RUTE ISABEL PAULO MARTINS

POST-TRANSCRIPTIONAL REGULATION OF HFE GENE EXPRESSION

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RUTE ISABEL PAULO MARTINS

POST-TRANSCRIPTIONAL REGULATION OF HFE GENE EXPRESSION

Thesis presented to obtain the Ph.D. degree in Biology (Molecular Genetics), by the Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia.

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Sumário

O ferro é um elemento essencial em diversos processos metabólicos celulares. O desafio que se coloca para a maioria dos organismos prende-se com o controlo do ferro absorvido de modo a suprir as necessidades destes processos evitando, no entanto, os danos causados pelo ferro livre. Na realidade, algumas das doenças humanas mais comuns estão relacionadas com a perturbação da homeostase do ferro. Entre estas, encontra-se a hemocromatose hereditária que, estando maioritariamente associada a mutações no gene HFE, origina a acumulação de ferro em vários órgãos. A proteína HFE actua na homeostase do ferro através da regulação da expressão da hepcidina no fígado. O principal transcrito HFE apresenta baixos níveis de expressão numa série de tecidos humanos, tendo sido descritos diversos transcritos adicionais.

O trabalho aqui apresentado aborda a caracterização dos transcritos alternativos de HFE, os mecanismos envolvidos na sua génese, assim como o seu possível papel fisiológico e regulação. A análise de diversos tecidos humanos permitiu identificar vários transcritos HFE resultantes de *splicing* alternativo. O estudo funcional de algumas proteínas correspondentes demonstrou que o processo de *splicing* alternativo pode gerar variantes não funcionais ou produzir uma variante HFE solúvel que é secretada pelas células associada à beta2-microglobulina. Esta proteína poderá desempenhar um papel crucial na homeostase do ferro, actuando como um agonista ou antagonista da HFE *full length*. Além disso, foi demonstrado que a expressão do transcrito HFE principal é fisiologicamente regulada pelo mecanismo de *nonsense-mediated mRNA decay* (NMD), dado que os seus níveis aumentam quando este mecanismo é inibido. A pesquisa realizada em tecidos humanos permitiu verificar que a expressão do mRNA HFE resulta da utilização de quatro locais de clivagem e poliadenilação alternativos. Este padrão de poliadenilação alternativa específico de tecido aparenta responder a estímulos de ferro, actuando coordenadamente com o NMD no ajustamento dos níveis de expressão de HFE.

Esta dissertação demonstra que a regulação da expressão do gene HFE é influenciada pós-transcricionalmente pelos mecanismos de *splicing* alternativo, poliadenilação alternativa e NMD. Este conhecimento poderá conduzir a novas perspectivas de investigação na área do metabolismo do ferro e contribuir para o delinear de novas estratégias terapêuticas a aplicar em patologias de homeostase do ferro através da regulação da hepcidina.

Abstract

Iron is a key element for numerous metabolic processes in living cells. The challenge for most organisms is to acquire the adequate amounts of iron for these processes yet avoiding the toxicity associated with free iron. In fact, disruptions of iron homeostasis account for some of the most common human diseases. Amongst these, lays hereditary hemochromatosis, which is mainly associated with mutations in the HFE gene, leading to iron overload in specific organs. HFE protein acts in iron homeostasis by regulating the expression of hepcidin in the liver. Besides the major HFE transcript, which is expressed at low levels in a wide range of human tissues, several additional alternative HFE transcripts have been described.

The work presented in this dissertation addresses the characterization of HFE alternative transcripts, the biological mechanisms involved in their genesis as well as their physiological significance and regulation. A variety of human tissues was analysed and shown to express several alternatively spliced HFE transcripts. Functional analysis of the corresponding proteins revealed that alternative splicing can either generate non-functional HFE protein variants or produce a soluble HFE variant that is secreted by cells associated with beta2-microglobulin. This soluble HFE may have a vital role in iron homeostasis by acting as an agonist or antagonist of the full length HFE. Furthermore, HFE transcripts were found to be physiologically regulated by the nonsense-mediated mRNA decay (NMD), since its levels are significantly increased when depleting human cells from a key NMD effector. Through the analysis of several human tissues, it is shown that HFE mRNA expression results from alternative cleavage and polyadenylation at four different sites. This tissue-specific polyadenylation pattern seems to respond to cellular iron status, acting coordinately with NMD to fine-tune HFE's expression levels.

The regulation of HFE gene expression is here shown to be post-transcriptionally influenced by alternative splicing, alternative polyadenylation and nonsense-mediated mRNA decay mechanisms. These findings may hint future directions in the active field of iron biology research and provide interesting cues that could be translated into new therapeutics for iron homeostasis disorders through the HFE-mediated regulation of hepcidin.

Abbreviations

A	adenosine
apo-Tf	iron-free transferrin
bp	base pairs
BMP	bone morphogenetic protein
C	cytidine
CBP	cap-binding protein
CD	cluster of differentiation of leukocytes
cDNA	mRNA complementary DNA
C/EBPα	CCAAT/enhancer binding protein α
Cyto	cytoplasmic tail
C-terminal	carboxyl-terminal
DAPI	4,6-diamidino-2-phenylindole
DcytB	duodenal cytochrome B
DMEM	Dulbecco's modified Eagle medium
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSE	downstream sequence element
EJC	exon junction complex
Epo-R	erythropoietin receptor
ER	endoplasmic reticulum
eRF	eukaryotic release factor
ERK 1/2	extracellular signal-regulated kinases 1/2
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FBS	fetal bovine serum
Fe2-Tf	diferric-iron-loaded transferrin
G	guanosine
GDF15	growth differentiation factor 15
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
GPS1	G protein pathway suppressor 1
HCP1	heme carrier protein 1
HFE	high Fe
HIF-1	hypoxia inducible factor-1
HIV	human immunodeficiency virus
HH	hereditary hemochromatosis
HLA	human leukocyte antigen

hnRNP	heterogenous nuclear RPN
HO1	heme oxygenase 1
holo-Tf	iron-loaded Tf
IL	interleukin
IP	immunoprecipitation
IRE	iron responsive element
IRP	iron regulatory protein
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
JAK	Janus kinase
kb	kilo bp
kDa	kilo dalton
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
Nef	negative factor
NMD	nonsense mediated mRNA decay
nt	nucleotide
NTBI	non-transferrin bound iron
OMIM	Online Mendelian Inheritance in Men
ORF	open reading frame
PABPC1	cytoplasmic poly(A)-binding protein 1
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCNA	proliferating cell nuclear antigen
Poly(A)	polyadenylation
PVDF	polyvinylidene fluoride
PTC	premature termination codon
qPCR	quantitative real-time PCR
RACE	rapid amplification of cDNA ends
RES	reticuloendothelial system
RNAP II	RNA polymerase II
RNP	ribonucleoprotein
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
ROS	reactive oxygen species
RT	reverse transcription
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sHFE	soluble HFE

siRNA	short interfering RNA
Steap3	six-transmembrane epithelial antigen of the prostate 3
sTfR1	serum TfR1
SMAD	this acronym results from the combination of two proteins: the *Caenorhabditis elegans* protein Sma (designated as mutations in this gene causes animals to be small) and the drosophila protein MAD ("mothers against decapentaplegic")
SMG	suppressor with morphogenetic effects on genitalia
snRNP	small nuclear RNP
ss	splice site
STAT3	signal transducer and activator of transcription 3
TBST	tris-buffered saline with triton
T	thimidine
Tf	transferrin
TfR	transferrin receptor
TGF-β	transforming growth factor β
Tm	transmembrane domain
Tris	tris(hydroxymethyl)aminomethane
TWSG1	twisted gastrulation 1
U	uridine
uORF	upstream ORF
UPF	up-frameshift
UTR	untranslated region
WT	wild type
Zip14	Zrt-Irt-like protein 14
β2M	β2-microglobulin

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CHAPTER 1

General Introduction

I. General overview of iron metabolism

Iron is crucial for life, being involved in a diversity of cellular processes such as oxygen transport, electron transfer and DNA synthesis. The biological relevance of iron is largely attributable to its chemical properties as a transition metal. This capability of readily engaging one electron, allows iron to coexist either in an oxidized insoluble (Fe^{3+}) or a reduced soluble (Fe^{2+}) form. The dark side of this element is that, in excess, free iron catalyzes the production of oxidative radicals that are able to damage the macromolecular components of cells. On the other hand, cellular iron deficiency arrests cell growth leading to cell death [Halliwell and Gutteridge 1984].

Iron is the fourth most abundant component in the Earth's crust, but most of the environmental iron exists in the ferric (Fe^{3+}) form, which is almost insoluble in water at neutral pH, severely compromising its biological utility. Being an essential trace element required by virtually all organisms (except for a few species of bacteria), they have developed complex systems of iron transport and management to deal with its poor bioavailability [Chua et al. 2007]. Even so, iron balance is tenuous, as both iron deficiency and iron overload are deleterious. These disorders of iron homeostasis are amongst the most common diseases in humans, affecting up to one-quarter of the world's population [McLean et al. 2009]. To get a glimpse of how iron balance is accomplished, the molecular mechanisms involved in the regulation of iron homeostasis will be exposed in the course of this dissertation.

I.1. Iron distribution, utilization and recycling

One astonishing feature of iron metabolism is the extent to which body iron is conserved. Although the adult human organism contains 3 to 5 g of iron, only 1-2 mg enters and leaves the body on a daily basis (Figure 1.1). Iron excretion is a rather unregulated pathway, as it is the result of mandatory losses through menstruation, sloughing of epithelial cells from the skin and from the mucosal cells of the gastrointestinal, biliary and urinary tracts [Cook et al. 1973; Andrews 1999]. Conversely, all cells require a small amount of iron but the precursors of red blood cells, the erythroblasts, are by far the most demanding. Under normal physiological conditions, about 20 mg of iron is daily consumed by the erythroblasts for

heme biosynthesis in the bone marrow to fulfill the production of more than 200 billion erythrocytes [Hentze et al. 2004; Chua et al. 2007]. In fact, more than two thirds (60-70%) of the total body iron content is present as hemoglobin in erythrocytes, whilst another 10% is contained in myoglobin, iron-containing enzymes and cytochromes. The remaining 20-30% is stored in the liver and macrophages as ferritin and hemosiderin [Cook et al. 1973; Andrews 1999].

The major source of iron for the erythroid precursors is plasma iron-transferrin (Fe2-Tf). But the circulating Fe2-Tf pool is 10 times smaller than the daily iron requirements, so a high turnover rate is necessary to ensure the adequate supply of iron to the bone marrow [Andrews 1999; Nemeth 2008]. This recycling process is carried out by macrophages of the reticuloendothelial system (RES) present in the spleen, liver and bone marrow. Through the phagocytosis of senescent erythrocytes, the iron within is recovered, transferred to the bone marrow and re-incorporated during the synthesis of new red blood cells [Knutson and Wessling-Resnick 2003].

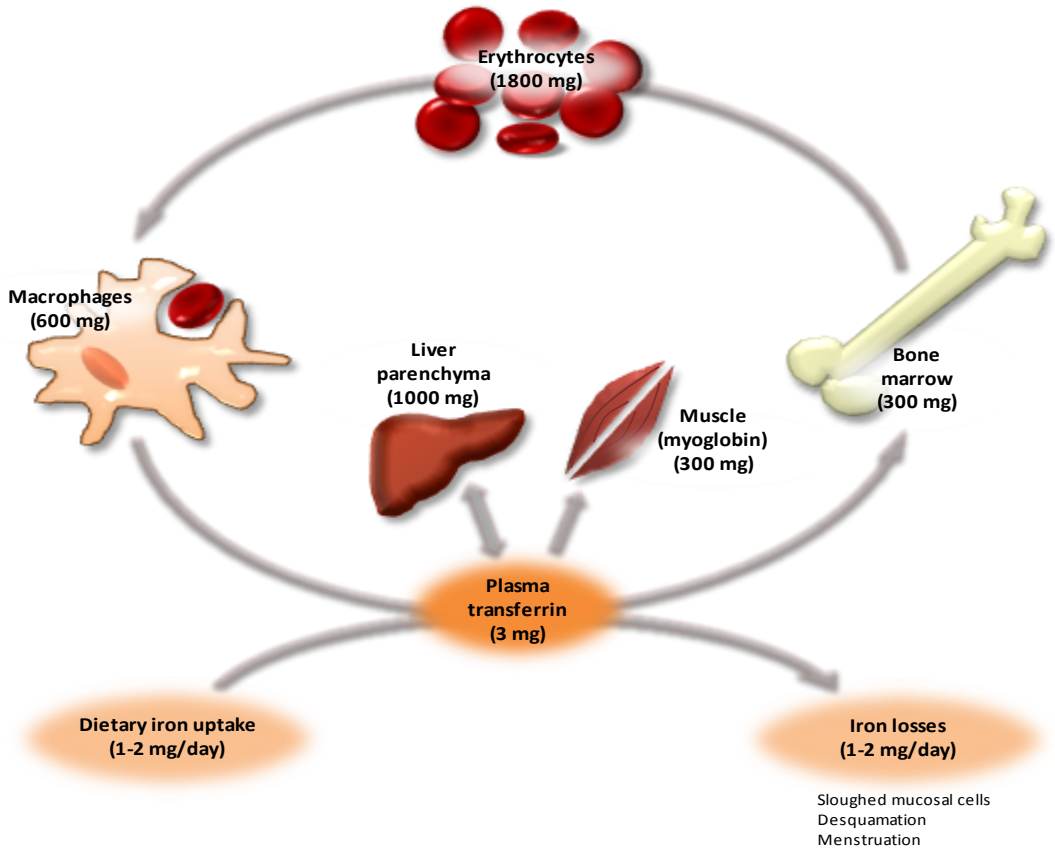

Figure 1.1. Distribution of iron within the body. In a balanced state, about 1-2 mg of iron is daily absorbed and a similar amount is lost. Most of the iron that circulates in the plasma is incorporated into hemoglobin in erythroid cells. As only about 0.1% of the total body iron content is found in the plasma, the recycling of the iron present in the senescent erythrocytes by the macrophages is crucial to meet the erythropoietic demands. The major iron storage compartments are the liver, the macrophages and the muscles. (Adapted from Pietrangelo 2004).

I.2. Iron acquisition, transport and storage

I.2.1. Iron absorption

Dietary iron absorption is achieved in the duodenum, where iron must traverse the apical and basolateral membranes of the absorptive cells (enterocytes) in order to reach the bloodstream (Figure 1.2). Iron is present in two forms in the diet: heme iron (derived from hemoglobin and myoglobin) and non-heme iron (present as iron hydroxides, salts and iron-containing proteins such as ferritin) [Carpenter and Mahoney 1992; Lopez and Martos 2004]. Heme and non-heme iron pass from the intestinal lumen to the enterocyte by distinct pathways, but once within the cell, iron from each source will be part of a common intracellular pool that can either be stored in the form of ferritin or transported into the bloodstream [Hentze et al. 2004; Chua et al. 2007].

Although heme iron is more efficiently absorbed than non-heme iron, this only accounts for about 10 to 15% of daily iron intake [Carpenter and Mahoney 1992]. The mechanism by which heme is taken up by duodenal enterocytes remains controversial. A candidate brush border heme transporter was described, the heme carrier protein 1 (HCP1), but it was further demonstrated that this protein transports folate far more efficiently than heme [Shayeghi et al. 2005; Qui et al. 2006]. After crossing the apical membrane, iron is excised from the heme porphyrin ring, under the action of heme oxygenase 1 (HO1), becoming part of the cytosolic iron [Raffin et al. 1974].

The majority of non-heme iron enters the gastrointestinal tract in the ferric form that must be converted into the ferrous form for bioavailability. Numerous dietary components are capable of reducing ferric iron, but the enterocytes have endogenous reducing activity [Lopez and Martos 2004]. It is currently accepted that this is achieved by the duodenal cytochrome B (DcytB) reductase that is expressed on the apical membrane [McKie et al. 2001]. However, the absence of an abnormal phenotype in DcytB knockout mice, suggests the presence of other brush border ferrireductases [Gunshin et al. 2005]. Once Fe^{2+} is formed, it becomes a substrate for the divalent metal transporter 1 (DMT1; also known as DCT1 or Nramp2), the intestinal iron importer, for transport across the membrane into the cytoplasm [Gunshin et al. 1997; Fleming et al. 1997]. The role of this molecule is supported by the animal models, where a defective DMT1 gene leads to ineffective iron uptake and microcytic anemia [Fleming et al. 1997; Fleming et al. 1998].

Once within the enterocyte, iron has two possible fates, depending on iron requirements. If the iron demand is low, it can be stored as ferritin, being eventually lost by sloughing of the villus tip [Geyer 1979]. Conversely, if there is a requirement to replenish the stores or an increased metabolic demand, iron will be transported across the basolateral membrane into the circulation [Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000]. This transport is assured by ferroportin (also known as IREG1, MTP1 or SLC40A1). Selective inactivation of the murine ferroportin in intestinal cells confirms that ferroportin is the major, and most probably, the only iron exporter [Donovan et al. 2005]. As ferroportin is selective for Fe^{2+}, the iron export depends on a multicopper oxidase to convert Fe^{2+} to Fe^{3+} for incorporation of iron into transferrin, the serum iron carrier protein [Schade and Caroline 1946]. Hephaestin is a membrane-bound homologue of the serum multicopper oxidase ceruloplasmin and most likely the responsible for the release of oxidized iron into the bloodstream [Vulpe et al. 1999]. It was also shown that ceruloplasmin may carry out the oxidase function at the basolateral membrane of the enterocyte [Cherukuri et al. 2005]. This hypothesis arose from the evidence that iron accumulation in enterocytes of the mouse model for sex-linked anemia is resolved after the neonatal period, suggesting that hephaestin is needed for iron stores during rapid growth, while ceruloplasmin may be required in adult stages [Edwards and Bannerman 1970; Cherukuri et al. 2005].

I.2.2. Cellular iron uptake

I.2.2.1. Transferrin bound iron

The major iron source for most tissues is transferrin bound iron. Transferrin (Tf) has two iron-affinity binding sites, keeping iron non-reactive in circulation and extravascular fluid, delivering it to cells bearing specific receptors [Bailey et al. 1988]. The classic transferrin receptor 1 (TfR1) is expressed in most cells, presenting a higher expression in rapid proliferating cells, activated lymphocytes and erythroid precursors [Ponka and Lok 1999; Ned et al. 2003]. The almost ubiquitous expression of this receptor reveals the importance of a constitutive pathway for iron acquisition by receptor-mediated endocytosis, the so-called transferrin cycle, which has become a paradigm in cell biology. Briefly, to initiate the cycle, diferric transferrin (or holo-transferrin; holo-Tf) binds to TfR1 at cell surface that will invaginate to form endocytic vesicles (Figure 1.2). Acidification of endosomes will take place

promoting iron release from Tf and Fe^{3+} will be reduced by the ferrireductase Steap3 (Six-transmembrane epithelial antigen of the prostate 3), allowing transmembrane transport by DMT1 [Dautry-Varsat et al. 1983; Fleming et al. 1998; Ohgami et al. 2005]. The iron is then utilized by the cell or stored as ferritin. The Tf cycle is completed when the endosome returns and fuses with the cell membrane, where the receptor becomes accessible and apo-Tf (iron-free transferrin) is released to circulation, allowing both molecules to start the cycle all over again.

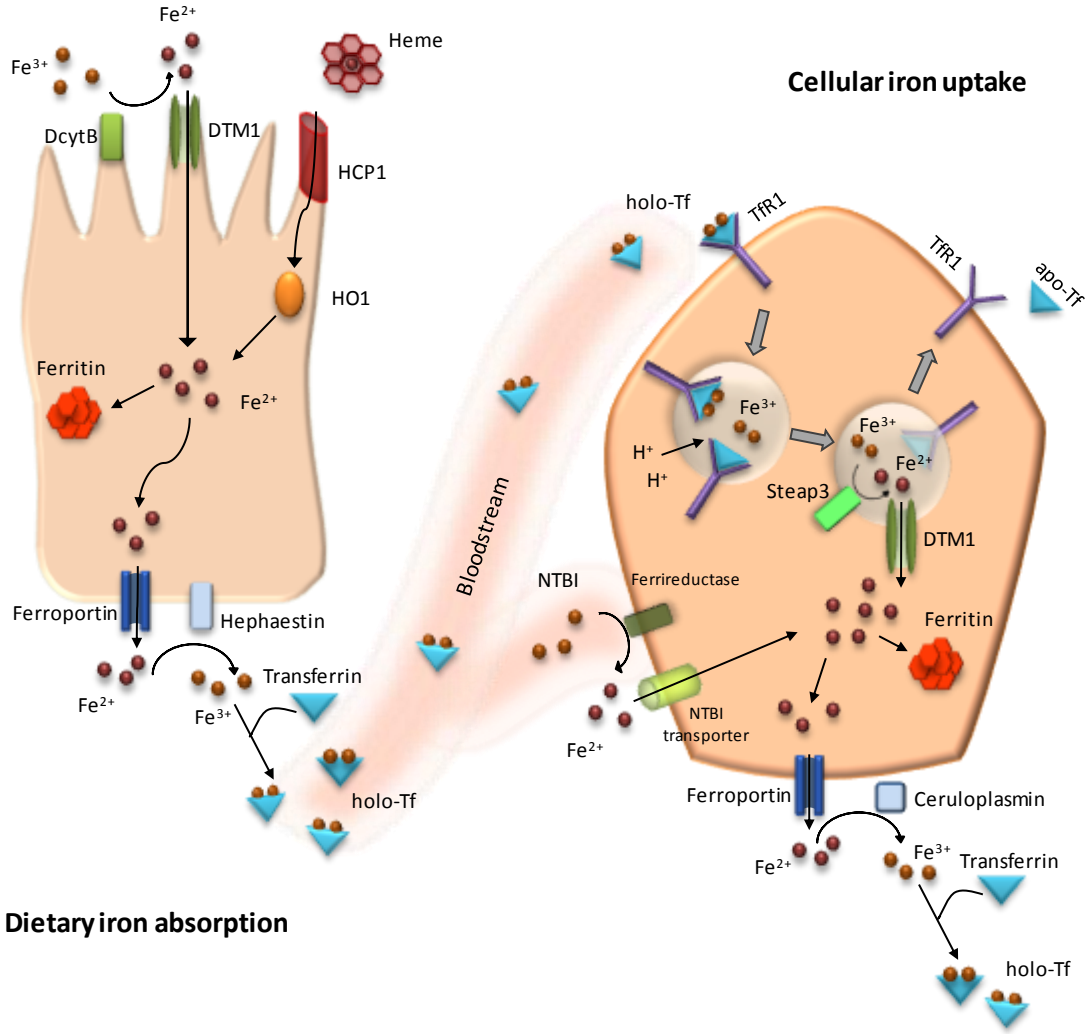

Figure 1.2. Iron acquisition, transport and storage. The absorption of dietary iron (heme and non-heme) is achieved by the enterocytes of the duodenum (on the left). Once within the enterocyte, iron may be stored or transported to the bloodstream. Then, iron is acquired by almost all human cells, so a generic cell is depicted on the right. Uptake of transferrin bound iron occurs through the transferrin cycle, whereas non-transferrin bound iron is mediated by specific transporters. See text for details.

It is currently accepted that there is an important molecule liable to affect the transferrin cycle, the HFE (high Fe) protein. The membranar association of HFE with TfR1, as well as its ability to compete with transferrin for binding to the receptor, brought new insights to the iron metabolism field [Parkkila et al. 1997a; Feder et al. 1998; Lebron et al. 1998]. A second transferrin receptor, TfR2, is strongly expressed in liver hepatocytes, but has a lower binding affinity for holo-Tf than TfR1 [Kawabata et al. 1999]. TfR2 is capable of mediating the internalization and recycling of Tf by a similar mechanism to that described for TfR1 [Kawabata et al. 1999; Graham et al. 2008]. Recent evidence suggests that both HFE and TfR2 are involved in a specific pathway with strong interactions with the uptake of iron by the TfR1-mediated endocytosis, which will be further explored in this thesis.

I.2.2.2. Non-transferrin bound iron

Iron can also be present in the plasma in a free form, generally designated as non-transferrin bound iron (NTBI). It actually consists in iron bound to low affinity molecules, with the major component identified as ferric citrate [Grootveld et al. 1989]. The concentration of NTBI is normally low but it increases when the binding capacity of transferrin becomes saturated. Since it easily penetrates into cells, particularly in the liver and heart, NTBI has great pathophysiological importance in iron overload disorders [Breuer et al. 2000; Chua et al. 2004]. The uptake of NTBI by the cells is still far from understood (Figure 1.2). It likely involves cell surface reduction by an unidentified ferrireductase to dissociate iron from its ligand, possibly by Steap3 [Chua et al. 2007]. Then, iron is delivered into the cell by a transporter [Trinder and Morgan 1998]. Several plausible candidate transporters for NTBI have emerged, including DMT1, Zip14 (Zrt-Irt-like protein 14) and calcium channels [Oudit et al. 2003; Chua et al. 2004; Liuzzi et al. 2006; Shindo et al. 2006]. The relative contribution of these transporters to NTBI uptake is poorly characterized, but it is likely that more than one of these transporters is involved [Chua et al. 2007].

I.2.3. Iron storage

Following delivery to the cells, iron enters an intermediate intracellular labile iron pool, where it can be incorporated into ferritin or heme, associated with other non-heme iron proteins in the cytosol or exchanged between the intracellular endosomal, lysosomal and mitochondrial compartments [Mulligan et al. 1986; Chua et al. 2007].

In general, the excess of iron will be stored in the form of ferritin, a water soluble molecule consisting of 24 subunits, capable of sequestering up to 4,500 atoms of iron (Figure 1.2) [Harrison 1977]. As the amount of iron in the cells increases, a larger percentage deposits in hemosiderin, an insoluble molecule thought to be a by-product of ferritin degradation [Munro and Linder 1978]. This has been suggested as a protective mechanism against oxidative damage, since iron stored in hemosiderin is more inaccessible and less effective in producing free radicals than iron stored in ferritin [O'Connell et al. 1986].

The main sites for body iron storage are the hepatic parenchyma (or hepatocytes) and the macrophages of the reticuloendothelial system [Cook et al. 1973; Andrews 1999]. In fact, iron accumulation in the reticuloendothelial cells of the liver, spleen and bone marrow, occurs when body iron stores are replete. Iron in the RES is a secondary accumulation due to the catabolism of the red cell heme acquired via erythrophagocytosis [Knutson and Wessling-Resnick 2003]. Stored iron in hepatocytes and macrophages can be mobilized to meet erythropoietic and cellular demands, when body iron stores are low [Andrews 1999; Hentze et al. 2004; Chua et al. 2007].

II. Regulation of iron homeostasis

Since iron loss is essentially an unregulated process, a tight balance between iron absorption, uptake, transport, storage and utilization is essential to maintain iron homeostasis. Among these compartments, a constellation of factors directly or indirectly related with iron regulation (the so-called iron-related genes) must be extensively controlled by a myriad of molecular mechanisms to achieve homeostasis.

II.1. Molecular mechanisms involved in the expression of iron-related genes

II.1.1. Transcriptional regulation

The transcriptional regulation of iron-related genes has been shown to respond to several stimuli like iron status, erythropoietic activity, inflammation and hypoxia. In fact, the major player in the regulation of iron homeostasis is the liver-derived hormone hepcidin, which is transcriptionally controlled by all the stimuli above indicated [Nicolas et al. 2002a; Nicolas et al. 2002b; Nemeth et al. 2003; Pinto et al. 2008]. It is known that the hepcidin promoter contains binding motifs for several recognized transcriptional factors [Courselaud et al. 2002; Truksa et al. 2007; Weizer-Stern et al. 2007; Casanovas et al. 2009; Truksa et al. 2009]. The expression of hepcidin is indeed regulated by a range of upstream molecules that culminates in the binding of factors to the hepcidin promoter and these pathways will be further exposed in this thesis.

Cytokines, such as interleukin-6 (IL-6), interleukin-1 (IL-1) and interferon-γ, have been shown to affect the messenger RNA (mRNA) expression of several iron-related genes. Such is the case of H-ferritin [each ferritin is composed by two chains, heavy (H) and light (L)], transferrin receptor 1, hepcidin and ferroportin genes [Wei et al. 1990; Fahmy and Young 1993; Tran et al. 1997; Lee et al. 2004; Nemeth et al. 2004a; Lee et al. 2005].

Hypoxia is intimately related with erythropoiesis. Under low oxygen tension, the transcription factor HIF-1 (hypoxia inducible factor-1) is activated and will interact with erythropoietin, increasing the iron required for erythropoiesis [Wang and Semenza 1995; Semenza 1999]. Consequently, hypoxia has been shown to affect the expression of transferrin, TfR1, ceruloplasmin, ferroportin, DcytB and hepcidin genes [Rolfs et al. 1997; Lok and Ponka 1999; Tacchini et al. 1999; McKie et al. 2000; Mukhopadhyay et al. 2000; McKie et al. 2001; Nicolas et al. 2002b].

As expected, iron levels may control the transcription of some iron-related genes. Indeed, McKie et al. [2001] have shown that in iron-deprived mice, the expression of DMT1 mRNA (with no iron responsive element) is increased. Interestingly, two reports have recently shown that the expression of the hypoxia inducible factor HIF-2α in the intestine altered both serum iron and tissue iron stores [Mastrogiannaki et al. 2009; Shah et al. 2009]. This is due to a strong effect on the transcription of DMT1, DcytB and ferroportin within the enterocyte. It was suggested that this HIF-2α-mediated mechanism may override the hepcidin-ferroportin regulatory axis on the control of iron absorption.

The processes of erythrophagocytosis and the recycling of heme have shown to induce changes in the macrophage gene expression, including variations in heme oxygenase 1, ferroportin and ferritin. Regarding transcription, it was recently shown that ferroportin expression is inhibited by Bach1 (btb and cnc homology 1) and activated by Nrf2 (nuclear factor erythroid 2-related factor 2), in a heme-dependent mechanism involving an MARE/ARE (Maf recognition elements/antioxidant response elements) sequence located 7 kb (kilo base pairs) upstream of the ferroportin promoter [Marro et al. 2010]. These authors suggest that the iron released from hemoglobin by HO1 activity is unlikely to be involved in this process since the transcription of ferroportin is activated by hemoglobin, hemin or the protoporphyrin ring alone.

Heme was also proven to regulate the gene transcription of HO1 and ferritin (both heavy and light) chains, through the transcriptional repressor Bach1 [Sun et al. 2002; Hintze and Theil 2005; Hintze et al. 2007; Marro et al. 2010].

II.1.2. Post-transcriptional regulation

II.1.2.1. Iron regulatory proteins

Iron-related genes display a specific mode of gene expression regulation. It involves the interaction of cytosolic iron regulatory proteins (IRPs) with structural elements in mRNA transcripts, designated iron responsive elements (IREs). The latter are conserved stem loop structures present in either 5' or 3' untranslated regions (UTR) of several iron-regulated genes [Hentze et al. 1988; Muckenthaler et al. 2008; Hentze et al. 2010]. The IRPs act as sensors of the cytoplasmic iron, controlling the expression of many of the proteins involved in iron homeostasis, such as ferritin, TfR1, ferroportin and DMT1, among others. Under iron depletion conditions, the binding of IRPs to IREs increases, resulting in an augmented mRNA stability in transcripts with multiple IREs located at the 3' UTR, such as TfR1 and DMT1 [Hentze and Kuhn 1996; Gunshin et al. 2001]. Conversely, the binding to single IREs in the 5' UTR of an mRNA will block translation, as observed in ferritin L and H chains, erythroid 5-aminolevulinic acid synthase, mitochondrial aconitase and ferroportin mRNAs [Hentze et al. 1987; Hentze and Kuhn 1996; Muckenthaler et al. 1998; Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000; Eisenstein and Ross 2003]. On the contrary, when iron is abundant, IRPs are devoid of mRNA binding activity and the target transcripts are freely accessible for degradation by

nucleases (3' UTR IREs) or to translation complexes (5' UTR IREs). This post-transcriptional mechanism is of extreme importance since it regulates the iron uptake via TfR1-Tf, a crucial process for almost all cells [Theil 1994; Hentze and Kuhn 1996]. There are two recognized IRPs, 1 and 2, which are structurally and functionally similar. Although both are capable of mRNA binding, only IRP1 possesses aconitase activity (ability to convert citrate to isocitrate). A recent study provided experimental proof of the cellular iron transport regulation by the IRE-IRP interaction through generating enterocyte-specific ablation of both IRP1 and 2 in mice. The resulting animals developed intestinal iron malabsorption, as consequence of a strong reduction in DMT1 expression and upregulation of ferroportin [Galy et al. 2008].

II.1.2.2. Alternative splicing

The role of alternative splicing in generating proteomic diversity has been extensively studied and considered the fail-safe mechanism by which organisms have survived and evolved. This mechanism will be explored latter on in this thesis, but for now, examples of how iron-related genes utilize alternative splicing forms to respond to certain stimuli will be given.

The DMT1 gene expresses multiple isoforms with and without 3' IREs [Gunshin et al. 1997; Fleming et al. 1998; Lee et al. 1998; Hubert and Hentze 2002]. These alternative transcripts result from the combination of 5' and 3' exons (1A or 1B and IRE or non-IRE, respectively). The outcome of 4 DMT1 isoforms (1A/+IRE, 1A/-IRE, 1B/+IRE and 1B/-IRE) have implications on iron regulation. Potentially, the two mRNA isoforms that are +IRE may be stabilized by an IRP. In fact, it was shown by Hubert and Hentze [2002] that the main isoform that increases during iron deficiency is the 1A/+IRE. But it was unclear if the transcription of the 1A form is upregulated, if the +IRE RNA is stabilized, or both. On one hand, IRP ablation on enterocytes was show to diminish +IRE DMT1 mRNA, suggesting that the IRE contributes to the stabilization of this mRNA during iron deficiency [Galy et al. 2008]. On the other hand, the overall gain in 1A isoforms in iron chelated Caco-2 cells is greater than the net gain in the +IRE isoforms, favoring the transcriptional regulation of the 1A promoter during iron deficiency [Hubert and Hentze 2002].

Ceruloplasmin is a multicopper oxidase present in plasma that promotes iron incorporation of ferric iron into transferrin [Holmberg and Laurell 1948; Osaki et al. 1966]. Recognition of an

essential role for this protein as a ferroxidase came with the identification of patients with aceruloplasminemia, who develop diabetes, neurodegeneration and parenchymal iron overload [Harris et al. 1995; Yoshida et al. 1995]. Studies in a murine model of aceruloplasminemia reveal a physiologic role for ceruloplasmin in determining the rate of iron efflux from cells with mobilizable iron stores [Harris et al. 1999]. There are now two recognized ceruloplasmin proteins resulting from alternative splicing events that occur downstream of exon 18. The secreted form includes solely the exon 19 to form the five C-terminal amino acids, whereas the glycosylphosphatidylinositol (GPI)-anchored form will only include the exon 20, adding 30 amino acids that encode for GPI-anchor addition [Hellman and Gitlin 2002]. These isoforms present distinct patterns of tissue expression. While serum ceruloplasmin is generally considered as secreted by the liver (although extra-hepatic expression has also been observed), the membrane-bound GPI-anchored ceruloplasmin is the predominant form in the brain [Klomp and Gitlin 1996; Patel and David 1997; Hellman and Gitlin 2002; Banha et al. 2008].

As previously stated, TfR2 is a member of the transferrin receptor family capable of binding transferrin, although with lower affinity than TfR1 [Kawabata et al. 1999]. TfR2 is expressed at high levels in hepatocytes and at low levels in peripheral blood mononuclear cells (PBMCs), spleen and erythroid progenitors [Kawabata et al. 1999; Kawabata et al. 2001; Forejtnikova et al. 2010]. Unlike TfR1, TfR2 has no IRE and it is not post-transcriptionally regulated by iron via the IRE-IRP pathway [Kawabata et al. 1999]. Interestingly, the TfR2 gene encodes for two main transcripts, a longer tissue-specific form (alpha) and a shorter one (beta), which utilizes a putative start codon in exon 4 and is in frame with the major transcript. The beta isoform lacks the intracellular and the transmembrane domains and is predicted to produce an intracellular/secreted protein with a still unclarified function [Kawabata et al. 1999]. Indeed, a recent work on murine models with a selective inactivation of the beta isoform, suggests a specific splenic function for this isoform by targeting ferroportin expression since it may act as a sensor of the iron recycled from erythropoiesis [Roetto et al. 2010]. Furthermore, the same study reinforces the role of the hepatic alpha-TfR2 in the proposed TfR2-HFE complex, whose formation is favored by increased diferric transferrin to activate hepcidin [Gao et al. 2009; Roetto et al. 2010].

Recently, an isoform of ferroportin lacking the IRE was identified in enterocytes and red blood cell precursors. The expression of this isoform revealed the capability of these cells to surpass translational repression during low body iron conditions [Zhang et al. 2009a]. Moreover,

5’ RACE (rapid amplification of cDNA ends) experiments performed during erythroid differentiation revealed multiple ferroportin transcripts, suggesting a tissue-specific mechanism of iron export, but this requires further clarification [Cianetti et al. 2005].

Several other alternative transcripts generated by genes associated with iron metabolism have been described, but their particular function and pattern of tissue expression remains to be clarified. This is the case of HFE, hemojuvelin, transferrin, among others [Jeffrey et al. 1999; Rhodes and Trowsdale 1999; de Arriba Zerpa et al. 2000; Thenie et al. 2001; Papanikolaou et al. 2004].

II.1.3. Post-translational regulation

Several mechanisms of post-translational regulation have been described in the iron metabolism field. Among these, regulation of ferroportin by hepcidin plays a pivotal role in controlling iron homeostasis. The hepcidin-ferroportin interaction induces the internalization and degradation of ferroportin, resulting in a diminished iron release from cells [Nemeth et al. 2004b]. Due to its importance, this mechanism and upstream pathways leading to hepcidin synthesis will be further developed in this thesis.

It is currently accepted that in hepatocytes, the membrane bound hemojuvelin acts to stimulate the pathway leading to hepcidin expression, whereas its soluble form acts to inhibit the same signaling pathway [Lin et al. 2005; Lin et al. 2008]. In fact, hemojuvelin itself has a quite complex mode of self-regulation which is far from understood. The production of the soluble form from the membrane-bound requires the action of the protease furin, whose activity can be increased by iron deficiency and hypoxia [Silvestri et al. 2008a]. On the other hand, in an iron loading situation, the inhibition of soluble hemojuvelin requires the neogenin protein [Zhang et al. 2007]. Moreover, the serine protease matriptase-2 is able to cleave membrane-bound hemojuvelin releasing peptides distinct from the soluble form(s), which are thought to be secreted by an intracellular mechanism [Silvestri et al. 2008b].

Several studies have enlightened the role of glycosylation in HFE protein processing, which allows proper intracellular trafficking and functional activity at the cell membrane [Gross et al. 1998; de Almeida et al. 2007a; Bhat et al. 2010].

Transferrin receptor 2 is regulated by transferrin saturation. In fact, diferric-Tf is a strong modulator of TfR2 trafficking since it increases TfR2 half life by favoring both recycling and

surface stabilization of the receptor and by inhibiting its lysosomal degradation [Johnson and Enns 2004; Robb and Wessling-Resnick 2004].

A study performed in Belgrade rats shows that the internalization of DMT1 protein in duodenal enterocytes may be an acute regulatory mechanism to limit iron uptake [Yeh et al. 2000].

The depicted mechanisms are the outcome of an iron-driven regulation with an essential role on systemic iron homeostasis. As stated before, the maintenance of systemic iron is only achieved by an integration and coordination of a number of complex regulatory pathways in which hepcidin is the central player.

II.2. Systemic regulation of iron homeostasis by hepcidin

Hepcidin, a hormone synthesized mainly by hepatocytes and secreted to the plasma, has been accepted as the key regulator of systemic iron homeostasis [Pigeon et al. 2001; Nicolas et al. 2001; Park et al. 2001]. Hepcidin production is stimulated by increased plasma iron and tissue iron stores [Pigeon et al. 2001; Nicolas et al. 2002a; Nicolas et al. 2002b]. Hepcidin regulation of iron occurs through its binding to ferroportin, the iron exporter required for iron efflux, present in enterocytes, macrophages as well as in other iron exporting cells, including placental syncytiotrophoblasts and hepatocytes [Abboud and Haile 2000; Donovan et al. 2000; McKie et al. 2000; Donovan et al. 2005]. Upon reaching its target tissues, hepcidin binds to ferroportin present at cell surface. It induces the phosphorylation of amino acids located at an intracellular loop of ferroportin, triggering the internalization of the hepcidin-ferroportin complex. Within the cell, ubiquitination of ferroportin and lysosomal degradation of both proteins will take place [Nemeth et al. 2004b; De Domenico et al. 2007]. Decreased expression of ferroportin at cell surface thereby reduces the iron efflux from cells into the plasma. In fact, hepcidin has been shown to restrict intestinal iron absorption and macrophage iron release, by these means reducing body iron stores and limiting the iron available for erythropoiesis [Laftah et al. 2004; Delaby et al. 2005].

The evidence that the role of hepcidin is fundamental was provided by both human disorders and animal models. Mice in which the hepcidin gene was inadvertently inactivated developed severe iron overload, whereas transgenic mice overexpressing hepcidin presented a severe iron deficiency anemia [Nicolas et al. 2001; Nicolas et al. 2002a].

Since its discovery in the beginning of the new millennium, hepcidin has been placed as the final target of diverse pathways. These regulatory pathways that control hepcidin gene transcription have the common purpose of managing iron availability. Iron storage, erythropoiesis, inflammation and hypoxia are the most extensively studied stimuli that influence hepcidin expression, but only the coordinated action between these positive and negative regulators will determine the net hepcidin level.

II.2.1. Hepcidin regulation by erythropoiesis, hypoxia and inflammation

It has been established for quite some time that the erythropoiesis rate influences iron absorption regardless of body iron stores, but only recent studies have disclosed the players involved in this communication. A strong candidate for this activity was serum TfR1 (sTfR1) levels, since it correlates well with erythropoietic mass and is responsive to iron deficiency [Cazzola et al. 1999]. In fact, about 80% of sTfR1 is generated by the maturation of erythroid cells [R'Zik et al. 2001]. Arguments against this hypothesis are given by the fact that sTfR1 is produced even when erythroid cells no longer require iron for hemoglobin synthesis and by the lack of response in iron absorption in mice overexpressing sTfR1 [Flanagan et al. 2006].

The hormone erythropoietin has been shown to be essential for erythroid differentiation, but the direct relationship between erythropoietin and the suppression of hepcidin in liver hepatocytes arose recently [Tan et al. 1992; Eckardt and Kurtz 2005; Fein et al. 2007; Pinto et al. 2008]. The erythropoietin receptor (Epo-R) belongs to the cytokine receptor superfamily and, among many other tissues, is expressed at the cell surface of hepatocytes (Figure 1.3). Here, erythropoietin may interact with Epo-R, triggering a decreased binding of the transcription factor C/EBPα (CCAAT/enhancer binding protein α) to a cognate site in the hepcidin promoter [Courselaud et al. 2002; Pinto et al. 2008]. Alternatively, Huang et al. [2009] proposed that erythropoietin can suppress hepcidin expression indirectly by the downregulation of the signal transducer and activator of transcription 3 (STAT3) and SMAD4 [the name SMAD is a combination of two proteins: the *Caenorhabditis elegans* protein Sma (designated as mutations in this gene causes animals to be small) and the drosophila protein MAD ("mothers against decapentaplegic")].

Recent research has considered the growth differentiation factor 15 (GDF15), a member of the transforming growth factor β (TGF-β) superfamily, as the erythroid regulator of hepcidin (Figure 1.3). This factor has increased expression and secretion during erythroid maturation and is highly increased in patients with defective erythroid expansion [Tanno et al. 2007; Tamary et al. 2008; Finkenstedt et al. 2009; Ramirez et al. 2009; Theurl et al. 2010]. Moreover, while in vitro studies reveal the suppressive effect of GDF15 on hepcidin expression, GDF15 expression itself was shown to be regulated by the iron status [Tanno et al. 2007; Lakhal et al. 2009]. Although some skepticism may arise from the fact that GDF15 and hepcidin levels do not correlate in patients undergoing hematopoietic stem cell transplant recovery (whereas other erythroid markers correlate with hepcidin), the recent findings are consistent with previous proposals in which erythropoiesis is positively related with iron absorption and mobilization [Kanda et al. 2008]. Moreover, they also reinforce the idea that erythropoiesis dominantly represses hepcidin expression in spite of iron overload [Tanno et al. 2007; Lakhal et al. 2009].

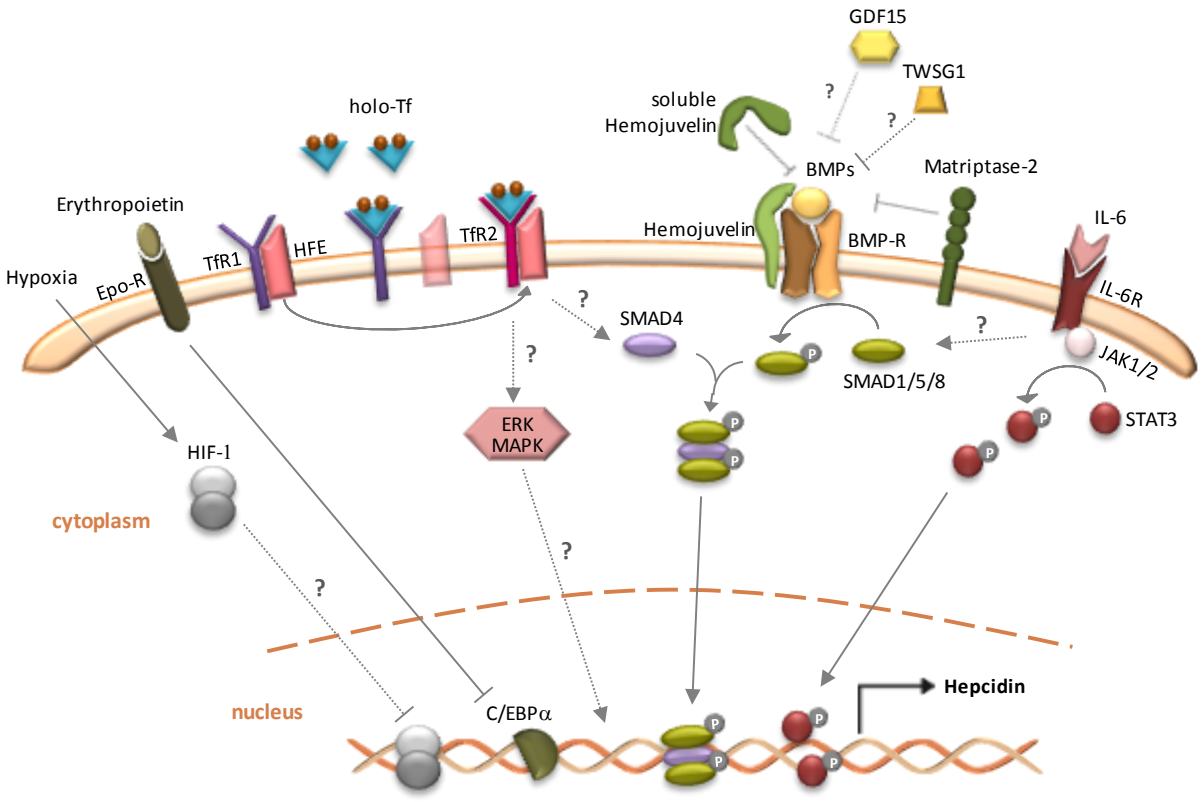

Figure 1.3. Transcriptional regulation of hepcidin expression. There are several upstream stimuli that through signaling pathways determine the expression levels of hepcidin. Inflammatory status and iron overload act as positive regulators, whereas hypoxia and erythropoietic demand operate as repressors of hepcidin expression. See text for details. (Adapted from Anderson et al. 2009).

The latest factor recognized as a putative erythroid regulator of hepcidin is a cytokine named TWSG1 (twisted gastrulation 1) (Figure 1.3). Contrarily to GDF15, TWSG1 is produced during the earlier stages of erythropoiesis [Tanno et al. 2009]. This study shows that TWSG1 suppresses hepcidin indirectly by inhibiting the bone morphogenetic proteins (BMPs) signaling pathway. Here, it is proposed that TWSG1 and GDF15 might act together to inappropriately inhibit hepcidin expression and deregulate iron homeostasis in thalassemia syndromes.

Hypoxia is another negative regulator of hepcidin expression, independently of body iron levels [Nicolas et al. 2002b; Choi et al. 2007; Peyssonnaux et al. 2007]. In fact, the transcriptional hypoxia-inducible factor pathway was shown to regulate hepcidin expression in mice [Peyssonnaux et al. 2007] (Figure 1.3). The liver-specific disruption of the von Hippel-Lindau gene, which encodes for an essential component of the complex that degrades HIF, led to decreased hepcidin mRNA levels in these mice. It has also been shown that HIF-1 is able to bind the hepcidin promoter, suggesting a direct repression of hepcidin by HIF-1 [Peyssonnaux et al. 2007]. Nevertheless, Choi and co-workers [2007] have brought disagreeing data, since either HIF-1 overexpression or knockdown fail to alter hepcidin expression in HepG2 cells. They have also shown that the increase in reactive oxygen species (ROS) in hypoxic cells impaired the binding of C/EBPα and STAT3 transcription factors to the hepcidin promoter, with a negative effect on its expression [Choi et al. 2007]. Moreover, Volke et al. [2009] also failed to find a direct transcriptional suppression of hepcidin by HIFs. So, whether or not HIFs directly bind to the hepcidin promoter is currently controversial.

Inflammation is a robust inducer of hepcidin expression, evoking its function as an antimicrobial peptide [Krause et al. 2000; Park et al. 2001; Nicolas et al. 2002b; Peyssonnaux et al. 2006; Sow et al. 2007]. Under inflammatory conditions, iron absorption is reduced and iron is sequestered in the macrophages, with a consequent hypoferremia in plasma [Nemeth et al. 2004a; Rivera et al. 2005]. A substantial body of evidence indicates that IL-6 is the predominant cytokine involved in the inflammatory regulation of hepcidin, but IL-1α and IL-1β are also able to stimulate hepcidin [Lee et al. 2004; Nemeth et al. 2004a; Lee et al. 2005] (Figure 1.3). This induction has been shown to occur through the Janus kinase (JAK) 1/2 signal transducer and STAT3 transcriptional mechanism [Wrighting and Andrews 2006; Verga Falzacappa et al. 2007; Truksa et al. 2007]. It has also become evident that the hepcidin response to IL-6 may require

cooperative activity of the BMP signaling pathway, possibly through the TGF-β/SMAD4 induction [Wang et al. 2005; Babbit et al. 2007; Yu et al. 2008].

Indeed, the BMP signaling pathway has a critical importance in the regulation of hepcidin transcription activation and the cohort of proteins involved upstream this response will now be exposed.

II.2.2. Hepcidin regulation by iron status

Dissecting the elegant mechanisms that allow systemic iron homeostasis maintenance through the modulation of hepcidin expression has been quite challenging. The study of genetic disorders of iron status has brought to light some of the most important players that take part of these pathways. This is the case of HFE, TfR2, hepcidin and hemojuvelin, among others [Feder et al. 1996; Camaschella et al. 2000; Roetto et al. 2003; Papanikolaou et al. 2004; Finberg et al. 2008]. Nevertheless, other proteins such as the BMPs, matriptase-2 and transferrin have been shown to be involved in the hepatic regulation of hepcidin driven by iron status. Although the mechanisms by which TfR2 and HFE act are only recently beginning to be untangled, the characterization of hemojuvelin has revealed a complex signal transduction pathway that regulates hepcidin expression, the bone morphogenetic protein pathway.

II.2.2.1. Hemojuvelin, BMPs and matriptase-2

Hemojuvelin's pivotal role in iron homeostasis has been demonstrated by both clinical and animal studies. Whereas homozygosity or compound heterozygosity for mutations in the human hemojuvelin gene are responsible for a juvenile form of hereditary hemochromatosis, disruption of both hemojuvelin-homologue alleles in mice result in marked iron deposition in the liver, heart and pancreas [Papanikolaou et al. 2004; Huang et al. 2005; Niederkofler et al. 2005]. The severe downregulation of hepcidin in these cases despite the presence of strong iron loading demonstrated that hemojuvelin is an essential upstream regulator of hepcidin.

Hemojuvelin is a member of the repulsive guidance molecule family of proteins that function as co-receptors of the bone morphogenetic proteins [Babitt et al. 2006]. Notably, recent studies have shown that hemojuvelin is able to bind several BMPs, such as BMP2, 4, 5, 6 and 9,

thereby increasing hepcidin synthesis [Babitt et al. 2006; Truksa et al. 2006; Babitt et al. 2007; Yu et al. 2008] (Figure 1.3). In general, BMPs are a subfamily of cytokines that belong to the TGF-β superfamily [Heldin et al. 1997; Derynck and Zhang 2003]. Individual members of the BMP subfamily are able to interact with type I and II receptors, therefore increasing the complexity of hepcidin regulation by the BMP pathway [Derynck and Zhang 2003]. Activated BMP receptors phosphorylate the SMAD1/5/8 protein complex which, in turn, will form a heteromeric complex with the DNA binding protein SMAD4 [Babitt et al. 2007]. This complex translocates into the nucleus and activates the transcription of target genes, such as hepcidin [Heldin et al. 1997; Derynck and Zhang 2003]. Evidence to support the BMP pathway in the regulation of hepcidin expression came out by the study of mice with liver-specific disruption of SMAD4 gene, which developed severe iron overload with almost no hepcidin expression [Wang et al. 2005]. Moreover, these mice failed to respond to iron loading or IL-6 injection. Recent studies on BMP6 knockout mice show the same iron overload phenotype as SMAD4 and hemojuvelin knockout mice [Andriopoulos et al. 2009; Meynard et al. 2009]. This confirms previous data according to which BMP6 expression is directly regulated by iron and essential for hepcidin upregulation [Kautz et al. 2008; Yu et al. 2008].

The task of hemojuvelin in the activation of hepcidin through the BMP-SMAD pathway is far from understood. In fact, hemojuvelin exists in at least two distinct forms: a transmembrane glycosylphosphatidylinositol (GPI)-linked form, which stimulates hepcidin, and a soluble form, which acts as an antagonist of the BMP signaling pathway [Lin et al. 2005; Zhang et al. 2005; Kuninger et al. 2006; Babbit et al. 2007]. This soluble form appears to be released from the mature hemojuvelin by furin or other pro-protein convertase [Silvestri et al. 2008a; Lin et al. 2008]. Evidence of the importance of this soluble hemojuvelin was given when its administration lowered hepcidin expression in mice and cultured cells [Lin et al. 2005; Babitt et al. 2007]. This led to the current model, in which soluble hemojuvelin can antagonize BMP signaling by binding to the BMPs and impair their association with the heteromeric BMP type I/II receptors [Lin et al. 2005; Babbit et al. 2007]. Importantly, the generation of soluble hemojuvelin was shown to be increased by iron treatment and hypoxia thereby repressing hepcidin, most likely by the increased activity of furin [Lin et al. 2005; Zhang et al. 2007; Silvestri et al. 2008a]. In parallel, work by Zhang and co-workers [2005] revealed that hemojuvelin interacts with neogenin on the cell membrane interfering with cellular iron levels. Recent studies confirm that neogenin may have two functions in the regulation of hemojuvelin, one in promoting hemojuvelin shedding in response to iron stimulus and another in inducting BMP-mediated hepcidin expression by

the neogenin-hemojuvelin interaction [Zhang et al. 2007; Zhang et al. 2009b]. However, conflicting data arise from two recent independent studies. Xia et al. [2008] have shown that the knockdown or overexpression of neogenin fails to induce changes in the hemojuvelin-induced BMP signaling and hepcidin expression, whereas Lee et al. [2010] indicate that neogenin enhances BMP signaling resulting in hepcidin upregulation but stating that this occurs by neogenin inhibition of hemojuvelin secretion.

The most recent partner shown to be involved in the hemojuvelin/BMP pathway is the membrane-bound serine matriptase-2 (Figure 1.3). Its importance in systemic iron regulation was firstly suggested by results obtained in two mouse models enclosing a mutated matripase-2, in which a marked increase in hepcidin levels was concomitant with iron deficiency anemia [Du et al. 2008; Folgueras et al. 2008]. These studies were promptly corroborated by the clinical studies in patients with iron-refractory iron deficiency anemia that were homozygous or compound heterozygous for mutations in matriptase-2 gene [Finberg et al. 2008; Melis et al. 2008]. The proposed role for matriptase-2 is the cleavage of membrane hemojuvelin into fragments, therefore inhibiting hepcidin expression activation [Silvestri et al. 2008b]. Furthermore, recent findings by Finberg et al. [2010] suggest that the involvement of matriptase-2 is required for the downregulation of the BMP/SMAD signaling, thus contributing to the regulation of systemic iron homeostasis.

II.2.2.2. HFE and transferrin receptors (TfR1 and TfR2)

It is now accepted that the iron present in the plasma and in the tissue stores enhances hepcidin synthesis which, in turn, inhibits the release of iron from macrophages and duodenal enterocytes to the plasma. The molecular details of this homeostatic loop are still incompletely understood. In fact, the most likely candidates able to act as iron sensors include the transferrin receptors, TfR1 and TfR2 (Figure 1.3). Although the molecular link between these receptors was characterized almost fifteen years ago, when the HFE protein was associated with hereditary hemochromatosis, the mechanism(s) by which HFE and the transferrin receptors affect hepcidin expression have only recently emerged [Feder et al. 1996; Schmidt et al. 2008; Gao et al. 2009; Ramey et al. 2009; Wallace et al. 2009; Gao et al. 2010; Poli et al. 2010].

Initial studies showed that HFE was associated with TfR1 at the cell membrane and that HFE could compete with Tf for binding to TfR1 [Parkkila et al. 1997a; Feder et al. 1998; Lebron et al. 1998].

This lead to the idea that iron-loaded Tf (holo-Tf) would release HFE to interact with other proteins. However, only the work of Schmidt et al. [2008] using mouse models with mutations that strengthen or weaken the interaction between HFE and TfR1 provided clear evidence of this. A TfR1 mutation that reduces Tf binding but maintains HFE affinity led to low hepcidin levels and iron overload, whereas mice expressing a mutant TfR1 with reduced HFE affinity had inappropriately high hepcidin and iron deficiency [Schmidt et al. 2008]. By this time, it was evident that the hepcidin expression is related to the amount of HFE not complexed with TfR1 but possibly available for TfR2. In fact, HFE and TfR2 had been previously shown to interact in human tissues and other mammalian cells overexpressing both these proteins. The binding sites were shown to be quite different from those involved in the HFE-TfR1 interaction [Griffiths and Cox 2003; Goswani and Andrews 2006; Chen et al. 2007]. Unlike the HFE-TfR1 interaction, there is no competition for holo-Tf, allowing the formation of the HFE-TfR2-Tf complex and giving the basis for hepcidin regulation by this complex [Bennett et al. 2000; Goswani and Andrews 2006; Chen et al. 2007]. Both holo-Tf and HFE were shown to stabilize TfR2 [Johnson and Enns 2004; Robb and Wessling-Resnick 2004]. Furthermore, mutations in the gene encoding for TfR2 originate iron loading symptoms very similar to those found in HFE-associated hemochromatosis [Feder et al. 1996; Camaschella et al. 2000]. Accordingly, TfR2 mutations lead to the same abnormally low hepcidin levels observed when HFE is disrupted, also suggesting that these proteins may be partners in the same regulatory pathway [Nemeth et al. 2005]. The direct evidence that an interaction between TfR2 and HFE is required for the signal transduction between holo-Tf and hepcidin was provided by Gao et al. [2009]. These authors showed that the stimulation of hepcidin transcription by holo-Tf requires both HFE and TfR2, in hepatic cell lines and primary hepatocytes. The use of HFE chimeras allowed to elegantly demonstrate that the interaction between HFE and TfR2 (but not TfR1) is necessary for this signal transduction [Gao et al. 2009]. This study provided strong evidence for the current model: under normal iron conditions, HFE is partitioned between TfR1 and TfR2, whereas an increase in Tf saturation results in the stabilization of TfR2 protein and degradation of TfR1 mRNA [Chen et al. 2007; Chen et al. 2009]. Under these conditions, HFE should shift away from TfR1 towards TfR2 and the HFE-TfR2-Tf complex might become part of the iron sensing complex leading to hepcidin induction [Schmidt et al. 2008; Fleming 2009; Gao et al. 2009; Wallace et al. 2009; Gao et al. 2010]. Recent work performed in mouse knockout models by Gao et al. [2010] confirm that both HFE and TfR2 are necessary for hepcidin regulation and suggests that HFE is the limiting factor in the formation of the complex. Furthermore, TfR2

has been shown to localize in membrane lipid rafts and to activate the ERK1/2 (extracellular signal-regulated kinases 1/2) and the p38 MAPK (mitogen-activated protein kinases) under holo-Tf stimulation so it was proposed that this could be *the via* followed by HFE-TfR2-Tf complex to regulate hepcidin synthesis [Calzolari et al. 2006; Gao et al. 2009; Ramey et al. 2009] (Figure 1.3). Accordingly, a very recent work by Poli and colleagues [2010] shows that the silencing of HFE and TfR2 in HepG2 cells reduces the phosphorylation of ERK1/2 under holo-Tf stimulus.

Two parallel studies have recently shown that the HFE regulatory function in the hepatocytes most likely traverse the BMP6 signaling pathway in directing hepcidin expression [Corradini et al. 2009; Kautz et al. 2009]. These authors have shown that in HFE knockout mice the levels of hepatic phosphorylated SMAD1/5/8 were unsuitably low for the body iron burden. Also, the BMP6 induction of hepcidin expression was reduced in HFE knockout hepatocytes when compared with normal hepatocytes, suggesting the involvement of HFE in the downstream signals of BMP6 [Corradini et al. 2009]. In agreement, a very recent report in HH patients (C282Y homozygotes) reveals that BMP6 hepatic expression is appropriately augmented, likely related to iron overload, but interestingly, SMAD6 and SMAD7 proteins, inhibitors of BMP signaling, are also upregulated [Ryan et al. 2010]. But several other recent reports have disclosed a cross-talk between the SMAD1/5/8 BMP-mediated pathway and the ERK/MAPK pathway triggered by HFE-TfR2-Tf complex [Ramey et al. 2009; Wallace et al. 2009; Poli et al. 2010]. Double null mice for HFE and TfR2 were shown to develop more severe iron loading than mice lacking either HFE or TfR2 and the results reveal that both molecules regulate hepcidin through parallel pathways involving ERK1/2 and SMAD 1/5/8 [Wallace et al. 2009]. Accordingly, Poli et al. [2010] have shown that TfR2-null mice have a marked reduction in SMAD1/5/8 and ERK1/2 phosphorylation levels similarly what occurs by silencing TfR2 in HepG2 cells, whereas the silencing of HFE only affected the ERK1/2 phosphorylation. Interestingly, these authors show that furin activity is also reduced in both these situations, strongly suppressing hepcidin mRNA, most likely due to the inhibition of BMP maturation [Poli et al. 2010]. Contradicting data are presented in a study performed by Truksa et al. [2006], where hepcidin stimulation by BMPs in hepatocytes from IL-6 and HFE knockout and TfR2 mutant mice was similar to the wild type, indicating that those pathways are not necessary for the BMP signaling down to hepcidin expression. Moreover, it was show by Gehrke et al. [2005] that in HFE-mutated hepatic samples from human and mice the regulation of hepcidin by iron is not completely abolished, and that the

expression levels of hepcidin, TfR2 and hemojuvelin suggest an HFE-independent regulation. Hence, the role of HFE and TfR2 in hepcidin activation and its potential relationship with the hemojuvelin/BMP pathway is still far from clarified and impels us to the idea that controlling hepatocellular iron sensing might be a group effort.

II.2.2.2.1. HFE biology and function

In spite of the fact that the role of HFE protein in the regulation of iron homeostasis is now accepted as a mandatory liver function with the final target being hepcidin, this was not always the case. As stated before, HFE was discovered in 1996 by Feder and co-workers as the long-sought protein responsible for hereditary hemochromatosis. Here, it was described as a 348 amino acid protein consisting of six distinct domains: the signal peptide (which is removed in the mature protein), three extracellular domains (α1, α2 and α3 loops), a transmembrane region and a short intracellular region [Feder et al. 1996; Lebron et al. 1998] (Figure 1.4).

The amino acid sequence and the requirement of the β2-microglobulin (β2M) chaperone for proper folding and function, led to the inclusion of HFE in the major histocompatibility complex (MHC) class I molecules (Figure 1.4). Therefore, it was firstly named HLA-H while presenting a greater resemblance to HLA-A2 and HLA-G (HLA or human leukocyte antigen designates the genes that are expressed at the cell surface of human leukocytes). It was soon realized that the groove formed by α1 and α2 antiparallel helices of HFE protein was too narrow to function as a peptide-binding groove [Feder et al. 1996; Lebron et al. 1998]. HFE structure also includes one of the most important conserved structural features in MHC class I molecules, which are the four cysteine residues that form the disulphide bridges in α2 and α3 domains [Feder et al. 1996; Lebron et al. 1998]. The correct conformation of the α3 domain is necessary for non-covalent interaction with β2M and proper cell-surface presentation [Bjorkman and Parham 1990].

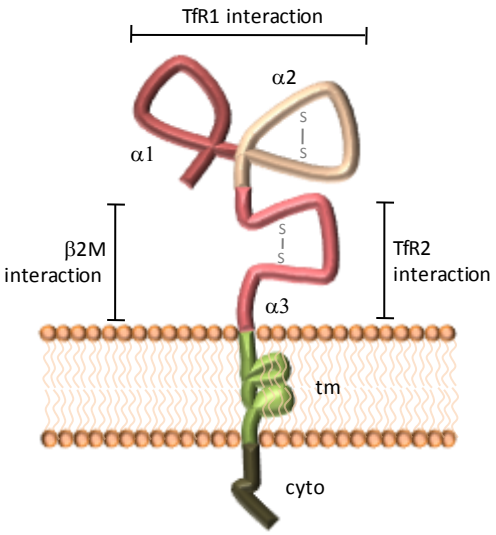

Figure 1.4. Schematic representation of the HFE protein at cell surface. The distinct colors depict HFE protein domains: the three extracellular domains (α1, α2 and α3), the transmembrane domain (tm) and the cytoplasmic tail (cyto). The domains required for β2M, TfR1 and TfR2 binding are indicated. (Adapted from Fleming 2009).

Hereditary hemochromatosis (HH) is characterized by abnormally high intestinal iron absorption, so a disrupted HFE would be responsible for this decontrol. But how do animal models help us to understand HFE's dysfunction in hemochromatosis? Many important conclusions were drawn from the study of HFE mutant mice. In fact, independent knockdown experiments have shown that HFE null mice absorb more iron than normal mice and present hepatic iron overload, fully mimicking the HH phenotype [Zhou et al. 1998; Bahram et al. 1999; Levy et al. 1999]. The vast majority of HH patients present the HFE mutation C282Y, which was shown to induce a conformational change in the HFE protein resulting in its inability to bind to β2M, therefore compromising HFE cell surface presentation [Feder et al. 1996; Feder et al. 1997; Waheed et al. 1997]. Accordingly, β2M knockout mice were shown to have an iron overload phenotype similar to hemochromatosis patients [Rothenberg and Voland 1996; Santos et al. 1996]. Comparison of HFE knockout mice with those homozygous for the orthologous C282Y mutation reveal that this missense mutation results in less iron loading that the null allele [Levy et al. 1999]. Studies in compound-mutant mice suggest that the iron loading in hemochromatosis is due to an increased iron flux involving both DMT1 and hephaestin [Levy et al. 2000].

Only recently the research regarding HFE's function was directed to the liver, since for quite some time it was thought to act in the maturing enterocytes of the duodenum. The initial studies on HFE protein localization confirm its intestinal and liver expression.

Immunohistochemistry of the gastrointestinal tract revealed a wide expression pattern, but the strongest staining was confined to the crypt cells of the small intestine [Parkkila et al. 1997b]. Here, HFE was found to be associated with TfR1 on the basolateral membrane and on the recycling endosomes of the immature enterocytes, confirming previous studies on placental tissue [Parkkila et al. 1997a; Waheed et al. 1999]. By comparing HFE and TfR1 expression in crypt and villus enterocytes, while measuring the uptake of transferrin-bound iron and ionic iron, these authors proposed the first mechanism of action of HFE protein. In the crypt enterocytes, HFE could regulate the uptake of Tf-bound iron from plasma, therefore sensing the level of body iron stores, triggering the programming mechanism by which the villus absorptive cells only take in the necessary dietary iron to maintain iron homeostasis [Waheed et al. 1999]. This model was supported by studies performed on HFE knockout mouse models and HH patients, in whom the augmented expression of the iron transporter DMT1 combined with decreased ferritin and higher duodenal IRP activities, reveal an iron deficient phenotype in duodenal enterocytes [Francanzani et al. 1989; Pietrangelo et al. 1995; Basclain et al. 1998; Fleming et al. 1999]. Only recently, the "crypt programming model" was defied. Using murine models with specific enterocyte ablation of HFE, Vujic Spasic et al. [2007] showed that hepatic iron stores and plasma iron levels were maintained, as well as hepcidin mRNA expression, therefore excluding the primary duodenal role of HFE in the pathogenesis of HH. In 2008, the same authors showed that only the hepatocyte-specific recombinant mice fully recapitulate the phenotype in HFE knockout mice, presenting severe iron accumulation and abnormally low hepcidin expression [Vujic Spasic et al. 2008].

Even before these recent intriguing discoveries, the mechanisms by which HFE would control intracellular iron levels brought conflicting results amongst the several studies that were performed. The overexpression of HFE in cells grown in culture was shown to reduce iron uptake and to lower intracellular ferritin levels [Gross et al. 1998; Corsi et al. 1999; Riedel et al. 1999; Roy et al. 1999]. The means by which HFE affected intracellular iron was a matter of discussion. While some authors stated that HFE could compete for Tf for binding to TfR1 or that it could affect the recycling of the receptor, others argued that this phenotype was not due to any effect on the Tf-mediated iron uptake via its receptor, but that HFE could interfere with DMT1-mediated iron influx [Feder et al. 1998; Lebron et al. 1999; Roy et al. 1999; West et al. 2001; Waheed et al. 2002; Zhang et al. 2003; Carlson et al. 2005]. The fact that overexpression of HFE leads to low intracellular iron are actually in a direct contradiction with the results of HH patients, who possess very little functional HFE and present an iron-deficient phenotype in the

duodenal enterocytes [Pietrangelo et al. 1995; Feder et al. 1996; Waheed et al. 1997]. Furthermore, HFE overexpression was shown to reduce cellular iron status in cell lines that express neither TfR1 nor TfR2 [Carlson et al. 2005].

On the other hand, HFE expression on macrophage and intestinal cell lines lead to increased intracellular iron stores, which was not due to any effect on Tf-mediated iron uptake, but related with the inhibition of iron efflux [Drakesmith et al. 2002; Davies and Enns 2004]. These authors suggest that the opposite effect of HFE in distinct cells lines may be due to the expression of the iron exporter ferroportin. In cells that do not export iron, such as HeLa and HEK293, intracellular iron levels decrease with HFE expression while in cells that export iron, such as HT-29 and THP-1, intracellular iron levels increase. Similarly, lack of functional HFE in humans causes opposite effects on iron levels in different cell types of affected tissues. In HH patients, Kupffer cells of the liver and intestinal enterocytes of the duodenum are iron poor, while liver hepatocytes are iron overloaded [Francanzani et al. 1989; Pietrangelo et al. 1995; Brunt et al. 2000]. The high IRP activity found in monocytes of hemochromatosis patients, and hence a low level of iron in the labile iron pool, is concordant with the increased iron content found in monocytes overexpressing HFE [Cairo et al. 1997; Drakesmith et al. 2002]. Despite the extensive studies, the mechanisms by which HFE modulates iron uptake and efflux from cells are only beginning to be understood [Chorney et al. 2003]. A recent work by Gao et al. [2008] show that HFE decreases the expression of Zip14, an iron and zinc transporter, inhibiting iron uptake in HepG2 cells. But so far, studies have failed to show a direct interaction of HFE with any iron transporter.

Besides providing iron for the erythropoiesis, the macrophages have been shown to work at the interface between iron and immunity [Theurl et al. 2005a]. It has been proposed that HFE may play an important role in macrophages. The expression of the wild type HFE normalizes the transferrin iron accumulation in macrophages from HH patients [Montosi et al. 2000]. Accordingly, macrophages derived from C282Y patients monocytes lose the ability to inhibit iron release leading to a relative macrophage iron deficiency [Drakesmith et al. 2002]. The transplantation of wild-type reticuloendothelial cells into HFE knockout mice was shown to increase hepcidin expression and to somehow revert the hepatic iron loading [Makui et al. 2005]. Accordingly, macrophages from HH patients infected with *Mycobacterium tuberculosis* exhibit a profound defect in their ability to acquire iron from exogenous transferrin and lactoferrin relatively to infected macrophages from normal controls, with consequent

growth impairment [Olakanmi et al. 2007]. In fact, it has been proposed by several authors that the relatively high prevalence of the C282Y mutation is due to a selective advantage given by the low RES iron during past endemic or epidemic infection [Moalem et al. 2004]. Similarly, Nef (negative factor) protein of HIV-1 was shown to control intracellular iron by impairing HFE surface presentation, whereas macrophages from HH patients failed to induce Nef-mediated iron and ferritin accumulation upon HIV-1 infection [Drakesmith et al. 2005]. The idea that HFE-mutated macrophages may confer some protection from HIV-1 infection complies with the description of a long-term survival in a patient with AIDS and hereditary hemochromatosis [Nielsen et al. 1999]. The first demonstration that HFE could be a target for viral proteins was given by the human cytomegalovirus protein US2 (unique short 2) that was shown to trigger HFE's degradation by the proteasome, leading to increased intracellular iron pool in HeLa and HEK293 cells [Ben-Arieh et al. 2001; Vahdati-Ben Arieh et al. 2003]. All this body of evidence confirms that HFE, not only is able to control the inner iron status of macrophages, but also has imperative effects in what regards infection progression.

Recent findings by Pinto et al. [2010] showing that human lymphocytes express hepcidin and control intracellular iron levels by regulating the expression of ferroportin, confirms the long-sought link between iron metabolism and the immune system. Regarding HFE, many direct data arose from the observation of abnormalities in the T lymphocytes of hemochromatosis patients., e. g., high CD4/CD8 (CD stands for "cluster of differentiation" of leukocytes) ratio due to low number of CD8+ T cells, decreased CD8-associated p56lck kinase activity, T cell receptor repertoire anomalies associated with C282Y mutation and the diminished cytotoxic activity of CD8+ cytotoxic T lymphocytes [Reimao et al. 1991; Arosa et al. 1994; Arosa et al. 1997; Porto et al. 1997; Cardoso et al. 2001]. Also, abnormalities in CD8+ T lymphocytes have been shown to be related with a more severe clinical expression of iron overload in hemochromatosis patients [Porto et al. 1997; Barton et al. 2005; Cruz et al. 2006]. Moreover, it has been shown that peripheral blood mononuclear cells containing the HFE C282Y mutation have a decreased presentation of MHC class I molecules at cell surface, subsequently proven to be due to the stimulation of an unfolded protein response [de Almeida et al. 2005; de Almeida et al. 2007b]. Whether this MHC class I expression defect associated with C282Y mutation is linked to the lymphocyte anomalies previously described is a matter for future studies. Although an immunologic function for HFE has not been described, all these data indicate that HFE may be a crucial player in the cross-talk between iron metabolism and immune response.

The means by which HFE affects intracellular iron is still unclear and most likely depends on the expression of other iron-related molecules. Its role on liver hepcidin expression is now undoubtedly accepted, but surely its effect on iron metabolism has broader implications, evidenced by the profound changes on intracellular iron levels in cultured cells overexpressing HFE, by its involvement in infection and possibly in the immune response.

II.2.2.2.2. HFE molecular genetics and expression

The HFE gene is located on chromosome region 6p21.3, approximately 4.6 megabases telomeric from HLA-A, encompassing approximately 12 kb of DNA [Feder et al. 1996]. The genomic structure of HFE is similar to other MHC class I-like molecules (Figure 1.5). Each of the first six exons encode one of the six distinct domains of the previously described protein, while the seventh exon is completely non-coding. The size of the sixth exon is 1056 base pair (bp) long, but only the first 41 bp are translated to amino acids. Therefore, the stop codon is located at the 5' part of this exon and the remaining downstream 1015 bp correspond to the HFE 3' untranslated region (UTR), along with exon 7 [Sanchez et al. 2001]. Many genes implicated in intracellular iron homeostasis display a post-transcriptional regulation based on the IRE/IRP system. However, the long-sought protein in iron metabolism, HFE, does not contain IREs nor is it known as being regulated by iron status.

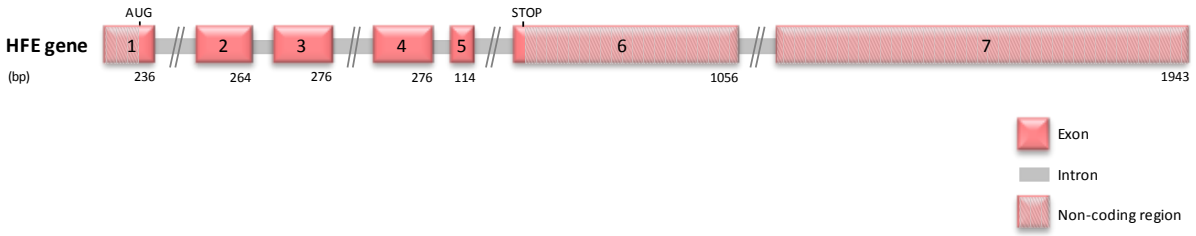

Figure 1.5. Schematic representation of the HFE gene. It is composed by seven exons, the first six exons encode for the six distinct domains of the immature HFE protein, while the seventh exon is non-coding. The size of the exons is given below the boxes in base pairs (bp).

In fact, very little is known regarding HFE transcriptional and post-transcriptional regulation. The comparison between the sequences of human, mouse and rat HFE promoter regions allowed the identification of several conserved transcription elements [Sanchez et al. 1998]. In vitro studies evidenced the *trans*-activation of HFE expression by liver-enriched C/EBPα, erythropoietic-specific GATA-1 [recognizes a consensus (T/A)GATA(A/G) motif] and ubiquitous Sp1 (named according to the original purification scheme that included sephacryl and phosphocellulose columns) transcription factors [Mura et al. 2004]. These data is consistent with the postulate that HFE acts for signaling iron status at the hepatic levels and discloses a coordinated expression to meet erythropoietic demands. Moreover, these authors found that the positive *cis*-regulating elements were characterized within the most proximal region of HFE (-1057/-8, relatively to the initiation codon), whereas a negative one extended upstream (-1485/-1057). Run off in vitro transcription revealed two major transcription initiation sites [Mura et al. 2004]. Intriguingly, in a failed attempt to characterize HFE transcription initiation site and promoter region, an HFE antisense mRNA comprising the 5' region of the gene was identified [Thenie et al. 2001]. This was found to be present in several human tissues and proven to negatively regulate HFE gene expression by in vitro coupled transcription-translation [Thenie et al. 2001].

The predominant HFE transcript is about 4.2 kb long. However, additional HFE mRNAs, both longer and shorter, have been reported [Jeffrey et al. 1999; Rhodes and Trowsdale 1999; Thenie et al. 2000; Sanchez et al. 2001]. Most of these transcripts were observed in human tissue mRNA Northern blots and further characterization is required [Thenie et al. 2000; Sanchez et al. 2001]. Reverse-transcription polymerase chain reaction (RT-PCR) allowed the identification of several alternative splicing forms in cell lines [Rhodes and Trowsdale 1999; Thenie et al. 2000; Sanchez et al. 2001]. These include the skipping of exon 2 (total or partial), the skipping of exon 3 (alone or combined with the skipping of exon 2), a partial skipping of exon 4 (alone or combined with the skipping of exon 3), among others. A study performed in HH patients allowed the identification of a putative soluble HFE variant due to the inclusion of intron 4 that was found in the Southern blot analysis of HFE RT-PCR products [Jeffrey et al. 1999]. Furthermore, a report by Sanchez and colleagues [2001] in which the complete exon 7 of HFE was described, also revealed alternative polyadenylation utilization in both intron 6 and exon 7 identified by 3' rapid amplification of cDNA ends (3' RACE) and expressed sequence tag analysis.

Human tissue HFE mRNA has a low ubiquitous expression, presenting higher expression levels in the liver, small intestine and spleen [Feder et al. 1996; Jeffrey et al. 1999; Thenie et al. 2000; Sanchez et al. 2001]. However, results are highly variable between studies. HFE expression levels in tissues such as the large intestine, pancreas, testis and lung, among others, are inconsistent. Even within the same report, the tissue HFE mRNA varies immensely depending for example on the probe used for Northern blot or in the ribonuclease protection assays [Feder et al. 1996; Sanchez et al. 2001]. On the other hand, immunohistochemistry studies indicate that HFE protein is expressed throughout the gastrointestinal tract (mainly in duodenal crypt cells), in the liver (hepatocytes, Kupffer cells, bile duct epithelial cells and sinusoidal lining cells), placenta (syncytiotrophoblasts), tissue macrophages, brain (capillary endothelial cells) and circulating monocytes and granulocytes [Parkkila et al. 1997a; Parkkila et al. 1997b; Bastin et al. 1998; Waheed et al. 1999; Parkkila et al. 2000; Zhang et al. 2004]. Once more, many differences can be observed among these studies. For instance, while some found no HFE expression in hepatocytes, other demonstrated that amongst the liver tissues, hepatocytes present the highest HFE protein level [Parkkila et al. 1997b; Bastin et al. 1998; Zhang et al. 2004]. There may be several reasons to explain these discrepancies, including factors affecting protein translation, stability and access of the antibody to its binding site on the HFE molecule. Also, intracellular localization of HFE protein can vary from cell surface to endosomal compartments to perinuclear staining and although TfR1 association can account for some explanation of cell-surface stability, many important questions remain unanswered.

As stated before, HFE protein has a dramatic impact on cell iron trafficking and on intestinal iron absorption, but interestingly, HFE gene expression is only modestly influenced by changes in cellular iron status [Ludwiczek et al. 2004; Theurl et al. 2005b]. All this bulk of information leads to questions that might be imperative towards understanding HFE post-transcriptional regulation. Which are the alternative HFE transcripts present in human tissues? And how abundant are they? What are the biological mechanisms involved in their genesis? How is the cellular distribution of these protein variants and how do they contribute to the maintenance of cellular and systemic iron homeostasis? The work developed throughout this thesis intends to answer these questions by characterizing the physiological significance of HFE variants and hopefully providing new insights towards HFE's function.

II.3. Disorders of disrupted iron homeostasis

The expression "iron metabolism" used worldwide to designate the combination of pathways involved in maintaining iron homeostasis is actually not correct, since iron itself is not metabolized in a classical sense. Accordingly, human iron disorders are invariably caused by imperfect iron balance or iron distribution. The most frequent are iron deficiency anemia, hemochromatosis and anemia of chronic disease.

Iron deficiency anemia results from unmet increased metabolic requirement or inadequate supply states, or the combination of the two. The depletion of the iron stores leads to poor hemoglobin synthesis in the maturing erythrocytes causing symptoms as pallor, fatigue and weakness [Andrews 1999]. Since our body is extremely dependent on the recycling of iron for red cell production, excessive blood loss is one of the most common causes of iron deficiency, disproportionally affecting young children and menstruating women [Andrews 1999; McLean et al. 2009]. In fact, iron deficiency anemia is a major public health problem, as the recent surveys show that it affects about one-quarter of the world's population [McLean et al. 2009]. Besides the common acquired condition, there are some rare forms of inherited iron deficiency anemia due to impairment of iron absorption or transport, which are caused by mutations in DMT1, transferrin or ceruloplasmin genes [Harris et al. 1995; Yoshida et al. 1995; Beutler et al. 2000; Priwitzerova et al. 2004; Mims et al. 2005; Aslan et al. 2007; Camaschella et al. 2007]. Anemia of chronic disease, also known as anemia of inflammation, has some features in common with iron deficiency anemia but is essentially a defect in iron recycling since macrophages that normally recycle iron are found to sequester it. Patients are affected with a wide variety of inflammatory conditions including arthritis, malignancies and infections. But only recently the underlying mechanisms of the iron sequestration have been found. The production of cytokines during inflammatory states leads to the overproduction of the iron-regulatory hormone hepcidin that will result in iron retention in the enterocytes and macrophages [Roy et al. 2003; Nemeth et al. 2004a].

On the other side of the coin, when the iron storage capacity is exceeded, the deposition of iron in the parenchymal tissue of organs will take place causing tissue damaging, a condition generally designated as iron overload. The affected patients present fatigue, depression and joint pain as complications that might eventually evolve to more severe phenotypes as liver cirrhosis, cardiomyopathies and diabetes [Beutler et al. 2003]. These iron overload disorders may be of primary (genetic) or secondary (acquired) cause. The latter, also considered

secondary hemochromatosis, are generally caused by problems of ineffective erythropoiesis (and worsened when blood transfusion is required), which may be inherited (e.g. thalassemia) or acquired (e.g. sideroblastic anemia) disorders [Beutler et al. 2003].

Amongst the inherited conditions causing primary iron overload, hereditary hemochromatosis is the most common. The recent discoveries concerning HH have provided new insights on iron homeostasis, so this common hereditary disease will now be discussed with some detail.

II.3.1. Hereditary hemochromatosis

The term hemochromatosis was firstly used in the 19th century to describe massive tissue iron deposition associated to diabetes, bronze pigmentation of the skin and cirrhosis [Trousseau 1865; von Recklinghausen 1889]. In the beginning of the 20th century the disease was considered hereditary and the work by Simon and co-workers in 1976 linked the disease to the major histocompatibility complex alleles HLA-A3 and B14 [Sheldon 1935; Simon et al. 1976]. Two decades later, through linkage-disequilibrium and full haplotype analysis, the HFE gene (originally named HLA-H) was identified and found mutated in the large majority of patients with hemochromatosis [Feder et al. 1996]. This autosomal recessive disorder is one of the most common genetic diseases in people of northern European descent, affecting about 1 in 300 individuals [Dadone et al. 1982; Edwards et al. 1988]. As mentioned, iron accumulation in the organs causes complications in the liver (cirrhosis and possibly hepatocellular carcinoma), heart (cardiomyopathies), joints (arthritis) and endocrine glands (diabetes and hypogonadotrophic hypogonadism) [Carthwright et al. 1979]. Therapeutic phlebotomies are generally used to reduce the iron burden from both plasma and stores, thereby decreasing disease morbidity and mortality if instituted early in the course of the disease [Bomford and Williams 1976].

The most common mutation associated with the disease is the substitution of cysteine for tyrosine at position 282 of the HFE immature protein (C282Y), accounting for about 85% of the patients [Feder et al. 1996; Adams et al. 2005]. However, the clinical penetrance of the C282Y homozygous individuals has been a topic of great debate, since many patients never develop clinical disease, and the proportion of who do remains controversial [Ryan et al. 2002; Ajioka and Kushner 2003; Beutler 2003]. In fact, the C282Y mutation is common in northwestern European populations, but also in the Portuguese population [Merryweather-Clarke et al. 2000; Cardoso et al.

2001]. Another mutation in HFE commonly associated to HH is the substitution of codon 63 from histidine to aspartic acid (H63D). Nonetheless, the clinical effects of H63D mutation appear to be limited [Feder et al. 1996; Gochee et al. 2002]. The risk of iron loading is dramatically reduced when comparing H63D homozygosity or H63D/C282Y heterozygosity (nearly 200-fold lower) to C282Y homozygosity [Risch 1997; Gochee et al. 2002]. Curiously, the mutation H63D is quite common in the general population, being present in 15-40% of Caucasians [Bacon et al. 1999; Merryweather-Clarke et al. 2000]. The high frequency of these mutations in the European population may be explained by selective advantage through the protection against anemia [Rochette et al. 1999]. Several other missense, nonsense and frameshift mutations have been described in the HFE gene, but, in general, a casual relationship between these mutations and the development of iron overload remains to be established [Barton et al. 1999; de Villiers et al. 1999; Mura et al. 1999; Piperno et al. 2000; Pointon et al. 2000; Beutler et al. 2002; Le Gac et al. 2003; Mendes et al. 2009; Pointon et al. 2009].

However, as it is often the case, the situation is more complex than originally thought. The clinical penetrance of HFE mutations is incomplete and both environmental and genetic factors can influence the course of the disease. Moreover, we now know that mutations in other genes, although more rarely, can cause hemochromatosis and HFE-associated hemochromatosis is frequently named classical or type 1 hemochromatosis [Pietrangelo 2004; Pietrangelo 2006]. The Online Mendelian Inheritance in Men (OMIM) data base currently lists four types of hemochromatosis, each caused by mutations involving a different gene. Type 3 hemochromatosis is caused by mutations in TfR2 gene and presents the higher resemblance to HFE-associated hemochromatosis in terms of age onset, iron accumulation distribution and clinical symptoms [Camaschella et al. 2000]. Type 2 or juvenile hemochromatosis is caused by mutations in hemojuvelin or hepcidin, thereby originating an earlier and more severe onset of the disease [Roetto et al. 2003; Papanikolau et al. 2004]. The type 4 (or ferroportin disease) is the only autosomal dominant and has more distinguishable features from the others, since it is not preceded by high transferrin saturation levels, iron accumulation is reticuloendothelial instead of parenchymal and there is a weak response to phlebotomy [Montosi et al. 2001; Njajou et al. 2001]. In fact, the inclusion of ferroportin as a type of hemochromatosis is a matter of discussion for some authors [Pietrangelo 2004; Andrews 2008]. But, in reality, all forms of HH result from either inappropriate levels of hepcidin or, in the case of mutations in the ferroportin gene, from resistance to hepcidin action [Bridle et al. 2003; Muckenthaler et al. 2003; Papanikolau et al. 2004; Kawabata et al. 2005; Porto et al. 2005]. It is now

accepted that the severity of the disease is directly associated with hepcidin levels. This hypothesis is favored by the fact that mutations in hepcidin aggravates the phenotype of C282Y homozygotes and from mice studies revealing that the overexpression of hepcidin can revert the iron overload in HFE knockout mice [Merryweather-Clarke et al. 2003; Nicolas et al. 2003; Jacolot et al. 2004; Viatte et al. 2006].

There are many inherited and environmental factors leading to hemochromatosis heterogeneity, but the comprehension of the complex pathways controlling hepcidin expression has allowed to consider its deregulation as the common etiology of the disease. The role of the genes involved in these pathways and in HH development still requires further investigation to allow comprehension of the immense variation in clinical presentation and hopefully lead to therapies based on hepcidin levels manipulation.

III. Post-transcriptional regulation of gene expression

Gene expression regulation allows genetically identical cells of a multicellular organism to produce the adequate proteins in the right cell at the correct time. In fact, each cell type is designed to have a specific role that contributes to the overall functioning of the organism [Orphanides and Reinberg 2002]. Eukaryotic gene expression begins with transcription, followed by multiple post-transcriptional processes that carry out the capping, splicing, polyadenylation and export of the mRNA to the cytoplasm for translation [Maniatis and Reed 2002]. This cascade of events that take place during gene expression, from the transcription of genetic information hoarded in the DNA down to the eventual protein production and post-translational processing, undoubtedly encloses some of the most significant biochemical pathways for the living organisms. As expected, numerous regulatory mechanisms have arisen at multiple points to preserve the accuracy of gene expression. Amongst them, lay two extremely important mechanisms that can act alone or combined to ensure the proper levels of transcripts and the profuse gene expression diversity [Black 2003; Green et al. 2003; Lewis et al. 2003; Maquat 2004]. These are the alternative splicing and nonsense-mediated mRNA decay (NMD), which will be further enlightened in this thesis due to their role in HFE's gene expression regulation.

III.1. General aspects of the gene expression pathway

The level of the gene expression is primarily regulated by transcriptional factors that bind to DNA regulatory sequences upstream the transcription start site. Following a determined cellular or environmental stimulus, transcription factors are activated and interact not only with the gene regulatory elements, but also with components of the transcription machinery to promote access to DNA and the recruitment of RNA polymerase II (RNAP II) to the transcription start site [Proudfoot et al. 2002]. Soon after the beginning of transcription (initiation), a cap structure is added to the 5' end of the nascent RNA that protects it from nuclease degradation, promotes its later export to the cytoplasm and also stimulates translation [Lewis and Izaurralde 1997]. Then, RNAP II moves 5' to 3' of the gene sequence to extend the transcript (elongation) and the non-coding sequences (introns) are removed by pre-mRNA splicing [Padgett et al. 1986; Uptain et al. 1997]. When reaching the end of the gene, RNAP II stops transcription (termination). The newly synthesized RNA is cleaved and a polyadenosine [poly(A)] tail is added to the 3' end of the transcript (polyadenylation) [Wahle 1995]. Since all these processes occur within the nucleus, the mature transcript must be transported to the cytoplasm for translation. This export is mediated by factors that bind mRNA molecules and interact with the proteins that line the nuclear pore [Zenklusen and Stutz 2001]. Once in the cytoplasm, the translation of the mRNA into protein takes place on large ribonucleoprotein complexes, the ribosomes. The translation process is mechanistically similar to transcription, commencing with the location of the start codon by translation initiation factors together with ribosome subunits, further evolving to elongation and termination phases [Moldave 1985; Gebauer and Hentze 2004]. The nascent polypeptide chain undergoes proper folding and frequently post-translational modifications to generate the final active protein [Han and Martinage 1992]. Although appearing as a quite straightforward multistep process, the underlying events from RNA transcription to post-translational protein modifications are highly complex and regulated processes, with frequent coupling among steps previously thought to be distinct in place and time [Maniatis and Reed 2002; Orphanides and Reinberg 2002; Kornblihtt et al. 2004]. We will now focus on those processes that can have a significant effect on gene expression regulation, specifically on the expression of the HFE gene.

III.2. Constitutive and alternative splicing

Pre-mRNA splicing is an essential step for the expression of eukaryotic genes, since the exons that will make up the mRNA product are interrupted by non-coding sequences (introns) present in the DNA and in the nascent pre-mRNA transcript. To generate correct mature mRNAs, intron removal and the concomitant joining of the exons is precisely and efficiently carried out by the spliceosome, a macromolecular ribonucleoprotein complex that assembles on the pre-mRNA [Padgett et al. 1986; Jurica and Moore 2003]. This complex assembly is guided by weakly conserved consensus sequences present at the ends of the introns: the 5' splice site, 3' splice site and the branch point site [Hertel 2008]. Briefly, the general mechanism of splicing is carried out by the spliceosome, an assembly of five small nuclear ribonucleoprotein (snRNP) particles (U1, U2, U4, U5 and U6) that are associated with a large number of additional structural proteins [Black 2003; Jurica and Moore 2003]. The stepwise initiation of splicing and coalition of the spliceosome begins with the recognition of the transcript 5' and 3' intronic splice site by the spliceosome components snRNP U1 and U2AF (U2 auxiliary factor) factors respectively, whereas the splicing factor 1 binds the branch point. This protein-RNA structure is designated the early (E) complex. Subsequently, the E complex recruits the branch point-bound U2 snRNP, being converted to the A complex. The B complex is formed when the tri-snRNP particle containing U4, U5 and U6 joins the spliceosome, which then undergoes a structural transformation and becomes the C complex [Black 2003; Jurica and Moore 2003; House and Lynch 2008]. This extensive remodelling of the C complex enables the production of an active site that is capable of catalyzing the transesterification reaction required for exon ligation and lariat release [Graveley 2000].

The specificity and activity of the spliceosome much relies on the recognition of the above mentioned motifs to define exon-intron boundaries. Nevertheless, it is not fully understood how splice sites (ss) are selected, essentially due to the degeneracy of the splicing regulatory regions such as the 5' and 3' ss, branch point and exonic/intronic adjacent sequence elements [Hertel 2008]. There are four additionally important classes of splicing motifs necessary for proper splice-site identification: ESEs (exonic splicing enhancers), ESSs (exonic splicing silencers), ISEs (intronic splicing enhancers) and ISSs (intronic splicing silencers). So, the inclusion of a given exon is under a combinatorial control of multiple regulatory RNA elements as well as the inherent strength or weakness of the flanking splice sites [Matlin et al. 2005; Hertel 2008]. The ESEs and ISEs provide binding sites for a handful of *trans*-acting factors

that promote exon inclusion, predominantly of the SR family of proteins, named due to their high serine-arginine content [Graveley 2000; Long and Caceres 2009]. ESSs and ISSs are recognized by splicing suppressors, generally members of the heterogeneous nuclear RNP (hnRNP) family of proteins, a structurally set of RNA-binding proteins [Black 2003]. Importantly, these *trans*-acting factors may have an ubiquitous or a tissue-specific expression and therefore encompass an especially important role in the regulation of alternative splicing. Although SR proteins and hnRNPs do not always correlate with enhancers and silencers, respectively, this simplification helps to illustrate the emergent concept of a "splicing code", in which the splicing pattern of a gene could be determined by the interplay of proteins along a nascent transcript [Matlin et al. 2005]. In fact, very recent work by Barash and colleagues [2010] intended to decipher the splicing code and revealed extremely important new data regarding widespread regulatory strategies for alternative splicing events, sustained by the combinations of hundreds of RNA features.

III.2.1. Alternative splicing and gene expression diversity

Alternative splicing greatly expands the genetic information content, since the versatility of the transcriptome allows that multiple different mRNAs arise from one individual gene, in many cases encoding for functionally distinct proteins [Modrek and Lee 2002; Stamm et al. 2005]. The progressive generation of larger sequence datasets has revealed that it is more the rule than the exception, indicating that 92-94% of human genes undergo alternative splicing [Pan et al. 2008; Wang et al. 2008]. Moreover, about 86% of these alternatively spliced genes have a minor isoform content of at least 15%, indicating a possible significant impact of these isoforms in gene expression diversity. Several alternative splicing events may occur starting from a single primary transcript (Figure 1.6). Most alternative splicing events may be classified into five basic splicing patterns: exon skipping, alternative 5' splice sites, alternative 3' splice sites, mutually exclusive cassette exons and intron inclusion. Other mechanisms may occur, changing the transcription initiation site or 3' end processing/termination sites. All of these are simplified patterns of alternative splicing and these events may be combined (and influence each other), generating more complex splicing patterns [Zavolan et al. 2003; Kornblihtt 2005]. Most of these alternative splicing events occur within the open reading frame, greatly expanding the human proteome [Kan et al. 2001; Modrek and Lee 2002]. When the sequence of the encoded proteins is affected, it may influence their

function and properties, such as stability, intracellular localization, binding properties, enzymatic activity and post-translational modifications [Wang and Cooper 2007]. On the other hand, when untranslated regions are involved, alternative splicing may affect mRNA stability, translation efficiency and mRNA localization [Wang and Cooper 2007]. The levels of gene expression may also be altered when the alternative splice variant gives rise to a premature translation termination codon (PTC), leading to the degradation of the transcript by the nonsense-mediated mRNA decay (NMD). There is growing evidence that the mechanistic coupling of alternative splicing and NMD provides an often-used means of regulating gene expression [Lewis et al. 2003]. Indeed, one third of the alternative splicing events generate PTCs [Green et al. 2003; Lewis et al. 2003]. The NMD mechanism and its role in regulating gene expression will now be discussed.

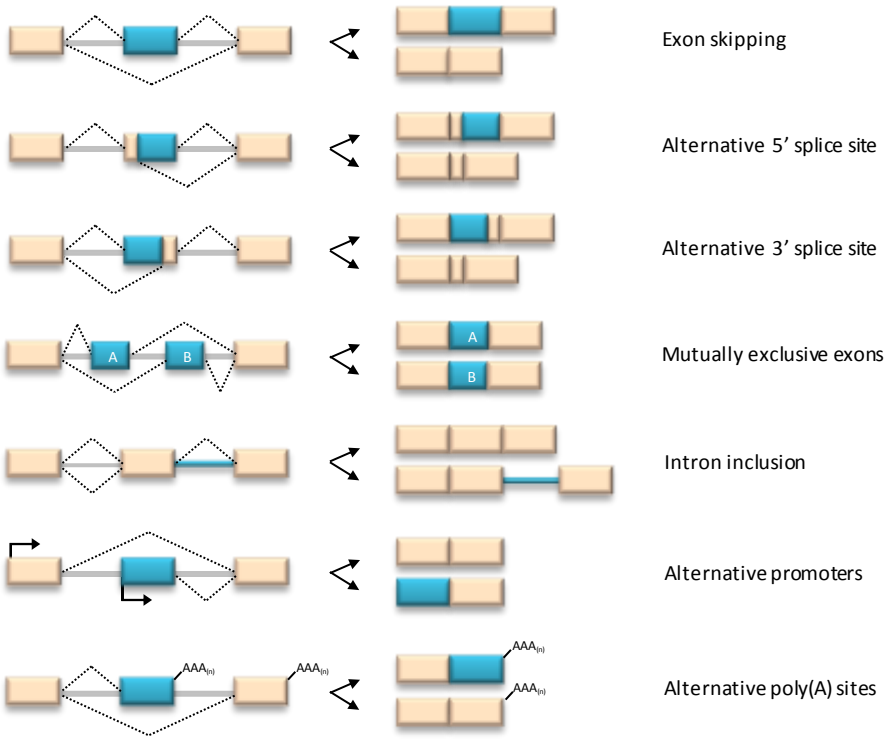

Figure 1.6. Elementary alternative splicing events. The depicted alternative splicing events generate functionally distinct transcripts. Dashed lines represent different possibilities for splice site joining. Constitutive exons are shown in beige and alternative ones in blue. (Adapted from Blencowe 2006).

III.3. Nonsense-mediated messenger RNA decay

Nonsense-mediated mRNA decay is a post-transcriptional eukaryotic mRNA surveillance mechanism responsible for the rapid degradation of transcripts harboring a PTC. Therefore, NMD limits the production of C-terminally truncated polypeptides and protects the cell from their possible deleterious dominant-negative or gain-of-function effects [Wagner and Lykke-Andersen 2002; Holbrook et al. 2004; Maquat 2004; Chang et al. 2007].

PTCs may be generated by various types of germline/somatic alterations in the DNA, as nonsense or frameshift mutations (leading to PTCs downstream of the shift) but also mutations occurring within either exons or introns that result in inaccurate pre-mRNA splicing (resulting in a shift of the open reading frame). Actually, it is estimated that about 30% of all known disease-associated mutations generates a PTC-containing mRNA. Errors during transcription or mRNA processing may also originate PTCs [Mendell and Dietz 2001; Holbrook et al. 2004; Rehwinkel et al. 2005].

Interestingly, PTCs can arise from regulated mRNA processes as well. The presence of an upstream open reading frame (uORF), the inability of incorporating a selenocysteine at a UGA codon, alternative splicing events or the presence of an intron in the 3' UTR, can all lead to PTCs recognized as such by NMD. So, on top of its role in clearing the cell of aberrant transcripts, recent evidence has disclosed an important function for NMD in controlling the expression levels of wild type genes, which are implicated in several essential biological processes [Holbrook et al. 2004; Mendell et al. 2004; Neu-Yilik and Kulozik 2008].

III.3.1. NMD rule and activation

One pivotal feature for NMD is to distinguish the termination codons that specify the end of an open reading frame from those that interrupt it. To achieve this, mammalian NMD relies on two pivotal spatially separated processes of gene expression, pre-mRNA splicing and translation. Although the mechanism to discriminate between premature and normal stop codons is only partially understood, the ability of a PTC to elicit rapid degradation of the mRNA depends upon a positional effect [Nagy and Maquat 1998; Conti and Izaurralde 2005; Silva et al. 2006; Muhlemann et al. 2008].

The fact that translation was shown to be mandatory for NMD triggering arose the possibility that a "mark" had been previously set on the mRNA. Studies in mammalian

systems led to the observation that stop codons are generally recognized as premature if they are located at more than 50-54 nucleotides (nt) upstream to the 3' most exon-exon junction [Nagy and Maquat 1998] (Figure 1.7). Consistent with the "50-54 nt boundary rule" is the fact that normal termination codons usually lay within the last exon of a gene or that naturally intronless genes are insensitive to NMD [Nagy and Maquat 1998; Maquat and Li 2001; Brocke et al. 2002]. Since PTC recognition is intrinsically dependent on exon-exon junctions in mammals, the splicing mechanism was associated to NMD. It is now accepted that the exon junctions are marked on the mRNA by a multiprotein exon-junction complex (EJC) deposited by the spliceosome at a position of 20-24 nt upstream of each exon-exon junction [Le Hir et al. 2000].

It is currently acknowledged that NMD activation depends on the EJC maintenance on the mRNA. As mentioned, during pre-mRNA splicing, the EJC is deposited 5' to the exon-exon junctions. While the mRNA is transported to the cytoplasm the up-frameshift (UPF) proteins 3 and 2 are recruited to the EJC. In a pioneer round of translation, the translating ribosome dislodges the EJCs when reaching the physiological termination codon and so the mRNA remains stable for multiple subsequent translational rounds [Dostie and Dreyfuss 2002; Lejeune et al. 2002; Alkalaeva et al. 2006]. However, when the translating ribosome encounters a PTC and at least one EJC is still bound downstream, the eukaryotic release factors 1 and 3 will bind to the ribosome and recruit the UPF1 protein, which promotes the binding of SMG1 (suppressor with morphogenetic effects on genitalia 1) factor [Czaplinski et al. 1998; Wang et al. 2001; Kashima et al. 2006]. UPF1 and SMG1 will interact with EJC-bound UPF2, triggering UPF1 phosphorylation by SMG1, event that promotes the recruitment of SMG5-SMG7 and (or) SMG6 [Anders et al. 2003; Chiu et al. 2003; Ohnishi et al. 2003; Fukuhara et al. 2005; Kashima et al. 2006]. These factors will promote UPF1 dephosphorylation by protein phosphatase 2A, enabling the recycling of NMD factors for another round of surveillance [Chiu et al. 2003; Ohnishi et al. 2003]. The rapid decay of the transcript is achieved by SMG7 alone or associated with SMG6 possibly through the recruitment of the decay machinery [Unterholzner and Izaurralde 2004; Glavan et al. 2006; Huntzinger et al. 2008; Eberle et al. 2009].

The degradation pathways of PTC-containing mRNAs are not fully understood in mammals. It is thought to involve both decapping with subsequent 5' to 3' exonucleolytic activity and deadenylation followed by 3' to 5' exonucleolytic degradation [Lykke-Anderson 2002; Chen and Shyu 2003; Lejeune et al. 2003; Couttet and Grange 2004]. But an additional decay mechanism has been brought to light, the endonucleolytic cleavage, very likely mediated by SMG6 [Stevens et al. 2002; Huntzinger et al. 2008; Eberle et al. 2009].

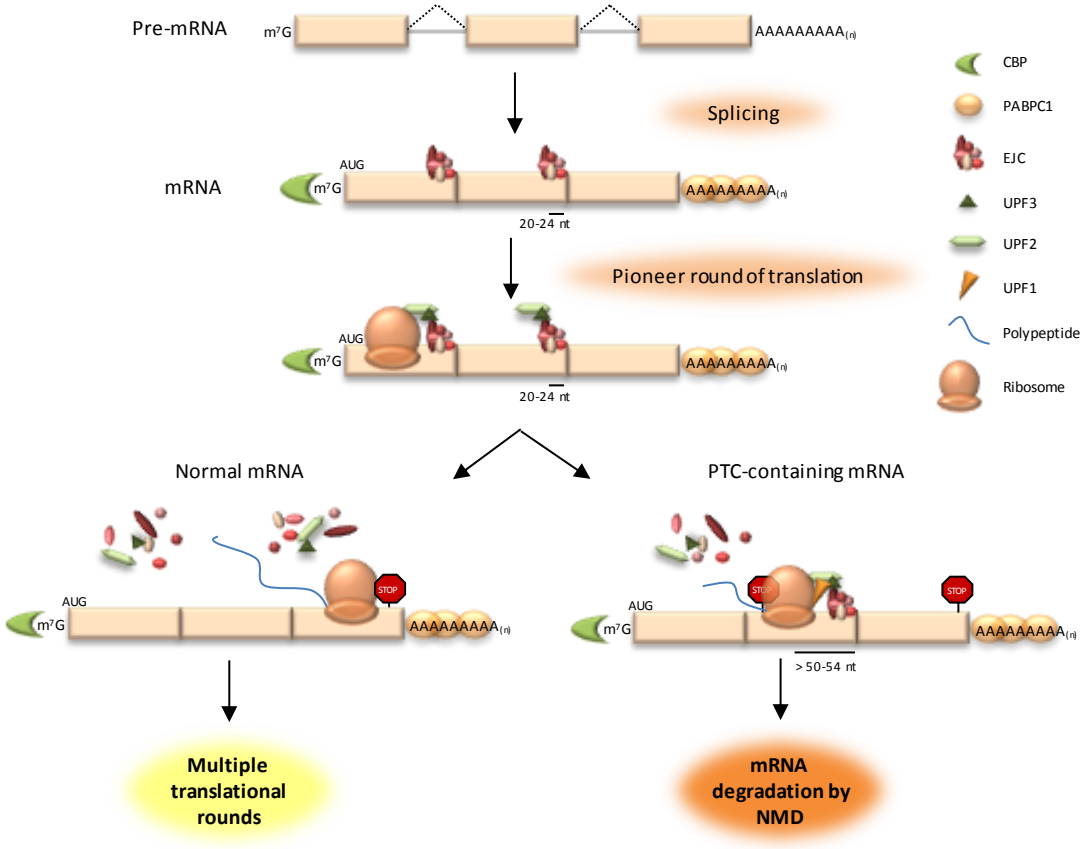

Figure 1.7. Premature stop codon recognition in mammals. When pre-mRNA splicing occurs, a multiprotein exon-junction complex (EJC) assembles 20-24 nt upstream to each exon-exon junction. During the pioneer round of translation, the ribosome will displace the EJCs as it proceeds with elongation of the polypeptide. In normal transcripts, the ribosome encounters the stop codon and all EJC have been displaced from the mRNA, so normal termination will take place. Conversely, if any EJC(s) is still bound to the mRNA when the ribosome reaches a PTC (located 50-54 nt upstream to the 3' most exon-exon junction), NMD will be triggered and the transcripts committed to rapid decay. CBP, cap-binding protein; PABPC1, cytoplasmic poly(A)-binding protein 1.

III.3.2. Gene expression regulation by NMD

As previously mentioned, NMD is an important contributor to the fidelity of gene expression, preventing translation of potentially harmful truncated proteins. Furthermore, in addition to disposing the cell of unintended aberrant transcripts, NMD also has a potential role to play in the normal regulation of gene expression [Rehwinkel et al. 2006].

Gene expression profiling in yeast, fruitfly and human cells have shown that NMD is able to regulate about 3-10% of the transcriptome [Lelivelt and Culbertson 1999; He et al. 2003; Mendell et al. 2004; Rehwinkel et al. 2005; Wittmann et al. 2006]. These studies have revealed a diverse repertoire of transcripts controlled by NMD, which are involved in a myriad of cellular processes such as cell cycle, transcription, intracellular transport, DNA repair, cytoskeleton organization and biogenesis, telomere maintenance, signal transduction, among others [He et al. 2003; Mendell et al. 2004; Rehwinkel et al. 2005; Rehwinkel et al. 2006; Wittmann et al. 2006]. So, it has become accepted that NMD can be applied to adapt protein expression to the physiological needs of cells and organisms. Under these circumstances, it is not clear if NMD is solely limiting protein expression or if, upon certain physiological stimuli, NMD itself can be regulated to allow the expression of functional truncated proteins [Bamber et al. 1999; Donnadieu et al. 2003; Neu-Yilik and Kulozik 2008].

It was proposed that about one-third of the alternatively spliced mRNAs contain a PTC, implying a widespread coupling of alternative splicing and NMD [Lewis et al. 2003]. This discloses a major effect of alternative splicing in the generation of an mRNA isoform that is targeted for degradation rather than translated into protein. Splicing factors can regulate gene expression by generating productive or unproductive splicing isoforms [Lewis et al. 2003]. In fact, it was shown that the splicing factors SC35 (so-called since it was firstly identified by the anti-spliceosome antibody alpha SC-35) and PTB (polypyrimidine tract binding) protein can downregulate their own expression by activating an alternative splice pattern that result in mRNA isoforms with PTCs [Sureau et al. 2001; Lewis et al. 2003; Wollerton et al. 2004]. Curiously, NMD factors themselves can exert an inhibition of their own expression, illustrated by the NMD regulation of SMG5 factor [Mendell et al. 2004; Rehwinkel et al. 2005; Chan et al. 2007].

A broader role for NMD has been unraveled through the studies that show that physiological mRNA transcripts that encode for functional full length proteins can be NMD substrates. Indeed, it was demonstrated that in mammalian cells depleted of the NMD factor UPF1 about 5% of the analyzed transcripts become upregulated [Mendell et al. 2004]. A similar result was observed by knocking down UPF2 since the microarray data revealed that about 1.5% of the mRNAs were consistently upregulated at least two-fold [Witmman et al. 2006]. In general, the upregulated transcripts shared similar features that enable them to be recognized as NMD substrates, such as uORFs in the 5' untranslated region, introns in the 3' untranslated regions, selenocysteine codons and, as expected, alternative splicing events that introduce nonsense codons or frameshifts. So, the context of a spliced intron at least 50 nucleotides downstream the initiation codon, required to trigger NMD, was obeyed in all these transcripts.

Considering NMD regulation of transcripts encoding for selenocysteine-containing proteins, it was shown in a few studies that mRNAs containing in-frame UGA triplets that encode for selenocysteine when selenium is abundant but are interpreted as PTCs under selenium deprivation [Moriarty et al. 1998; Sun et al. 2001].

The implication of NMD in the regulation of genes with uORFs has been addressed for quite some time now, mainly from studies in yeast [Pinto et al. 1992; Oliveira et al. 1993]. These mRNAs appear consistently as NMD targets, with NMD being triggered or impaired according to the cellular needs [Gaba et al. 2005; Neu-Yilik and Kulozik 2008]. Not surprisingly, uORF-containing transcripts have surfaced in gene expression profiling analyses after NMD silencing in yeast, fruitfly and human cells [Messenguy et al. 2002; Mendell et al. 2004; Wittmann et al. 2006; Hansen et al. 2009].

The presence of an intron in the 3' UTR of a pre-mRNA also potentially targets the mature mRNA to NMD. This was shown to be the case of some human genes, either by expression of the normal transcript or due to alternative splicing events [Mendell et al. 2004; Banihashemi et al. 2006; Wittmann et al. 2006]. All these mRNAs are unified by the presence of a spliced intron at least 50 nucleotides downstream of a termination codon, a context sufficient to initiate NMD. Actually, this specific architecture is present in HFE mRNA, since the native stop codon present in the major HFE transcript is located 1015 nt from the downstream non-coding exon-exon boundary [Sanchez et al. 2001]. We therefore hypothesize that the HFE gene can be included in this group of NMD-regulated genes. The work developed in this thesis addresses

this hypothesis by analyzing the expression levels of HFE transcripts when NMD is impaired, providing novel perspectives in what regards HFE's gene expression regulation.

CHAPTER 2

Differential HFE gene expression regulation by alternative splicing in human tissues

Author's note

The data presented in this chapter has been submitted for publication, with minor modifications, to the PLoS ONE Journal. This work was performed by Rute Martins, Bruno Silva, Daniela Proença and Paula Faustino. The first two authors have contributed equally to this work. I was involved in the identification and characterization of the HFE splice isoforms by RT-PCR and qPCR and performed the transfections to the immunoprecipitation analysis of the HFE splice variants. Moreover, I have contributed to the design of the experiments and to the writing of the article. Bruno Silva was involved in the identification and characterization of the HFE splice isoforms by RT-PCR and qPCR and carried out the transfections for the immunofluorescence assays of the HFE splice variants. Daniela Proença has done most of the RT-PCR and qPCR analysis. Paula Faustino was responsible for the designed and supervision of the work as well as for the writing of the manuscript.

I. Abstract

The pathophysiology of HFE-derived hereditary hemochromatosis and the function of HFE protein in iron homeostasis remain uncertain. Also, the role of alternative splicing in HFE gene expression regulation and the possible function of the corresponding proteins are still unknown. The aim of this study was to gain insights into the physiological significance of these alternative HFE variants.

Alternatively spliced HFE transcripts in diverse human tissues were identified by RT-PCR, cloning and sequencing. Total HFE transcripts, as well as two alternative splicing transcripts were quantified using a real-time PCR methodology. Intracellular localization, trafficking and protein association of GFP-tagged HFE protein variants were analyzed in transiently transfected HepG2 cells by immunoprecipitation and immunofluorescence assays.

Both level- and tissue-specificity are presented by the alternatively spliced HFE transcripts. Concerning the exon 2 skipping and intron 4 inclusion transcripts, the liver has the lowest relative level, while the duodenum presents one of the highest amounts of these isoforms. The protein resulting from exon 2 skipping transcript is unable to associate with β2M and TfR1 revealing a retention at the endoplasmic reticulum. On the other hand, the intron 4 inclusion transcript gives rise to a truncated soluble protein HFE that is mostly secreted by cells to the medium in association with β2M.

HFE gene post-transcriptional regulation is clearly affected by a tissue-dependent alternative splicing mechanism. Among the corresponding proteins, a soluble HFE isoform stands out, that, upon being secreted into the bloodstream, may act in remote tissues. It could be either an agonist or antagonist of the full length HFE, through hepcidin expression regulation in the liver or by controlling dietary iron absorption in the duodenum.

II. Introduction

Maintaining iron homeostasis is essential, as both iron deficiency and iron excess are associated with cellular and organismal dysfunction. This homeostasis is dependent upon a tight link between body iron requirements, iron recycling from macrophages and intestinal iron absorption. However, how this complex mechanism is controlled remains largely to be understood.

HFE is a major histocompatibility complex class I-like protein that is mutated in Hereditary Hemochromatosis (OMIM 235200), a common autosomal recessive disorder of iron metabolism [Feder et al. 1996]. The disease is characterized by excessive intestinal iron absorption and iron deposition in organs such as liver, heart and pancreas, potentially leading to cirrhosis, hepatocellular carcinoma, diabetes, cardiac failure and arthritis [Cartwright et al. 1979]. HFE is a transmembrane protein formed by six distinct domains: a signal peptide, three extracellular domains (α1, α2 and α3), a transmembrane region and a short cytoplasmic tail [Feder et al. 1996; Lebron et al. 1998]. It assembles with β2M to form a heterodimer expressed at the cell surface. The most common HH-associated HFE mutation, C282Y, abrogates the disulfide bond in the protein α3 domain and prevents its binding to β2M and cell surface presentation [Feder et al. 1997].

HFE is therefore a key component of human iron homeostasis but its precise role is still undefined. In fact, HFE protein has been detected in various cell types. It is expressed throughout the gastrointestinal tract as well as in macrophages and monocytes [Parkkila et al. 1997b; Parkkila et al. 2000]. In the liver, HFE was shown to be present on Kupffer cells, endothelium and hepatocytes [Bastin et al. 1998; Zhang et al. 2004]. In a variety of transfected cells, HFE co-localizes with transferrin receptor 1 at the cell surface and in a perinuclear compartment, namely the endosomal compartment [Gross et al. 1998; Griffiths et al. 2000; Ramalingam et al. 2000]. At cell surface, HFE can interact with TfR1 that binds diferric-iron-loaded transferrin (Fe2-Tf) [Bennett et al. 2000]. These complexes are endocytosed within vesicles, releasing iron into the cell. HFE also interacts with the liver-specific TfR1 homologue, TfR2 [Chen et al. 2007]. Recently, it was proposed that, in the hepatocyte, HFE is partitioned between TfR1 and TfR2, whereas an increase in Fe2-Tf saturation results in stabilization of TfR2 protein and degradation of TfR1 mRNA [Chen et al. 2009]. Under these conditions, HFE should shift away from TfR1 towards TfR2, so the TfR2-HFE complex is likely

to be part of the iron sensing complex involved in the induction of the iron regulatory hormone hepcidin [Schmidt et al. 2008; Fleming 2009; Gao et al. 2009; Gao et al. 2010].

The HFE gene (formerly known as HLA-H) is located at 6p21.3 and its genomic structure resembles other MHC class I molecules [Feder et al. 1996]. It is known that alternative splicing mechanism is a widespread mean for producing polypeptide diversity from a single gene [Modrek and Lee 2002; Johnson et al. 2003]. Accordingly, alternative splicing is a common process of producing MHC class I protein isoforms. For instance, HLA-G, which is a non-typical MHC class I protein that presents significant structural homology to HFE, shows alternative splicing expression regulation and some of the isoforms produced have specific biological functions [Riteau et al. 2001; Hviid 2006; Sangrouber et al. 2007]. Also, alternative splicing is frequently observed in the expression of iron metabolism-related genes, e.g. the IRE and non-IRE mRNA isoforms of the DMT1 gene which are differentially expressed in the duodenum and other tissues. It was postulated that the switching between these two mRNAs allows the regulation of iron uptake by this transporter into the enterocyte [Lee et al. 1998].

Previous studies have shown that HFE gene expression is subjected to alternative splicing [Jeffrey et al. 1999; Rhodes and Trowsdale 1999; Thenie et al. 2000; Sanchez et al. 2001]. The predominant HFE transcript has about 4.2 kb, but additional transcripts have also been reported, which seem to differ in both level- and the tissue- or cellular-specificity. However, the identification of HFE alternative transcripts, their tissue-specificity and abundance, as well as the biological significance of the corresponding isoforms, remains to be clarified.

As a consequence of alternative splicing soluble protein isoforms can be originated assuming, in some cases, an important regulatory role in physiological processes. Actually, a splice isoform of HFE mRNA was described and, although not studied at protein level, it was suggested that the corresponding soluble peptide might regulate cellular iron transport [Jeffrey et al. 1999]. Additionally, functional analysis with an artificially created β2M-HFE monochain [mimicking a soluble HFE (sHFE)], was shown to effectively reduce Tf uptake into cells [Laham et al. 2004]. However, this did not correlate to any changes in TfR1 or ferritin synthesis, in contrast to the normal HFE-induction. These findings suggest that a soluble β2M-HFE monochain acts differently that the full length protein. Nevertheless, the existence and the biological function of a putative sHFE isoform remained elusive.

In this study, we have characterized several HFE alternatively spliced transcripts in a variety of human tissues, their relative abundance and tissue-specificity. Raising the hypothesis that some of the corresponding protein variants might have a biological role, we analyzed those resulting from exon 2 skipping and intron 4 inclusion. By studying their intracellular localization, trafficking and assembly, we gained insights about their physiological significance. Therefore, we demonstrated that a sHFE isoform is secreted into the medium, maintaining its association to the chaperone β2M. So, we suggest that a sHFE produced in a variety of human tissues may be secreted into the bloodstream and thus act by association with cell surface expressed transferrin receptors in remote tissues. There, playing a role as an agonist or antagonist of the wild type HFE, it might modulate hepcidin expression in the liver or regulate dietary iron absorption in the duodenum.

III. Materials and Methods

III.1. First strand cDNA synthesis and polymerase chain reaction

First strand cDNA synthesis was performed using 3 μg of total RNA from eight human tissues [small intestine, spleen, liver, testis, ovary, duodenum, heart and kidney (BD Clontech or Ambion)] and HepG2 cell line with the SuperScript® II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. A 1:1 mixture of random hexamers (Invitrogen) and oligo (dT) were used as primers.

A PCR covering the entire HFE coding region was performed to synthesize cDNAs using primers #1 and #2. All primers used in this chapter are listed in Table 2.1. A specific PCR to amplify the region between HFE's exon 4 to 5 was done using primers #3 and #4.

All products obtained in both PCRs were cloned into the pCR®-TOPO-XL® vector (Invitrogen), sequenced with BigDye® Terminator v1.1 Sequencing Standard kit using M13 reverse and T7 promoter primers and analyzed with the ABI Prism 3100 automatic sequencer (Applied Biosystems).

III.2. Quantitative real-time PCR

The quantification of the alternative splicing HFE transcripts was conducted using real-time PCR performed on an ABI Prism 7000 Sequence Detection System. Primers were designed using the ABI Primer Express software to amplify specific amplicons for the total HFE (exon 6, primers #5 and #6), for the exon 2 skipping (exon 1-3 boundary, primers #1 and #7) and for the inclusion of intron 4 (intron 4 - exon 5 boundary, primers #8 and #9).

Synthesis of cDNA from each tissue was carried out as before. Each cDNA sample was diluted 5-fold to guarantee accurate pipetting and 5 μL added to 5 μmol primers and SYBR Green Master Mix (Applied Biosystems). The cycling parameters used in all transcripts tested were: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 65°C. Quantification of gene expression was performed by the absolute standard curve method using serial dilutions of plasmids carrying the corresponding cDNA.

Table 2.1. DNA oligonucleotides used in the current work.

Primer	Location	Sequence (5' → 3')
#1	Exon 1	ATGGGCCCGCGAGCCAGGCCG
#2	Exon 6	GTCTCCTTCCCACAGTGAGTCTGCAGGCTG
#3	Exon 4	GAACATCACCATGAAGTGGCTGAAGG
#4	Exon 5	GAACAAAATTCCAATGAACAAGATGACG
#5	Exon 6	CTACGTCTTAGCTGAACGTGAGTGA
#6	Exon 6	TGTCTCCTTCCCACAGTGAGTCT
#7	Exon 1 / 3	TGCAGGGTGTGGGACTGCAGCAAGCG
#8	Intron 4	GGCAATCAAAGGCTTTAACTTGCTTTT
#9	Intron 4 / Exon 5	CCAGACGGTGAGGGCTCTAA
#10	Exon 1	TTTTGGTACCATGGGCCCGCGAGCC
#11	Exon 6	TTTTGGATCCCACTCACGTTCAGCTAAGACGTAGTGCCC
#12	Exon 4	GGGAAGAGCAGAGATATACGTACCAGGTGGAGCACCC
#13	Exon 4	GGGTGCTCCACCTGGTACGTATATCTCTGCTCTTCCC
#14	Intron 4	TTTTGGATCCCACATACCCCAGATCACAATGAGG

III.3. Plasmid constructs

The cloning of HFE cDNA into the pEGFP-N1 (Clontech) took advantage of the RT-PCR products previously cloned into pCR®-TOPO-XL vector. Therefore, we used a construct already containing the total HFE cDNA and performed an amplification of the entire HFE coding sequence with primers #10 and #11, containing the KpnI and BamHI linkers inserted immediately next to the translational start and stop codons (that was modified in order to allow fusion to GFP open reading frame), respectively. Both pEGFP-N1 vector and PCR product were digested with the KpnI and BamHI endonucleases to clone the full length HFE

cDNA fused to GFP (pEGFP_HFE_full length). The same method was performed to create pEGFP_HFE_skip2 construct, to mimic the exon 2 skipping splicing transcript. To clone the C282Y mutant control, the pEGFP_HFE_full length was amplified using mutagenic primers #12 and #13, along with QuickChange® Site-Directed Mutagenesis Kit (Stratagene), as indicated by the manufacturer. In order to clone the splice variant in which the intron 4 is included, an antisense primer containing a BamHI linker (primer #14), along with primer #10 were used to amplify this exon 1 - intron 4 fragment using cDNA from small intestine as a template for the PCR. Once more, the KpnI and BamHI endonucleases were used to clone this cDNA fragment fused to GFP, creating the pEGFP_HFE_ivs4 construct. Final sequence analysis was performed to confirm that all constructs contained the correct sequence.

III.4. Cell culture and transient transfections

HepG2 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) in a 37^{o}C/5% CO2 atmosphere. For transient transfections, cells were seeded in 35 mm plates at a confluence of 5x10^{5} or 8x10^{5} cells per well, for immunofluorescence or immunoprecipitation assays, respectively. Twenty-four hours after seeding, 2 μg of the pEGFP_HFE constructs were used together with Lipofectamine™ 2000 Transfection Reagent (Invitrogen) or Lipofectamine™ LTX and PLUS™ Reagents (Invitrogen), for immunofluorescence and immunoprecipitation assays, respectively. Transfections were performed following the manufacturer's instructions. For immunoprecipitation and Western blot analyses, cells and supernatant were harvested 48 hours post-transfection, whereas for immunofluorescence assays, cells were analyzed 24 hours after transfection.

III.5. Immunofluorescence assays

Twenty-four hours after transfection, cells were washed in PBS, fixed in methanol at -20^{o}C and then washed again. Subsequently, cells were permeabilized and blocked simultaneously in a PBS solution containing FBS 10% and Triton X-100 0.5% for 30 min at room temperature. Incubation with the selected primary antibodies [rabbit anti-β2M (Abcam) at 1:200 dilution; mouse anti-TfR1 (Zymed) at 1:100 dilution and rabbit anti-calnexin (Santa Cruz Biotechnology) at 1:50 dilution] was performed for one hour at room temperature.

Afterwards, another incubation was made using cyanine3-conjugated secondary antibodies, anti-mouse or anti-rabbit (Jackson ImmunoResearch Laboratories), both at 1:100 dilution. Cells were washed again in PBS and nuclei stained with 10 μg/mL DAPI (Sigma). Coverslips were then mounted in VectaShield (Vector Laboratories) and sealed. Images were acquired with the 405-nm, 488-nm and 532-nm laser lines using a Leica DMI 4000B confocal microscope and processed with Leica Analysis Software.

III.6. Immunoprecipitation assays

Forty-eight hours after transfection, both cell media (≈2 mL) and platted cells were harvested. Firstly, cell media were centrifuged for 5 min at 2000 rpm. To their supernatant and cells, 150 μL of lysis buffer [50 mM Tris-HCl at pH 7.5, 1% (v/v) Nonidet P40 (Roche), 100 mM NaCl, 10% (v/v) glycerol, 10 mM $MgCl_2$ and a protease inhibitor cocktail (Sigma)] was added on ice. The media and cell lysates were cleared by centrifugation at 5000 rpm for 10 min and an aliquot of 20 μL (pre-IP) transferred to 2X Laemmli buffer. To the remaining supernatant, 5 μL of mouse GFP monoclonal antibody (Roche) was added. After one hour of incubation at 4°C, 60 μL of G-agarose beads slurry (1:1 in lysis buffer; Roche) was added and incubated overnight. Beads were spun down and a 20 μL aliquot of the supernatant (post-IP) was added to 2X Laemmli buffer. Beads were washed three times with 500 μL of lysis buffer and protein resuspended in 2X Laemmli buffer. These lysates, together with pre- and post-IP aliquots were analyzed by Western blot.

III.7. Western blot analysis

Proteins from cell lysates or from cell culture supernatants were resolved in a 12% SDS-PAGE according to standard protocols and transferred to PVDF membranes (Bio-Rad), which were blocked using a 15% (m/v) TBST-Milk solution. Membranes were probed using mouse anti-GFP (Abcam) at 1:10000 dilution, rabbit anti-β2M (Abcam) at 1:500 dilution or mouse anti-TfR1 (Zymed) at 1:500 dilution. For pre-IP lysates, a mouse anti-PCNA antibody (Calbiochem) was used as a loading control. Detection was carried out using secondary peroxidase-conjugated anti-mouse IgG (Bio-Rad) at 1:4000 dilution or anti-rabbit IgG (Bio-Rad) at 1:3000 dilution antibodies, followed by chemiluminescence assays.

IV. Results

IV.1. HFE mRNA is alternatively spliced in different human tissues

Total RNA from eight human tissues (heart, duodenum, small intestine, liver, spleen, kidney, ovary and testis) and from a HepG2 cell line were retrotranscribed to cDNA. A PCR using primers encompassing the previously predicted coding region of HFE gene (Figure 2.1A and B) was carried out to amplify HFE transcripts. At least eight bands could be observed in some lanes of the representative gel photograph (Figure 2.1B). To identify the corresponding HFE transcripts, the RT-PCR products from all samples were cloned and sequenced. As expected, we found the full length HFE transcript represented by the 1081 base pair (bp) fragment. Additionally, we identified two transcripts resulting from the skipping of a single exon: one corresponds to the HFE exon 2 skipping (817 bp fragment) and the other to the exon 3 skipping (805 bp fragment). Three other transcripts were found as a result of multiple exon skipping: exon 2-3 (541 bp fragment), exon 2-4 (265 bp fragment) and exon 2-5 (151 bp fragment).

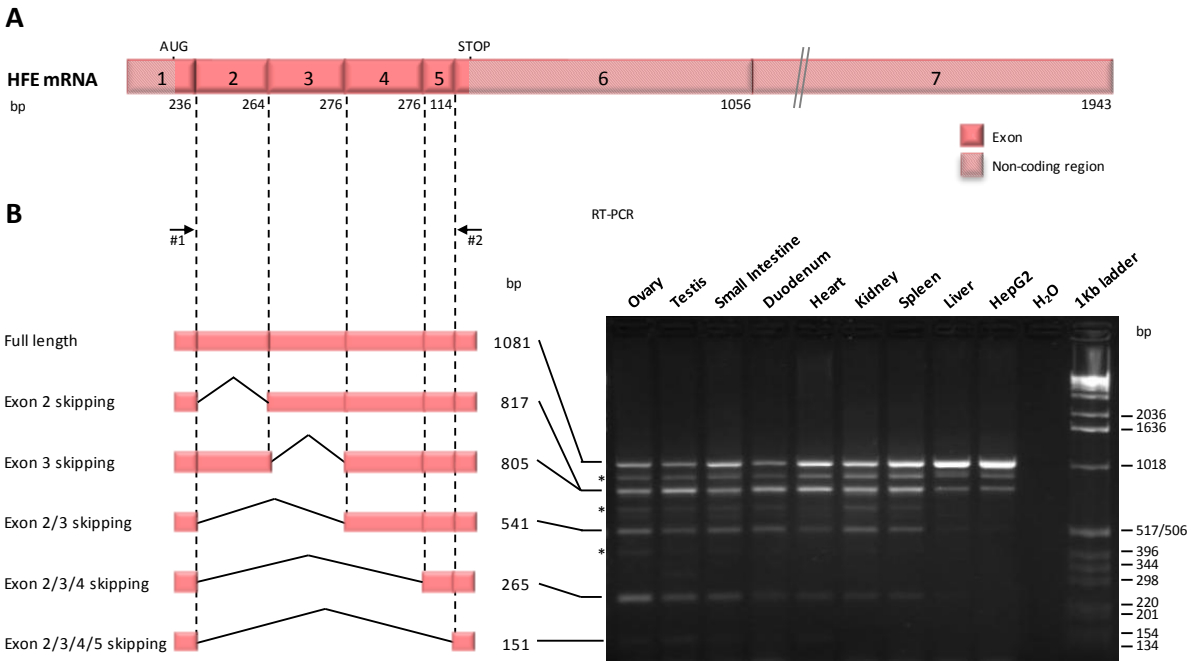

Figure 2.1. Splicing forms of HFE gene in several human tissues. (**A**) Schematic representation of the full length HFE transcript. The size of exons is presented in base pairs (bp). (**B**) An RT-PCR using total RNA from eight human tissues and HepG2 cell line was performed using primers #1 and #2 (their relative position is indicated with arrows) and results are shown on the right. The products obtained for each tissue were cloned into the pCR®-TOPO-XL® vector and sequenced. On the left are schematic representations of the alternative splicing forms identified as well as their length in bp. The asterisks (*) identify bands corresponding to PCR artifacts as a result from DNA hybrid chains.

Moreover, three other bands (indicated with an asterisk) could be seen in most of the lanes of the gel (Figure 2.1B). However, they were proved by direct sequencing to be artifact fragments of DNA hybrid chains formed during PCR assays.

The full length as well as most of the alternatively spliced HFE transcripts, were found in all the analyzed tissues (Figure 2.1B). As exon 2 and exon 3 skipping transcripts were not distinguished in the gel due to their similar molecular weight, their presences in all tissues were confirmed by additional RT-PCRs (data not shown). Only the exon 2-5 skipping seems to be tissue-specific, since it is only present in the gonads, small intestine, duodenum and heart (Figure 2.1B). Most of the alternative isoforms were also found in HepG2 cells, with the exception of the exon 2-4 and exon 2-5 skipping transcripts. All alternative transcripts that were identified present exons totally skipped without generating any frameshifts. However, in some of them, a single amino acid change occurs in the new exon-exon junction. As an example, the alternative transcript with a complete deletion of exon 2 results in a 260-aa protein variant where the arginine 26 changes to glutamine.

In order to improve the screening of alternatively spliced HFE transcripts, we performed a search for a previously described isoform resulting from the inclusion of intron 4 [Jeffrey et al. 1999]. So, a specific RT-PCR using primers located at exons 4 and 5 was performed in all tissues and HepG2 cells (Figure 2.2). The amplified products were cloned and sequenced. Besides the normally spliced, two additional HFE transcripts were identified, one resulting from the total intron 4 inclusion (438 bp fragment) and other, not previously published, resulting from the inclusion of the first 66 bp of intron 4 (346 bp fragment) (Figure 2.2). These alternative transcripts were observed in all analyzed tissues as well as in HepG2 cells. On the other hand, an additional band was present in the gel (indicated with an asterisk) that was once again proved to be a PCR artifact by direct sequencing.

Interestingly, these transcripts containing partial or total intron 4 inclusion both present a TGA sequence six nucleotides 3' from the exon 4 boundary (coding for a premature stop codon). A RT-PCR encompassing exon 1- intron 4 was also performed in all tissues to confirm the correct splicing of their upstream coding region (data not shown). So, potentially both transcripts encode the same and putatively soluble HFE peptide since it would not have the transmembrane domain (encoded by exon 5) and the cytoplasmatic tail (encoded by exon 6). Therefore, after α3 domain it would have two extra C-terminal amino acids, glycine and methionine, encoded by the 5' sequence of intron 4.

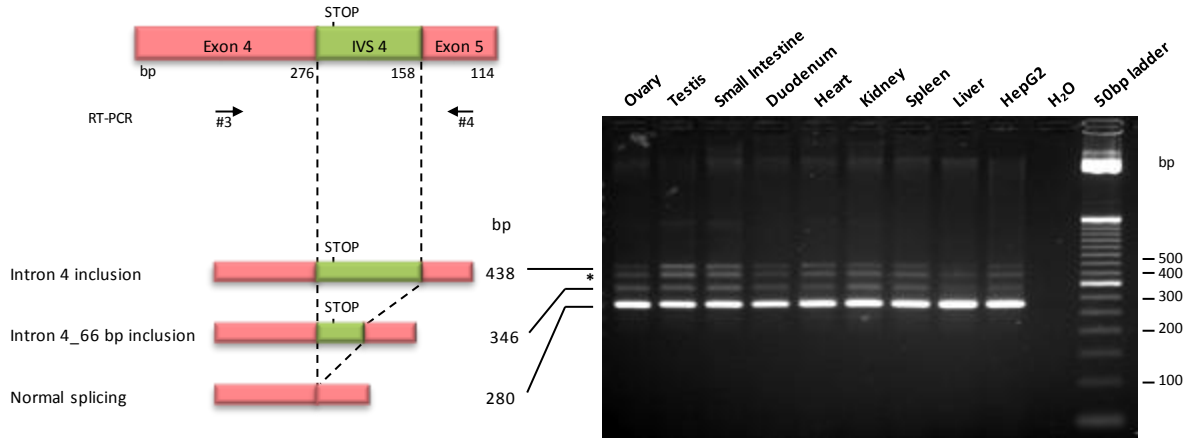

Figure 2.2. Expression of the intron 4 inclusion HFE splice transcript in several human tissues. A specific RT-PCR to amplify the region between HFE exon 4 to 5 using total RNA from eight tissues and HepG2 cell line was performed. These amplified products were cloned into the pCR®-TOPO-XL® vector for automated sequencing and further identification. A schematic representation of the HFE gene exon 4 to 5 is presented on the left. The intron 4 (IVS4) contains a stop codon (TGA) six nucleotides from the exon 4 boundary. The position of the primers (#3 and #4) used in the PCR and the schematic representations of the identified alternative splicing forms are revealed. Correspondence between these splicing forms and the PCR amplification products is shown, along with their length in base pairs (bp). The asterisks (*) identify bands corresponding to PCR artifacts as a result from DNA hybrid chains.

IV.2. Absolute and relative quantification of HFE exon 2 skipping and intron 4 inclusion transcripts reveal a tissue-specific pattern

In order to further characterize two of the alternatively spliced transcripts identified, absolute quantification of the total HFE mRNA along with the transcripts resulting from exon 2 skipping or intron 4 insertion was performed. The approach used was an accurate two-step quantitative real-time PCR (RT-qPCR) described for appropriate measurement of several low-abundance mRNA splice isoforms [Walton et al. 2007]. Reverse transcription using total RNA from each tissue was carried out in quadruplicate. Since HFE exon 6 is present in the full length as well as in all the abnormally spliced transcripts, primers located in this exon were used to quantify the total amount of HFE transcripts. For the same reason, in order to corroborate this quantification data, a similar procedure was made using primers located in exon 1 (data not shown). Specific primer sets were designed based on differences in the splicing pattern of each alternative transcript, where the sequence is unique. For instance, to quantify the exon 2 skipping transcript, a specific primer was designed spanning the exon 1-3 boundary, whereas to quantify the intron 4 inclusion a primer comprising the intron 4-exon 5 boundary was used. An absolute quantification method was performed using serial dilutions of plasmid constructs as standards ($8x10^5$ – 80 copies), previously obtained by

cloning the RT-PCR fragments into the pCR®-TOPO-XL vector. Each reaction was done in triplicate. Linear regression analysis of each standard curve from all plates was used to quantify transcript levels. The correlation coefficients ranged from 0.986 to 0.999, indicating low intra-assay variation. Quantification of total and alternative transcripts was also done in triplicate for each cDNA, whereas all standard deviations were less than 0.38 Ct.

The qPCR methodology performed allowed the quantification of total HFE mRNA as well as the two alternative HFE transcripts in all the tissues analyzed (Figure 2.3). Absolute quantification (presented as HFE copy number/μg total RNA) showed that ovary and liver have the highest level of total HFE mRNA while, on the contrary, the smallest amount is present in duodenum (Figure 2.3A). When comparing the two tissues thought to be targets for HFE's function, the liver presents an amount of total HFE mRNA approximately 4.3-fold higher than the duodenum.

Similarly, the relative quantification of the exon 2 skipping and intron 4 inclusion transcripts also revealed a differential expression in the tissues studied. For instance, the liver presents the lowest amount of both transcripts (3 and 6%, respectively), whereas the testis (26 and 35%, respectively) and the duodenum (20 and 25%, respectively) are the tissues where these variants are more prevalent (Figure 2.3B). Furthermore, the spleen, which may also be a potential site of action for HFE, shows a total of 31% of relative abundance for these splice variants (Figure 2.3B).

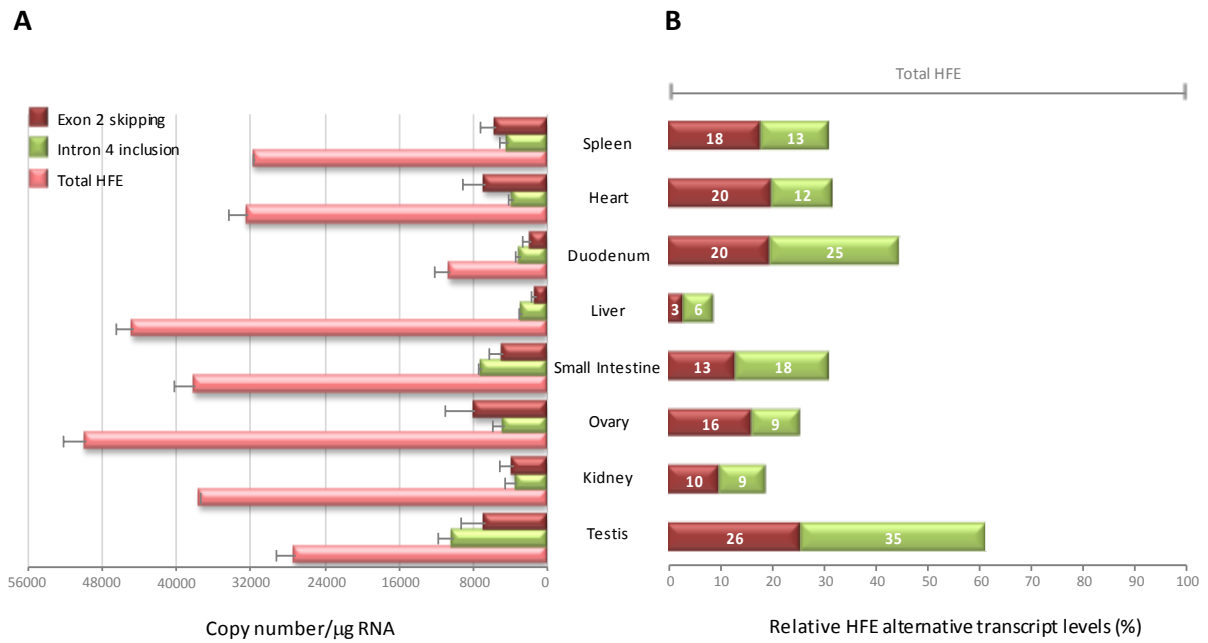

Figure 2.3. Absolute and relative quantification of the exon 2 skipping and intron 4 inclusion HFE splice transcripts. Total RNA from each tissue was used to synthesize cDNA and each cDNA sample was utilized as template for qPCR, which was performed using the SYBR Green Master Mix (Applied Biosystems) along with primers that specifically amplify the total HFE (exon 6), the exon 2 skipping (exon 1-3 boundary) and the inclusion of intron 4 (intron 4-exon 5 boundary). (**A**) Absolute quantification of total HFE and of two alternative splicing transcripts was performed by the absolute quantification method using serial dilutions of plasmids as standards. The histogram shows the mean and standard deviations from four independent experiments. (**B**) Relative quantification of splice transcripts resulting from exon 2 skipping and intron 4 inclusion using the absolute quantification data. The histogram represents each variant as a percentage of the total HFE designated as 100%.

IV.3. HFE variants present distinct subcellular localization

In an attempt to characterize the cellular localization of the corresponding HFE protein splice variants, HFE cDNAs (corresponding to the full length HFE mRNA, exon 2 skipping and intron 4 inclusion transcripts) were tagged to the GFP gene in the pEGFP-N1 vector (Clontech). In addition, a construct containing the full length HFE C282Y mutant was made to be used as a dysfunctional control.

Since we previously have shown that HepG2 cells endogenously express the HFE exon 2 skipping and intron 4 inclusion transcripts, they provide a suitable model to further characterize the corresponding transgenic proteins. Therefore, these cells were transiently transfected with the mentioned constructs. Confocal microscopy analysis of the subcellular localization of (i) the HFE_full length protein, (ii) proteins related to the two splice transcripts and (iii) the mutated HFE_C282Y was performed using antibodies against β2M, calnexin [an endoplasmic reticulum (ER) marker] and TfR1 (Figure 2.4). Nuclei were stained with DAPI.

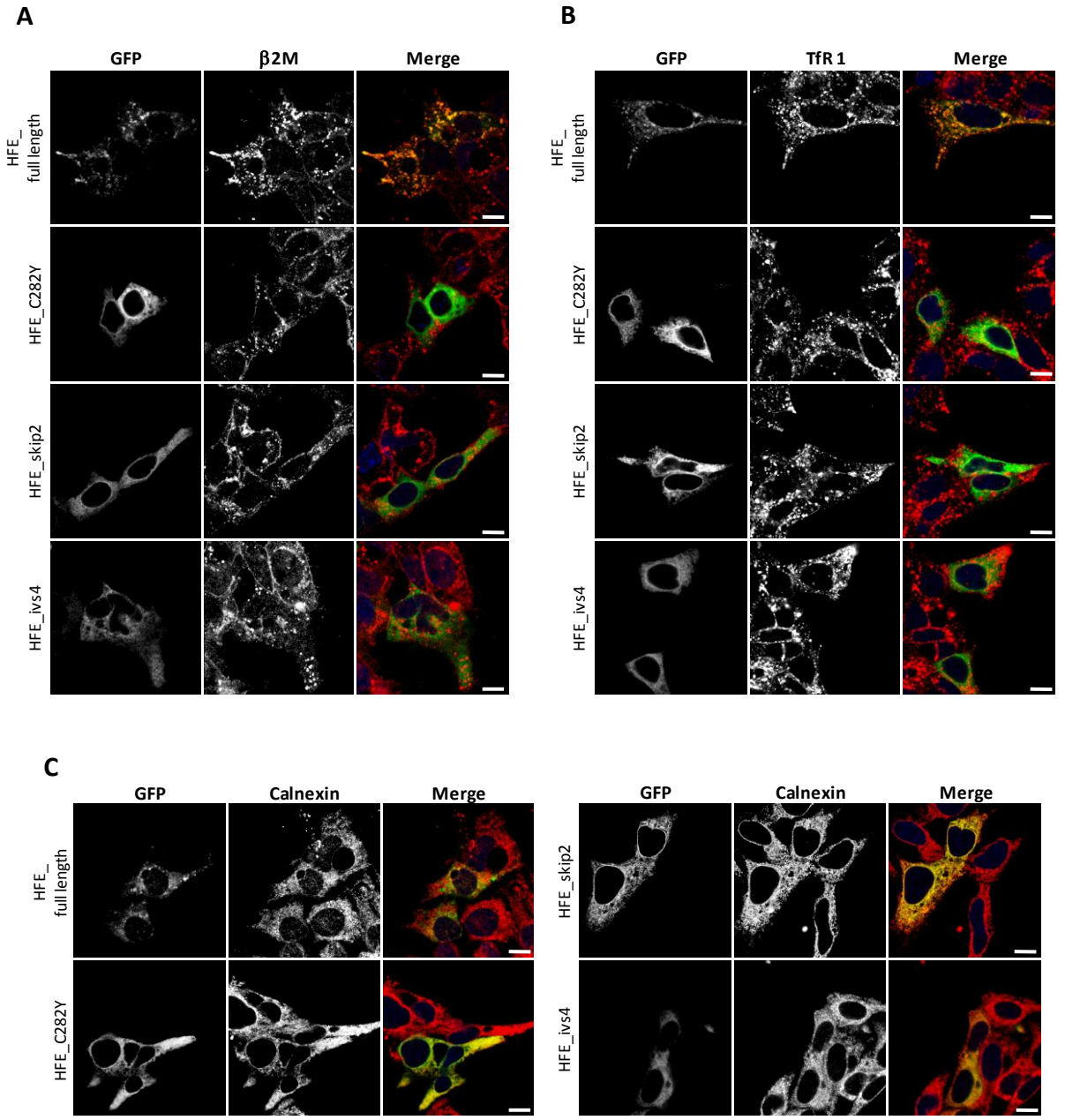

Figure 2.4. Cellular localization of HFE splice variants by immunofluorescence analysis. HepG2 cells were transfected with 2 μg of pEGFP_HFE_full length, pEGFP_HFE_C282Y, pEGFP_HFE_skip2 and pEGFP_HFE_ivs4 constructs. Twenty-four hours later, cells were submitted to an immunofluorescence assay. HFE protein variants distribution was compared with the location for (**A**) β2-Microglobulin (rabbit anti-β2M polyclonal antibody), (**B**) Transferrin Receptor 1 (mouse anti-human TfR1 monoclonal antibody) and (**C**) Endoplasmic Reticulum (rabbit anti-calnexin polyclonal antibody). Images were acquired using a 488-nm laser for GFP (green) and a 532-nm laser for the previously described antibodies (red). Nuclei were stained with DAPI (blue). White bars represent 10 μm.

HFE_full length protein presents mostly a perinuclear and cell membrane distribution. As expected, it co-localizes with β2M and TfR1 proteins. On the contrary, also as expected, HFE_C282Y is not present at the cell surface and has a diffuse cytoplasmic localization. It does not co-localize with either β2M or TfR1, being retained in the ER, as revealed by the calnexin co-localization (Figure 2.4). As well, the intracellular distribution of the HFE_skip2 variant is similar to the one obtained for the HFE_C282Y variant, since it co-localizes with calnexin, but not with β2M and TfR1. Concerning the HFE_ivs4 variant, it presents a scattered intracellular distribution and is apparently absent from the cell membrane. It seems not to co-localize with either β2M or TfR1 and to be present in the ER (Figure 2.4). To further clarify these results, immunoprecipitation assays were performed to all the HFE variants.

IV.4. Immunoprecipitation assays reveal a soluble and secreted HFE protein isoform

To confirm our protein co-localization data observed in immunofluorescence assays, HepG2 cells were transfected with 2 μg of pEGFP_HFE_full length, pEGFP_HFE_C282Y, pEGFP_HFE_skip2 or pEGFP_HFE_ivs4 constructs. Proteins obtained from cell lysates as well as from cell culture supernatants were subjected to immunoprecipitation assays using a mouse anti-GFP antibody (Figure 2.5). In cell lysate experiments, the HFE_full length protein is bound to β2M and TfR1, while HFE_C282Y does not co-immunoprecipitate with either one of these proteins. Similarly, the HFE_skip2 variant is not able to bind either β2M or TfR1 (Figure 2.5B). In addition, it can be observed that HFE_ivs4 variant seems to be present at low level in cell lysates in association with β2M but not with TfR1 (Figure 2.5B). These results are in agreement with those obtained by immunofluorescence experiments (Figure 2.4). The same procedures carried out in the corresponding cell culture supernatants reveal that HFE_full length, HFE_C282Y and HFE_skip2 are absent from the culture media (Figure 2.5C). Conversely and interestingly, the HFE_ivs4 variant is clearly shown in the culture supernatant in association with its chaperone β2M. This result reveals for the first time a soluble form of HFE protein (sHFE) which is secreted to the cell medium, since it lacks the transmembrane and cytoplasmic domains. This observation was confirmed in HuH7, HeLa and HEK293 cell lines (data not shown). In all cases, this isoform is largely secreted to cell medium remaining linked to the β2M, as shown by immunoprecipitation experiments.

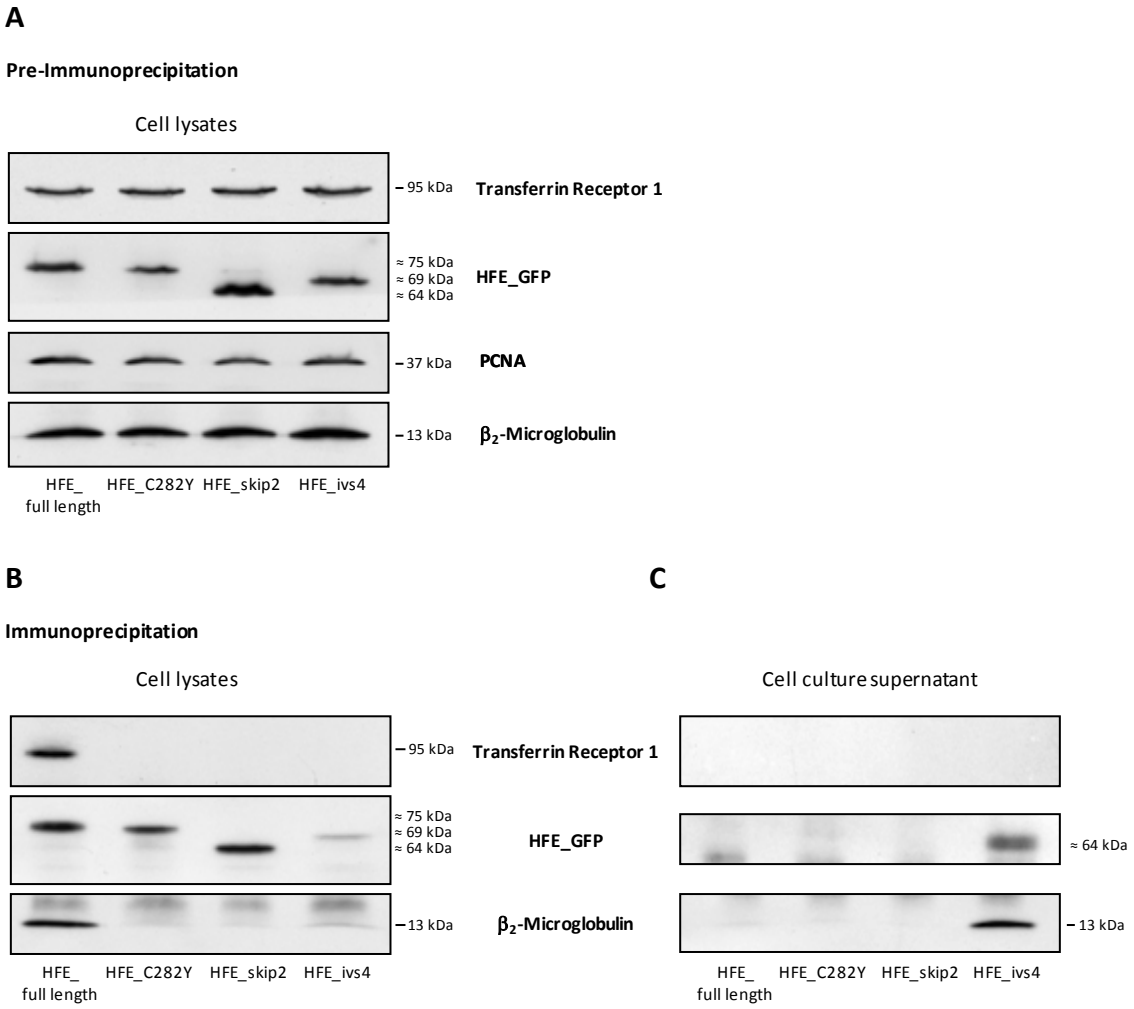

Figure 2.5. Immunoprecipitation assays of transfected HFE splice variants. HepG2 cells were transfected with 2 μg of pEGFP_HFE_full length, pEGFP_HFE_C282Y, pEGFP_HFE_skip2 or pEGFP_HFE_ivs4 constructs. Forty-eight hours later, cells and media were harvested for immunoprecipitation assays. (**A**) One aliquot (pre-immunoprecipitation) was collected for Western blot analysis using the indicated antibodies. Anti-PCNA was used as a specific antibody to control for protein loading. The predicted molecular mass of the proteins is indicated in kDa (kilodalton). (**B**) Immunoprecipitation of HFE transfected variants was done using mouse GFP monoclonal antibody and G-agarose beads. TfR1 and β2M were tested for co-immunoprecipitation with HFE_GFP variants by Western blot analysis. (**C**) The cell media were also subjected to immunoprecipitation as described. Western blot analysis was done to check for possible secretion of the HFE_GFP variants, together with putative binding partners.

V. Discussion

Alternative mRNA splicing is a complex post-transcriptional mechanism that enables the generation of multiple mRNA products from a single gene, increasing transcriptome and proteome complexity. By this way, a single gene can produce proteins with different properties and functions, which might differ in a tissue- or developmental stage-specific manner [Modrek and Lee 2002; Johnson et al. 2003; Stamm et al. 2005]. Here we report that, in addition to full length HFE, at least seven alternatively spliced HFE transcripts are expressed in several human tissues, differing in its level- and the tissue-specificity. Some studies had already shown that HFE gene is subjected to alternative splicing processes [Jeffrey et al. 1999; Rhodes and Trowsdale 1999; Thenie et al. 2000; Sanchez et al. 2001]. However, the precise characterization of the alternative transcripts, their tissue-specificity and abundance, as well as the intracellular localization and biological significance of the corresponding protein isoforms remained largely to be clarified.

In order to obtain an absolute and a relative quantification of total HFE transcripts and of two alternatively spliced transcripts in diverse tissues, we took advantage of a RT-qPCR strategy as it is the most sensitive method to ascertain gene expression levels. It offers a substantially higher sensitivity than other conventional methodologies previously used for HFE transcripts quantification, as Southern blot of RT-PCR products, RT-PCR and Northern blot [Jeffrey et al. 1999; Rhodes and Trowsdale 1999; Thenie et al. 2000; Sanchez et al. 2001]. Using this approach, we observed that total HFE mRNA expression varies amongst tissues. Apart from the gonads, we found that the liver has the highest total HFE expression. On the contrary, duodenum presents the lowest expression of the tissues tested. The liver, although presenting a high quantity of total HFE mRNA, has the lowest level of the studied HFE alternative transcripts. It is expected that, in the liver, the full length HFE is playing an important role in iron metabolism. On the contrary, in the duodenum, the tissue where the total HFE expression is the lowest, the studied alternative transcripts present a high relative level (approximately 45% of the total). One may speculate that these alternative transcripts may have a significant function in this tissue. Having in mind two models of HFE possible action, the crypt model and the hepcidin regulation model, we attempted to further understand the different tissue-level of HFE transcripts by studying the cellular localization of the corresponding proteins [Fleming and Britton 2006; Schimdt et al. 2008; Fleming 2009; Gao et al. 2009; Wallace et al. 2009].

Concerning the HFE exon 2 skipping transcript, which was already described in hepatic, colon and ovary cell lines [Rhodes and Trowsdale 1999], here we demonstrate its presence at different levels in several human tissues. Regarding its corresponding protein, the extracellular α1 domain encoded by exon 2 is lacking, and therefore it is unable to bind to TfR1 [Feder et al. 1998]. Our immunofluorescence and immunoprecipitation results, besides confirming that HFE_skip2 is not associated with TfR1, also revealed no interaction with β2M chaperone and consequent ER retention. We therefore conclude that this HFE variant apparently does not have any cellular function, being probably degraded by the cell proteolytic system. Possibly, the fact that the level of its corresponding mRNA is elevated in different tissues reveals part of the complex post-transcriptional HFE gene expression regulation.

It is known that, as a consequence of the alternative splicing mechanisms, soluble protein isoforms can be originated, assuming in some cases, an important regulatory role in physiological processes. In fact, some years ago, an alternatively spliced HFE transcript due to the intron 4 inclusion was described [Jeffrey et al. 1999]. It was detected at relatively high level in duodenal biopsies of normal individuals or with secondary iron overload. On the contrary, absence or low presence was observed in duodenal biopsies of HH-C282Y patients [Jeffrey et al. 1999]. Although not studied at protein level, it was suggested that the corresponding soluble peptide might regulate cellular iron transport. Here, we positively show for the first time, a soluble HFE protein isoform lacking the transmembrane domain and the cytoplasmic tail, due to the in frame premature stop codon present at intron 4 (Figure 2.2). Since it has an intact α3 domain (encoded by exon 4), it can bind its chaperone β2M, be correctly folded, conducted to the cell surface and secreted to the cell medium (Figure 2.5). Curiously, other non-typical MHC class I protein encoding gene (HLA-G) that presents significant structural homology to HFE, shows precisely the same structural alternative splicing pattern. This soluble HLA-G protein has an important biological function, which is distinct from that of the full length protein [Sangrouber et al. 2007].

Tissue-specific regulation of this HFE alternative splicing form is demonstrated here, since a relatively more abundant level of intron 4 inclusion transcript is found in the testis and the duodenum than in other tissues. Conversely, the lowest level is found in the liver. Also, a previous study showed apparently similar levels of this transcript in these two HFE target tissues [Jeffrey et al. 1999]. Altogether, the results allow us hypothesize that sHFE plays a role in the regulation of iron metabolism. For instance, a sHFE produced at a high level in duodenal

enterocytes may be secreted into the bloodstream and thus act in remote tissues, binding to the cell surface expressed transferrin receptors (TfR1 or TfR2). There, it might act as an agonist or an antagonist of the wild type HFE on hepcidin expression activation in the liver, by controlling the dietary iron absorption in the duodenum (modulating the expression of iron-related transporters) or by exerting a role on iron recycling by macrophages.

The physiological effects of a sHFE require further investigation to test these hypotheses. Also, it is important to investigate if sHFE serum levels vary with changes in body iron stores, as in iron overload disorders (such as HH) or in iron deficiency disorders. In this context, a preliminary study has already reported that a sHFE was reduced in the serum of iron loaded rats when compared to normal control rats [Li et al. 1998]. Additionally, intron 4 inclusion transcript tissue levels were found differentially expressed in duodenal and liver biopsies of control and iron overload patients [Jeffrey et al. 1999].

So, we propose that the sHFE is playing a crucial role in systemic iron metabolism regulation, establishing a communication bridge between duodenum and liver, and possibly other tissues. If so, the sHFE isoform might be developed as a useful therapeutic agent in the treatment of iron-related disorders.

VI. Acknowledgments

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CHAPTER 3

Alternative polyadenylation and nonsense-mediated decay act in concert to fine-tune HFE mRNA levels in response to the cellular iron status

Author's note

The data presented in this chapter has been submitted for publication, with minor modifications, to the RNA Journal. The authors of this work are Rute Martins, Daniela Proença, Bruno Silva, Ana Luísa Silva, Paula Faustino and Luísa Romão. I have performed most of the experimental work, from the 3´RACE experiments, assemblage of plasmid constructs, cellular transfections, Western blots and RT-qPCR assays. Daniela Proença was involved in the construction of some plasmids and optimization of the qPCR experiments. Bruno Silva has contributed to the optimization of the qPCR experiments and cell culture handling. Ana Luísa Silva has contributed to the planning of the assays and to the analysis of the data. Paula Faustino has participated in the supervision of the work. Luísa Romão has designed the research, supervised the work, analyzed the data and wrote the manuscript.

I. Abstract

Nonsense-mediated decay is an mRNA surveillance pathway that selectively recognizes and degrades defective mRNAs carrying premature translation termination codons. However, several studies have shown that NMD also targets physiological transcripts that encode full length proteins, modulating their expression. Indeed, some features of physiological mRNAs can render them NMD-sensitive.

Human HFE is an MHC class I protein mainly expressed in the liver that, when mutated, is associated to hereditary hemochromatosis, a common genetic disorder of iron metabolism. The HFE gene structure comprises seven exons; although the sixth exon is 1056 base pairs long, only the first 41 bp encode for amino acids. Thus, the remaining downstream 1015 bp sequence corresponds to the HFE 3' untranslated region, along with exon 7. Therefore, this 3' UTR encompasses an exon-exon junction, a feature that can make the corresponding physiological transcript NMD-sensitive.

Here, we demonstrate that in UPF1-depleted HeLa and HepG2 cells the HFE transcripts are significantly upregulated, meaning that, in fact, the physiological HFE mRNA is an NMD-target. Besides, it is shown, by 3' RACE analysis in several human tissues, that HFE mRNA expression results from alternative cleavage and polyadenylation at four different sites – one located at exon 6 and three located at exon 7. Remarkably, our data further reveal that treatment of HepG2 cells with holo-transferrin increases the amount of NMD-resistant HFE transcripts, which appears to be due to the shortening of their 3' UTRs by preferential/alternative cleavage and polyadenylation at exon 6. These results reveal that NMD and alternative polyadenylation can coordinately be used to adapt levels of human hepatocellular HFE mRNA to the needs of the cell in response to the iron status.

II. Introduction

It has been estimated that one third of hereditary genetic diseases, as well as many forms of cancer, are caused by mutations that lead to the generation of transcripts bearing a premature translation termination codon. Most of these PTC-containing mRNAs are targets for the nonsense-mediated mRNA decay pathway [Isken and Maquat 2007; Muhlemann et al. 2008; Silva and Romao 2009]. NMD is an evolutionarily-conserved post-transcriptional surveillance mechanism that selectively detects and degrades transcripts bearing PTCs. PTCs or nonsense codons can either be generated by various types of germline/somatic alterations in the DNA or be originated as a result of routine errors in gene expression. In mammalian cells, NMD depends on the interaction of the translation termination complex with a dynamic multiprotein assembly, the so-called exon junction complex [Lejeune and Maquat 2005; Chang et al. 2007]. These protein complexes can assist to discriminate a premature translation termination event from a normal one. According to the classical model for mammalian NMD, the EJC, or a critical subset of EJC components, is deposited 20-24 nucleotides upstream of the exon-exon junction(s) during splicing and remains associated with the mRNA during its transport to the cytoplasm [Le Hir et al. 2000]. Translating ribosomes subsequently displace EJCs from the open reading frame during the initial ('pioneer') round of translation [Ishigaki et al. 2001; Lejeune et al. 2002]. However, if an mRNA contains a PTC located more than 50-54 nt upstream of at least one exon-exon junction, the ribosome will fail to displace these distal EJC(s). In this case, when the ribosome reaches the PTC, the translation eukaryotic release factors eRF1 and eRF3 at the PTC interact in *cis* with the retained EJC(s) via a multiprotein bridge [Kashima et al. 2006]. Of central importance in this reaction is the interaction of UPF1 with the terminating complex and with the UPF2/UPF3 components of the retained EJC(s) [Kashima et al. 2006]. This interaction marks the mRNA for rapid decay. Nevertheless, identification of a stop codon as a PTC depends on the physical distance between the PTC and the cytoplasmic poly(A)-binding protein 1. Since PABPC1 and UPF1 both compete for the interaction with the eRF3 at the terminating ribosome, if PABPC1 is in close proximity to the PTC, it seems to function as an NMD repressor; on the other hand, when the interaction between PABPC1 and the termination complex is not favorable, then UPF1 can interact with eRF3 in the termination complex to induce NMD [Eberle et al. 2008; Ivanov et al. 2008; Silva et al. 2008; Singh et al. 2008].

NMD is an important contributor to the fidelity of gene expression as it prevents translation of potentially harmful truncated proteins from faulty mRNAs. However, it has become clear during recent years that many physiological mRNAs are also NMD substrates, indicating a role for NMD beyond mRNA quality control, as a translation-dependent post-transcriptional regulator of gene expression. In effect, a group of NMD substrates includes physiological transcripts that encode functional full length proteins, as shown in several microarray studies [He et al. 2003; Mendell et al. 2004; Rehwinkel et al. 2005; Wittmann et al. 2006]. The comparison of mRNA levels of normal with NMD-deficient cells, by genome-wide RNA microarray expression profile studies, revealed that the expression of approximately 10% of the human transcriptome is regulated by NMD [Mendell et al. 2004; Wittmann et al. 2006]. These physiological substrates have one feature in common with their pathological counterparts: they possess a translation termination codon that is, by NMD standards, conceived as premature. This applies, for example, to the termination codons of upstream ORFs, to termination codons that are introduced into an ORF as the result of somatic DNA rearrangements, alternative splicing, ribosomal frameshifting, mRNA editing or to termination codons that are followed by splice events in the 3' untranslated region [Mendell and Dietz 2001; Holbrook et al. 2004; Rehwinkel et al. 2005]. In some cases, these features are exploited for self-regulatory mechanisms. For example, when a gene product induces the alternative splicing of its own transcript, a PTC may be introduced into its ORF or a splice junction may be generated 3' to the termination codon, thus directing the resulting alternative transcript to NMD. Moreover, it is suspected that potentially NMD-sensitive physiological transcripts can stand at crossing points of pathways or networks and thus modulate such pathways as a whole. A common feature of all these processes is that NMD can potentially be used to adapt protein expression to the physiological needs of the cell. The large and diverse repertoire of transcripts controlled by NMD reflects the significant influence of NMD on the metabolism of the cell and consequently in many human diseases [Neu-Yilik and Kulozik 2008].

When mutated, the human HFE gene may be involved in hereditary hemochromatosis, a common genetic disorder of iron metabolism characterized by excessive intestinal iron absorption that leads to iron deposition in cells and subsequent dysfunction of several organs [Cartwright et al. 1979; Feder et al. 1996]. HFE protein has been recognized as a key component of human iron homeostasis machinery but its precise role is still unknown. HFE is capable of forming protein complexes with both transferrin receptors 1 and 2 in the hepatocyte membrane [Parkkila et al. 1997b; Chen et al. 2007]. It was recently proposed that HFE

is partitioned between TfR1 and TfR2, and under increasing iron concentrations, HFE should shift away from TfR1 towards TfR2, triggering the signaling transduction pathway that leads to induction of the iron regulatory hormone hepcidin [Schmidt et al. 2008; Gao et al. 2009].

The HFE genomic structure is similar to other human MHC class I-like molecules. Each of the first six exons of the corresponding mRNA encode for one of the six distinct domains of the protein: a signal sequence, three extracellular domains, a transmembrane region and a short cytoplasmatic tail [Feder et al. 1996]. Although the sixth exon is 1056 base pairs long, only the first 41 bp are translated to amino acids. In fact, the native translation termination codon is located at the 5' part of this exon and the remaining downstream 1015 bp correspond to the HFE 3' UTR, along with exon 7, which is 1943 bp long [Feder et al. 1996; Sanchez et al. 2001]. The HFE transcript has about 4.2 kb long, being essentially expressed in the liver, duodenum, small intestine, spleen or heart. Furthermore, several additional alternative HFE transcripts are also present in a wide variety of human tissues [Feder et al. 1996; Jeffrey et al. 1999; Rhodes and Trowsdale 1999; Thenie et al. 2000; Sanchez et al. 2001]. All mRNA isoforms are expressed at low levels and HFE gene expression seems to be modestly influenced by changes in cellular iron status. In fact, experimental iron loading or deficiency has been associated with minor changes in HFE expression in the small intestine [Frazer et al. 2001; Ludwiczek et al. 2004]. It was also reported that mouse hepatic HFE mRNA increases approximately 2-fold in response to iron loading [Theurl et al. 2005b]. The above mentioned alternative HFE transcript species have been attributed to alternative splicing events or alternative usage of two polyadenylation [poly(A)] signals located within exon 7, at 1455 and 2958 nt downstream of the stop codon [Thenie et al. 2000; Sanchez et al. 2001]. Nonetheless, the identification of HFE alternative transcripts, their tissue-specificity and abundance, as well as the biological significance of the corresponding isoforms, remain to be clarified.

As indicated above, previously published data regarding the specific architecture of the human HFE mRNA have shown that it comprises seven exons, and the native translation termination codon is located at exon 6 at more than 50-54 nt upstream of the exon 6/exon 7 junction [Thenie et al. 2000; Sanchez et al. 2001], which is a feature that could make this transcript a physiological target for NMD. In addition, the fact that it presents a long 3' UTR might also result in mRNA destabilization due to NMD, as it was already shown for other transcripts [Eberle et al. 2008; Singh et al. 2008]. To explore this hypothesis, we first analyzed the HFE mRNA 3' end processing in several tissues to characterize the potential alternative polyadenylation

isoforms. This analysis revealed the usage of two novel alternative polyadenylation sites, located at exons 6 and 7. Then, we demonstrated that those HFE isoforms specifically using poly(A) signals at exon 7 are in fact physiological NMD-targets. To understand how the NMD mechanism could play a role in the regulation of the HFE mRNA levels in response to iron challenge, we next examined whether human HFE transcripts could be physiological substrates for the UPF1-dependent NMD pathway after treatment of hepatic HepG2 cells with human holo-transferrin, the circulating form of physiological iron. Our results have shown that after cellular holo-transferrin treatment, the amount of NMD-resistant transcripts increases, which seems to be due to the shortening of the HFE 3' UTR by activation of the poly(A) signal located at exon 6. Our data support the conclusion that both post-transcriptional mechanisms of alternative polyadenylation and NMD coordinately fine-tune hepatocellular HFE mRNA levels in response to the cellular iron status.

III. Materials and Methods

III.1. 3' Rapid amplification of cDNA ends

3' RACE experiments were performed using the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech) according to the manufacturer's instructions. Briefly, 1 μg of total RNA from each tissue [small intestine, spleen, liver, testis, ovary, duodenum, heart, kidney and PBMCs (BD Clontech or Ambion)] was retrotranscribed using 3' RACE CDS primer as well as the kit specific components for cDNA synthesis (90 min at 42°C). To cover the entire human HFE 3' UTR, four parallel PCR reactions were performed with an HFE specific forward primer (primer #1, #2, #3, or #4) and the universal primer of the 3' RACE amplification kit as reverse primer. All primers used in this chapter are listed in Table 3.1. A touchdown PCR program was done as indicated in the user manual. Then, a nested PCR was performed by using one forward internal primer (primer #5: EX6F; primer #6: EX6G; primer #7: EX7G; primer #8: EX7H; Figure 3.1A) and the nested universal primer from the kit. The PCR products from the nested PCR were separated on agarose gels, cloned into the pCR®2.1-TOPO (Invitrogen) and sequenced with BigDye® Terminator v1.1 Sequencing Standard kit (Applied Biosystems), using M13 forward and reverse primers and analyzed with the ABI Prism 3100 automatic sequencer (Applied Biosystems).

Table 3.1. DNA oligonucleotides used in the current work.

Primer	Sequence (5' → 3')
#1	AGT GAC ACG CAG CCT GCA GAC TCA C
#2	TGG TGC CTT CAT TTG GGA TGC TAC TC
#3	TTC AAC TGT GGT AGC CGA ATT AAT CGT G
#4	GAA TCA CAG GCC ATT GCT GAG CTG CC
#5	TTT CTG AGT TCC TGC ATG CCG GTG ATC C
#6	AGT GAA GTA GGC CGG GCA CGG TGG C
#7	GGC TTC ACT TAC TCT TCT ACC TCA TAA GG
#8	GAT TGA GGA CTG CTG AGA GGT ACA GGC C
#9	TTT TGC GGC CGC ATG GGC CCG CGA GCC AGG CCG
#10	TTT TAT CGA TAG GTC CCA TCC CCA TTG GGC
#11	GAA CAT CAC CAT GAA GTG GCT GAA GG
#12	GGG GTG TTT CTT GAA ATC TCA GCC C
#13	GAA GGG CAG GTG CTT CAG GAT ACC
#14	TTT TTC CGG AAC ATG GTA ACT GTT GCC
#15	GGG CGC TCT TCC GCT TCC TTC CGG ACG CTC ACT GAC GAC TCG
#16	GCG AGT CAG TGA GCG TCC GGA AGG AAG CGG AAG AGC GCC C
#17	CCG AGG GCT ACT GGA AGT AGG GGT ATG ATG GGC AGG
#18	CCT GCC CAT CAT ACC CCT ACT TCC AGT AGC CCT CGG
#19	GGG CTC TAG GGG GTA TCC TCC GGA CCA CGC GCC CTG TAG C
#20	GCT ACA GGG CGC GTG GTC CGG AGG ATA CCC CCT AGA GCC C
#21	CTA CGT CTT AGC TGAACG TGA GTG A
#22	TGT CTC CTT CCC ACA GTG AGT CT
#23	AAG CAT TCT GTC TTG AAG GGC A
#24	CTG AGC TGT ATA TGG TAT CCT GAA GC
#25	GGA GAA ACT GGA CAG CAC AGA CTT
#26	TCA TTC AGC AGC TTG ATG GTG
#27	CGA GTC CAA GTA CGC CTC ATG
#28	GGT TGT CCT TCA TCT CGT CCA
#29	CCA CTG CTT ACT GGC TTA TCG A
#30	GGG TCT CCC TAT AGT GAG TCG TAT TA
#31	CGA CCA CCA AGC GAA ACA T
#32	GCT TCC ATC CGA GTA CGT GC

III.2. Plasmid constructs

The HFE minigenes used in this work comprise all human HFE exons and introns 4, 5 and 6. The normal HFE minigene (WT) was obtained by sequentially cloning and ligating three distinct human HFE fragments. The first fragment, encompassing exon one (from AUG codon) to exon four, was PCR amplified from HFE cDNA by using primers #9 (with a NotI restriction site linker) and #10 (with a ClaI restriction site linker). An 812 bp fragment was isolated on an agarose gel and cloned into the pCR®2.1-TOPO vector (Invitrogen). Then, this fragment was inserted into pTRE2pur (Clontech) using the NotI and ClaI restriction enzymes. The second fragment of the HFE minigene encompasses exon 4 to the 5' end of exon 7 of the human HFE gene. It was PCR amplified with the Expand Long Template PCR System (Roche) and primers #11 and #12 using human genomic DNA as template. The corresponding 4455 bp fragment was purified and cloned into pCR®2.1-TOPO vector and subsequently inserted into the pTRE2pur already containing the first HFE fragment, by using BstBI (restriction site located in HFE exon four) and EcoRV (restriction site present in HFE exon 7) enzymes. The

third HFE fragment encompasses the entire exon 7 plus a 401 bp downstream fragment. It was PCR amplified by using the Expand Long Template PCR System with primers #13 and #14 (with a Kpn2I restriction site linker) and human genomic DNA as template. The resulting 2210 bp fragment was isolated in an agarose gel and purified, cloned into the pCR®2.1-TOPO vector and subsequently subcloned into the pTRE2pur carrying the two previously cloned HFE fragments, by using EcoRV and Kpn2I restriction enzymes (the Kpn2I site was previously inserted into the pTRE2pur vector at position 1770, by directed mutagenesis using primers #15 and #16). All cloned fragments were confirmed through automated sequencing with several human HFE specific primers. The nonsense-mutated minigene (Y138X) carrying the naturally-occurring nonsense mutation TAC→TAG at codon 138 [Mendes et al. 2009] was obtained by site-directed mutagenesis using primers #17 and #18 and the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). The NMD-resistant minigene (Del_IVS6) was obtained from the WT minigene, by replacing the BsgI and BsmI fragment containing intron 6 with the corresponding BsgI/BsmI cDNA fragment. All the HFE minigenes where finally removed from the pTRE2pur vector and subcloned into the pcDNA3 (Invitrogen) using NotI and Kpn2I restriction enzymes. The Kpn2I site was previously introduced into pcDNA3 vector at position 1310 by site-directed mutagenesis using primers #19 and #20, therefore removing the poly(A) site of the bovine growth hormone gene present in the vector.

III.3. Cell culture and transfections

HepG2 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS. Transient transfections were performed in HeLa cells using Lipofectamine™ 2000 Transfection Reagent (Invitrogen), following the manufacturer's instructions, in 35 mm plates, using 1 μg of the test construct DNA and 1 μg of pEGFP vector (BD Biosciences) to control for transfection efficiency. Cells were harvested for RNA and protein lysates 24 hours later.

III.4. Transient transfections of siRNAs

Transfections of HeLa cells with short interfering RNAs (siRNAs) were carried out using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions, in 60 mm plates, using 200 pmol of siRNA oligonucleotides and 10 µL of transfection reagent. Cells were harvested for RNA and protein extracts at 24, 48 and 72 hours post-transfection. When HeLa cells were also transiently transfected with plasmid constructs, siRNAs transfections were performed with 100 pmol of siRNAs and, 24 hours later, with 50 pmol of siRNAs concomitantly with the plasmids transfections. The siRNA oligonucleotides used for transfections [luciferase (5'-CGUACGCGGAAUACUUCGA-3') and UPF1 (5'-UUACCGCGUUCUGUGUGAA-3')] were purchased as annealed, ready-to-use duplexes from MWG. HepG2 cells were transfected with the same siRNAs using 200 pmol of each oligonucleotide and 10 µL of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen), following the reverse-transfection protocol indicated by the manufacturer. For the iron challenging assays, 24 hours after siRNAs transfection, the cell media was replaced by fresh RPMI 1640 medium supplemented with 10% (v/v) FBS or RPMI supplemented with 10% (v/v) FBS and 30 µM of holo-transferrin (Sigma). Cells were incubated for additional 24 hours and then collected for RNA and protein extracts.

III.5. RNA isolation

Total RNA from small intestine, spleen, liver, testis, ovary, duodenum, heart and kidney was purchased (BD Clontech or Ambion). To isolate total RNA from peripheral blood mononuclear cells (PBMCs), cells were separated using Lymphoprep^{TM} (Axis-Shield PoC AS) and RNA extraction was performed using the RNeasy Mini Kit (Qiagen). RNA from transfected cells was prepared using the RNeasy Mini Kit (Qiagen) following the manufacturer's indications. RNA samples were treated with RNase-free DNase I (Ambion) and purified by phenol/chloroform extraction. Before further analyses, mRNA samples isolated from cultured cells transfected with test plasmids were assessed by RT-PCR to reject the hypothesis of activation of cryptic splicing pathway(s) that might affect the human HFE mRNA sequence. From all the studied transcripts, a single full length product was amplified (data not shown), demonstrating a normal splicing pattern.

III.6. First strand cDNA synthesis and quantitative real-time PCR

Synthesis of cDNA was carried out using 3 μg of total RNA and SuperScript® III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System using SYBR Green Master Mix (Applied Biosystems). Primers were designed using the ABI Primer Express software. Primers were specific for the following transcripts: HFE (exon 6: primers #21 and #22; exon 7: primers #23 and #24); transferrin receptor 1 (primers #25 and #26); G protein pathway suppressor 1 (normalization control; primers #27 and #28); heterogeneous 5' UTR common to all transfected HFE minigenes (primers #29 and #30) and neomycin resistance gene (primers #31 and #32). The following cycling parameters were used in all transcripts tested: 10 min at 95ºC, and 40 cycles of 15 sec at 95ºC and 1 min at 65ºC. Quantification of each transcript was performed by the absolute quantification method using serial dilutions of plasmids carrying the corresponding cDNA. These plasmids were generated by introducing PCR fragments into pCR®2.1-TOPO vector. For the quantification of the transferrin receptor 1 the relative standard curve method was used (ΔΔCT method; Applied Biosystems).

III.7. Western blot analysis

Protein lysates were resolved in 10% SDS-PAGE according to standard protocols and transferred to PVDF membranes (Bio-Rad). Membranes were probed using goat polyclonal anti-UPF1 (Bethyl Labs) at 1:250 dilution, mouse monoclonal anti-α-tubulin (as loading control; Sigma) at 1:10000 dilution, or with mouse monoclonal anti-transferrin receptor 2 (Santa Cruz Biotechnology) at 1:250 dilution. Detection was carried out using secondary peroxidase-conjugated anti-mouse IgG (Bio-Rad) or anti-goat IgG (Sigma) antibodies followed by chemiluminescence assays. For densitometric analysis, films from at least three independent experiments were digitalized and analyzed using ImageJ software.

III.8. Statistical analysis

Results in histograms are expressed in means and standard deviations from three independent experiments, corresponding to three independent transfections. Student's t

test was used for estimation of statistical significance (unpaired, two tailed). Significance for statistical analysis was defined as $p<0.05$.

IV. Results

IV.1. Usage of two novel alternative polyadenylation sites in the human HFE transcripts located at exons 6 and 7

Besides the two alternative polyadenylation signals, previously identified as being recognized for 3' end cleavage and polyadenylation of the human HFE mRNA [Sanchez et al. 2001], this transcript contains several potential polyadenylation signals downstream of the native translation termination codon. To determine which poly(A) signals are in fact active in the human HFE transcript 3' end processing, we carried out 3' rapid amplification of cDNA ends experiments (Figure 3.1) using nested forward primers located at HFE exons 6 and 7 (Figure 3.1A). This analysis was conducted in several human tissues, using total RNA from small intestine, spleen, liver, testis, ovary, duodenum, heart, kidney and peripheral blood mononuclear cells. After cloning and sequencing the obtained fragments, we were able to confirm that all primers generated 3' RACE products containing poly(A) tracts that begin 4-23 bp downstream of a polyadenylation signal (Figure 3.1C) that are not present in genomic DNA. More specifically, a primer located at the 5' part of exon 6 (primer EX6F; Figure 3.1A) generated two HFE specific 3' RACE products with a size of 679 and 1277 bp, respectively (Figure 3.1B). These correspond to transcripts where the poly(A) signals GAUAAA and AAUAAA are recognized, respectively inducing polyadenylation at 857 and 1455 nt downstream of the stop codon [poly(A) signals 1 and 2; Figure 3.1C]. The usage of the first polyadenylation signal was observed in transcripts isolated from duodenum, liver, testis, spleen and small intestine, whereas the usage of the second polyadenylation signal was observed in mRNA extracted from all tissues, except from PBMCs (Figure 3.1C). The primer located at the 3' part of exon 6 (primer EX6G; Figure 3.1A) allowed the detection of only one specific 3' RACE product with a size of 483 bp (Figure 3.1B). This product corresponds to transcripts in which the second poly(A) signal is recognized to induce 3' end cleavage and polyadenylation at 1455 nt downstream of the stop codon (Figure 3.1C). These results confirm those obtained with primer EX6F in what concerns the usage of the second polyadenylation signal located at 1455 nt downstream of the stop codon.

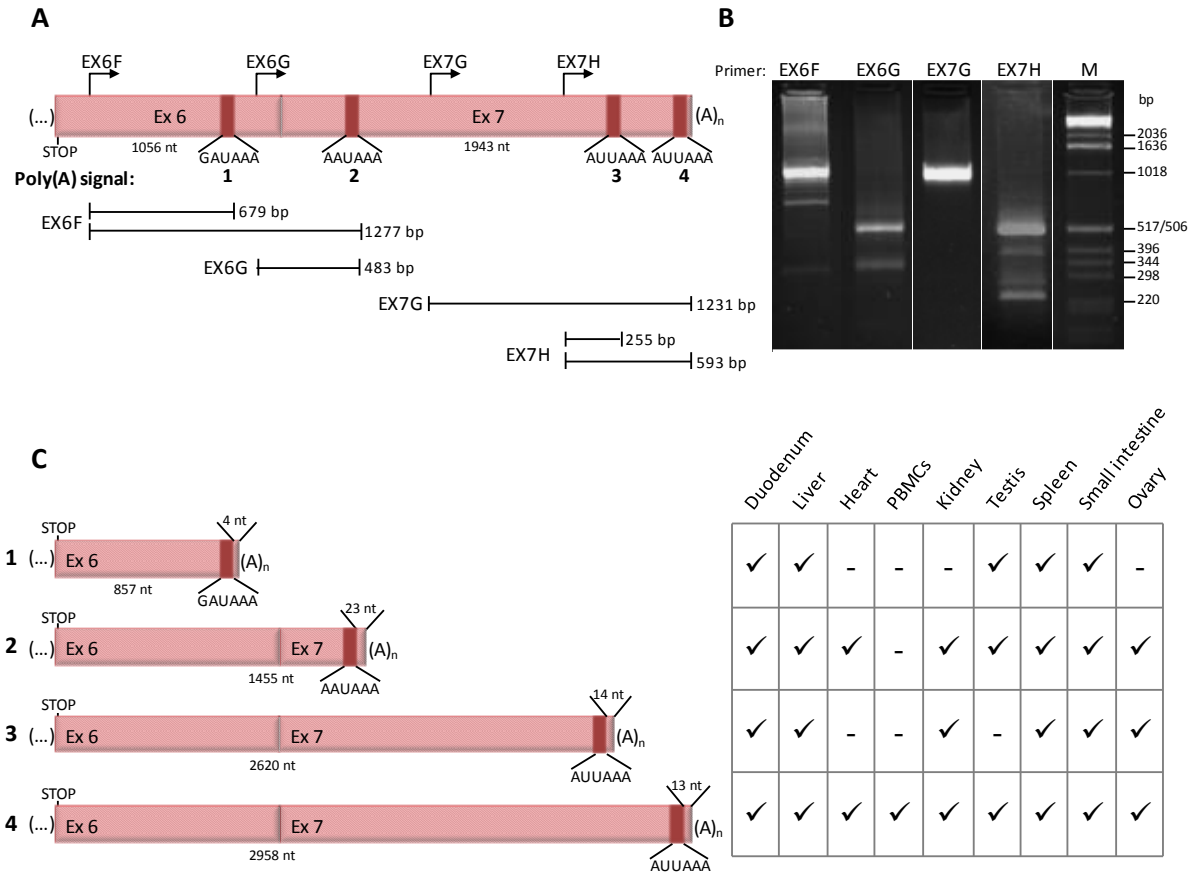

Figure 3.1. Usage of four alternative poly(A) sites for 3' end cleavage and polyadenylation of human HFE transcripts. (**A**) The diagram shows the human HFE 3' untranslated region comprising exons (Ex) 6 and 7. The length of the exons is shown in base pairs (bp). The relative position of the four different forward primers (EX6F; EX6G; EX7G and EX7H) used in 3' RACE experiments is shown. The vertical dark pink bars represent the polyadenylation [poly(A)] signals (numbered from 1 to 4) that were found to be used in the HFE mRNA 3' end processing. Their sequence is also shown. Bellow, the thin lines represent the 3' RACE products obtained by each primer (on the left). The correspondence between each 3' RACE product, its poly(A) site and its length in bp is also represented. (**B**) Representative agarose gel electrophoresis showing 3' RACE products from human liver total RNA. (**C**) Schematic representation of the four human HFE 3' untranslated regions identified and characterized by 3' RACE products obtained from total RNA, isolated from duodenum, liver, heart, peripheral blood mononuclear cells (PBMCs), kidney, testis, spleen, small intestine and ovary. Again, vertical black bars represent the poly(A) signal that is used in each isoform, with the corresponding sequence depicted and the distance from the poly(A) signal to the cleavage site given in nucleotides (nt). The size of each 3' untranslated region is shown below in nt. The table on the right shows the presence (✓) or absence (-) of the HFE alternative poly(A) isoform in each tissue analyzed.

The primer located at the 5' part of exon 7 (primer EX7G; Figure 3.1A) generated a 3' RACE product of 1231 bp (Figure 3.1B) which corresponds to an HFE transcript where the poly(A) signal 4 (AUUAAA) is recognized, inducing 3' end cleavage and polyadenylation at 2958 nt downstream of the stop codon (Figure 3.1A and C). This poly(A) signal recognition was confirmed in the 3' RACE analysis performed with primer EX7H (Figure 3.1 A). Here, we found two specific products with 255 and 593 bp in length (Figure 3.1 B): the longest fragment corresponds to the recognition of poly(A) signal 4; the smallest product corresponds to the recognition of an additional AUUAAA poly(A) signal [poly(A) signal 3; Figure 1A], which allows processing of HFE mRNAs with 3' end cleavage and polyadenylation at 2620 nt downstream of the stop codon (Figure 3.1B and C). Besides, we observed that poly(A) signal 3 is recognized in HFE transcripts expressed in duodenum, liver, kidney, spleen, small intestine and ovary (Figure 3.1C). On the other hand, poly(A) signal 4 is recognized in mRNAs isolated from all tissues studied, which might confirm previous results showing that this signal is the main HFE poly(A) signal [Feder et al. 1996; Sanchez et al. 2001]. In addition to the four identified and characterized 3' RACE products, some other fragments were also obtained when using primers EX6F, EX6G and EX7H (Figure 3.1B). However, the corresponding sequencing analysis has shown that they are not HFE-specific fragments. These non-specific amplifications might reflect the presence of many A + T-rich sequences in exon 7 [Sanchez et al. 2001]. Comparing our results with previously described data [Feder et al. 1996; Sanchez et al. 2001], showing the usage of two poly(A) signals at 1455 and 2958 nt downstream of the stop codon of the human HFE transcript, we can conclude that its 3' end processing machinery also recognizes two novel poly(A) signals – poly(A) signals 1 and 3 – which allow 3' end cleavage and polyadenylation at 857 and 620 nt downstream of the stop codon (Figure 3.1). These two novel polyadenylation signals are recognized in mRNA from several tissues, including liver, duodenum, spleen and small intestine.

IV.2. The physiological human HFE mRNA is a natural NMD-target

NMD is an mRNA surveillance mechanism that rapidly degrades mRNAs carrying PTCs [Muhlemann et al. 2008; Neu-Yilik and Kulozik 2008; Silva and Romao 2009; Nicholson et al. 2010]. Albeit its important role in mRNA quality control, it has become clear that the NMD mechanism also plays a role in regulating the steady-state level of a set of wild-type transcripts [He et al. 2003;

Mendell et al. 2004; Rehwinkel et al. 2005; Wittmann et al. 2006]. These physiological NMD substrates structurally mimic nonsense transcripts as they possess a translation termination codon that is recognized as premature. In face of this knowledge, and considering the position of the human HFE poly(A) signals that are used for its 3' end processing (Figure 3.1), it seems evident that a percentage of the alternatively polyadenylated human HFE mRNA species – those using the poly(A) signals 2, 3 or 4 (Figure 3.1) – will comprise an exon-exon junction located more than 55 nt downstream of the natural stop codon, a context that can be sufficient to define the natural stop codon as a "premature stop codon" and to induce NMD. On the other hand, those transcripts resulting from cleavage and polyadenylation by usage of the poly(A) signal 1 must be NMD-resistant as no splicing event occurs downstream of the stop codon. To examine whether HFE transcripts could be physiological substrates for the UPF1-dependent NMD pathway, we quantified the endogenous HFE mRNA levels after siRNA-mediated depletion of UPF1 in HeLa and HepG2 cells and results were compared to those obtained in NMD-competent cells transfected with non-specific control (luciferase) siRNAs (Figure 3.2). At three different time points (24, 48 and 72 hours) after siRNAs transfection, the Western blot analysis demonstrated a decrease in UPF1 protein levels induced by siRNA of about 80-85% or 60-65%, in HeLa or HepG2 cells respectively, when compared with results obtained after treatment with luciferase siRNAs (Figure 3.2A). Under these conditions, the HFE mRNA levels were quantified by RT-qPCR assays, relatively to the HFE mRNA levels obtained in cells treated with the control siRNA (luciferase siRNA). To exclusively measure the abundance of mRNA that could be a natural NMD-target, we used oligonucleotides for qPCR that specifically hybridize the 5' end of HFE exon 7 (Figure 3.2B). Our data have shown that depletion of UPF1 for 72 hours in HeLa cells, results in a 1.6-fold increase of the abundance of those HFE mRNAs using poly(A) signals 2, 3 or 4. The same analysis in HepG2 cells resulted in a 2.6-fold increase of the abundance of the same HFE mRNAs. These results are consistent with the mRNAs using poly(A) signals 2, 3 or 4 being natural substrates for NMD in both cell lines. However, it is interesting to note that in hepatic HepG2 cells, there is a higher amount of HFE NMD-targets, relatively to what occurs in HeLa cells, which might indicate that regulation of physiological levels of HFE mRNA by NMD is fine-tuned in a tissue-specific manner.

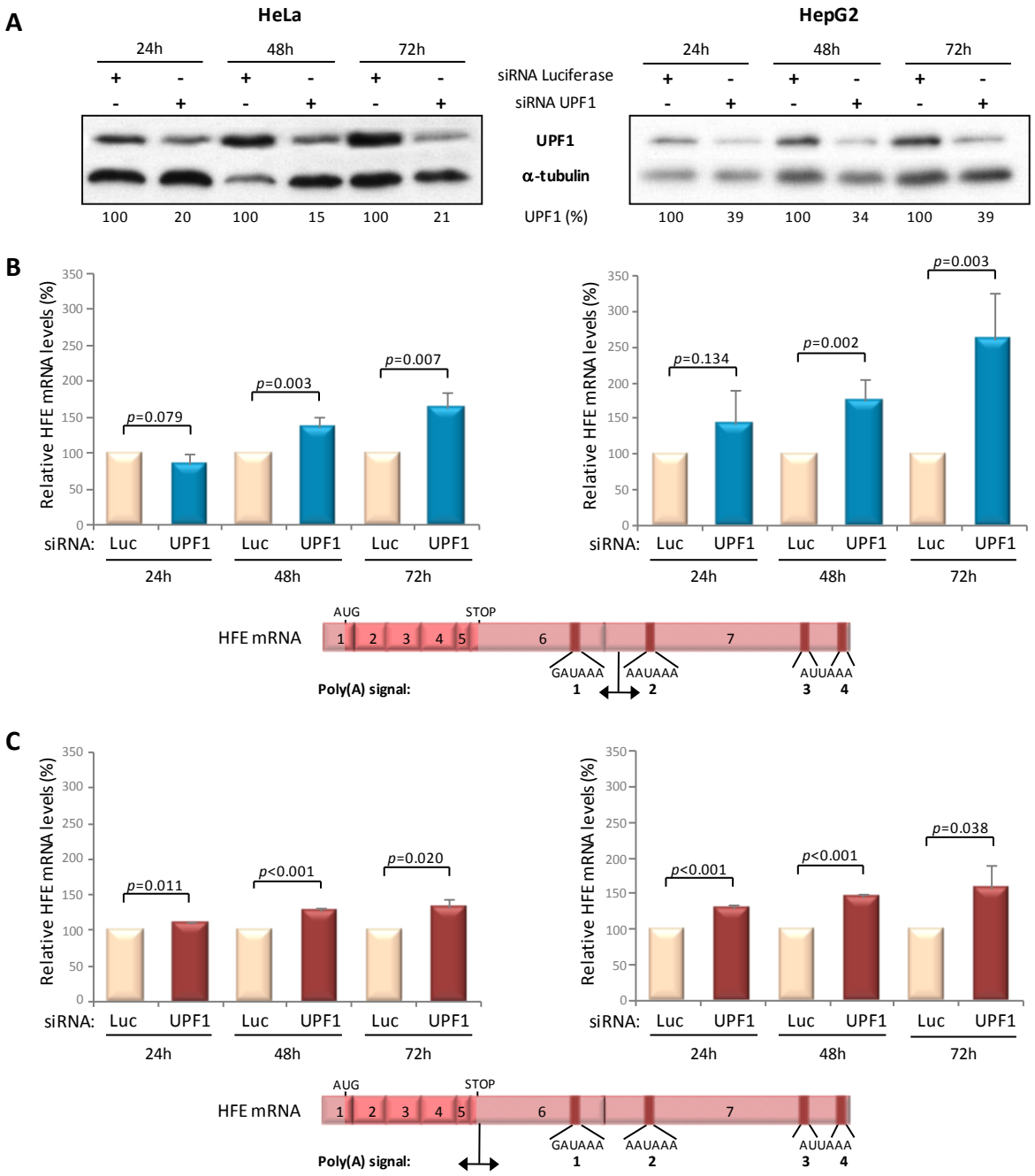

Figure 3.2. Downregulating UPF1 from HeLa or HepG2 cells results in an upregulation of the endogenous HFE transcripts indicating that the physiological HFE mRNA is a natural NMD-target. HeLa and HepG2 cells were transiently transfected with siRNA duplexes directed to human UPF1 or to a non-endogenous control (Luc). Cells were harvested for protein and RNA at three time points (24, 48 and 72 hours) after siRNA treatment. (A) Western blot analysis of the HeLa and HepG2 cells extracts using UPF1 and α-tubulin (loading control) specific antibodies. The percentage (%) of UPF1 protein expressed in the cells after siRNA treatment is indicated below each lane. (B) Relative changes in HFE mRNA levels were analyzed by RT-qPCR, normalized to the levels of endogenous GPS1 mRNA. cDNAs were synthesized from total RNA and each cDNA sample was used as template for qPCR, which was performed using primers that specifically hybridize to the 5' end of HFE exon 7. Levels of HFE mRNA with UPF1 siRNA treatment were compared to those obtained after Luc siRNA treatment. Below there is a schematic representation of the human HFE mRNA structure showing the position of the poly(A) signals used in its 3' end processing, while the double arrow represents the localization of the amplicon obtained in the qPCR. (C) Relative changes in HFE mRNA levels were quantified by RT-qPCR as in B but using primers that specifically hybridize to the 5' end of the HFE exon 6. Levels of HFE mRNA with UPF1 siRNA treatment were compared to those obtained after Luc siRNA treatment. Below there is a schematic representation of the human HFE mRNA structure showing the position of the poly(A) signals used in its 3' end processing as well as the localization of the amplicon obtained in the qPCR.

To examine if the effect of the UPF1-dependent mechanism that modulates levels of HFE mRNA species using poly(A) signals 2, 3 or 4, is important in the context of the total amount of HFE mRNAs, we have also measured levels of HFE mRNA by RT-qPCR, using specific primers located at exon 6 upstream of the poly(A) signal 1 (Figure 3.2C). This approach allowed quantification of all mRNAs polyadenylated at any one of the four signals. As in the previous experiment, the increase in HFE mRNA levels was more noticeable after 72 hours of UPF1 siRNAs treatment. Under these conditions and in agreement with the preceding experiment, the expression of endogenous HFE mRNA, in both cell lines treated with UPF1 siRNAs, was significantly increased to about 1.3- and 1.6-fold in HeLa and HepG2 cells, respectively (Figure 3.2C). Although the amount of the total HFE mRNA species increased upon UPF1 siRNAs treatment (Figure 3.2C), they did not reach the high abundance of those HFE mRNAs specifically using poly(A) signal 2, 3 or 4, found to be upregulated in the previous experiment (Figure 3.2B). These data confirm the presence of the NMD-resistant HFE mRNA isoforms resulting from 3'-end cleavage and polyadenylation at exon 6.

The results of this full set of studies demonstrate that human HFE mRNAs with polyadenylation at exon 6 are in fact expressed in HeLa and HepG2 cells and their levels contribute for the total amount of the cellular HFE mRNA. On the other hand, it seems that the amount of HFE mRNA isoforms resulting from cleavage and polyadenylation at exon 7, although present in both cell lines, is more representative in HepG2 cells. Nevertheless, it is noteworthy that the physiological HFE mRNA isoforms resulting from 3' end cleavage and polyadenylation at exon 7 behave as natural targets for the UPF1-dependent NMD mechanism in both HeLa and HepG2 cells.

IV.3. The HFE transcripts carrying a nonsense mutation are also committed to NMD

The preceding study has revealed that the expression of physiological human HFE transcripts, most specifically of those species with 3' end cleavage and polyadenylation at exon 7, are downregulated by the UPF1-dependent NMD mechanism. To determine whether human HFE mRNAs carrying a nonsense mutation show a parallel NMD profile, we investigated the effect of the TAC→TAG nonsense mutation at codon 138 (Y138X) of the human HFE gene, which was previously described in association with an iron overload phenotype in Portuguese individuals [Mendes et al. 2009]. With that aim, we first cloned, into

the mammalian expression pcDNA3 vector, a normal (WT) HFE minigene that encompasses all exons and introns 4, 5 and 6 of the human HFE gene (Figure 3.3A). Then, by using the normal HFE minigene as template for site-directed mutagenesis, we cloned the Y138X minigene (see Materials and Methods). In view of the higher transfection efficiency observed in HeLa cells, as compared with that obtained in HepG2 cells, the former cell line was chosen for these studies. Thus, plasmids harboring the WT or Y138X minigenes above described were transiently transfected into HeLa cells, previously treated with luciferase siRNAs (Figure 3.3B). Levels of the encoded mRNAs were determined by RT-qPCR, with specific primers for the 5' UTR that is transcribed by the cytomegalovirus promoter of the pcDNA3 vector and is specific for all mRNAs encoded from transfected HFE minigenes. Expression of these genes was normalized to the expression of the neomycin resistance gene and compared to those levels of mRNA encoded by a minigene similar to the normal HFE construct in which intron 6 was deleted by site-directed mutagenesis (see Materials and Methods; Del_IVS6 minigene; Figure 3.3A). This construct was used as a negative control for NMD-commitment since, in the corresponding transcript, splicing is abrogated downstream of the native translation termination codon, and thus it becomes NMD-resistant. Results from three independent experiments showed that expression of the normal transcript is at about 15% of the Del_IVS6 NMD-resistant mRNA (Figure 3.3C). On the other hand, quantification of the Y138X steady-state mRNA levels showed that this transcript accumulates at lower level than those of the wild-type mRNA, at about 10% of the Del_IVS6 mRNA (Figure 3.3C). These results indicate that both mRNAs seem to be degraded by the NMD pathway, although the Y138X mRNA seems to be induced to decay with a slightly higher efficiency. To unequivocally prove that WT and Y138X mRNAs are committed to the NMD mechanism, and knowing that NMD is a UPF1-dependent pathway, the effect of inhibiting NMD on both mRNAs was examined by treating the transfected cells with UPF1 siRNAs during 48 hours. Western blot analysis demonstrated a decrease in UPF1 protein level induced by siRNA of about 70-80%, when compared with results obtained after treatment with luciferase siRNA (Figure 3.3B). At this level of UPF1 downregulation, mRNA was quantified by RT-qPCR, relatively to Del_IVS6 mRNA expression, as before.

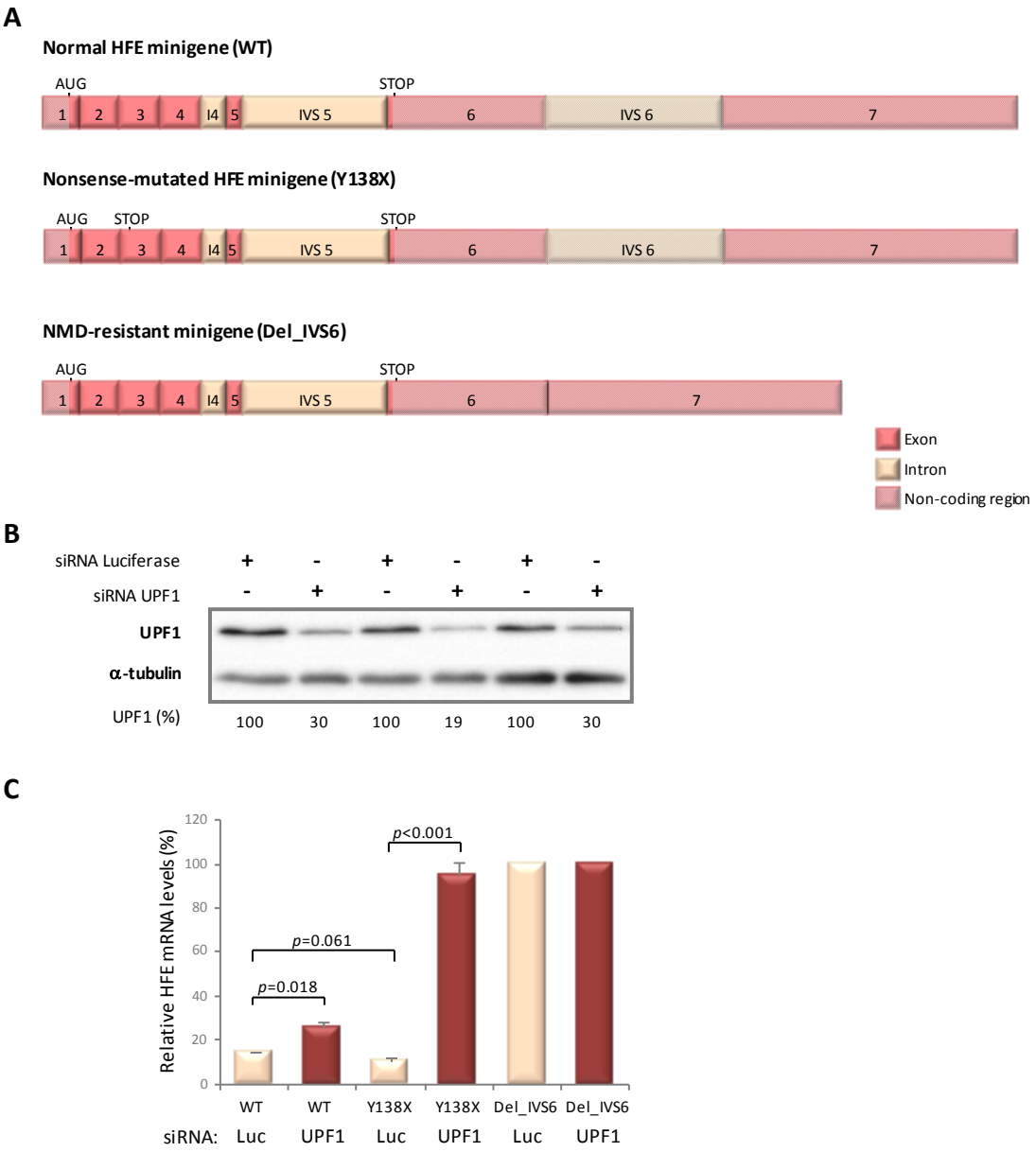

Figure 3.3. Normal and nonsense-mutated human HFE transcripts show low levels of expression when compared to those obtained for an NMD-resistant HFE transcript. (A) Schematic representation of the studied HFE minigenes designed to mimic human HFE genes that respectively encode normal (WT), nonsense-mutated (Y138X), and NMD-resistant (Del_IVS6) human HFE mRNAs. The position of the initiation (ATG) and termination (STOP) codons is represented. (B) Western blot analysis of HeLa cells extracts transfected with human UPF1 siRNA or a control siRNA target (luciferase). Twenty-four hours after siRNA treatment, cells were co-transfected with the plasmids encoding the normal, nonsense-mutated, or NMD-resistant mRNAs and with a second dose of siRNAs (UPF1 or luciferase). Twenty-four hours later, cells were harvested for protein and RNA. Immunoblotting was performed using a human UPF1 and α-tubulin (control for variations in protein loading) specific antibodies. The percentage (%) of UPF1 protein remaining expressed in the cells after siRNA treatment is indicated below each lane and was achieved by densitometric analysis using ImageJ software. (C) HFE mRNA quantification was performed by RT-qPCR as in Figure 3.2B but using primers specific for the heterologous 5' UTR common to all transfected genes. Neomycin resistance transcript was used as a normalization control. Quantification of the transcript levels was performed by the absolute quantification method. Levels of HFE mRNA obtained after cellular UPF1 siRNA treatment were compared to those obtained after luciferase siRNA treatment at the same conditions. The histogram shows the mean and standard deviations from three independent experiments, corresponding to three independent transfections. Statistical analysis was performed using Student's t test (unpaired, two tailed).

As shown in Figure 3.3C, depletion of UPF1 resulted in a 2-fold increase in the WT mRNA (p=0.018), while the increase in the Y138X transcripts was at about 6.7-fold (p<0.001), reaching values similar to those observed for the NMD-resistant Del_IVS6 transcripts expressed in the same conditions. These data confirm that WT and Y138X transcripts are both regulated by the NMD mechanism, although Y138X mRNA seems to be at some extent more efficiently degraded than the normal transcript (p=0.061). The fact that WT transcripts expressed in UPF1-depleted cells did not reach high levels comparable to those observed for Y138X and Del_IVS6 mRNAs expressed in UPF1-depleted cells, may reflect the presence of a proportion of WT HFE transcripts that use a poly(A) signal at exon 6 for 3' end processing and consequently, their NMD-resistance. Taken together, our data show that all alternatively polyadenylated mRNA isoforms encoded from a HFE gene that carries a PTC are regulated by the NMD mechanism; however, in what concerns the WT HFE transcripts, only those resulting from 3' end cleavage and polyadenylation at exon 7 behave as NMD-targets. Yet, our results indicate that the NMD mechanism might play an important role in regulating the levels of the HFE translated protein.

IV.4. Holo-transferrin seems to induce preferential recognition of a poly(A) signal at exon 6, shortening the 3' UTR, and thus it mitigates the effect of NMD on HFE gene expression regulation

To understand how the NMD mechanism could play a role in the regulation of HFE gene expression in response to iron challenge, we next decided to investigate whether human HFE transcripts could be physiological substrates for the UPF1-dependent NMD pathway after cell treatment with human holo-transferrin. For that, we quantified the endogenous HFE mRNA levels after siRNA-mediated depletion of UPF1 in HepG2 cells untreated or treated with 30 μM human holo-Tf. Results were compared to those obtained in NMD-competent cells transfected with luciferase siRNAs, also treated or untreated with 30 μM human holo-Tf during the same time (Figure 3.4). After 48 hours of siRNAs transfection and 24 hours of holo-Tf treatment, the Western blot analysis demonstrated a decrease in UPF1 protein levels induced by siRNA of 80-90%, when compared with results obtained after treatment with luciferase siRNA (Figure 3.4A). The Western blot analysis also demonstrated an increase in TfR2 protein levels induced by the holo-Tf treatment (Figure 3.4A), which ensures that the iron sensing is being activated by this treatment in our experimental system. To confirm that

the cellular holo-Tf treatment can also affect the mRNA levels through the iron regulatory system, we quantified the TfR1 mRNA levels by RT-qPCR. Its levels were significantly decreased ($p<0.001$) with holo-Tf addition (Figure 3.4B), which is in accordance with previously published data [Rao et al. 1985]. Under these conditions, the endogenous HFE mRNA levels were quantified by RT-qPCR assays, relative to the HFE mRNA levels obtained in cells treated with the control (luciferase) siRNA. To exclusively measure the effect of holo-Tf on the abundance of mRNAs that could be natural NMD-targets, we first used oligonucleotides for qPCR that specifically hybridize to the HFE exon 7 (Figure 3.4C). Our data have shown that depletion of UPF1 in HepG2 cells results in a significant 1.8-fold increase (p=0.003) of the abundance of those HFE mRNAs using a poly(A) signal at exon 7. This result is in accordance with the data presented in Figure 3.2B. On the other hand, the cellular UPF1-depletion carried out simultaneously with holo-Tf treatment, resulted in a much weaker 1.3-fold increase of the HFE mRNA levels. Also, it must be noted that in UPF1-depleted cells, addition of holo-transferrin induces a significant decrease in HFE mRNA levels (from 1.8-fold to 1.3-fold decrease; p=0.025), while the same treatment in luciferase siRNA-treated cells does not significantly affect HFE mRNA levels (p=0.314) (Figure 3.4C). Together, these results indicate that the addition of holo-Tf to the cell culture medium might induce preferential polyadenylation at exon 6 of the human HFE mRNA, which makes these transcripts NMD-resistant. To further confirm these results, we also measured, by RT-qPCR, the levels of HFE mRNA using specific primers for HFE exon 6 upstream of the poly(A) signal 1 (Figure 3.4D), which allowed quantification of the total amount of HFE mRNA isoforms alternatively polyadenylated at any one of the four signals. As in Figure 3.2C, levels of HFE mRNA, in cells treated with UPF1 siRNAs, increased about 1.4-fold (p=0.007) relative to the corresponding levels observed in cells treated with luciferase siRNA (Figure 3.4D). However, it must be referred that this increase is not as high as that observed when transcripts with polyadenylation at exon 7 are specifically quantified (Figure 3.4D versus Figure 3.4C). These results point out the presence of NMD-resistant HFE transcripts with 3' end cleavage and polyadenylation at exon 6.

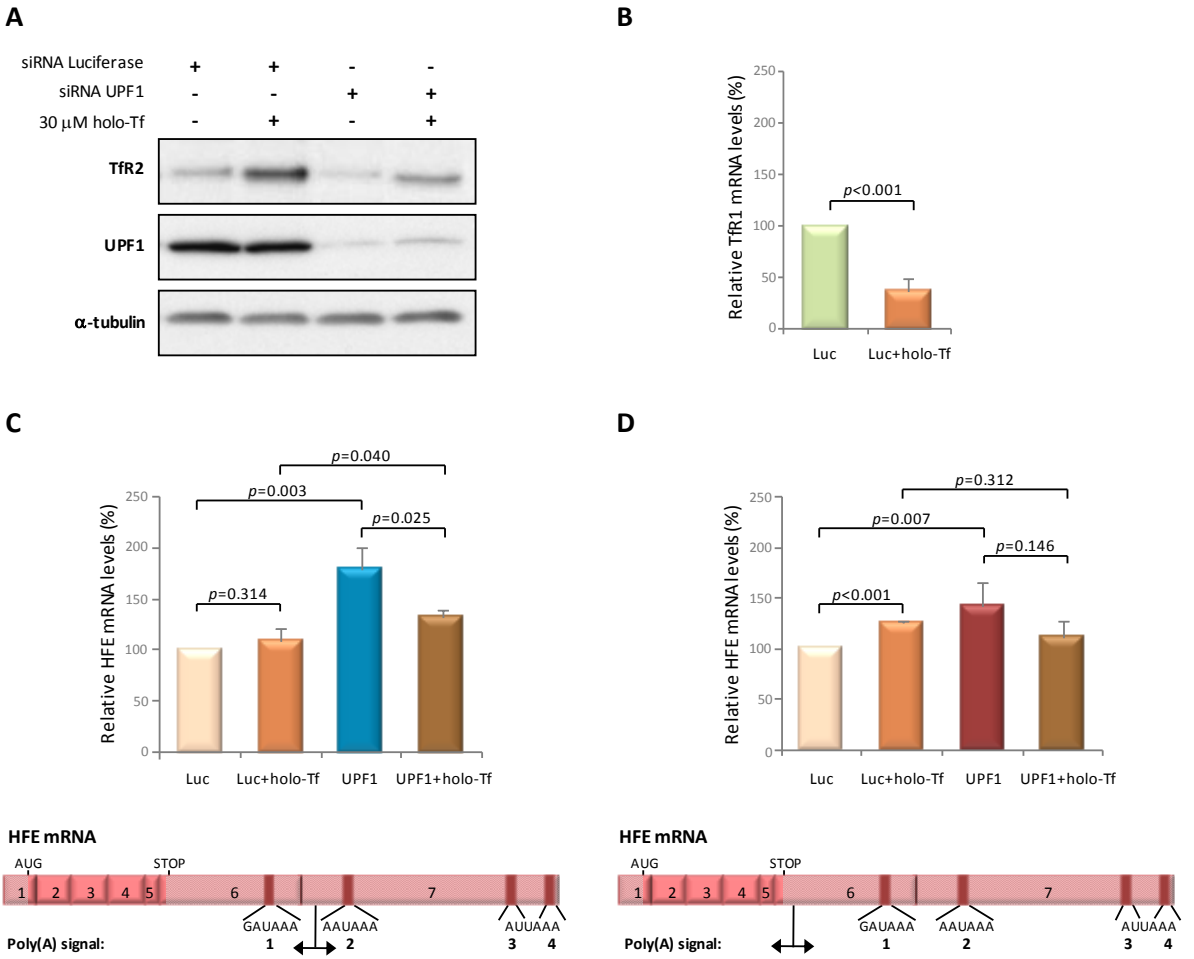

Figure 3.4. Holo-transferrin treatment of HepG2 cells increases endogenous HFE mRNA levels by inducing a preferential recognition of a poly(A) signal at exon 6, which makes the transcripts NMD-resistant. HepG2 cells were reverse-transfected siRNA (siRNA) duplexes directed to human UPF1 or to a non-endogenous target (luciferase) used as control. Twenty-four hours later, the cell media was replaced by fresh medium supplemented with 10% serum with or without 30 μM of holo-Tf. Cells were harvested for RNA and protein extracts 24 hours after holo-Tf treatment. (**A**) Western blot analysis was performed with specific antibodies for UPF1 and TfR2 proteins, using an α-tubulin antibody to control for protein loading. (**B**) HepG2 cells previously transfected with luciferase (Luc) siRNAs were treated with holo-Tf and relative changes TfR1 mRNA levels were assessed by RT-qPCR, normalized to GPS1 mRNA. (**C**) Relative levels of HFE mRNAs with 3' end cleavage and polyadenylation at exon 7, were specifically assessed by RT-qPCR, using primers that specifically hybridize to the 5' end of the HFE exon 7, as in Figure 3.2B. Levels of HFE mRNA obtained after cellular UPF1 siRNA treatment were compared to those obtained after luciferase (Luc) siRNA treatment, with or without holo-transferrin (holo-Tf) treatment. Below the histogram, there is a schematic representation of the human HFE mRNA, as in Figure 3.2B. (**D**) Relative levels of HFE mRNAs with 3' end cleavage and polyadenylation at exon 6 or 7, were assessed by RT-qPCR, using primers that specifically hybridize to the 5' end of the HFE exon 6, as in Figure 3.2C. Levels of HFE mRNA obtained after cellular UPF1 siRNA treatment were compared to those obtained after luciferase (Luc) siRNA treatment, with or without holo-Tf treatment. Below the histogram, there is a schematic representation of the human HFE mRNA, as in Figure 3.2C.

In addition, the total amount of HFE transcripts that includes those NMD-resistant isoforms due to polyadenylation at exon 6, increases 1.2-fold (p<0.001) in response to holo-Tf, which might reflect their higher stability. These data are in accordance with the fact that in cells treated with holo-Tf, the inhibition of NMD by UPF1 depletion does not significantly affect levels of the general pool of HFE transcripts (p=0.312; Figure 3.4D). Taken together, the results of this full set of experiments demonstrate that physiological human HFE mRNAs expressed in the hepatic HepG2 cell line and resulting from 3' end cleavage and polyadenylation at exon 7 are a natural target for the UPF1-dependent NMD mechanism, as above shown in Figure 3.2. On the other hand, in response to holo-Tf, it seems that polyadenylation at exon 6 becomes preferentially active which negatively impacts on the proportion of HFE transcripts that are NMD-targets, resulting in increased levels of total HFE mRNA in the cell. Therefore, our data show that both post-transcriptional mechanisms of alternative 3' end cleavage and polyadenylation and NMD might play an important task in fine-tuning HFE mRNA and protein levels, in response to iron challenge.

V. Discussion

The human HFE gene reference structure comprises 7 exons [Sanchez et al. 2001]. Although the sixth exon is 1056 bp long, only the first 41 bp encode for amino acids, so the remaining downstream 1015 bp sequence corresponds to the HFE 3' UTR, along with exon 7. The data presented in this study show that the splicing event occurring in the 3' UTR of the HFE transcript is in fact an attribute that makes the physiological transcript NMD-sensitive. In addition, we also show that besides the usage of the two polyadenylation signals for 3' end cleavage and polyadenylation previously described [Sanchez et al. 2001], HFE mRNA expression can also result from cleavage and polyadenylation at two other alternative sites – one located at exon 6 and the other one located at exon 7. Nevertheless, under cellular iron challenge, we observe an increase in the amount of NMD-resistant HFE transcripts, which appears to be due to shortening of the 3' UTRs resulting from alternative cleavage and polyadenylation at exon 6. These results show that alternative polyadenylation has functional consequences, with the shorter mRNA isoforms exhibiting increased stability, as they lose the feature that could make them physiological NMD-targets. Our results show how alternative polyadenylation and nonsense-mediated mRNA decay coordinately play a role in the fine-tuning the human HFE mRNA levels in response to the cellular iron status.

HFE protein has a well-recognized role in the regulation of iron homeostasis. HFE is capable of forming protein complexes with both TfR1 and TfR2 in the cellular membrane [Parkkila et al. 1997b; Chen et al. 2007]. It was recently proposed that HFE is partitioned between TfR1 and TfR2, and under increasing iron concentrations, HFE should shift away from TfR1 towards TfR2, triggering the signaling transduction pathway that leads to induction of the iron regulatory hormone hepcidin [Schmidt et al. 2008; Fleming 2009; Gao et al. 2009]. Here, we clearly show that under physiological conditions, human HFE transcript expression is regulated by the NMD mechanism, which can explain its relative low levels of expression observed in all tissues studied, including the liver cells [Feder et al. 1996; Fleming and Britton 2006]. Although there are inconsistent results on the effect of changes in cellular iron status on the gene expression of HFE [Feder 1999; Han et al. 1999; Theurl et al. 2005b], our results show that alternative polyadenylation and nonsense-mediated mRNA decay mechanisms coordinately act to fine-tune levels of HFE mRNA in response to changes in the cellular iron status. To further corroborate the importance of these post-transcriptional mechanisms in the regulation of HFE mRNA in response to iron challenge, there is the fact that the TfR2 mRNA, which is thought to be the principal partner of HFE protein in the regulation of the iron metabolism in the liver, is also a natural target for NMD [Wittmann et al. 2006]. As a result, NMD might be potentially used in a concerted way to adapt HFE and TfR2 protein expression to the physiological needs of the hepatocyte.

To our knowledge, there are a few nonsense mutations reported in the human HFE gene [Piperno et al. 2000; Beutler et al. 2002; Mendes et al. 2009; Pointon et al. 2009]. Yet, only one of these mutations has been studied in what concerns its ability to commit the transcript to NMD [Pointon et al. 2009]. This mutation consists in a single nucleotide deletion (c.del478) causing a frameshift that introduces a PTC in exon 4. The authors have demonstrated that the mutant transcript is degraded by NMD [Pointon et al. 2009]. Although these results seem to be in some extent in discordance with those presented here, as the c.del478 allele seems to account for only 2% of the total cytoplasmic HFE mRNA [Pointon et al. 2009], which indicates that this transcript is efficiently degraded by NMD, this might reflect the fact that the condition of iron overload observed in the patient can induce alternative polyadenylation in exon 6. Thus, these transcripts do not behave as physiological NMD-targets and, when carrying a PTC, they are all committed to NMD.

HFE mRNA metabolism has been poorly studied. Even the description of the specific structure of its 3' UTR has been disputed. The first reported Northern blot analysis showed that the human HFE gene is expressed as a 4.2 kb mRNA [Feder et al. 1996]. Nevertheless, the corresponding reported cDNA was only 2.7 kb long (GenBank U60319); in fact, the remaining 1.5 kb were more recently described by Sanchez et al. [2001] as being part of the HFE exon 7. Also, these authors have demonstrated by 3' RACE experiments that human HFE mRNA can result from the usage of two alternative polyadenylation signals located at exon 7, at 1455 and 2958 nts downstream of the stop codon [poly(A) signals 2 and 4, Figure 3.1] [Sanchez et al. 2001]. The observation that this 3' UTR structure presents additional putative polyadenylation signals downstream of the native translation termination codon, led us to further investigate their possible usage for 3' end processing. In fact, our results have shown that two novel poly(A) signals are also recognized in several human tissues. These poly(A) signals allow 3' end cleavage and polyadenylation at exon 6 or 7, respectively at 857 or 2620 nt downstream of the stop codon. These poly(A) signals are recognized in mRNAs from several tissues, including liver, duodenum, spleen and small intestine, which are tissues where HFE mRNA is mainly expressed and where HFE protein is expected to have a key role in the regulation of the iron metabolism. This observation might reflect their involvement in modulating HFE mRNA levels in the tissues were it is specifically expressed.

Cleavage and polyadenylation is required for the maturation of most mRNA transcripts [Proudfoot 1991; Colgan and Manley 1997]. Usually, the formation of mature mRNAs in vertebrates involves the cleavage and polyadenylation of the pre-mRNA at about 10-30 nt downstream of an AAUAAA or AUUAAA signal sequence. Although a strong polyadenylation signal is usually located within the 3' UTR, nearly in all transcripts there are single-base variants of the AAUAAA sequence that can also be recognized as polyadenylation signals [Beaudoing et al. 2000; Proudfoot 2004; Nunes et al. 2010]. A large scale analysis has shown that at least ten single-base variants of the AAUAAA sequence can also be found with a highly significant occurrence rate, potentially representing about 15% of all polyadenylation signals [Beaudoing et al. 2000]. In addition, Beaudoing and colleagues have revealed that about 29% of the mRNAs display two or more polyadenylation sites. In these mRNAs, the poly(A) signals proximal to the coding sequence tend to use variant signals more often, while the 3' most sites tend to use a canonical signal. Also, it has been suggested that variant signals (including the common AUUAAA) are processed less efficiently than the canonical signal and could therefore be selected for regulatory purposes [Beaudoing et al. 2000]. In the present work, we show that the

human HFE mRNA constitutes an example of a transcript where four poly(A) signals (one AAUAAA hexamer, two AUUAAA hexamers, and one GAUAAA hexamer) can be recognized for its 3' end cleavage and polyadenylation. These results confirm those obtained when we used the polyadq program [http://rulai.cshl.org/tools/polyadq/polyadq_form.html; Tabaska and Zhang 1999] to evaluate potential poly(A) signals in the human HFE 3' UTR – this program predicted that the AAUAAA and AUUAAA sites here described in the HFE exon 7 [poly(A) signals 2, 3 and 4; Figure 3.1] would be the active signals. It is interesting to note that according to the published data [Beaudoing et al. 2000], also in the HFE 3' UTR there is the recognition of more non-canonical than canonical AAUAAA poly(A) signals, the 5' most upstream poly(A) signal being, among the four poly(A) signals that are alternatively recognized, the hexamer less frequently recognized in mammalian cells [Beaudoing et al. 2000]. The data presented here is in fact in accordance with the usage of the four described alternative poly(A) signals to control the HFE mRNA levels.

The recognition of the non-canonical poly(A) signals by the 3' end processing machinery is not completely understood. It is currently believed that auxiliary sequences located either upstream or downstream (DSEs) of the non-canonical poly(A) sites may be able to compensate for a degenerated hexamer. Such sequences may serve to stabilize the poly(A) complex assembly by providing alternative binding for components of the 3' end processing machinery [Venkataraman et al. 2005]. A wide scale analysis of human poly(A) signals has in fact shown that many non-canonical poly(A) signals contain upstream A-rich sequences and tend to have a higher frequency of U and GU nucleotides in their DSEs with canonical poly(A) signals [Nunes et al. 2010]. Knowing that the human HFE 3' UTR has a very high A + U content, probably the recognition of its non-canonical poly(A) signals might indeed take advantage of potential A-, U- and/or GU-rich elements [Sanchez et al. 2001].

As indicated above, it is known that mRNAs with multiple poly(A) signals tend to use non-canonical poly(A) signals (including the common AUUAAA) more often than mRNAs with a single poly(A) hexamer. It has been also shown that the occurrence of non-canonical poly(A) signals mediates variation in poly(A) efficiency, thus enabling developmental, physiological and pathological regulation of gene expression [Edwalds-Gilbert et al. 1997; Graber et al. 1999; Beaudoing et al. 2000; Hughes 2006]. The occurrence of alternative poly(A) can also enable regulation of the ability of genes to respond to physiological stimuli [Sellers et al. 2004; Hughes 2006]. Recently, it has been shown that alternative polyadenylation can be a mechanism by

which transcripts can lose repressive 3' UTR elements which are associated to promotion of oncogenic transformation or immune cell activation [Sandberg et al. 2008; Mayr and Bartel 2009]. These authors have shown that cell proliferation conditions are associated with widespread reductions in the 3' UTRs by alternative poly(A), in which shorter mRNA isoforms exhibit increased stability resulting in augmented protein production, in part through the loss of microRNA-mediated repression [Sandberg et al. 2008; Mayr and Bartel 2009]. Our data show that the human HFE mRNA expression results from alternative poly(A) at a GAUAAA non-canonical poly(A) signal at exon 6, or at two AUUAAA sites or one AAUAAA site located at exon 7 of the HFE 3' UTR. On the other hand, we have observed that the extended HFE 3' UTR isoforms also encompass an intron located more than 54 nt downstream of the native stop codon, which is a feature that induces mRNA destabilization by the NMD mechanism. It is remarkable that in cells under iron challenge, our results show that the amount of NMD-resistant HFE transcripts increases, which appears to be due to shortening of the 3' UTR by alternative poly(A) at exon 6. The present work provides another example of how the shortening of the 3' UTR by alternative cleavage and polyadenylation can have the functional consequence of increasing mRNA levels through the loss of the feature that can make this transcript an NMD-target, in response to the cellular iron status. Which mechanism underlies the recognition and increased utilization of the proximal poly(A) signal located in exon 6 of the human HFE mRNA in response to iron challenge will be the purpose of further studies.

VI. Acknowledgments

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CHAPTER 4

General Discussion

I. General discussion and concluding remarks

The maintenance of iron homeostasis in the human body is a tightly regulated process only achieved by the feedback regulation between the sites of iron absorption, utilization and storage. Among these compartments, the proteins involved directly or indirectly with iron regulation must be coordinately controlled by signals and regulatory mechanisms that orchestrate their expression to achieve iron homeostasis [Muckenthaler et al. 2008; Hentze et al. 2010]. There are several molecular mechanisms of gene expression regulation that may take place at transcriptional, post-transcriptional and post-translational levels [Maniatis and Reed 2002; Orphanides and Reinberg 2002; Chua et al. 2007]. In fact, many of these processes affect the expression of the so-called iron-related genes and there are two that have a great deal of relevance in the expression control of their respective proteins [Chua et al. 2007; Hentze et al. 2010]. One of these mechanisms is the IRE/IRP system, a cellular pathway specific of the iron metabolism sphere, in which the presence of hairpin structures (IREs) found in 5' or 3' untranslated portions of mRNAs may, respectively, block translation or stabilize these mRNAs upon IRP binding [Hentze and Kuhn 1996; Muckenthaler et al. 1998; Hentze et al. 2010].

Another thoroughly exploited mechanism since the discovery of the hepatic hormone hepcidin in the beginning of the 21st century is its transcriptional control [Courselaud et al. 2002; Truksa et al. 2007; Weizer-Stern et al. 2007; Casanovas et al. 2009; Truksa et al. 2009]. Hepcidin in circulation negatively regulates the iron egress from cells by binding to ferroportin and inducing its degradation, consequently leading to iron retention in the intestinal epithelium and in macrophages of the reticuloendothelial system [Laftah et al. 2004; Nemeth et al. 2004b; Delaby et al. 2005; De Domenico et al. 2007]. Why is the transcription of hepcidin so important? Essentially because the rapid excretion of this hormone and lack of a regulated proteolytic processing implies that its regulation occurs at the transcription modulation level [Andrews 2008; Kemna et al. 2008]. It has been shown that hepcidin controls iron availability in response to iron levels, erythropoiesis, hypoxia and inflammation, and that all these upstream stimuli ultimately act on hepcidin transcription activation or repression at the hepatic level (Figure 1.3) [Nicolas et al. 2002a; Nicolas et al. 2002b; Nemeth et al. 2003; Pinto et al. 2008].

Regarding the hepcidin transcriptional control mediated directly by iron status, two very important mechanisms have been proposed, one mediated by BMP-signaling and another driven by the HFE-TfR2 complex (for details see Chapter 1). In fact, the identification of most of the participants in both these mechanisms that affect the expression of hepcidin, and

therefore the regulation of iron homeostasis, arose from genetic analyses of patients presenting primary iron overload. By far, the most common of the heritable forms of iron overload is the autosomal recessive disorder caused by mutations in the HFE gene, designated HFE-associated hemochromatosis or type 1 hemochromatosis [Feder et al. 1996; Pietrangelo 2004; Andrews 2008]. Nevertheless, several other effectors of the iron regulatory pathway have been identified associated with a determined form of the HH disease, namely TfR2, hemojuvelin, ferroportin and hepcidin (see Chapter 1 for details) [Camaschella et al. 2000; Montosi et al. 2001; Njajou et al. 2001; Roetto et al. 2003; Papanikolau et al. 2004]. All these forms of hemochromatosis have in common the abrogation of hepcidin function in modulating ferroportin activity due to hepcidin inappropriate expression (in the case of HFE, TfR2, hemojuvelin and hepcidin mutations) or to ferroportin mislocalization or insensitivity to hepcidin regulation (in the case of ferroportin mutations) [Bridle et al. 2003; Muckenthaler et al. 2003; Papanikolau et al. 2004; Kawabata et al. 2005; Porto et al. 2005].

Patients with mutations in HFE and TfR2 generally have a midlife presentation of elevated transferrin saturation, parenchymal iron loading and macrophage iron deficiency [Carthwright et al. 1979; Feder et al. 1996; Camaschella et al. 2000]. All these features may be attributable to the modest hepcidin production in these individuals. The inadequate regulation of ferroportin at the basolateral membrane of enterocytes impels the increased dietary iron uptake, whereas ferroportin maintenance at the macrophage renders their relative iron deficiency. The increasing knowledge of how HFE and TfR2 mutations affect hepcidin inappropriate expression has provided little insights into the specific functions of these molecules. Concerning HFE's mode of action, it has been proposed by several studies that HFE's function possibly varies from tissue to tissue, since a strong effect is observed on the intracellular iron levels, but often opposite effects arise according to the analyzed tissues or cell line [Gross et al. 1998; Corsi et al. 1999; Riedel et al. 1999; Roy et al. 1999; Drakesmith et al. 2002; Davies and Enns 2004; Enns 2006]. Moreover, very little is known about the transcriptional and post-transcriptional control of HFE gene expression, but several HFE alternative transcripts have been formerly described [Jeffrey et al. 1999; Rhodes and Trowsdale 1999; Thenie et al. 2000; Sanchez et al. 2001]. Nevertheless, since their specific structure, abundance and physiological function in human tissues remain to be characterized, these were the objectives pursued in the second chapter of this thesis.

Given the low level of HFE transcripts and consequently of its corresponding protein(s), a strategy of RT-PCR combined with cloning and sequencing of the obtained fragments were the methods utilized to identify the alternatively spliced HFE transcripts present in different human tissues. Most of them were in-frame exon skippings (alone or combined) that will potentially generate different arrangements of the translated protein. In fact, survey estimations reveal that approximately 75% of alternative splicing events take place within the translated regions of mRNAs that will affect protein-coding region [Okazaki et al. 2002; Zavolan et al. 2003]. Modifications in the primary structure of proteins may alter their binding properties, influence their intracellular localization, modify their enzymatic activity and/or affect their stability by diverse mechanisms [Kriventseva et al. 2003; Stamm et al. 2005; Blencowe 2006]. The scale of changes evoked by alternative splicing can range from very subtle modulations in the function of a given protein to a complete loss of function [Kriventseva et al. 2003; Stamm et al. 2005; Blencowe 2006]. In our study, we found singular exon skippings that, by eliminating the correspondent domain of the HFE full length protein (such as the skipping of exon 2 or exon 3), have the potential of altering HFE's protein binding capacity to its known ligands such as β2M, TfR1 or TfR2. Since the skipping of exon 2 transcript had been previously described as being present in hepatic, colon carcinoma and ovary cell lines, and due to the inability to observe the native protein owed to its low expression, we decided to further investigate this variant by fusing its cDNA to GFP [Rhodes and Trowsdale 1999; Sanchez et al. 2001]. The initial suspicion was that, by lacking the α1 domain of the protein, its interaction to TfR1 would be impaired. However, it was found that this modification affected an even upstream process, its binding to β2M and consequently, the TfR1 interaction was also affected. This was observed by both immunofluorescence and immunoprecipitation assays. The co-localization of HFE_skip2 protein with calnexin discloses a possible endoplasmic reticulum retention and eventual triggering of the unfolded protein response mechanism, as it has been described for the C282Y mutant [de Almeida et al. 2005; de Almeida et al. 2007b]. Curiously, studies performed in the human genome have shown that exon skipping is more likely to occur when exons are flanked by long introns, while experimental and computational analyses have revealed that the length of the upstream intron is more important in inducing alternative splicing than the length of the downstream intron [Fox-Walsh et al. 2005]. This is in accordance with the relatively high abundance levels of the HFE exon 2 skipping variant observed in human tissues, since the upstream intron one has about 3 kb long, whereas the intron two encompasses about 200 bp.

In parallel, we also found alternative transcripts containing partial and total inclusion of HFE's intron 4 that, by including an in-frame stop codon, could preclude the presence of the transmembrane and cytoplasmic domains of the full length protein. Again, as this HFE splicing isoform had been previously reported and regarding its potential feature as a soluble protein, we further investigated this variant as indicated before [Jeffrey et al. 1999]. The study of the intron 4 inclusion splice variant, lead us to interesting new data by revealing that this GFP tagged-soluble HFE is somehow able to maintain its conformational structure and be secreted by the cells, properly bound to β2M, on the contrary to the exon 2 skipping variant. Actually, several studies have underscored the significance of proteins that are affected by alternative splicing through the regulation of membrane binding [Blum et al. 1996; Tomida 1997; Diez et al. 2001; Kuramoto et al. 2001; Riteau et al. 2001; Meshorer et al. 2004; Stamm et al. 2005; Hviid 2006; Sangrouber et al. 2007]. In the majority of these cases, the membrane localization is an obvious property of the protein and non-membrane bound variants are generated by alternative splicing that may delete or interrupt transmembrane or membrane-association domains. These soluble proteins can be released from the cell, e.g. to the bloodstream or extracellular space, or otherwise, translocate into to a different cellular compartment. Moreover, they may acquire new functions, exerting dominant negative effects over the membrane bound forms or modulate the function of the latter [Stamm et al. 2005]. In this study, we propose that this soluble HFE can be released from some tissues such as the duodenum or the spleen, since those are the tissues presenting the highest relative amount of this isoform by qPCR (apart from the gonads), possibly by exerting an agonist or antagonist function of the membrane-bound HFE. This may happen immediately at the extracellular space of these cells (in the duodenal enterocytes or splenic macrophages), thereby possibly controlling iron export through ferroportin. This theory is to some extent supported by previous studies in which HFE overexpression has a particular effect in impairing iron export in cells where ferroportin is expressed, such as HT-29 (a cell line that mimics duodenal characteristics) or THP1 (a monocyte/macrophage-derived cell line) [Drakesmith et al. 2002; Davies and Enns 2004]. On the other hand, this soluble HFE variant may have a faraway effect, possibly acting on the liver hepatocytes by wielding an effect on the signaling transduction pathway conducted by the HFE-TfR2 complex in the expression of hepcidin. Again, any hint whether this soluble HFE may act as an agonist or antagonist of the membrane-bound HFE requires further studies. Although in numerous cases the exact function of the soluble variants has not been determined, various reports have indicated that some of these proteins may affect the

signaling transduction ability, as is the case of IL-6 or CD40 molecules [Kestler et al. 1995; Tone et al. 2001; Alberti et al. 2005].

Besides the role here emphasized for the alternative splicing mechanism, one should take into account the possibility of the use of alternative transcription or poly(A) sites in increasing the diversity of HFE transcripts reported. In what concerns HFE's transcriptional regulation, a study performed by Mura et al. [2004], where the mapping of the initiation sites indicated by both run off in vitro transcription and 5' RACE experiments, revealed a window of initiation within the -265 to -10 nt upstream of the first coding nucleotide. So, this relatively small distance between the putative initiation sites encourages the theory that the wide variety of HFE transcripts observed in reported Northern blots, ranging from 0.9 to 6.1 kb, may be attributable to either alternative splicing or alternative polyadenylation [Thenie et al. 2000; Sanchez et al. 2001]. In agreement, the discrepancies began when the HFE gene was first discovered and expression analysis by the Northern blot indicated a 4.2 kb transcript, whereas the cDNA report was only 2.7 kb long [Feder et al. 1996]. Only the studies by Sanchez et al. [2001] brought light to this problem, proving that the missing 1.5 kb of mRNA is in fact part of exon 7, which presents two polyadenylation signals, giving rise to both 2.7 and 4.2 kb transcripts. This report also identifies two additional polyadenylation sites in intron 6 [Sanchez et al. 2001].

Thus, in chapter 3, we began by confirming the structure of HFE's 3' UTR and, in addition to the alternatively polyadenylated transcripts found by Sanchez et al. [2001], we found two novel poly(A) signals for 3' end cleavage and polyadenylation affecting the post-transcriptional regulation of HFE, one in exon 6 and another in exon 7. The corroboration of the HFE's 3' UTR structure was mandatory to follow the investigation in what concerns HFE's regulation by NMD. NMD is a post-transcriptional eukaryotic mRNA surveillance mechanism responsible for the rapid degradation of transcripts harboring a PTC [Isken and Maquat 2007; Muhlemann et al. 2008; Silva and Romao 2009; Nicholson et al. 2010]. These stop or nonsense codons are generally recognized as premature if they are located at more than 50-54 nt upstream to the final exon-exon junction (Figure 1.7) [Nagy and Maquat 1998]. In this way, NMD limits the production of truncated polypeptides and protects the cell from their possible deleterious effects [Wagner and Lykke-Andersen 2002; Holbrook et al. 2004; Maquat 2004; Chang et al. 2007]. Besides shielding the cells from these truncated proteins generated by nonsense mutations, very significant since about one third of hereditary genetic diseases are caused by mutations

generating PTC-bearing transcripts, a wider role has been attributed for NMD as a contributor to the fidelity of gene expression [Mendell et al. 2004; Wittmann et al. 2006]. In effect, a group of NMD substrates includes physiological transcripts that encode functional full length proteins, produced generally as a consequence of alternative splicing, presence of an upstream ORF or an intron in the 3' UTR [Lelivelt and Culbertson 1999; He et al. 2003; Lewis et al. 2003; Mendell et al. 2004; Rehwinkel et al. 2005; Wittmann et al. 2006]. In fact, this latter specific architecture is also present in the HFE mRNA, since the native stop codon present in the major HFE transcript is located in exon 6, 1015 nt from the downstream non-coding exon-exon boundary [Sanchez et al. 2001]. We therefore hypothesized that the HFE gene can be included in the group of these NMD-regulated genes. As we have shown in chapter 3, there is a very significant effect of NMD in downregulating HFE physiological transcripts both in HeLa and HepG2 cells. As expected, this occurs more evidently when measuring those transcripts that include exon 7. As this increase in HFE mRNA levels was measured by the knockdown of UPF1, one should consider that, somehow due to technical difficulties, the abrogation of UPF1 expression level is incomplete, with an average of 70% reduction in all experiments performed. So, most likely the decay mechanism acting on HFE mRNA physiological levels is much stronger than the approximately 2.5-fold increase revealed at the 72 hours of UPF1 knockdown in HepG2 cells. Next, we were interested in analyzing the effect of NMD in nonsense mutations found in the HFE gene.

Homozygosity for the HFE C282Y mutation accounts for about 85% of patients with typical hemochromatosis [Feder et al. 1996; Adams et al. 2005]. However, other very rare or private HFE mutations have been reported, specially on southern Europe where C282Y is less frequent, contributing to the HH genetic heterogeneity [Barton et al. 1999; de Villiers et al. 1999; Mura et al. 1999; Merryweather-Clarke et al. 2000; Piperno et al. 2000; Pointon et al. 2000; Beutler et al. 2002; Le Gac et al. 2003; Mendes et al. 2009; Pointon et al. 2009]. In fact, the great majority of these novel HFE mutations that are associated with a strong iron burden are generally in *trans* with C282Y, and therefore fully explaining this HH phenotype. In agreement with this, we analyzed the Y138X nonsense mutation that we had previously found in a Portuguese hemochromatosis patient who presented a C282Y/Y138X genotype [Mendes et al. 2009]. As shown in chapter 3, NMD can also exert its role in diminishing the stability of transcripts with nonsense mutations, as we proven for the Y138X-containing transcript. On a recent preceding study where the effect of a frameshift mutation leading to a PTC was analyzed, it was also proved that this mutation leads to the destabilization of the HFE nonsense-mutated mRNA [Pointon et

al. 2009]. As NMD plays an evident role in controlling the levels of the nonsense mutated transcripts, one may speculate whether its action has a positive or negative effect in the development of the hemochromatosis disease. If NMD was to be absent, could those truncated polypeptides have some function in controlling hepcidin expression, as it is the case of the full length protein? Probably not, as those nonsense mutations (or frameshift generating PTCs) described in patients are usually located in exons 2, 3 or 4, which may lead to obvious conformational changes in the proteins' structure, possibly exerting a negative effect on the residual cell surface presentation of the HFE C282Y molecule [Levy et al. 1999]. Of course, these theories require further investigation.

So, NMD has a double task in what concerns controlling the levels of HFE transcripts both physiological and nonsense-mutated. When combining both studies performed on chapters 2 and 3, some interesting questions remain to be addressed. Do all the HFE transcripts with alternative poly(A) usage maintain the 5' coding region enabling the production of the full length HFE production? It has been proposed that alternative splicing and alternative polyadenylation may be combined in order to produce mRNAs with distinct stabilities (altering the 3' UTR enables the action of micro-RNAs or NMD), to influence rates of translation synthesis or affect the transport of the processed mRNA from the nucleus to the cytoplasm [Colgan and Manley 1997; Zhao et al. 1999; Maniatis and Reed 2002; Sandberg et al. 2008; Mayr and Bartel 2009]. Moreover, once the polypeptide is produced, many questions remain in what regards its cellular localization, binding capacity, protein stability or post-translation modification sensivity. So, it would be of great significance to try to merge the 5' and 3' structures of the alternative splicing and polyadenylation transcripts to further understand their function and possibly disclose novel regulatory mechanisms acting on HFE post-transcriptional expression. Regarding the alternative polyadenylation transcripts 2 and 4 described of chapter 3, since these isoforms were previously described by others as cDNA clones or Northern blot transcripts, one may assumed that they encompass HFE's total coding sequence. So, these transcripts' structure is probably post-transcriptionally regulated merely by the influence of their 3' end processing. But in what concerns the novel alternative polyadenylated transcripts (signals 1 and 3), further experiments are necessary to confirm their upstream structure.

The presence of a soluble HFE also poses a curious intersection at the production of this alternative splicing variant and the now known HFE's expression regulation by NMD. In

chapter 2, we have shown that this soluble protein variant may arise from two distinct splicing isoforms (by the total or partial inclusion of intron 4), that were observed in all the tissues tested. Interestingly, in both cases, a premature stop codon will be generated six nucleotides 3' from the exon four boundary. Proposing a parallel situation to what occurs with HLA-G splicing isoform, we assumed the production of a soluble HFE peptide, since it would not have the transmembrane domain and the cytoplasmatic tail. However, it is unknown whether the levels of this peptide can be regulated by NMD. Since an NMD expression control has been shown for both HFE physiological and nonsense transcripts, at first, there is no reason to believe that these isoforms containing intron 4 may escape the NMD mechanism. Still, it cannot be disregarded that these transcripts could be specifically cleaved and polyadenylated at exon 6 and therefore behave as NMD-resistant transcripts. Nevertheless, if that is the case, its expression levels should be highly increased when compared to the total HFE, which is not in accordance with the obtained results. Hence, most likely, this variant uses a polyadenylation signal at exon 7, and suffers a similar NMD-induced regulation as the normal transcript.

Since both alternative polyadenylation and NMD were shown to control the levels of normal HFE transcripts, we were interested in testing whether the cellular iron conditions could affect this regulation. The iron challenging assays carried out in HepG2 cells expose that the amount of NMD-resistant HFE transcripts is increased, which appears to be due to the shortening of their 3' UTRs by preferential/alternative cleavage and polyadenylation at exon 6. This is in agreement with other reports where it was shown that the occurrence of alternative polyadenylation can also enable regulation of the ability of genes to respond to physiological stimuli [Sellers et al. 2004; Hughes 2006]. Moreover, the use of non-canonical polyadenylation signals mediates variation in polyadenylation efficiency, thus enabling developmental, physiological and pathological regulation of gene expression [Edwalds-Gilbert et al. 1997; Graber et al. 1999; Beaudoing et al. 2000; Hughes 2006]. These results hint the relevance of iron stimulation on the production of alternative mRNA species, and it would be extremely interesting to observe an effect of the iron status on the relative abundance of the HFE alternative splice transcripts here analyzed. In fact, reports regarding other iron-related genes disclose a physiological adaptation in the production of their splicing isoforms to respond to cellular demands, as it has been proposed for DMT1 and ferroportin transcripts [Hubert and Hentze 2002; Cianetti et al. 2005; Zhang et al. 2009a].

Mechanisms of gene expression regulation are essential for the correct functionality of a multicellular organism as the complex human body. In particular, the myriad of processes involved in post-transcriptional regulation have been regarded as extremely important throughout the recent decades of research. In this thesis, we propose that alternative splicing, alternative polyadenylation and nonsense-mediated mRNA decay act together in controlling HFE's expression in a variety of human tissues. Moreover, tissue-specific patterns have been disclosed and a coordinated action of these mechanisms with iron stimulation may be foreseen. The body of knowledge arising from the role that post-transcriptional mechanisms exert in HFE's expression may hint future directions in the active field of iron biology and provide interesting cues that may translate into new therapeutics of iron homeostasis disorders.

II. Future perspectives

At this point, one may underscore that there are several post-transcriptional mechanisms acting on the control of HFE gene expression. Moreover, this may be of particular importance to elucidate HFE's role in the iron metabolism but they also have broader implications, evidenced by its involvement in infection and in the immune response.

As stated before, it would be of great interest to analyze the coding region associated with the novel alternative polyadenylated mRNA species found in this study. Ideally, due to the low abundance of these transcripts, deep-sequencing would be a more reliable approach. This would provide novel insight towards the post-transcriptional mechanism in the genesis of these mRNAs and the possible function of the produced peptides. Given their potential biological activity, it would be of particular interest to investigate the 3' structure of the splicing variant including intron 4 that gives rise to a soluble HFE. On the other hand, as the iron intake by the cell seems to induce preferential polyadenylation at exon 6, it would be interesting to explore the effect of environmental iron scarcity in the production of the polyadenylated species. These iron challenging assays would allow studying the structural and abundance variations on the HFE mRNA species. This might be achieved by measuring the specific levels of the alternative polyadenylation HFE transcripts by means of specific Northern blot probes using mRNA from distinct cell lines. To understand the tissue-specific 3' end cleavage and polyadenylation, the identification of the auxiliary sequences located either upstream or downstream of the poly(A) sites would be of crucial interest. It is

accepted that such sequences may serve to stabilize the poly(A) complex assembly by providing alternative binding for components of the 3' end processing machinery [Venkataraman et al. 2005]. So, it is possible that the endogenous expression of putative binding factors could somehow untangle this tissue-specific regulation.

In this thesis, relevance was mainly given to two HFE alternative splice transcripts, but others (e.g. the skipping of exon 3) may have an important physiological role as well. As for future experiments, the modulation of iron levels in different cells and measurement of the relative abundance of the HFE alternative splice transcripts could provide pertinent insights about their possible function. Moreover, the biological elements that have given the basis for the tissue-specificity of the HFE splice isoforms remain to be recognized, as the exon selection is known to be influenced by a number of activating and regulatory elements. The variation in splice site, exon/intron architecture, number of silencers and enhancers and secondary structures are all crucial for efficient exon definition and have a profound effect on the splicing pattern of a gene [Hertel 2008; House and Lynch 2008]. So, the analysis of these combinatorial actions should be addressed towards understanding the tissue-specificity of HFE splicing pattern, especially in what concerns spliceosomal components and splicing activators/repressors. For that, the expression of HFE minigenes with specific deletions of the putative binding sites of these splicing factors could provide a productive approach to unveil the origin and abundance of the HFE splicing isoforms.

It has been proposed in a study by Floreani et al. [2005] that the HFE exon 2 skipping may be caused by a presence of a polymorphism in intron two (IVS2+4 C→T) when in frame with the S65C mutation (a mutation that has been found in HH patients), and associated to an iron overload phenotype. However, others have defied that hypothesis since the above described polymorphism has a high frequency in the normal population or by showing that it is a neutral polymorphism in what regards the risk of developing iron overload [de Lucas et al. 2005; Curcio et al. 2008]. Curiously, among the several haplotypes where the H63D mutation (which has a very controversial role in causing iron overload) has arisen, the most frequent is, in fact, in linkage disequilibrium with the IVS2+4 polymorphism [de Lucas et al. 2005; Rochette et al. 1999]. Hence, we are currently testing whether this polymorphism, associated with H63D or not, may trigger the skipping of exon 2. If this is the case, interesting new data may arise in what regards the functional consequences of H63D in the HFE protein.

The identification of a soluble HFE associated to β2M, which we proved to be secreted to cell media in a variety of transfected cell lines, actually provides an enormous deal of interest. As previously stated, it may act at the nearby extracellular space of the cells where it is produced. Here, it can be an agonist or antagonist of the membrane-bound HFE. To test this hypothesis, transfection of both full length HFE and soluble HFE and evaluation of the intracellular iron status in various cell lines would disclose whether these molecules act in accordance or in conflict. By performing parallel assessments of the levels of the iron transporters (e.g. TfR1, DMT1 and ferroportin), we could have a hint of the action mode of each HFE variant. This would provide novel insights towards the role of both full length and soluble HFE in a variety of tissues, particularly in intestinal enterocytes or splenic macrophages.

On the other hand, the soluble HFE may have the ability of travelling through the bloodstream operating in remote tissues. Again, it may act as a positive or negative regulator of membrane-bound HFE. One may hypothesize that soluble HFE may interact with cell surface expressed receptors (TfR1/TfR2) in hepatic cells and, thus, regulate the systemic net iron levels by controlling hepcidin expression. To elucidate the physiological role of this HFE variant on hepcidin expression, stable transfections of the full length and soluble HFE proteins should be done under iron stimulus, whereas immunoprecipitation experiments would allow to identify its putative binding partner(s) (e.g. TfR1 or TfR2). The evaluation of phosphorylation status of ERK1/2 and p38 MAPK molecules concomitantly with hepcidin mRNA levels of expression should indicate if this is *the via* implicated in hepcidin regulation by the soluble HFE, as it has been recently proposed for the membrane-bound form [Calzolari et al. 2006; Gao et al. 2009; Poli et al. 2010].

The results obtained in previously suggested experiments may unveil new insights into the devise of new therapeutic strategies for iron-related disorders, based on manipulation of hepcidin levels or on the use of the soluble HFE isoform as a therapeutic agent. To understand the physiological effects of a circulating soluble HFE, it should be very enlightening to investigate if its serum levels are altered in individuals with changes in body iron stores. By analyzing patients with primary or secondary iron overload and comparing them with normal or iron deficiency individuals, the quantification results of the soluble form could indicate the therapeutical approach to follow. The phlebotomy treatment performed on hemochromatosis patients is difficult to supplant since it is inexpensive, easy,

safe and effective. Even so, a soluble HFE therapy may be successful in the C282Y homozygote HH patients, where there is very little HFE at the cell surface and the soluble HFE could somehow substitute its deficiency. Likewise, novel treatment strategies may be pursued in patients in the secondary iron overload disorders or iron deficiency patients, according to the function of the soluble HFE and its expression in these patients with iron deregulation.

CHAPTER 5

References

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