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Chapter 4

The UPR, Cellular and Systemic Iron Homeostasis

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Abstract

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. 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; high Ca^{2+} stores necessary for optimal function of the former members and oxidizing milieu compatible with disulphide bond formation. The accuracy of the process is ensured by stringent quality control mechanisms, coupled to ER-associated degradation (ERAD) of aberrant proteins. Operating together, both systems guarantee that only proteins whose native conformation was reached are delivered to the Golgi apparatus towards their final destinations. When this operational control fails, the cell senses and protects itself from the ensuing insult with a coordinated set of actions designated as the unfolded protein response, the UPR. The more widely known response involves induction of ER chaperones and foldases to meet the exceptional folding demands, improvement of ERAD of irreparably unfolded proteins and global suppression of translation to minimize the burden of new clients entering the ER. Recently, however, we and others have shown that the UPR influences the cell surface expression of MHC class I molecules and the transcriptional profile of iron genes with cellular and systemic regulatory roles. In this review we present the work that has revealed the ER stress-iron metabolism axis, also discussing possible

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implications for understanding the maintenance of cellular and systemic iron homeostasis both in physiological and pathological contexts. The review is concluded with the consideration of the implications of the wider role of the UPR in cell physiology, iron overload, inflammation and hepatic disease.

Introduction

Many basic biological discoveries experience three phases of development. In an infant phase one person or small group of people find something new that they alone feel is important. With time such feeling may be vindicated or dissipated and transformed into something else very different, depending on the progress, understanding and confirmation by others. In a third phase, if vindicated, a finding may enter basic general knowledge and/or gain significance in the medical world. This latter step brings the original finding into a limelight that has resulted often in the distinction by great international prizes. Protein folding is no exception. Two of the first papers published in Nature on the biological significance of protein folding date of 1950 [1] and 1951 [2]. One of the first reviews on the control of protein exit from the ER dates from 1989 [3]. In the same year Ellis *et al.* published a review on the molecular chaperone complex [4].

In universes with over 41,000 publications listed in Pubmed for protein folding and close to 14,000 for chaperones, one approaches any new contribution to the field with awe if not apologetically. But knowledge moves from the inspiration of great discoveries to touch models that seem small drops in its large ocean. We have reason to believe that recent observations with protein folding in cellular iron homeostasis contribute to illustrating how basic knowledge can benefit from the study of new genetic disease models and move in the reciprocal direction to influence understanding of the original disease. The disease model reviewed in this chapter is HFE hemochromatosis. The reciprocal influence resides in finding the effect of the UPR associated with an HFE mutation on MHC-class I expression and iron gene expression. In addition, the chapter will review other disease models that have helped to place the Unfolded Protein Response in the biomedical limelight.

Visiting the Endoplasmic Reticulum

The multitasking nature of the endoplasmic reticulum (ER), underpinned by its major role in cellular processes as diverse as lipogenic reactions [5], Ca^{2+} homeostasis [6] and organelle biogenesis [7], is consolidated by its crucial activity in the secretory pathway. More than a mere transit compartment for secretory and membrane-spanning proteins, the ER is responsible for their biosynthesis, folding and maturation [8]. Since ~30% of the cellular proteome is processed through the ER, such functions represent a remarkable challenge to the organelle whose large scale achievements closely depend upon specialized luminal conditions [9].

Within the ER, client proteins fold to adopt the biologically active three-dimensional structure, an energetically stable conformation known as native state [10]. Although in the early 1960's Anfinsen and others have postulated that instructions for the native structure are

codified in the amino acid sequence [11-12], spontaneous self-folding capacity is hampered *in vivo* by the intracellular crowded environment, making assistance from molecular chaperones and catalysts vital for the folding efficiency. A particularly thorough assistance must be 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. A growing list of ER folding factors, acting *in vivo* in a network-like manner, has emerged in the last decades. Among the most prominent are "classical" molecular chaperones (eg. BiP and GRP94), lectin chaperones (eg. CNX and CRT), redox enzymes (eg. PDI) and peptidyl-prolyl isomerases. As part of a stringent quality control system [13], these components assist, accelerate and monitor the protein processing events which, coupled to ER-associated degradation (ERAD) of aberrant proteins [14], guarantee that only those whose native conformation was met are delivered to the Golgi apparatus towards their final destinations.

Stressing Out the ER and the Unfolded Protein Response

Notwithstanding the zealous 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 [15-16], whereas expression of mutant substrates or ER folding components [17], viral infection and even the potent secretory activity of certain cell types [18-19] are examples of chronic stress insults. To contain and reverse the accumulation of misfolded clients under these deleterious scenarios, cells have evolved specialized ER-to-nucleus signaling circuits referred to as the Unfolded Protein Response (UPR). A coordinated array of strategies encompassing ER expansion, global slowdown of protein synthesis to attenuate the ER load, transcriptional induction of ER chaperones and foldases to face the increased folding demands and improvement of ERAD machinery to bolster the clearance of ill-fated proteins is set in motion by the UPR [16, 20]. Although eminently displaying a pro-survival goal, if the UPR efforts fail to restore ER homeostasis, pro-apoptotic programs are evoked instead [21].

In mammalian cells, three ubiquitous ER-resident transmembrane proteins operate as proximal sensors and define the major UPR signaling branches – double-stranded RNA-dependent protein kinase-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) [16, 20]. Despite this diversity, interaction with the chaperone immunoglobulin heavy chain-binding protein (BiP) has been proposed as a common regulator of the ER transducers. Under unstressed conditions, BiP binds the luminal domains of all sensors, rendering them inactive, whereas massive accumulation of unfolded proteins leads to BiP sequestration and allows initiation of the PERK-, IRE1- and ATF6-dependent cascades [22-23]. Alternative ER stress sensing mechanisms, involving either direct interaction of the IRE1's major histocompatibility complex (MHC)-like groove with

misfolded proteins [24-26] or the redox status of the ATF6 luminal portion [27], have been likewise suggested and are still a matter of debate.

The aforementioned description of the UPR network was given from a "traditional" perspective, according to which the UPR appears as a stress response triggered by and aimed to mitigate misfolding events in the ER. Over the last few years, however, as the molecular details of its signaling pathways came into sharp focus, a broader than anticipated spectrum of action for the UPR-derived output was unveiled. In fact, signals emanating from the ER via the UPR have been involved in multiple cellular processes, including cell differentiation [28-29], glucose and lipid homeostasis [30-31], inflammatory and oxidative stress responses [32-33]. Also reflecting this wide scope is the activation of the UPR by physiological stimuli unrelated to the presence of unfolded proteins in the ER. Such an example occurs during B-cell differentiation, in which the UPR-mediated ER expansion precedes massive immunoglobulin production [34]. Owing to these findings, the UPR is increasingly recognized as a proactive program, rather than a reactive emergency plan for extreme intracellular conditions[35].

Given its pleiotropic effects, the implication of the UPR in a variety of disease states comes as no surprise. Protein misfolding and neurodegenerative disorders are among the most obvious, but the list extends to metabolic diseases (eg. diabetes and obesity) [36-37], viral infections and cancer [38], just to mention a few. Despite these correlations, whether the UPR is the underlying cause or, instead, a consequence of disease remains undefined in the majority of cases. Positing that the UPR does play an active role in the pathological process, may it establish and help to bring into light new disease paradigms?

Is the UPR Changing the Paradigm of Disease? HFE Hemochromatosis as Model

Emerging from faulty regulation of duodenal iron absorption in the face of increasing iron stores, HFE-associated hereditary hemochromatosis (HH) is a heritable condition of iron overload caused by mutant forms of *HFE* [39]. The product encoded by this gene shares structural homology to a MHC-class I protein, requiring association with β_2-microglobulin (β_2m) for cell surface expression [40-41]. Unlike conventional MHC-class I molecules, however, HFE is not coupled to antigen presentation functions, probably due to the narrow dimensions of its peptide binding groove [41]. The C282Y point mutation of HFE, in which a G-A transition replaces the amino acid cysteine by tyrosine at position 282, is carried by the majority of HH patients (>80%) [40]. The demonstration that the HFE-β_2m heterodimer competes with diferric transferrin for binding to transferrin receptor (TfR)1 was the definitive evidence implicating HFE in iron homeostasis [42-43]. With the binding capacity of HFE later extended to TfR2 [44], a partnership involving HFE, TfR1 and TfR2 has emerged as a critical pathway governing systemic iron balance [45-46]. According to this model, when iron stores are replete, circulatory holotransferrin binds avidly to TfR1. Displaced HFE becomes therefore available to interact with TfR2, initiating a signaling program that culminates with the induction of hepcidin expression [45, 47]. Hepcidin, a 25-residue peptide hormone first described as an antimicrobial molecule, is presently acknowledged as the seminal orchestrator of systemic iron homeostasis and the iron exporter ferroportin its cognate receptor [48].

Mainly secreted by hepatocytes [49], hepcidin targets the membrane-anchored ferroportin, triggering its internalization, ubiquitination and lysosomal degradation [50]. Iron egress from enterocytes and macrophages is thereby inhibited, ultimately restricting the availability of the biometal in circulation.

Blocking the formation of a disulphide bond in the α_3 domain of HFE, the C282Y mutation prevents assembly with β_2m [51-52]. As a consequence, transport of the mutant protein through the secretory pathway is impaired and its accumulation in the ER as high molecular weight aggregates undergoing proteasome-dependent degradation ensues [52]. Due to compromised cell surface presentation, the C282Y variant of HFE fails to bind TfR1 or stabilize TfR2 according to the prevailing transferrin saturation levels.

Besides loss of protein function [53-54], the C282Y mutation was recently coupled to UPR activation [55-56]. The possibility that novel regulatory mechanisms imparted by the C282Y-mediated ER stress events might play a role in HH disease expression is thus foreseeable. A number of findings support such hypothesis: i) a causal relation between diminished MHC-class I cell surface expression and HFE C282Y-triggered UPR activation was disclosed [55, 57], an effect later broadened to other ER insults (eg. palmitate and glucose starvation) [58]; ii) a correlation between calreticulin (CRT) expression levels and the clinical phenotype of HH-individuals was reported, with a protective role being attributed to this ER-chaperone [59]; iii) poor penetrance and high phenotypic heterogeneity among C282Y carriers are remarkable features of HH [60-62], strongly suggesting the existence of additional factors modifying this genetically determined disorder. These observations, tied to the progressive course of HH and onset of symptoms at middle age, has encouraged the classification of HH as a conformational disorder [63-64]. A reasonable explanation for the clinical variability referred above would rely, at least in part, on disparate individual abilities to mount an appropriate protective response towards the C282Y mutant client. Although conceptually interesting, this hypothesis is far from consensual. One argument militating against it relates to the low tissue levels of HFE expression, recently estimated below 0.53 nmol/g of total protein in human liver [65]. Nonetheless, and despite some controversial data [66], increased hepatic mRNA expression of Hfe was reported in iron-supplemented mice [67-68], a trend also recapitulated by microglia derived cells subjected to stressor agents and serum deprivation [69]. Accordingly, one could envisage a scenario in which the basally innocuous HFE pool may accumulate with time to levels that congest the ER with the C282Y misfolding variant as iron overload progresses in HH, thus favoring ER stress conditions, an explanation compatible with the more frequent expression of the disease in the late thirties in men and in postmenopausal women. Alternatively, it is conceivable that the presence of the C282Y mutant protein sensitizes cells to nuanced but persistent ER stress over which independent stimuli might exert cumulative or synergetic effects throughout life. Whether and how this model influences the *in vivo* pathophysiology of HFE-linked HH remains elusive. While no definitive answers are provided, one has certainly benefited from the recently uncovered interplay between iron homeostasis and UPR activation [70-71].

The UPR-Iron Metabolism Axis: Putative Physiological Significance

Compelling evidence extending the UPR beyond the realm of protein misfolding and proteotoxicity attenuation has been provided. An intersection with iron metabolism was first suggested by differential gene expression screenings. Two such examples are the increased transcript levels of the ER chaperones CRT and BiP found in iron-burdened astrocytoma cells [72] and the transferrin gene down-modulation reported in stable transfectants of the stress-inducible transcription factor CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) [73]. Proteomic analysis also revealed increased hepatic BiP expression in dietary iron-loaded mice [74], but details of this putative interconnectivity have remained inconclusive.

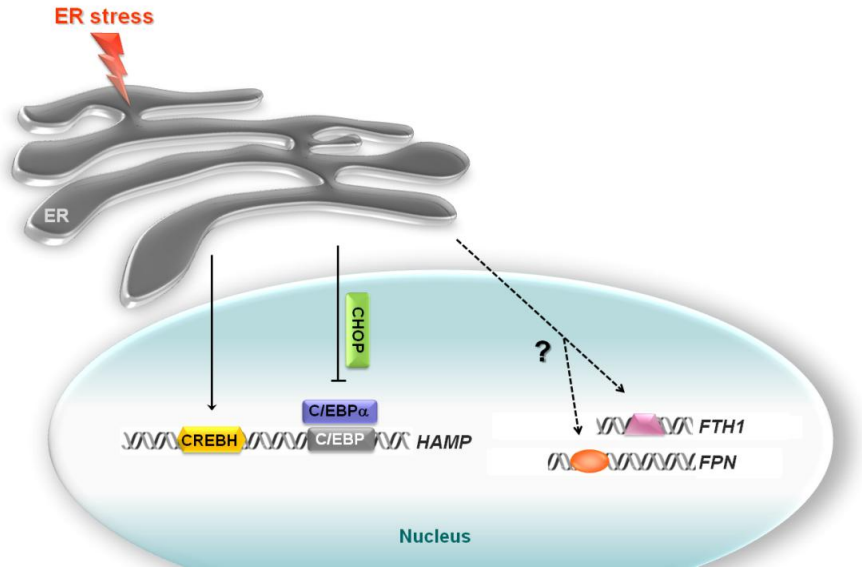

Figure 1. UPR-induced modulation of iron-related genes. Upon UPR activation, increased levels of CHOP expression deplete the C/EBPα protein pool, therefore limiting its availability to stimulate hepcidin (HAMP) promoter. With the maintenance of the stress response, CHOP levels return to basal values, enhancing the C/EBPα-binding capacity to HAMP promoter. Increased expression of HAMP under stress conditions was also attributed to CREBH-dependent stimulation of its promoter. Both ferritin H (FTH1) and ferroportin (FPN) harbor putative binding sites recognized by ER stress-inducible transcription factors, which might confer some UPR-responsiveness to these genes.

Challenging a hepatocytic cell line with chemical agents interfering with disulphide bond formation (dithiothreitol and homocysteine) in ER client proteins, ongoing ER stress was shown to significantly reshape the expression profile of iron-related genes, namely hepcidin, ferroportin and ferritin H. Using this experimental approach, the molecular mechanism underlying the biphasic modulation of hepcidin was likewise deciphered, with the nuclear factors C/EBPα and CHOP appearing as suspected important mediators [70]. The interplay between iron metabolism and the UPR signaling pathways was independently reported by Vecchi et al. that after stressing hepatoma-derived cells with the ER-to-Golgi transport

inhibitor brefeldin A, calcium ionophore A23187 and tunicamycin reported increased levels of hepcidin transcripts, a pattern also detected in the liver of tunicamycin-treated mice [71]. The stimulation of hepcidin was linked to cAMP response element-binding protein H (CREBH)-dependent activation of its promoter [71]. The two proposed mechanisms for hepcidin induction under stress scenarios are not incompatible and could likely coexist (Figure 1).

The systemic impact of the UPR has been underscored by the connection to insulin secretion and peripheral resistance [36, 75], glucose homeostasis [76] and inflammation [77]. With the UPR-induced hepcidin modulation [70-71], a new piece can be added to this puzzle. By limiting duodenal iron absorption, hepcidin up-regulation in stressful states may be part of an anticipatory "strategy" to evade extra sources of stress, as those associated with iron-generated ROS. In line with hepcidin's antimicrobial role [49], consequences on the innate immunity are expected as well, thereby furthering the scope of the recently uncovered ER stress-mediated inflammatory responses [32, 78]. In keeping with the cell context-dependent nature of the UPR [35, 79-80] and since production of hepcidin has been also reported in macrophages, neutrophils [81] and lymphocytes [82], the qualitative and quantitative output of UPR signaling pathways on these extra-hepatic sources merits closer scrutiny.

Apart from the systemic influence driven by hepcidin, repercussions of UPR activation are also evident at the cellular iron metabolism level, as suggested by the modulation of ferroportin and ferritin H expression imposed by ER insults [70]. 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, respectively (Figure 1).

The UPR-Iron Metabolism Axis: Possible Link to Pathological Conditions

The novel association between ER stress and iron homeostasis may provide an interesting framework to further understand the pathogenic mechanism(s) behind selected disorders other than HFE hemochromatosis. To illustrate this idea we focus on the following examples.

Iron accumulation in affected brain regions is a commonality of various neuropathologies, including Alzheimer's disease (AD) and Parkinson's disease (PD) [83]. Regardless of the yet uncertain mechanisms driving this deposition, the significance of inherent oxidative stress to neuronal damage has been vastly recognized [84]. Although a causal relationship between these disorders and the UPR awaits definitive confirmation, protein misfolding and aggregation are hallmarks of AD and PD, probably potentiating neuronal cell death [85-86]. The neurodegeneration field may be therefore worth exploring for the dialogue between iron homeostasis and ER stress. It is tempting to speculate, for example, that the transcriptional modulation triggered by UPR activation takes part on the brain iron imbalance observed in AD and PD.

Another foreseeable repercussion of these new findings touches on the virus-iron metabolism-UPR defined triad. The ability of viruses to usurp the biochemical machineries of host cells to mass-replicate themselves is a longstanding concept. One of the widely studied processes is the viral interference with multiple steps of MHC-class I antigen presentation route, thought to evolve to elude immune surveillance [87]. 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 [88-89]. 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 [90-91], 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 [92]. The UPR, whose activation has been proven in infected cells [93-95], emerges as a plausible common denominator of the aforementioned viral strategies. In fact, by exploiting the UPR pathways, viruses might simultaneously: i) guarantee ER expansion to accommodate massive production of viral proteins; ii) impair MHC-class I presentation [55, 58], 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.

Diabetes and cancer, two additional platforms of convergence between iron (dys)regulation and UPR activity [36, 96-101], may also benefit from the ER stress-iron homeostasis crosstalk recently uncovered. Its biological relevance in pathological contexts must be thoroughly characterized, warranting promising research directions.

Table 1. Knowledge of the effects of C282Y mutation compared to other UPR eliciting gene mutations

Mutation	**Affected protein**	**Disease**	**Documented effects**	**Correcting**
C282Y	HFE	HFE-hemochromatosis	- ER retention - Proteasomal degradation - Diminished MHC-class I surface expression - Iron genes expression	Yes (*in vitro*) [56, 113]
ΔF508	CFTR	Cystic fibrosis	- ER retention - Proteasomal degradation - Reduced endogenous WT CFTR mRNA levels	Yes (*in vitro*) [114-115]
Z variant	α1-antitrypsin	α1-antitrypsin deficiency	- ER retention - UPR activation dependent on additional insults - Activation of ER caspases	Yes (*in vitro*) [116]

α1-antitrypsin deficiency and cystic fibrosis (see Table 1) are examples of two other diseases with gene mutations evoking the UPR [102-105] in which links of severity of disease expression to the presence of HFE mutations are just being unveiled [56, 106-109]. Such interactions involving the UPR will inevitably come to pose new challenges to the crosstalk between the consequences of HFE mutations reviewed in this chapter and the variability seen in expression of other diseases, enlarging the biomedical stage and enhancing the position of the UPR in the biomedical limelight referred to in the introduction.

Conclusion

Originally described as a check-and-balance program focused on the recovery of stress-corrupted ER folding environment, compelling evidence has revealed that the UPR is co-opted for the maintenance of key basic cell functions [35]. The connection between the UPR and the modulation of genes relevant for iron metabolism reviewed in the present chapter extends this networking model, highlighting further the multi-tasking nature of the UPR.

Clearly, such interaction with cellular physiology has placed the UPR at the crossroads of multiple pathological conditions as well. Over the last years, substantial effort to decipher details of these associations was set in motion. In the future, the challenge will be to discriminate between the role of UPR in disease causality and/or its activity as a secondary disease manifestation. Once clarified, the UPR may incite a paradigm switch of certain disorders. One aspect of the importance of changing disease paradigms in light of novel findings is that it may inspire the design of new therapeutic approaches. The HFE-linked HH is no exception. With the standard therapy relying on blood-letting, early initiation of treatment efficiently prevents organ failure due to iron toxicity and restores normal lifespan [39], although the immunological abnormalities consistently found in HH patients remain unresponsive to phlebotomies [110]. Such anomalies encompass decreased counts of circulating $CD8^+$ T cells in comparison to control individuals [111], accompanied by defective cytotoxic activity [112]. A link between these observations and the impaired MCH-class I expression imposed by the C282Y-evoked UPR [55] has been hypothesized [63] (Figure 2). Together, the protective role against oxidative stress recently attributed to CRT in HFE C282Y stable transfectants, plus the negative correlation between expression of this ER chaperone and the number of clinical manifestations of HH subjects [59], convey the rationale for considering that pharmacological chaperones might be valuable in the context of HFE hemochromatosis. This possibility, already studied *in vitro* for the chemical chaperones taurine-conjugated ursodeoxycholic acid (TUDCA) and sodium 4-phenylbutyrate (4PBA) [56, 113], awaits *in vivo* assessment if we aim to understand the broad impact of UPR activation (and mitigation) in the context of HH. An attractive approach in this regard would be the *Hfe* C282Y knock-in murine model.

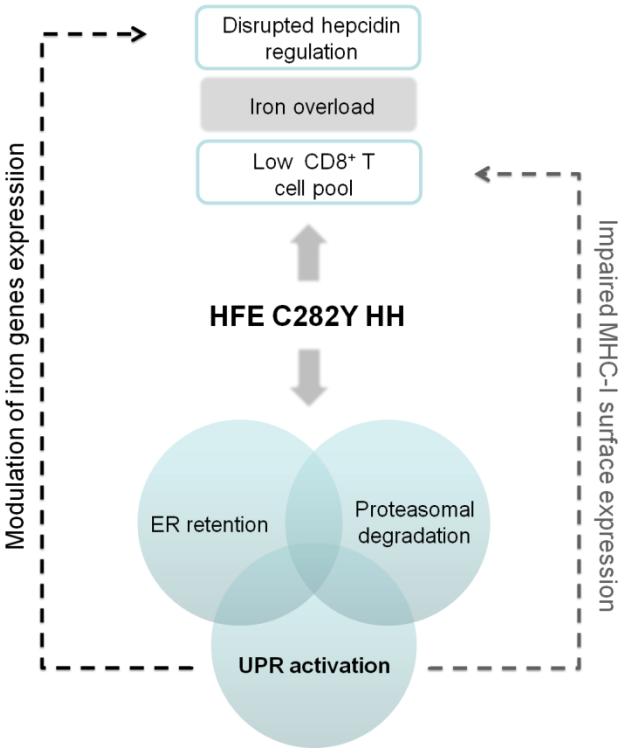

Figure 2. The UPR-iron metabolism axis: insights from HFE-hemochromatosis. The C282Y mutation of HFE accounts for the majority of cases of the iron overload disorder hereditary hemochromatosis (HH). This mutation, blocking the formation of an intramolecular disulphide bond, triggers two major consequences. From a functional point of view, the ability of HFE to interact with transferrin receptor (TfR)1 or TfR2 and in so doing regulate hepcidin levels is lost in the C282Y mutant form. Furthermore, C282Y molecules remain in the endoplasmic reticulum (ER) as high molecular weight aggregates that undergo accelerated proteasomal degradation and activate an Unfolded Protein Response (UPR). Activation of UPR signaling pathways was shown to down-regulate the cell surface expression of MHC-I molecules, also reshaping the mRNA expression prolife of iron-related genes. The possibility of a C282Y-mediated interplay between the UPR cascades and iron homeostasis influencing disease progression is therefore attractive. The impaired MHC-I cell surface expression triggered by UPR activation may also contribute to the low numbers of CD8+ T lymphocytes found in HH patients.

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