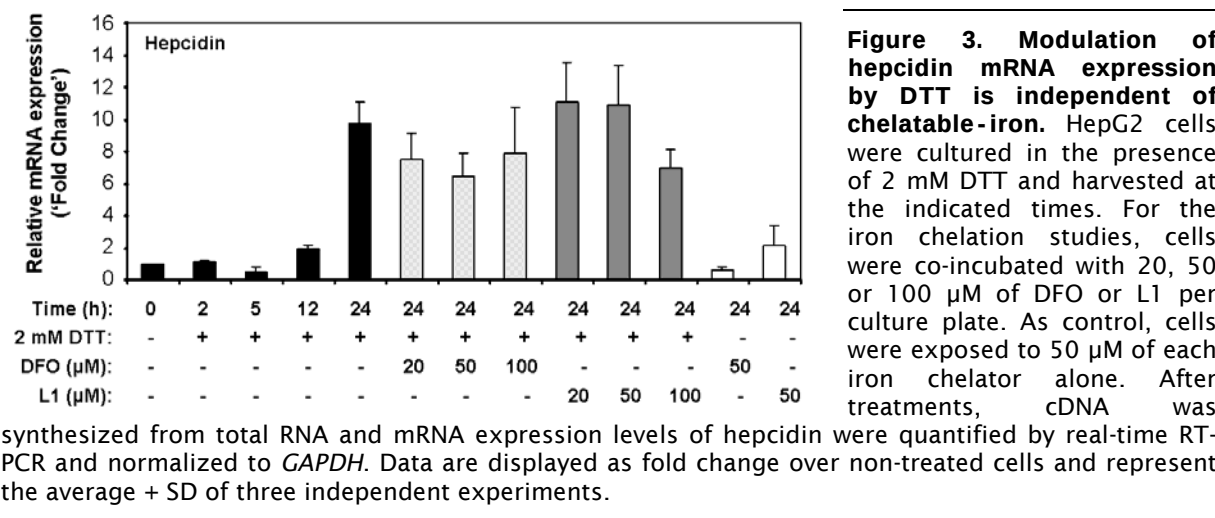
Expression levels of albumin, herein employed as control gene, were examined on cells exposed to DTT, with no differences detected in either time- or dose-dependent assays (Fig. 2A and C).

Modulation of hepcidin expression upon DTT-elicited UPR is chelatable iron-independent

The central role of hepcidin in iron homeostasis, coupled to its marked modulation in HepG2 cells undergoing ER stress, prompted us to focus on this molecule. Therefore, the subsequent experiments were designed to dissect the regulatory mechanisms underlying hepcidin expression pattern in the presence of an active UPR.

Although DTT has proven to be a useful UPR inducer [Braakman et al. 1992], being a reducing agent its spectrum of action is broad and relatively unspecific. Hence, we hypothesized that DTT could accelerate Fe^{3+} reduction, thereby modulating cellular iron traffic and, consequently, regulating the expression of the genes under study [reviewed in Chua et al. 2007]. To assess the contribution of iron to the differential expression of hepcidin in response to DTT, a kinetic analysis of HepG2 cells exposed to this drug was conducted in the presence of two distinct iron chelators: desferrioxamine (DFO) and deferiprone (L1). As displayed in Fig. 3, the 24 h up-regulation of hepcidin transcripts was preserved in the co-incubation assays,

ruling-out the contribution of chelatable iron to our observations. As an additional control, BiP and CHOP expression levels were measured upon 24 h of combined treatment, with no significant differences found at this point (data not shown).



C/EBP α and CHOP mediate the early down-modulation of hepcidin upon UPR induction

The liver-enriched nuclear factor C/EBP α has been implicated in both up- [Courselaud et al. 2002] and down- [Pinto et al. 2008b] regulation of hepcidin in a variety of contexts. As a first step to delineate the molecular mechanism(s) involved in the UPR-dependent modulation of hepcidin expression, the levels of this transcription factor were evaluated during the time- and dose-response assays. Western blot analysis of HepG2 cells exposed to the stressor agents allowed the detection of two products with the expected sizes of 42 and 30 KDa (Fig. 4A and B), corresponding to the two C/EBP α isoforms. The lowest protein levels of C/EBP α (one half the control values) were observed after 5 h of DTT treatment (Fig. 4A), which coincides with the decreased expression previously found for hepcidin (Fig. 2A). Hence, we hypothesized that reduced amounts of C/EBP α protein might determine a poorer stimulation of the hepcidin promoter, therefore accounting for the down-modulation of its transcript levels at this time-point. The mRNA levels of C/EBP α remained unaffected over the time-course assays (Fig. 4C), suggesting that the 5 h-

repression of this nuclear factor is post-translationally determined. Since CHOP protein levels also peaked 5 h post-stimulation with DTT (Fig. 1D), the possibility of CHOP participating in the modulation of C/EBP α protein described above was considered. This hypothesis was reinforced by the dose-response experiments, in which the same range of DTT concentrations (2-10 mM) accommodated both induction of CHOP (Fig. 1B) and down-modulation of C/EBP α (Fig. 4B) proteins. Once again, C/EBP α protein changes did not mirror those of the correspondent mRNA (Fig. 4D). Employing Hcys as ER-stress activator, a similar set of results was obtained. In terms of kinetics, the down-modulation of hepcidin initiated after 5 h of treatment (Fig. 2B) also overlapped with both induction of CHOP (Fig. 1D) and reduction of C/EBP α (Fig. 4A) proteins. Overall, data provided by the dose-response experiments with Hcys (Fig. 1B and 4D) were in close agreement with the C/EBP α -CHOP interplay suggested by the DTT assays.

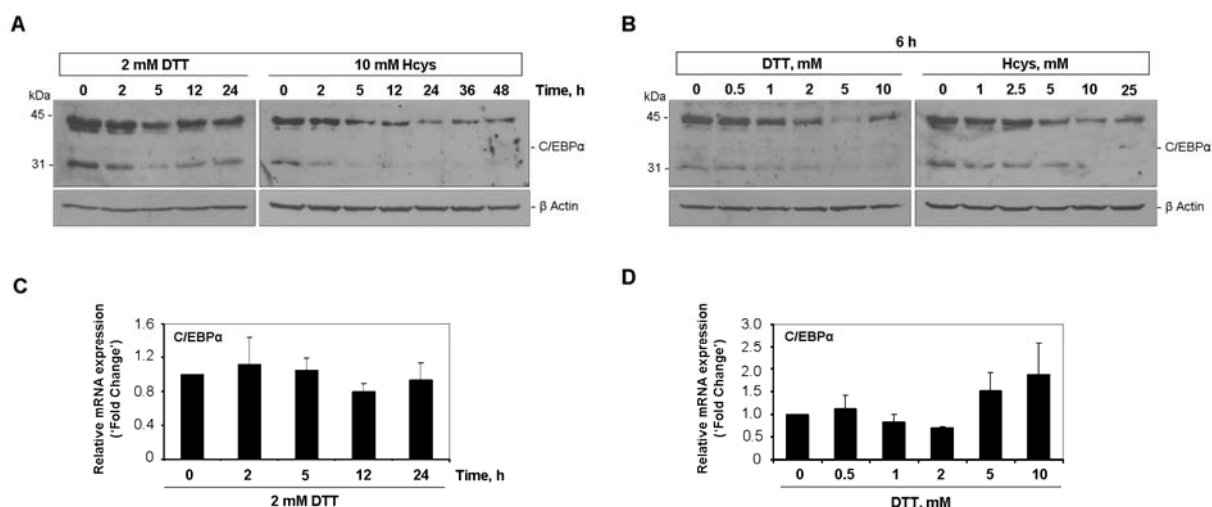


Figure 4. C/EBP α protein but not mRNA levels are modulated by the UPR. HepG2 cells were exposed to 2 mM DTT (**A** and **C**) or 10 mM Hcys (**A**) for different time periods. Control cells were left untreated. Increasing concentrations of DTT (**B** and **D**) or Hcys (**B**) were used to treat HepG2 cells during 6 h. Control cells were incubated with vehicle alone. **A** and **B**, After cell lysis, C/EBP α protein content was examined in whole extracts by immunoblot. Two forms of C/EBP α with the expected sizes (42 and 30 KDa) were detected. Molecular weights are indicated in the left side of the panels. To confirm equal lane loading β -actin was measured. Representative blots of three independent experiments are shown. **C** and **D**, Quantification of C/EBP α mRNA levels in DTT-treated HepG2 cells was performed by real-time RT-PCR. Data were normalized to *GAPDH* as endogenous control. Fold change relative to control cells was calculated and is displayed as the average + SD of three independent experiments.

To further test the hypothesis of a C/EBP α protein repression dependent on CHOP induction we used the DTT model of UPR activation. HepG2 cells were transfected with siRNA oligonucleotides targeting CHOP prior to supplementation of 2 and 5 mM DTT. Confirming the silencing efficiency, transfection of HepG2 cells with CHOP siRNAs impaired the DTT-stimulation of CHOP (Fig. 5). Importantly, down-modulation of C/EBP α protein in response to DTT (2 and 5 mM) was significantly prevented, thus supporting our initial hypothesis.

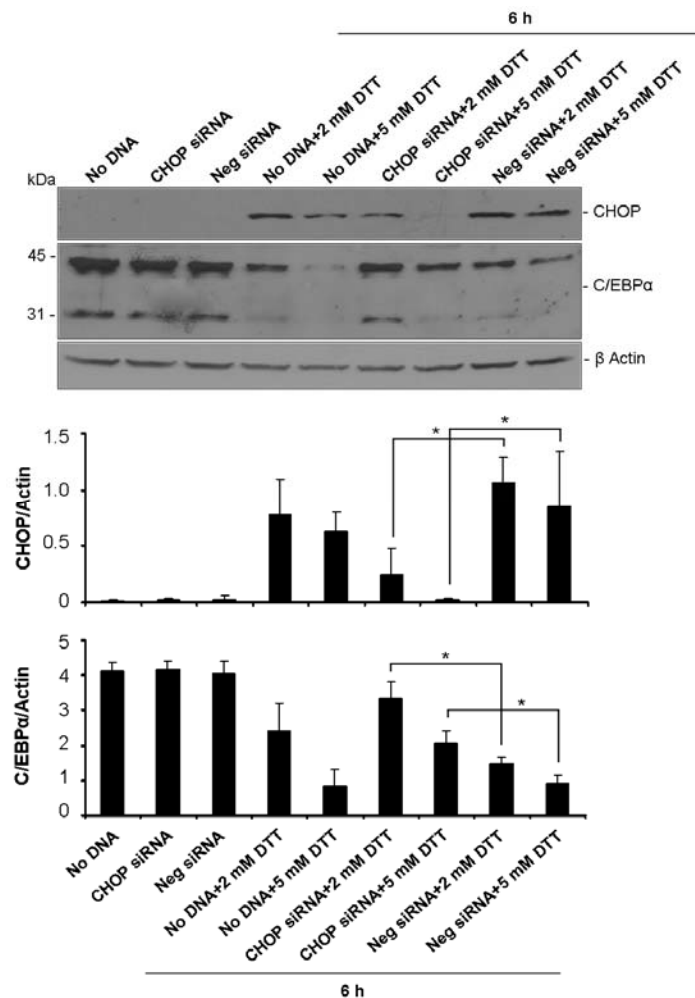


Figure 5. Reduction of C/EBP α protein levels depends on CHOP up-regulation. HepG2 cells were transfected with CHOP or scrambled (Neg) siRNAs for 24 h or left untreated (No DNA). DTT was added at the indicated dosage in the last 6 h of the transfection period. Efficacy of CHOP silencing was assessed in total cell lysates by western blot with anti-CHOP antibody (upper panel). Blots were also probed to C/EBP α and the two expected products (42 and 30 KDa) were detected (middle panel). β -actin was employed as loading control. The intensity of bands relative to CHOP and the 42 KDa-form of C/EBP α was quantified by densitometry, normalized to β -actin and graphically represented as average + SD of three independent experiments. * $p < 0.05$.

Differential C/EBP α binding to hepcidin promoter mediates the late up-regulation of hepcidin by the UPR

Maintenance of DTT- and Hcys-induced UPR for longer than 5 or 24 h respectively, was accompanied by an up-regulation of hepcidin expression (Fig. 2A and B). Since this stimulation of hepcidin could not be directly attributed to quantitative changes in the C/EBP α protein pool (Fig. 4A), we asked whether in this phase the UPR would be modulating the binding activity of C/EBP α to the hepcidin promoter. This question was addressed *in vitro* by performing a fluorescent variant of EMSA (fEMSA), in which nuclear extracts of DTT-treated HepG2 cells were combined with a DNA probe containing the consensus binding sequence recognized by C/EBP α [Ryden and Beemon 1989]. The single retarded complex detected was efficiently competed by an excess of unlabeled probe (Fig. 6A, *lanes 4 and 6*) and partially supershifted upon addition of an anti-C/EBP α antibody (Fig. 6A, right panel), which attested its specificity. The intensity of the C/EBP α -DNA complex, reflecting the binding activity of this nuclear factor to its consensus sequence, enhanced from 4 to 11 h and returned to baseline values beyond this time-point (Fig. 6A). Although the kinetics of C/EBP α binding did not strictly match the expression profile of hepcidin, our fEMSA results suggest that modulation of C/EBP α binding to hepcidin promoter might underlie the up-regulation of its expression detected after 24 h of DTT treatment.

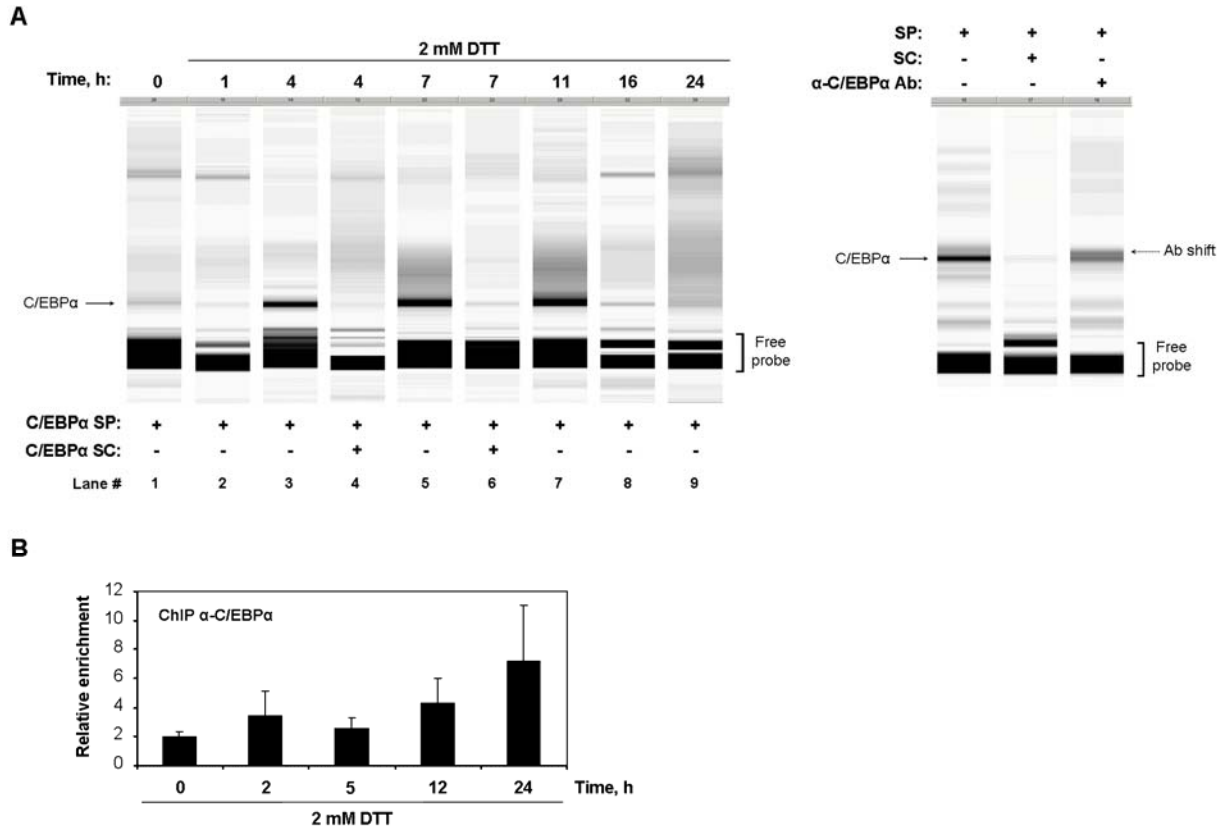


Figure 6. DNA-binding activity of C/EBPα is modulated over the time-course of UPR activation. HepG2 cells cultured in the presence of 2 mM DTT for the indicated intervals were subjected to fEMSA (**A**) and ChIP (**B**) analyses. **A**, Nuclear protein extracts from DTT- or control-treated cells were combined with the Cy5-labeled specific probe containing the C/EBPα consensus binding site (C/EBPα SP) and electrophoresed under native conditions. For competition assays, a 50-fold molar excess of unlabeled probe (C/EBPα SC) was used (lanes 4 and 6). The specific DNA-protein complex is indicated by an arrow. Free-probe is also shown in the bottom of the gel. The fEMSA gel view displayed is representative of four independent experiments (left panel). A specific antibody against C/EBPα was added for the supershift reaction (right panel). The partial shift is highlighted in the right side of the figure. **B**, Cross-linked chromatin was immunoprecipitated with anti-C/EBPα or anti-IgG (serum control) antibodies. The recovered chromatin samples were analyzed by quantitative real-time PCR with primers flanking the C/EBPα binding site found within the -136/+9 region of human *HAMP* promoter. Results depict the enrichment relative to serum control immunoprecipitation normalized to ChIP input values and are expressed as average + SD of three independent experiments.

To better examine how DTT affected the binding of C/EBPα to the hepcidin promoter *in vivo*, ChIP analysis was carried out. As Fig. 6B shows, following 24 h of culture in the presence of this ER stressor, the recruitment of C/EBPα to the hepcidin promoter was increased, which may account for the up-regulation of hepcidin mRNA levels detected at the same time-point.

To conclusively confirm the involvement of C/EBP α in the 24 h-increase of hepcidin transcript levels, a RNA interference approach was employed. Transfection of HepG2 cells with siRNA oligonucleotides against C/EBP α successfully attenuated the expression of the corresponding protein ($71 \pm 20\%$ of silencing relative to negative control siRNA-transfected cells; Fig. 7A). Moreover, C/EBP α silencing significantly abolished the responsiveness of hepcidin to the ER-stressor DTT upon 24 h of incubation (Fig. 7B), thus confirming C/EBP α as a relevant intermediary in the transcriptional regulation of hepcidin by the UPR.

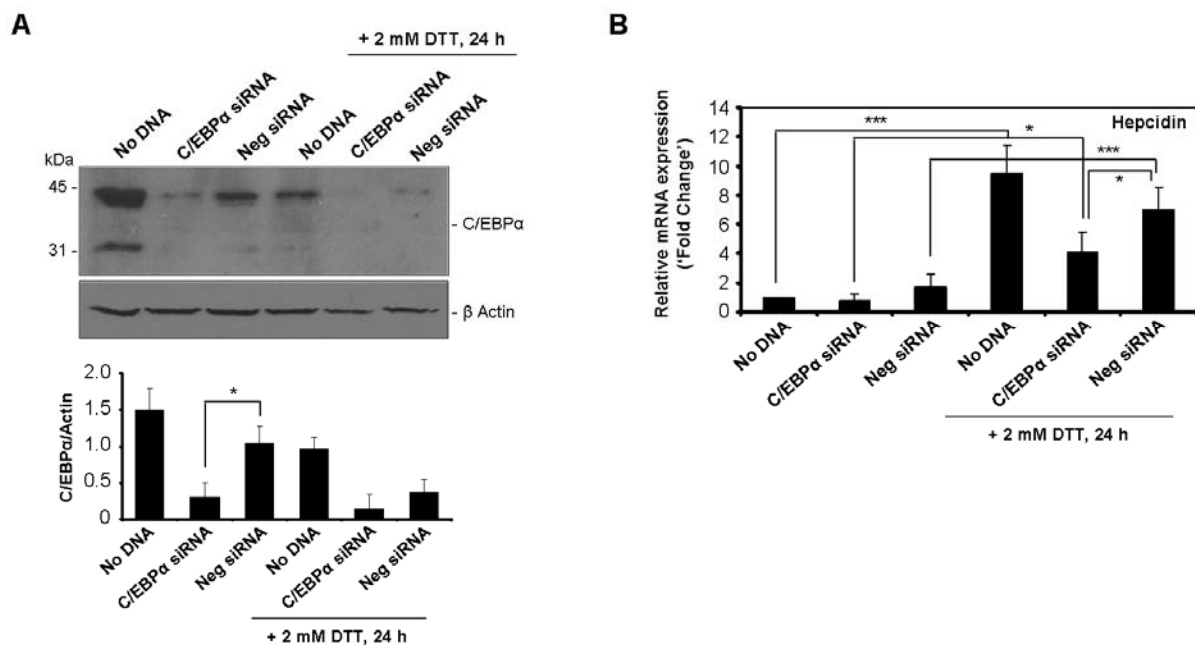


Figure 7. Silencing of C/EBP α partially prevents the late increase of hepcidin induced by the UPR. HepG2 cells were transfected with C/EBP α or scrambled (Neg) siRNAs for 48 h or left untreated (No DNA). Cells were exposed to DTT during the last 24 h of transfection procedure. **A**, Efficiency of C/EBP α knock-down was evaluated in whole cell extracts by western blot using an antibody against C/EBP α . The typical 42 and 30 KDa products were detected. To equalize lane loading β -actin was probed. A representative blot is shown. Intensity of the 42 KDa-band of C/EBP α was quantified by densitometry, normalized to β -actin and plotted as average + SD of three independent experiments. **B**, Total RNA of siRNA-transfected or untreated (No DNA) HepG2 was isolated. Using real-time RT-PCR, mRNA expression levels of hepcidin were measured and normalized to the *GAPDH* house-keeping gene. Data are expressed as fold change over No DNA-treated cells. * $p < 0.05$, *** $p < 0.001$.

Discussion

Compelling evidence extending the UPR beyond its classical role in the mitigation of protein misfolding and proteotoxicity has been provided through its connection to obesity [Ozcan et al. 2009] and type 2 diabetes [Oyadomari et al. 2002], the crosstalk with inflammation [reviewed in Zhang and Kaufman 2008] and its impact on the cell surface expression of major histocompatibility complex (MHC) class I molecules [de Almeida et al. 2007a; Granados et al. 2009]. Moreover, previous reports have also suggested the existence of interplay between the UPR and iron metabolism [You et al. 2003; Nishina et al. 2008; Harrison-Findik et al. 2007]. Details of this association, however, remain unclear, prompting us to exploit whether the expression of iron-related genes is influenced by ER stress-dependent mechanisms.

Hepcidin, a liver-derived peptide hormone, binds to the iron exporter ferroportin, causing its internalization and degradation, thereby blocking cellular iron efflux and intestinal iron absorption [Nemeth et al. 2004]. The pivotal role of hepcidin in the maintenance of systemic iron balance led us to focus on the regulation of its expression in the context of an active UPR. In our model, mRNA levels of hepcidin were significantly influenced by the UPR elicited by DTT and Hcys. In addition, the results enabled us to identify C/EBP α as an important mediator of such modulation. Our interest on this transcription factor was driven by two previous findings: i) C/EBP α is a liver-enriched nuclear factor known to regulate hepcidin transcription [Courselaud et al. 2002] and ii) C/EBP α can heterodimerize with other members of the same family, some of which induced by the UPR signaling program [Ron and Habener 1992]. CHOP is one of these members, whose higher protein levels consistently overlapped with the down-modulation of both C/EBP α protein and hepcidin transcripts in the experimental conditions used. This observation allowed us to hypothesize a concerted regulation between CHOP and C/EBP α , successfully supported by the CHOP silencing assays (Fig. 8 depicts a schematic representation of the proposed model). According to these data, the rise of CHOP levels upon ER stress leads to a decrease of C/EBP α protein content, which may limit the availability of this nuclear factor to stimulate hepcidin transcription, with the concomitant down-modulation of its mRNA levels. An analogous interplay between C/EBP α and CHOP

was previously described in pre-adipocytes, where CHOP up-regulation prevented C/EBP α expression [Tang and Lane 2000; Batchvarova et al. 1995]. Similarly, the negative regulation of C/EBP α expression by CHOP was observed in livers from tunicamycin-challenged mice and proposed as a key mechanism linking ER stress to the disruption of hepatic lipid metabolism [Rutkowski et al. 2008].

We found substantial differences in the time-course expression of the UPR downstream effectors. While increased protein levels of BiP were sustained, the up-regulation of CHOP was more transient. This pattern is in agreement with the concept of chronic *vs* acute stress, distinguished by detectable levels of BiP or CHOP, respectively [Rutkowski et al. 2006]. In addition, the loss of CHOP expression is likely on the basis of hepcidin recovery observed in the long-term incubation with both stressors. Accordingly, our ChIP analysis revealed that the endogenous hepcidin promoter of HepG2 cells was enriched in the C/EBP α transcription factor after 24 h of DTT-evoked UPR. One can speculate that this occurs as an attempt by the cell to compensate for the deficit of hepcidin expression imposed by CHOP up-regulation. The modulation of C/EBP α binding activity throughout the ER-stress response was likewise evidenced by the fEMSA experiments, although with different kinetics. The discrepancy between the fEMSA and ChIP data may reflect the inability of the former method to efficiently mimic binding conditions found *in vivo*. It can also be explained by differential affinity of C/EBP α for the binding sites used in each assay: a C/EBP α consensus binding sequence in fEMSA and the native C/EBP α -responsive elements within hepcidin promoter in ChIP.

The contribution of iron to the up-regulation of hepcidin here reported was excluded when the UPR was chemically elicited by DTT under iron chelating conditions. Furthermore, it has been shown that hepatocytes in culture fail to increase hepcidin production in response to iron loading [Nemeth et al. 2003; Jacolot et al. 2008].

Our *in vitro* model system of a pharmacologically-elicited UPR could render valuable clues when extrapolated to a physiological context. Hepcidin levels are known to be abnormally low in HFE-linked HH [Bridle et al. 2003]. Considering that markers of an active UPR were formerly found in individuals homozygous for the C282Y mutation of HFE [de Almeida et al. 2007a], it is tempting to speculate that the CHOP-C/EBP α interconnection here described may contribute to the impaired

induction of hepcidin expression regardless of the iron burden. The intersection between the UPR signaling pathways and the transcriptional regulation of hepcidin could therefore contribute to explaining some of the phenotypic variation amongst HH patients [Pietrangelo 2004].

Regarding the other two genes examined in the present study, up-regulation of ferroportin and ferritin H was found in HepG2 following the UPR activation, both in dose- and time-response assays. Although beyond the scope of this work, some hypotheses can be drawn concerning the molecular pathways involved in such outcome (summarized in Fig. 8). Apart from the supra-mentioned post-translational regulation by hepcidin [Nemeth et al. 2004], expression of ferroportin is also controlled by a well established Iron Responsive Element (IRE)/Iron Regulatory Protein (IRP) mechanism [Lymboussaki et al. 2003]. Nonetheless, elevated hepatic mRNA levels of ferroportin were detected in HFE C282Y homozygous despite iron overload [Bridle et al. 2003; Gleeson et al. 2007], thus suggesting that transcriptional events also contribute to its regulation [Zoller et al. 2002]. In line with this, *in silico* analysis of ferroportin gene and promoter showed the presence of a number of putative binding sites for transcription factors commonly induced during the UPR (e.g. ATF/CRE, AARE, CHOP-binding sequence; <http://www.genomatix.de>). Hence, we reasoned that the presence of such regulatory elements might confer some UPR-responsiveness to ferroportin.

Ferritin is an iron-storage protein whose regulation also depends on the IRE/IRP post-transcriptional system [Hentze and Kühn 1996]. Interestingly, the existence of an antioxidant-responsive element (ARE) activated through binding to Nrf2, a nuclear factor induced by the UPR program [Venugopal and Jaiswal 1998], was reported for the H- and L- isoforms of ferritin gene [Pietsch et al. 2003]. Taken together, this report and our experimental data are indicative of a complementary/additive action driven by both iron excess and UPR activation towards ferritin mRNA up-regulation.

The boost of ferroportin and ferritin H can be envisaged as a mechanism to prevent intracellular accumulation of free iron. Our data of albumin expression seem to exclude a mere UPR-“side effect” as the reason behind our observations, supporting the likelihood of their physiological relevance. Whether the modulation of iron-related genes is part of a general UPR strategy aiming at maintaining cell

viability and homeostasis or, instead, it represents a novel pathway to specifically control the expression of a subset of genes related to iron metabolism, remains the subject for further study.

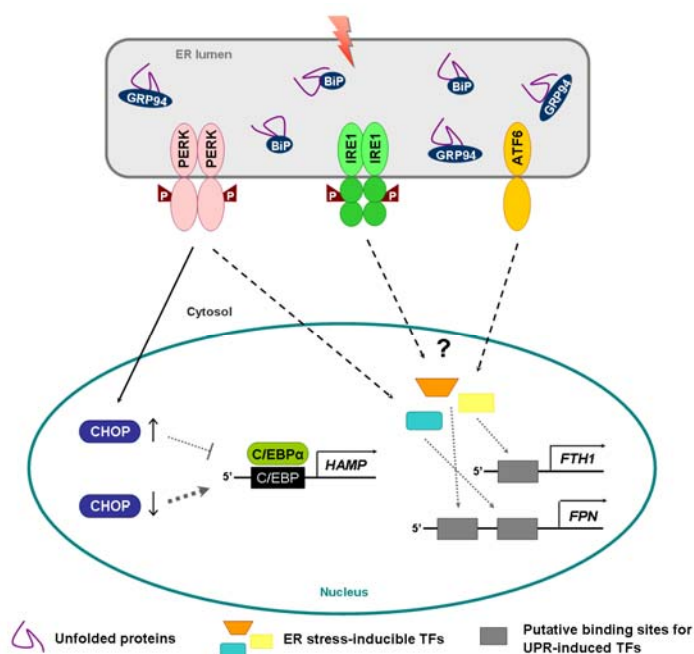


Figure 8. Schematic overview of the mechanisms underlying the UPR-induced modulation of iron-related genes. Activation of the PERK-dependent branch of the UPR modulates CHOP levels. This will in turn affect the C/EBP α protein pool and ultimately the stimulation of the hepcidin (*HAMP*) promoter. Regarding the other iron-genes, both ferritin H (*FTH1*) and ferroportin (*FPN*) display putative binding sites recognized by transcription factors (TFs) activated during the UPR. The presence of these regulatory elements may confer some UPR-responsiveness to ferritin H and ferroportin, increasing their expression in response to ER stress.

CHAPTER V

Does the nature of the ER stress-producing agent influence the transcriptional response of iron-related genes?

Specific aims

Work from our group, described in the previous chapter, has uncovered the connection of UPR to iron homeostasis through the transcriptional modulation of iron-related genes [Oliveira et al. 2009]. Parallel, near-simultaneous observations were reported by others using an animal model [Vecchi et al. 2009]. Both studies relied on the chemical induction of ER stress. In the former, DTT and Hcys were employed to abrogate oxidative protein folding within the ER, whereas in the second accumulation of unglycosylated clients was favored by challenging mice with Tm. The impact of the late condition, however, was essentially evaluated in terms of hepcidin expression, while the consequences of Tm on other iron metabolism-associated genes remain largely uncharacterized. Furthermore, distinct stressor agents were found to differentially activate the UPR signaling cascades [Benedetti et al. 2000; Lemin et al. 2007], also diverging on the transcriptional regulation of common target genes [Labunskyy et al. 2009].

Based on the above premises we sought to determine how the expression of iron-related genes is tuned in the HepG2 cell line upon Tm supplementation. The purpose of this approach is two fold:

- a. to test whether the response of iron-genes to ER stress is a universal effect or, instead, restricted to thiol-containing compounds;
- b. to extend the data achieved *in vivo* with Tm to other relevant genes partaking in iron metabolism.

Results and Discussion

Experimental model of ER stress

Similarly to the strategy delineated in Chapter IV, we started by setting up the experimental conditions of ER stress. The HepG2 cell line was maintained as *in vitro* model while Tm, with its ability to impair protein N-glycosylation, was chosen to unbalance the ER homeostasis. Fixing a time interval of 6 h and exposing HepG2 cells to Tm in the range of 1-50 µg/mL up-regulated the steady-state mRNA level of both BiP and CHOP, two commonly used markers of UPR activation (Fig. 1A). The dose-dependent effect was lost for the highest drug concentration (50 µg/mL; Fig. 1A), probably because cell viability was already compromised at this stage, as assessed by the Trypan blue exclusion test (data not shown). These changes were mirrored by the western blot results, with CHOP exhibiting the sharpest modulation and varying from undetectable to greatly stimulated levels during Tm treatments (5-20 µg/mL; Fig. 1B). Unlike BiP, a dose-response relationship was not obvious for GRP94, the other ER-chaperone detected by the anti-KDEL antibody (Fig. 1B).

As minimal concentration required for simultaneously inducing BiP and CHOP mRNA and protein expression, 5 µg/mL Tm was selected to carry out the kinetic studies. Although BiP transcripts increased gradually throughout the assays, statistical significance was only obtained upon 12 and 24 h of incubation with Tm (Fig. 1C). Equivalent outcome was found in terms of protein, with immunoblots depicting up-regulated levels of BiP at the same time-points. Albeit modest, enhanced expression of GRP94 was also detectable at 24 h (Fig. 1D). A time-course stimulation of CHOP mRNA was found between 5 and 24 h of culture in the presence of Tm, with peak values occurring after 12 h of drug exposure (Fig. 1C). Such profile was further validated by western blot analysis (Fig. 1D).

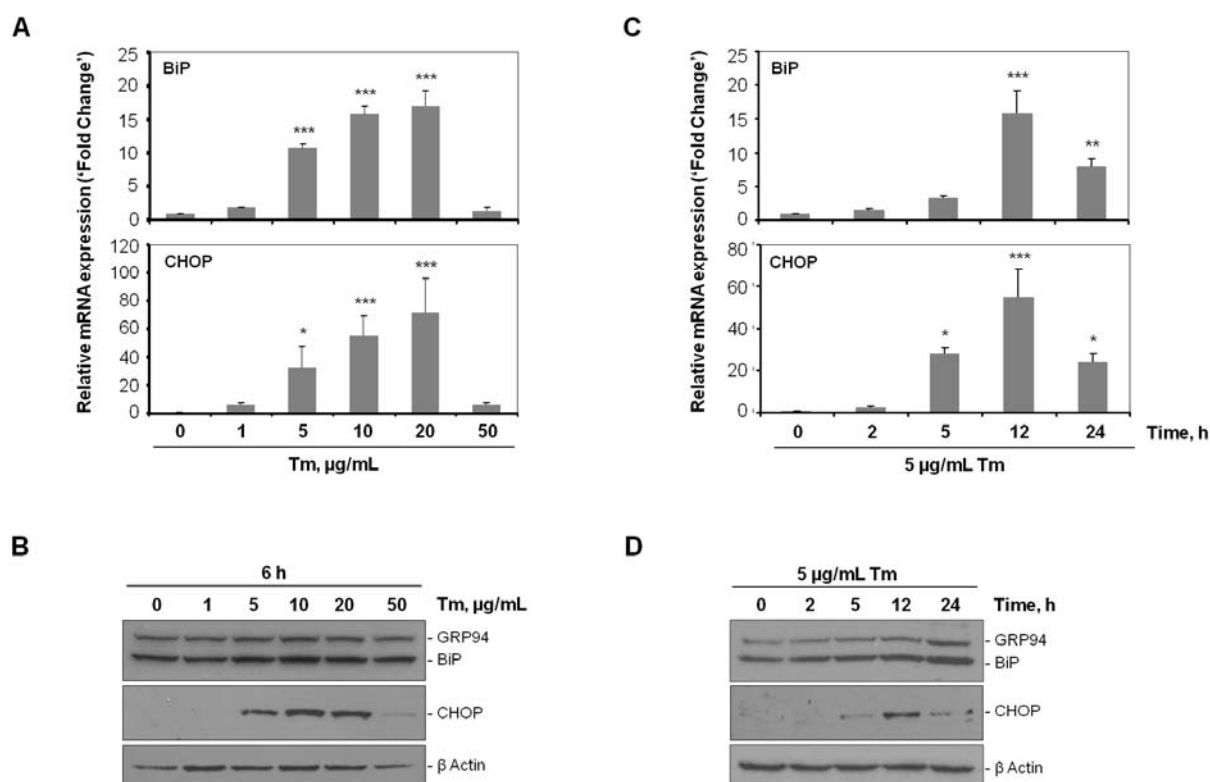


Figure 1. Characterization of UPR activation markers in HepG2 cells challenged with Tm. Dose-response assays (**A** and **B**): HepG2 cells were treated for 6 h with increasing concentrations of Tm, as indicated. Control cells received vehicle alone. Time-course assays (**C** and **D**): HepG2 cells were exposed to 5 $\mu\text{g/mL}$ Tm for the indicated times. Control cells were left untreated. **A** and **C**, BiP and CHOP mRNA levels were measured by real-time RT-PCR and normalized to *GAPDH* expression. Results are expressed as fold change over control-treated cells and represent the average + SD of three independent experiments. **B** and **D**, GRP94, BiP (using an anti-KDEL antibody) and CHOP were analysed by western blot in whole cell lysates of HepG2 cells. As loading control, membranes were stripped and reprobed for β -actin. Representative blots of three independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Tm-induced ER stress influences the temporal expression of iron metabolism-associated genes

Applying the preselected dose of 5 $\mu\text{g/mL}$, time-course expression of some iron-related genes was monitored in Tm-challenged HepG2 cells. Globally, the presence of Tm in the culture medium increased hepcidin mRNA ~2 to 3.5-fold above the basal values. The maximal effect was observed 5 h post-stimulation (Fig. 2), thus coinciding with the recent data provided by Vecchi et al. using the same experimental scheme and cell line [Vecchi et al. 2009]. From this time-point onwards hepcidin expression decreased, reaching the lowest level at 24 h (Fig. 2). The gene

profiling described here fails to resemble that triggered by DTT and Hcys (Chapter IV), where an early down-modulation of hepcidin was counterbalanced in the long-term UPR activation. Such stressor-dependent divergences have been reported elsewhere and might stem from a selective or preferential activation of the UPR signaling branches, ultimately affecting the behavior of target genes. Supporting this viewpoint, the transcriptional activation of *c-myc* by DTT was found to involve PERK, whereas Tm did not [Liang et al. 2006]. Moreover, XBP1 splicing and ATF6 α transcription were shown to proceed differently in the presence of an UPR imposed by faulty glycosylation or redox imbalance [Lemin et al. 2007]. Another possibility relies on the kinetics of UPR activation associated with assorted ER stressors [DuRose et al. 2006]. As judged by the time-course expression of both BiP and CHOP (compare Fig. 1C and D of Chapters IV and V), DTT is a faster and more severe ER stress inducer than Tm, which may also underlie the apparent discrepancy in hepcidin response. An identical hypothesis was drawn to explain the disparate impact of Tm and DTT on the regulation of the thioredoxin-like protein Sep15 [Labunsky et al. 2009].

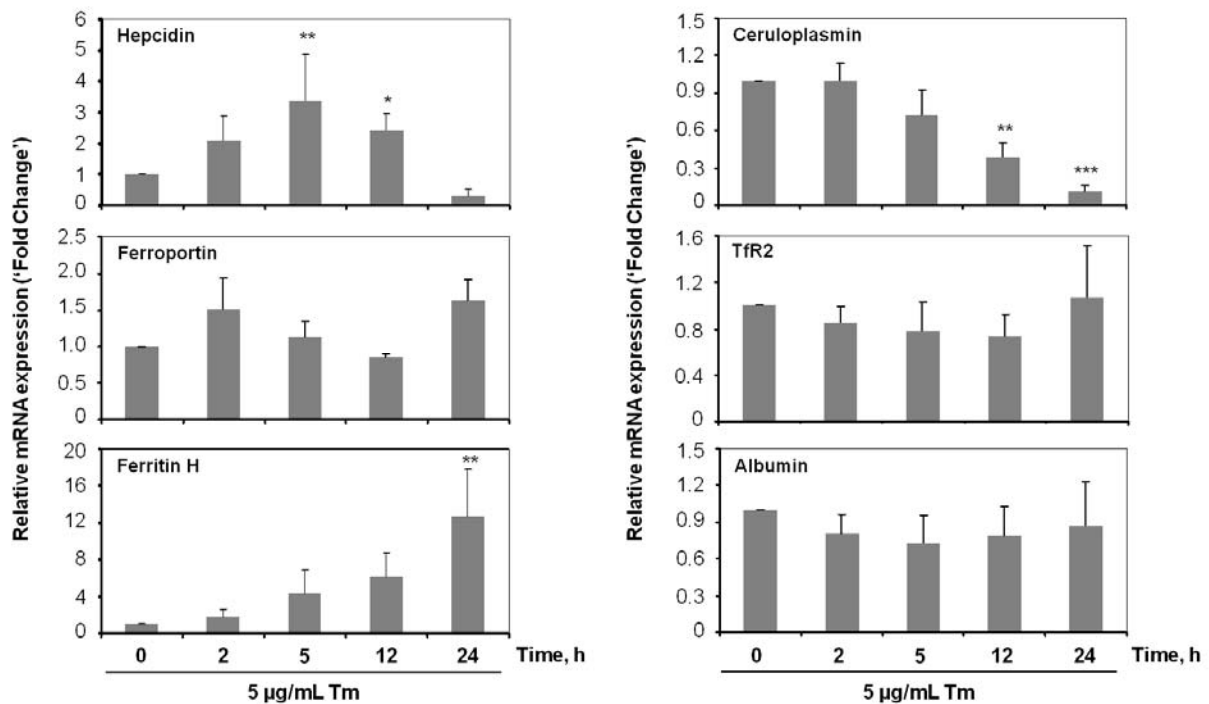


Figure 2. Differential expression of iron-related genes in response to Tm-elicited UPR activation. HepG2 cells were cultured in the presence of 5 µg/mL Tm for the indicated intervals. Untreated cells were used as control. After treatments, total RNA was isolated and mRNA levels of the “iron-genes”

hepcidin, ferroportin, ferritin H, ceruloplasmin and TfR2 assessed by real-time RT-PCR. Expression of albumin, a non-iron-related gene, was evaluated as control. Data were normalized to *GAPDH* and are expressed as fold change over control-treated cells. Each bar displays the average + SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

In contrast with the stimulatory action driven by thiol-containing drugs, the presence of Tm had no effect on the mRNA expression pattern of ferroportin (Fig. 2). The first argument presented above in the context of hepcidin might account for the variable outcome of ferroportin as well.

Considering the predominance in hepatocytes and the role as upstream regulator of hepcidin [Deaglio et al. 2002; see Chapter I for review], TfR2 was included in the current study, displaying unaltered transcript levels throughout the Tm-triggered UPR (Fig. 2). On the contrary, ferritin H was markedly responsive to Tm treatment, being up-regulated in a time-dependent fashion (Fig. 2) and recapitulating the trend noted for DTT and Hcys insults (Chapter III).

Ceruloplasmin mRNA expression was significantly reduced over the time-course assays with Tm (Fig. 2). Inhibition of ceruloplasmin protein secretion was also attributed to Tm in previous work [Yeo et al. 1989], lending credence to our results. The ferroxidase activity towards ferrous ions renders ceruloplasmin essential in the mobilization of iron from cells [Harris et al. 1995]. Accordingly, decreased levels of this molecule are suggestive of enhanced cellular iron retention, hypothesis compatible with the concomitant rise of ferritin H observed in the same time-frame (Fig. 2). Moreover, down-regulation of both ceruloplasmin content and enzymatic activity has been documented in HH patients homozygous for the HFE C282Y mutation [Cairo et al. 2001; Lainé et al. 2002]. The absence of this phenotype among individuals with acquired iron overload led Cairo et al. to discard the contribution of such element. On the contrary, Lainé et al. argued that the ceruloplasmin response involves excess iron-mediated repression of HIF-1 [Mukhopadhyay et al. 2000]. In the light of recent findings, another possibility can be raised. Expression of the C282Y mutant protein was shown to stress the ER [de Almeida et al. 2007a; Lawless et al. 2007; Chapter III], which might also underlie the low ceruloplasmin levels found in HH subjects [Cairo et al. 2001], as judged by the data presented here. Aside from participating in iron homeostasis, ceruloplasmin is an acute-phase reactant as well [Gitlin 1988]. The outcome pattern of ceruloplasmin under our experimental

conditions may therefore be envisioned as part of the crosstalk between innate immunity and UPR unveiled in recent years [Zhang et al. 2008; Vecchi et al. 2009]. This possibility deserves further investigation.

Expression of the control gene albumin remained unchanged during the incubation of cells with Tm (Fig. 2).

C/EBP α - a promising target regulated by the Tm-induced UPR

The longstanding recognition of C/EBP α as regulator of hepcidin expression [Courselaud et al. 2002] was recently extended to the UPR context [Oliveira et al. 2009]. Expression levels of this transcription factor were therefore examined in HepG2 cells exposed to Tm. As assessed by immunoblot analysis, expression of the two C/EBP α isoforms (42 and 30 KDa) decreased in the course of the experiment (Fig. 3A), trend also mirrored by the corresponding mRNA transcripts (Fig. 3C). To further confirm the effect of Tm on C/EBP α , the impact of different drug concentrations was evaluated. A dose-dependent down-regulation of C/EBP α was detected both in terms of protein (Fig. 3B) and mRNA (Fig. 3D) levels. An identical response of C/EBP α to Tm challenge was described by Lefterova et al. in adipocytes [Lefterova et al. 2009].

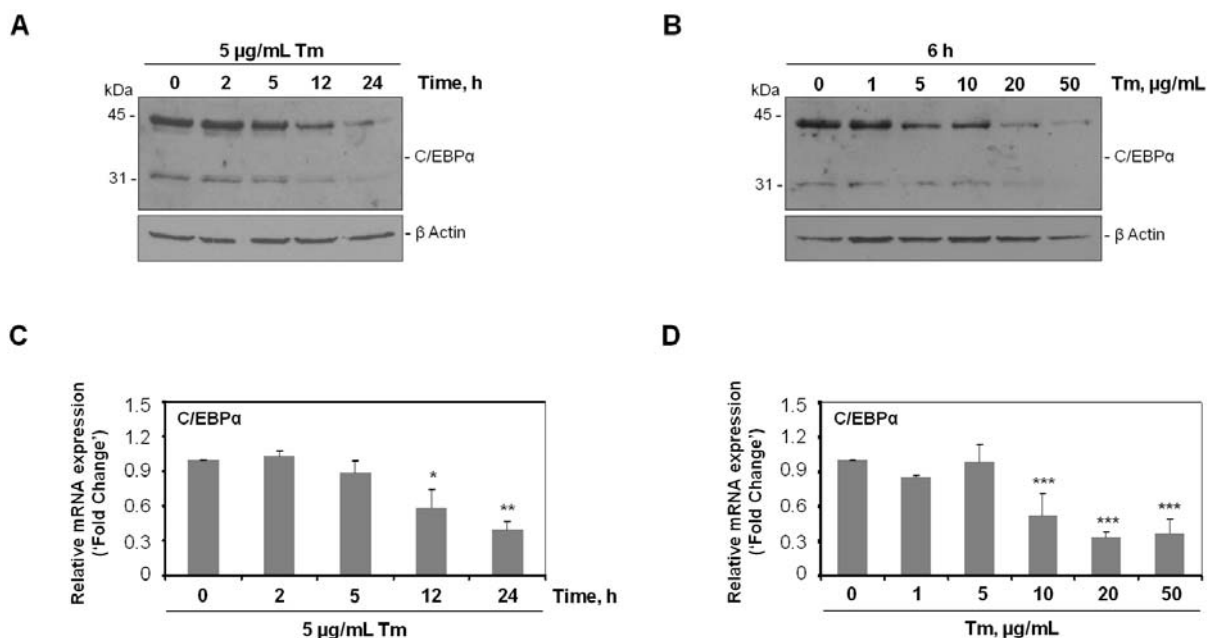


Figure 3. C/EBPα is modulated by the Tm-induced UPR. HepG2 cells were exposed to 5 µg/mL Tm for different time periods (**A** and **C**). Control cells were left untreated. Increasing concentrations of Tm were used to treat HepG2 cells during 6 h (**B** and **D**). Control cells were incubated with vehicle alone. **A** and **B**, After cell lysis, C/EBPα protein content was examined in whole extracts by immunoblot. Two forms of C/EBPα with the expected sizes (42 and 30 KDa) were detected. Molecular weights are indicated in the left side of the panels. To confirm equal lane loading β-actin was measured. Representative blots of three independent experiments are shown. **C** and **D**, Quantification of C/EBPα mRNA levels in HepG2 cells was performed by real-time RT-PCR. Data were normalized to *GAPDH* as endogenous control. Fold change relative to control cells was calculated and is displayed as the average + SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control.

Comparison of the respective expression profiles (Fig. 2 and 3A) reveals that hepcidin mRNA changes were not paralleled by those of C/EBPα protein, except for the last time-point recorded. Although the underlying mechanism(s) were not explored in the current study, some hypotheses can be anticipated regarding the up-regulation of hepcidin between 2 and 12 h post-treatment. Independently of the steady-state protein levels of this nuclear factor, certain ER stress conditions were shown to enhance the DNA-binding activity of C/EBPα to the hepcidin promoter [Oliveira et al. 2009]. A similar effect might be elicited by Tm incubation, thereby contributing to the increase of hepcidin transcripts. Another possibility relies on CREBH, an ER stress-responsive transcription factor recently proposed to mediate the stimulatory effect of Tm on hepcidin expression [Vecchi et al. 2009]. The two scenarios are not mutually exclusive and a cooperative model is reasonable as well.

Interestingly, C/EBP α has also been implicated in the transcriptional regulation of ceruloplasmin [Bingle et al. 1993]. In our experimental conditions, down-modulation of ceruloplasmin mRNA levels (Fig. 2) coincided temporally with the depletion of C/EBP α pool (Fig. 3A and C), thus suggesting a causal relationship between both observations. Such hypothesis should be dissected in further work.

Concerning our original objectives, the data herein presented: a) reinforce the newly disclosed association between chemically-induced ER stress and iron homeostasis, via modulation of iron-genes expression; b) support that the UPR consequences depend on the type of insult used to imbalance the ER folding environment and c) suggest that a single ER stressor-based model may not capture all the possible final outcomes.

CHAPTER VI

General Discussion

Originally described as a check-and-balance program focused on the recovery of stress-corrupted ER folding homeostasis, the boundaries of UPR have been steadily broadened to cell differentiation, metabolic and inflammatory processes [Reimold et al. 2001; Zhang et al. 2006a; Zhang et al. 2006b]. Suggestive evidence for an intersection with iron metabolism was first provided fortuitously by differential gene expression screenings. Two such examples are the up-regulation of CRT and BiP transcripts found in iron-burdened astrocytoma cells [Ye and Connor 2000] and the *Tf* gene down-modulation reported in stable CHOP transfectants [You et al. 2003]. Likewise, proteomic analysis revealed increased hepatic levels of BiP in dietary iron-loaded mice [Petrak et al. 2007]. Despite all these cues, the interplay of UPR and iron homeostasis has remained a hitherto unexplored field. With a renewed interest fueled by recent findings linking the C282Y mutation of HFE to ER stress phenomena, the current thesis addressed whether expression of iron-related genes is targeted by the UPR. Discussed below are the main results of our studies, including their relevance in light of both the original question and the future research possibilities.

One mutation, many outcomes

The discovery that C282Y faulty HFE elicits UPR activation conveyed fresh vistas for an old mutation and set the foundation for the present work. Pioneering models, however, relying on HEK293T-overexpressing systems and *ex vivo* peripheral blood mononuclear cells (PBMCs) [de Almeida et al. 2007a; Lawless et al. 2007] were unsuited to monitor expression of genes encoding iron management proteins. Such a task rather demanded hepatocytic cells, dedicated producers of critical iron metabolism-related molecules [reviewed in Graham et al. 2007]. Interesting contributions were yielded by this experimental constraint. First and foremost, it underlined the differential behavior of cells engineered to stably or transiently express mutant proteins. This issue, formerly discussed by others with respect to HLA-B27 [Penttinen et al. 2004; Turner et al. 2005], is particularly relevant when assaying pathways that, as the UPR, display well distinct chronic and acute facets [Rutkowski et al. 2006]. Whilst constitutive expression of HFE C282Y represents a better mimic of physiological conditions, the selection process may have biased this model by maintaining only the cells optimally adapted to the mutant

client. With transient expression, on the contrary, since cells had no time to “adapt” to the proteotoxic insult, early stages of the ER stress response could be captured without interference of “tolerance” events (Chapter III). Identical concerns were shared by Hidvegi et al. in the context of mutant α_1 -antitrypsin and handled through inducible expression systems [Hidvegi et al. 2005].

The lack of a pre-existing platform to address our question drew further attention to the influence of cell background on the UPR extent. The stronger induction of ER stress prototype targets in HEK293T [de Almeida et al. 2007a; de Almeida et al. 2007b; Lawless et al. 2007] suggests an increased susceptibility of these cells to the C282Y mutant HFE as compared to HepG2 counterparts (Chapter III). Evidence for the cell-specific nature of UPR has accumulated in recent years [Benedetti et al. 2000; DuRose et al. 2006], although details of the phenomenon await clarification. This differential character has been attributed to unique molecular machineries and/or to intrinsic abilities to cope with ER congestion. The former argument is supported by the finding of novel UPR transducers distributed in a tissue-restricted manner, such as OASIS, Tisp40 and CREBH [Kondo et al. 2005; Nagamori et al. 2005; Zhang et al. 2006a] (reviewed in Chapter I). The second assumption reflects the highly evolved ER network distinctive of professional secretory cells. As members of this family [Ji and Kaplowitz 2006], hepatocytes might have an exceptional capacity to naturally buffer the C282Y protein accumulation, thus explaining the milder response mounted by transient HepG2 transfectants (Chapter III). Together with protein energetics, the critical influence of local “chaperomes” accompanied by unique ER export and degradation activities, was quantitatively modeled by Wiseman et al. and used to explain why assistance of the same mutant protein is cell type-specific [Wiseman et al. 2007]. Moreover, such biochemical factors have been implicated in the tissue-selective pathology of amyloidosis [Sekijima et al. 2005] and Gaucher disease [Wiseman et al. 2007], likely accounting for the tissue-restricted UPR induction observed in HLA-B27 transgenic rats as well [Turner et al. 2005].

Also reflecting the disparate outcomes imparted by the HFE C282Y protein was the downstream effect on hepcidin gene expression (Chapter III), with our lentiviral-based system blunting the down-modulation previously noticed [Pinto et al. 2008a]. To foresee this response as a consequence of the ER stress caused by

transient C282Y mutant expression is an appealing hypothesis deserving further investigation.

Lessons from chemical models of ER stress

In light of the current paradigm, the iron-dependent tuning of hepcidin is governed by the HFE/TfR1/TfR2 partnership [Gao et al. 2009]. Due to compromised cell surface presentation, the C282Y variant of HFE fails to bind TfR1 or to stabilize TfR2 according to the prevailing Tf saturation levels. Widely accepted, this loss-of-function model challenges the identification of novel regulatory mechanisms mediated by the C282Y mutation, as those involving its ER retention. Central to this thesis, the last topic was addressed beyond the influence of HFE by exposing cells to pharmacological ER-disturbing conditions. While anticipating that this experimental design certainly exacerbates the ER stress insults faced *in vivo*, it could also provide valuable clues as prior studies have shown [Lefterova et al. 2009; Ozcan et al. 2009].

By demonstrating that ongoing ER stress modulates the gene expression of hepcidin, ferroportin, ferritin H and ceruloplasmin in HepG2 cells, our data unveiled a previously unrecognized interplay between iron metabolism and UPR signaling pathways (Chapters IV and V). Such intimate relationship was independently corroborated by Vecchi et al. that stressing hepatoma-derived cells with the ER-to-Golgi transport inhibitor brefeldin A, calcium ionophore A23187 and Tm reported up-regulated levels of hepcidin transcripts, a pattern also detected in the liver of Tm-treated mice [Vecchi et al. 2009]. The two pieces of work, ours and Vecchi's, have merited the close attention of others [De Domenico and Kaplan 2009; Messner and Kowdley 2010; Gnana-Prakasam et al. 2010; Babitt and Lin 2010].

The second key lesson was that the overall outcome of the UPR depends on the nature of the stressor agent itself. Similar observations have been recorded elsewhere [DuRose et al. 2006; Lemin et al. 2007], but the underpinning mechanisms remain obscure. Distinct kinetic determinants likely contribute to this variability [Labunskyy et al. 2009]. In fact, relying upon *de novo* synthesis of proteins, Tm may display delayed activation kinetics as compared to DTT and Hcys, whose targets are disulphide bonds of pre-existing clients. These divergences were also coupled to differential sensitivities of the UPR branches towards assorted forms of ER insults [DuRose et al. 2006]. One can therefore envision the customizable trait

of the UPR as a strategy to tackle ER luminal disequilibria with the best-suited response.

Our DTT-induced ER stress model also provided a glimpse on the molecular basis behind hepcidin gene modulation, uncovering C/EBP α and CHOP as important mediators of the process [Oliveira et al. 2009]. Vecchi et al., on the other hand, attributed the stimulation of hepcidin under stress scenarios to CREBH-dependent activation of its promoter [Vecchi et al. 2009]. The two proposed mechanisms are not incompatible and could conceivably coexist.

The ER stress-iron metabolism axis: unraveling its putative physiological significance

Given the complexity of the whole organism, extrapolation of *in vitro* data to physiological scenarios is often challenging and should proceed cautiously. Nevertheless, results obtained by Vecchi et al. using a mouse model of ER stress [Vecchi et al. 2009] fostered this predictive exercise.

Primarily designed to ensure the accurate folding of proteins under adverse intracellular conditions, a systemic interference of the UPR has been disclosed through the connection to insulin secretion and peripheral resistance [Lipson et al. 2006; Özcan et al. 2004], glucose homeostasis [Gonzales et al. 2008] and inflammation [Zhang and Kaufman 2008]. With the modulation of hepcidin herein described, a new item can be coupled to this picture. By limiting duodenal iron absorption, hepcidin up-regulation in this context may be part of a protective “plan” to evade extra sources of stress, as those associated with iron-generated ROS. In line with hepcidin’s antimicrobial role, consequences on the innate immunity are expected as well, thereby furthering the scope of the recently uncovered ER-stress mediated inflammatory responses [Zhang et al. 2006a; Martinon et al. 2010]. With the present work, the already intricate coordination of hepcidin by iron stores, erythroid factors and inflammation was extended to the ER stress chain of events.

Besides the systemic impact underlined by hepcidin, repercussions of UPR activation are also manifested at the cellular iron metabolism level, as suggested by the modulation of ferroportin and ferritin H expression (Chapter III). The mRNA enrichment of both genes in cells enduring ER stress may reflect an attempt to circumvent intracellular deposition of free iron either via its sequestration or export.

Possible link to pathological conditions

Building evidence has coupled the ER stress response circuitries to multiple pathologies, namely diabetes, obesity and neurodegeneration [Oyadomari et al. 2002; Ozcan et al. 2009; Matus et al. 2008]. Encouraged by ER retention of the C282Y HFE protein and the subsequent UPR activation, categorization of HH as a conformational disorder has been claimed [de Almeida and de Sousa 2008; Gray et al. 2009]. In fact, the phenotypic heterogeneity, combined with low clinical penetrance, strongly suggest the existence of additional factors modifying this genetically determined disease. A reasonable explanation for such inconsistencies might rely on dissimilar individual abilities to mount an appropriate protective response towards the C282Y mutant client. Albeit attractive, this hypothesis is far from consensual. One argument militating against it relates with the low tissue levels of HFE expression, recently estimated below 0.53 nmol/g of total protein in human liver [Chloupková et al. 2010]. Nonetheless, and despite some controversial data [Bondi et al. 2005], increased hepatic mRNA expression of Hfe was reported in iron-supplemented mice [Ludwiczek et al. 2004; Theurl et al. 2005], a trend also recapitulated by microglia derived cells subjected to stressor agents and serum deprivation [Lee and Connor 2005]. Accordingly, one could envisage a scenario in which the basally innocuous HFE pool may accumulate to levels that clog the ER with the C282Y misfolding variant as iron overload progresses in HH, thus favoring ER stress conditions. Whether and how this vicious cycle-based model influences the *in vivo* pathophysiology of HFE-linked HH definitely deserves assessment.

In keeping with our findings it can be argued that hepatocytes are relatively resilient to the deleterious effects imposed by the HFE C282Y mutant. However, since production of hepcidin has been reported in macrophages, neutrophils [Peyssonnaud et al. 2006] and lymphocytes [Pinto et al. 2010], the impact of UPR signaling pathways on these extra-hepatic sources merits closer scrutiny.

Moreover, the results here presented may prove useful in furthering the understanding of neurodegenerative processes. In fact, iron accumulation in affected brain regions is a commonality of various neuropathologies, including Alzheimer's disease (AD) and Parkinson's disease (PD) [Zecca et al. 2004]. Regardless of the yet uncertain mechanisms driving such deposition, the significance of inherent oxidative stress to neuronal damage has been increasingly recognized [Altamura and

Muckenthaler 2009]. Belonging to the class of conformational disorders, protein misfolding and aggregation are also hallmarks of both AD and PD, probably potentiating neuronal cell death [Matus et al. 2008]. The neurodegeneration field may be therefore worth exploring for the dialogue between iron homeostasis and ER stress. One can conceive, for example, that the transcriptional reshaping triggered by UPR activation takes part on the brain iron imbalance observed in AD and PD.

Another foreseeable repercussion of our findings touches on the virus-iron metabolism-UPR defined triad. The ability of viruses to co-opt the biochemical machineries of host cells to mass-replicate themselves is a longstanding concept. One of the widely studied is the viral interference with multiple steps of MHC-class I antigen presentation route, likely evolved to elude immune surveillance [Hewitt 2003]. Because iron availability is critical for efficient proliferation, an additional subversive approach triggered by viruses includes manipulation of host iron status. Despite our still tangential understanding of this strategy, progress has been made by demonstrating that TfR1 might be engaged in the viral entry process [Parker and Parrish 2000; Parker et al. 2001]. Furthermore, US2 and Nef proteins encoded in the genomes of human cytomegalovirus (HCMV) and human immunodeficiency virus (HIV)-1, respectively, were shown to down-regulate the cell surface expression of HFE [Ben-Arieh et al. 2001; Drakesmith et al. 2005], presumably with the consequence of replenishing intracellular iron stores and benefit viral growth. Also supporting this interaction, repressed hepcidin synthesis was attributed to hepatitis C virus (HCV) infection [Nishina et al. 2008]. The UPR, whose activation has been proven in infected cells [Tardif et al. 2004; Isler et al. 2005; Li et al. 2007], emerges as a plausible common denominator of the aforementioned viral strategies. In fact, by exploiting the UPR, viruses might simultaneously: i) guarantee ER expansion to accommodate massive production of viral proteins; ii) impair MHC-class I presentation [de Almeida et al. 2007a; Granados et al. 2009], thus helping in the immune evasion endeavor and iii) tune the activity of host proteins involved in iron metabolism to ensure adequate supply of this biometal.

The biological relevance of the UPR-induced modulation of iron homeostasis in the context of neurodegeneration and viral infection must be thoroughly characterized, warranting promising research directions.

Concluding remarks and future perspectives

Over the last decades, substantial effort to elucidate the molecular components and mechanistic details of the UPR canonical cascades was set in motion, with remarkable insights being achieved. As part of this collective endeavor, our work helped to consolidate intrinsic features of the UPR, underscoring its cell type- and stressor-specific traits.

Largely propelled by its implication in varied pathological conditions, understanding the interface between ER stress phenomena and other cellular signaling pathways has become a popular research field. The connection to iron metabolism disclosed by the present thesis adds an extra piece to this networking model, further highlighting the multi-tasking nature of the UPR. Probably more important than providing solutions, our work paved the way for future research, inspiring a myriad of novel questions. How is the geographical distribution of iron within the body affected under ER stress conditions? To what extent is the ER processing of iron-related proteins conditioned by prevailing stressful circumstances? Is the ER cleavage of HJV targeted upon UPR activation and how can this process modify the downstream regulation of hepcidin? Do viruses hijack the UPR as a strategy to deliberately manipulate host iron metabolism or does it arise as a mere side-effect of UPR induction?

Persistence of these and other inquiries reflects how far we are from a complete comprehension of the UPR-iron metabolism defined crosstalk. However, by establishing a correlation between two apparently unrelated processes, it also shows how far we have come. Now that the “tip of the iceberg” was revealed, endless possibilities of exciting and innovative research lie ahead.

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