HHS Public Access

Author manuscript

Gene. Author manuscript; available in PMC 2019 July 26.

Published in final edited form as:

Gene. 2015 December 15; 574(2): 179–192. doi:10.1016/j.gene.2015.10.009.

HFE gene: structure, function, mutations, and associated iron abnormalities

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Abstract

The hemochromatosis gene *HFE* was discovered in 1996, more than a century after clinical and pathologic manifestations of hemochromatosis were reported. Linked to the major histocompatibility complex (MHC) on chromosome 6p, *HFE* encodes the MHC class I-like protein HFE that binds beta-2 microglobulin. HFE influences iron absorption by modulating the expression of hepcidin, the main controller of iron metabolism. Common *HFE* mutations account for ~90% of hemochromatosis phenotypes in whites of western European descent. We review *HFE* mapping and cloning, structure, promoters and controllers, and coding region mutations, HFE protein structure, cell and tissue expression and function, mouse *Hfe* knockouts and knockins, and *HFE* mutations in other mammals with iron overload. We describe the pertinence of *HFE* and HFE to mechanisms of iron homeostasis, the origin and fixation of *HFE* polymorphisms in European and other populations, and the genetic and biochemical basis of *HFE* hemochromatosis and iron overload.

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Concluding Comments
Studies of *HFE* and HFE protein in humans and mice have greatly advanced knowledge about non-classical MHC class I genes and proteins, iron homeostasis in mammals, and *HFE* hemochromatosis and other iron overload disorders in humans.

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Keywords

hemochromatosis; iron; major histocompatibility complex; transferrin receptor

1. Introduction

The French clinicians Trousseau and Troisier described the rare triad of darkening of the skin, diabetes mellitus, and cirrhosis in the latter half of the 19th C [1,2]. Three decades later, von Recklinghausen, a German pathologist, named the condition *hämochromatose* [3]. In a 1935 monograph, English gerontologist Joseph Sheldon reported his analysis of 311 cases from the literature and speculated that hemochromatosis is heritable [4]. In the 1970s, Simon and colleagues reported that hemochromatosis is relatively common, is linked to human leukocyte antigen (HLA) markers, and is transmitted as an autosomal recessive trait [5–8]. In 1996, Rothenberg and Voland hypothesized that non-classical class I MHC genes that bind β2M also control iron absorption and that β2M knockout mice (*beta2m*$^{-/-}$) would develop iron overload [9]. They and others confirmed this hypothesis [9–11], but humans with iron overload phenotypes did not have explanatory mutations in *B2M* [12]. In 1996, Feder and colleagues used positional cloning to identify *HFE*, the hemochromatosis gene, linked to the major histocompatibility complex (MHC) on chromosome 6p [13].

The membrane protein HFE is similar to MHC class I-type proteins and binds beta-2 microglobulin (β2M) [13]. HFE binds transferrin receptor (TFRC) in its extracellular α1-α2 domain [14,15]. HFE is required for normal regulation of hepatic synthesis of hepcidin, the main controller of iron metabolism [16]. Common *HFE* mutations account for ~90% of hemochromatosis phenotypes in whites of western European descent.

2. *HFE* mapping and cloning

In studies of French subjects, Simon et al. first reported that hemochromatosis is a heritable condition linked to MHC alleles HLA-A*03, B*07, and B*14 on the short arm of chromosome 6 (chromosome 6p) [5–8]. There is strong linkage disequilibrium within the MHC over a physical distance of 6 Mb in which there is a lack of recombination in most hemochromatosis patients [17]. Consequently, estimates of the position of the hemochromatosis locus relative to HLA-A and HLA-B [7,18–20] and HLA-F [21,22] were inconsistent. Polymorphic short tandem repeat markers within chromosome 6p enhanced the ability to conduct positional cloning [23–26]. Multipoint mapping within hemochromatosis families indicated that the gene was <1 cM proximal and ~0.5 cM distal to HLA-A [27]. D6S105(8), significantly associated with hemochromatosis, was the closest marker to the gene known at the time [27–31]. The gene was located telomeric to D6S105 [32]. D6S105(8) occurred in 30% of hemochromatosis-associated haplotypes in Italians [33]. Hemochromatosis ancestral haplotypes in Australians extended telomeric of HLA-A as far as D6S105 [34]. Yeast artificial chromosome LD5–1 hybridized with the hemochromatosis gene [35].

In 1996, Feder et. al. identified a 250 kb region between D6S2238 and D6S2241 that contained the hemochromatosis gene [13]. Within this region, they identified a MHC class I-

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like gene telomeric to the classical MHC that contained two missense mutations. Homozygosity for a c.845G→A mutation (cysteine→tyrosine at amino acid 282, p.C282Y) was found in 83% of hemochromatosis patients. This mutation was detected in 3.2% of control chromosomes. The remaining patients had c.187C→G (histidine→aspartic acid at amino acid 63; p.H63D) either in compound heterozygosity with p.C282Y or as p.H63D homozygosity [13].

Feder and colleagues named the gene HLA-H [13], although the name had been published earlier to designate a presumed pseudogene in the HLA class I region [36]. Bodmer and Mercier appealed for a more appropriate designation [37,38]. Both the WHO Nomenclature Committee for Factors of the HLA System and the HuGO Genome Nomenclature Committee approved the symbol *HFE* (*H* = high; *FE* = iron) (OMIM *613609). The cytogenetic location of *HFE* is 6p22.2 (genomic coordinates (GRCh37): 6:26,087,421–26,096,437).

3. *HFE* gene structure

HFE contains 7 exons spanning 12 kb [13]. *HFE* encompasses 9,609 bp of DNA on chromosome 6p within the extended HLA class I region. Histone genes are present on both sides of *HFE* [39]. Exon 1 corresponds to the signal peptide and exons 2–4 to the α1, α2, and α3 domains, respectively. Exon 5 accounts for the transmembrane domain. The cytoplasmic tail is encoded by the 5' portion of exon 6 that includes a native stop codon. Thus, the full-length transcript represents 6 exons [39].

3.1. *HFE* promoters and controllers

Alignment of the promoters of the human, rat, and mouse *HFE* genes reveals highly conserved elements, including binding sites for the transcription factors GATA, NF-IL6, AP1, AP2, CREB, PEA3, gamma-IRE, GFI1, HNF-3beta, and HFH2 [40].

The 5' end of HFE mRNA includes two major initiation sites directed by TATA-like sequences and a window of initiation upstream of the first coding nucleotide [41]. *HFE* is activated by liver-enriched C/EBPalpha, erythropoiesis-specific GATA-1, and Sp1 transcription factors [41]. An inverted repeat sequence near the *HFE* promoter can bind poly (ADP-ribose) polymerase 1 (PARP1) and repress *HFE* promoter. Knockdown of PARP1 or treatments with either hemin or $FeCl_3$ increase *HFE* mRNA and protein, leading to up-regulation of hepcidin mRNA [42]. An antisense transcript originating from *HFE* spans exon 1, exon 2, part of intron 1, and 1 kb upstream of it. The transcript was polyadenylated, had no open reading frame, and was expressed at low levels in all tissues and cell lines tested. This antisense RNA decreased *HFE* expression [43].

3.2. Alternative splicing

HFE expression is subjected to alternative splicing [44–47], like other MHC class I proteins [48,49]. Nine *HFE* splicing variants have been reported [39]. The predominant *HFE* full-length transcript is ~4.2 kb [47,50]. Other transcripts lack exon 2 or exon 3, or exons 2–3, 2–4, or 2–5 [50].

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The full length and most of the alternatively spliced *HFE* transcripts were detected in ovary, testis, duodenum, heart, kidney, spleen, and liver [50]. Ovary and liver have the highest levels of total *HFE* mRNA. Duodenum has smallest amount. Exon 2–5 skipping was detected only in the gonads, duodenum and heart [50].

In HepG2 cells, full-length HFE appears in a perinuclear and cell membrane distribution and co-localizes with β2M and TFRC [50]. The intracellular distribution of HFE protein derived from a transcript lacking exon 2 is similar to that of p.C282Y. HFE protein transcribed from exons 4 and 5 with inclusion of intron 4 has a scattered intracellular distribution, is absent from the cell membrane, does not co-localize with either β2M or TFRC, and is present in the endoplasmic reticulum [50]. A soluble HFE isoform lacking transmembrane and intracellular domains (sHFE) was found predominantly in the duodenum, spleen, breast, skin, and testis [44], and was secreted into HepG2 cell culture medium in association with β2M [50]. Alternative HFE splice variants may play regulatory roles in specific cells or tissues [50].

3.3. Evolution of *HFE*

Proteins with partial orthology to human HFE occur in primitive animals and plants [51]. The earliest animals that express MHC class I genes or proteins are branchiostomes [52]. Six cosmids from amphioxus (*Branchiostoma floridae*) contained genes orthologous to those of human MHC-linked regions. The genes mapped to a single chromosome [52]. In sharks and bony fishes, there are some orthologs of human MHC class I proteins, especially α3 domains [53–56].

There is no positive evidence of *HFE* orthologs in seabass (*Dicentrarchus labrax*) [57], in zebrafish (*Danio rerio*) [58], or in other fish species for which sequence data are available [59], although fish MHC class I proteins with distant homology to HFE have been identified [59]. *HFE* has not been described in amphibians, reptiles, or birds, but has been characterized in numerous mammals. These observations suggest that *HFE* arose later than MHC genes.

Basic Local Alignment Search Tool (BLAST) comparisons demonstrate that human HFE is ~100% similar to chimpanzee HFE and 61–67% similar to HFE proteins of dogs, rats, cattle, and mice. HFE C282 is conserved because cysteine 282 is essential to β2M binding and extracellular presentation of HFE. H63 is also conserved. Histidine 63 forms a salt bridge in the α2 domain that binds TFRC, suggesting that the salt bridge is important for HFE function [60]. Histidines 116 and 145 and tyrosine 140 are widely conserved. A cluster of four histidine residues (H109, H111, H116, H145) is associated with Y140 in the α1 domain. This configuration resembles functional sites in other iron-binding proteins [61].

Proline 188 is highly conserved. The function of MHC class I molecules depends on the interaction of the α1–α2 ligand binding superdomain with nonameric peptides presented to αβ T-cell receptors [62]. P188 occurs in the α1 domain and is associated with a kink necessary for peptide binding by MHC molecules [61,63]. In human HFE, amino acids 307–329 in the transmembrane domain are partially conserved, suggesting that normal function of this domain may not depend on a high degree of amino acid conservation.

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4. *HFE* coding region mutations

4.1. Common *HFE* mutations

The three most common coding-region mutations of *HFE* are: p.C282Y (exon 4; c.845G→A; rs1800562); p.H63D (exon 2; c.187C→G; rs1799945); and p.S65C (exon 2; c.193A→T; rs1800730) [64]. The p.C282Y mutation disrupts a critical disulfide bond in the α3 domain of HFE, abrogating its binding to β_2M and limiting its localization mostly to the cytoplasm [65,66]. p.H63D and p.S65C affect the α1 binding groove but do not prevent HFE presentation on cell surfaces.

4.2. *HFE* mutations and iron phenotypes

Mean serum iron, transferrin saturation (TS), and serum ferritin levels are higher in adults with p.C282Y homozygosity than in adults with other common *HFE* genotypes [67]. Demonstration of small differences in mean levels of these blood iron measures in adults with *HFE* genotypes C282Y/wt, H63D/wt, C282Y/H63D and H63D/H63D usually requires large cohorts [64,67].

"Classical" hemochromatosis typically occurs in adults who are p.C282Y homozygotes [64]. The prevalence of this genotype in many western European [68] Caucasian control populations is 0.002–0.005 (2–5/1000) [64]. Lower prevalence estimates have been reported from control populations in northern Spain (Catalonia) [69], among Basques in Guipuzcoa, Spain [70], and in central Italy [71]. The combined prevalence of C282Y homozygosity in 404 control subjects in Northern Ireland [72] and 249 control subjects in the northwestern Republic of Ireland [73] was 0.012 [95% confidence interval 0.006, 0.025]. Penetrance of iron overload phenotypes is variable and often greater in men [64].

Liver is the predominant target organ of iron overload, although arthropathy, diabetes mellitus and other endocrinopathy, and additional manifestations consequent to severe iron overload occur in some p.C282Y homozygotes [64]. A small proportion of adults with p.C282Y/p.H63D or p.C282Y/p.S65C compound heterozygosity or p.H63D homozygosity develop mild iron overload, usually in the presence of concomitant liver disease [64,74–77]. Prevalences and characteristics of adults with *HFE* hemochromatosis are reviewed in detail elsewhere [64].

4.3. *HFE* alleles and hemochromatosis

Diverse mutations involving *HFE* introns and exons discovered in persons with hemochromatosis or their family members cause or probably cause high iron phenotypes. Other mutations are either synonymous or their effect on iron phenotypes, if any, has not been demonstrated. Most of these mutations are rare and many have been discovered in Caucasians, although interest in identifying *HFE* mutations is great in regions where large numbers of Caucasians reside. These mutations and their phenotypes are displayed in Table 1.

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4.4. *HFE* hemochromatosis modifiers

HFE p.C282Y homozygosity is necessary but not sufficient to cause hemochromatosis phenotypes [119]. Several investigators reported that iron phenotypes were more severe in cohorts of hemochromatosis patients who inherited the common hemochromatosis ancestral haplotype [30,120–122]. Other genetic attributes reported to be "modifiers" of iron phenotypes in large cohorts of p.C282Y homozygotes include common alleles of *TF* [123,124]; *BMP2* (rs235756) [125,126]; single-nucleotide polymorphisms (SNPs) at *ARNTL* and *TFR2* [123]; *CYBRD1* (rs235756) [127]; *GNPAT* (rs11558492) [128]; and a microhaplotype on chromosome 6p [129].

4.5. Digenic hemochromatosis

Iron overload has been reported in persons who have digenic inheritance of one or more *HFE* mutations and a mutation of a non-*HFE* gene that is also involved in iron metabolism [79,102,130–135]. An example is the development of hemochromatosis in persons who are double heterozygotes for one or more *HFE* mutations and a mutation of the hepcidin gene (*HAMP*) [102,132]. Iron loading has occurred in persons with digenic inheritance of a *HFE* mutation and either a mutation of the hemojuvelin gene (*HJV*) [102,128,135] or the TFR2 gene (*TFR2*) [79]. Iron loading is often interpreted to be a synergistic effect of the two mutations because it is unlikely that either mutation alone would cause iron overload. Regardless, evidence is usually inadequate to prove that mutations of the two genes account for additive or multiplicative effects on iron absorption and retention within the same individual.

4.6. *HFE* alleles and porphyria cutanea tarda

Sporadic porphyria cutanea tarda (S-PCT), the most common of the porphyrias, is characterized by decreased activity of uroporphyrinogen decarboxylase (URO-D) in hepatocytes, accumulation of uroporphyrinogen I, photosensitivity dermatitis, and increased storage iron [136,137]. In persons with decreased URO-D activity, increased storage iron causes oxidation of uroporphyrinogen, resulting in the production of uroporphomethene. Uroporphomethene inhibits the decreased activity of URO-D further [138,139]. Prevalences of p.C282Y and p.H63D are much greater in persons with PCT [136,140–143]. Some persons with S-PCT or familial PCT and severe iron overload are homozygous for *HFE* mutations [144].

4.7. *HFE C282Y* frequency and geography

Molecular studies demonstrate that p.C282Y arose ~4000 years ago in the Neolithic Age [145,146] in Europe [147], possibly in Celtic people [148,149]. Although Vikings may have dispersed p.C282Y [146,149–151], especially in the late 8th-11th C, p.C282Y arose much earlier than the Viking era and thus may have also been spread in Europe by earlier seafarers [150]. Today, there are clines of decreasing p.C282Y frequency from Northwestern Europe to more eastern and southern regions of the continent [64,150]. Allele frequencies of p.C282Y in ethnically diverse western European white populations are 5–14% [152,153] and in North American non-Hispanic whites are 6–7% [154]. p.C282Y exists as a

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polymorphism only in Western European white and derivative populations, although p.C282Y may have arisen independently in non-whites outside Europe [155].

HFE p.C282Y arose on an ancestral chromosome 6p haplotype that included either HLA-A*03, B*07 or -A*03, B*14 and other alleles [5,6]. Part or all of the ancestral haplotypes is detectable in a majority of p.C282Y homozygotes with hemochromatosis phenotypes in Northwestern European and derivative countries [72,156–159]. Haplotypes A*03, B*35 and A*01, B*08, presumed to be linked to p.C282Y by the effects of migration and recombination, are common in subjects with p.C282Y homozygosity in northern Italy [160,161] and Sweden [156,162], respectively. Relative frequencies of haplotypes A*02, B*12 and A*09, B*05 are increased in Portuguese subjects with p.C282Y homozygosity [163].

4.8. *HFE H63D* frequency and geography

p.H63D is cosmopolitan but its frequency is greatest in whites of European descent [164,165]. Allele frequencies of p.H63D in ethnically diverse western European populations are 10–29% [166] and in North American non-Hispanic whites are 14–15% [154]. European haplotypes bearing p.H63D are typically associated with intronic haplotype TTG [114]. In northern Portuguese subjects, there is linkage disequilibrium between p.H63D and HLA-A*29-containing haplotypes [167]. In 19 populations of Central Eurasia, p.H63D was associated with three intronic haplotypes [168]. p.H63D, common in Indians, is associated with the European haplotype [169]. In Chinese subjects, p.H63D was detected on a variety of HLA haplotypes, indicating that p.H63D may predate the more genetically and geographically restricted p.C282Y mutation [170]. In Australian Aborigines, p.H63D was associated with HLA haplotypes common in Caucasians, suggesting that p.H63D was introduced by admixture. p.H63D (and p.C282Y) is absent in Brazilian Amerindians [171]. These observations suggest that p.H63D originated in Europe although multicentric origin, especially in Asia, cannot be excluded.

4.9. *HFE p.S65C* frequency and geography

In European whites, p.S65C is typically linked to intronic haplotype CCA [114]. In the Azores, p.S65C occurred in linkage disequilibrium with HLA-A*29 and -B*44 and with haplotype A*29, B*44 [172]. In Alabama whites with iron overload, p.S65C was linked to HLA-A*32 [91].

Allele frequencies of p.S65C in French and Basque cohorts were 2.5% and 2.9%, respectively [70,74]. In Sweden and Lithuania, allele frequencies were 1.6% and 1.9%, respectively [151,173]. In Canadian blood donors, the frequency of p.S65C was 2.0% [174]. In the Hemochromatosis and Iron Overload Screening (HEIRS) Study, the allele frequency of p.S65C in North American whites without high iron phenotypes was 0.7%. p.S65C was very uncommon in populations in Spain and the Mediterranean basin [175,176] and was not detected in Roma-Gypsies or Chinese men [177,178]. In the HEIRS Study, p.S65C was not detected in Hispanics, blacks, or Asians [102]. These observations suggest that p.S65C also arose in Europe.

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4.10. Advantages of common *HFE* mutations

HFE appears to be an example of a non-classical MHC locus that evolved a novel function, but what function? "The mutations of the *HFE* gene have all of the hallmarks of a balanced polymorphism…one in which the beneficial effect of the heterozygous state balances the deleterious effect of the homozygous state" [179]. It is plausible that protection against deficiency of iron or other trace metals absorbed by the same pathways, especially divalent metal-ion transporter (DMT) [180], and resistance to infectious disease are advantages that may have accrued to p.C282Y heterozygotes and resulted in fixation of p.C282Y in European Caucasian populations.

Women heterozygous for p.C282Y had higher values of hemoglobin, serum iron, and TS than women homozygous for the wild-type *HFE* allele in a small study [181]. In a larger study of similar design, a protective role against iron deficiency was not detected [179]. Prevalence of iron deficiency without anemia was lower among women heterozygous for p.C282Y than women homozygous for the wild-type *HFE* allele. There was a small but significant upward shift in the mid-range of the hemoglobin distribution among p.C282Y heterozygotes, consistent with an increased mean hemoglobin level without significant changes in the anemia range [182]. In one study, there was no demonstrable effect of p.C282Y on absorption of either heme or non-heme iron [183]. Taken together, these results suggest that putative evolutionary benefits of p.C282Y heterozygosity with respect to iron absorption, if any, are too small to measure. p.C282Y homozygotes absorb or retain increased amounts of zinc, manganese, and cobalt [184–186]. It has not been reported that p.C282Y heterozygotes absorb or retain greater proportions of non-ferrous metals with physiologic function.

It has been postulated that HFE is a receptor for microorganisms and that p.C282Y would protect against infection, although no specific microorganism(s) was proposed [155]. Although malaria in Europe was more common in areas adjacent to the Mediterranean Sea, malaria was endemic and epidemic in areas adjacent to the North Sea from late Antiquity until the latter half of the 19th C [187–189]. The larger erythrocytes of p.C282Y heterozygotes than those of *HFE* wild-type subjects [182,190] may have provided relative protection against malaria, although this is unproven. Mice lacking one or both *Hfe* genes were protected from *Salmonella typhimurium* septicemia because loss of Hfe induced the iron-capturing peptide LCN2 [191]. In northern Portuguese subjects, there is linkage disequilibrium between p.H63D and all HLA-A*29-containing haplotypes. Persons who have both p.H63D and A*29 have higher blood $CD8^+$ T-lymphocyte counts [167].

5. HFE protein structure, cell and tissue expression, and function

5.1. HFE structure

HFE is a protein of 343 amino acids that includes a signal peptide, an extracellular transferrin receptor-binding region (α1 and α2), an immunoglobulin-like domain (α3), a transmembrane region, and a short cytoplasmic tail [13] (Fig. 1). HFE binds β_2M to form a heterodimer expressed at the cell surface [13]. HFE is glycosylated at asparagine residues

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110, 130 and 234 during transport to the cell membrane [192]. Glycosylation is important for normal intracellular trafficking and function [192]. HFE interacts with TFRC [193].

The 2.6 Å crystal structure of HFE revealed that its ligand TFRC binds in a 2:1 TFRC:HFE molar ratio [61]. Most class I MHC molecules have a peptide-binding groove. Because the α1 and α2 helices are closer in HFE, the analogous site in HFE is too narrow for peptide binding [61]. TFRC and HFE bind tightly at the basic pH of cell surfaces, but not at the acidic pH of intracellular vesicles [61]. The 2.8 Å crystal structure of a complex between the extracellular portions of HFE and TFRC reveals that binding alters configurations of both HFE and its ligand [194]. The structures of TFRC alone and TFRC complexed with HFE differ in their domain arrangements and dimer interfaces [194].

Studies of cultured 293 cells overexpressing HFE wild-type proteins revealed that HFE forms stable complexes with TFR. In 293 cells overexpressing *HFE* C282Y, the association of HFE protein with TFR was markedly decreased [195]. Normal HFE protein decreased the affinity of TFRC for TF by inhibiting TFRC:TF-Fe interaction in an assay using purified proteins and a biosensor chip [61]. When HFE binds to TFRC in vitro, HFE changes the conformation of the Tf-Fe binding site as detected by biosensor assays, decreasing iron entry into Chinese hamster ovary cells [14]. In co-immunoprecipitation or surface plasmon resonance-based assay experiments using soluble HFE and TFR2, no evidence of binding of HFE and TFR2 was detected [196]. The pertinence of these in vitro results to iron homeostasis in vivo, if any, is unclear.

5.2. HFE function

Numerous factors, including HFE, act as upstream regulators of hepcidin transcription [197] (Fig. 2). The expression of *HAMP* was significantly lower in untreated patients with hemochromatosis, C282Y homozygosity, and iron overload than controls [198]. There was a significant correlation between hepatic iron concentration and expression of *HAMP* and *SLC40A1* in untreated hemochromatosis patients [198]. In iron-loaded *Hfe* knockout mice, liver hepcidin expression is relatively decreased [199]. These observations indicate that HFE plays an important part in the regulation of hepcidin expression in response to iron overload and that the liver is important in the pathophysiology of *HFE*-associated hemochromatosis [198]. These results also suggest that ferroportin could facilitate removal of excess iron from the liver [198]. Thus, *HFE* C282Y homozygosity results in decreased hepcidin responsiveness to iron and relative or absolute hepcidin deficiency [197].

5.3. HFE in cells and tissues

Small amounts of HFE are expressed in almost all normal cells and tissues [200]. Antibodies used to localize HFE in cells and tissues in several studies were raised against protein-specific peptides, not intact HFE [201–204]. Accordingly, uncertainty remains about the utility of these antibodies in localizing intact HFE protein in cells and tissues. HFE in human enterocytes appears in a decreasing gradient from villous crypts to villous tips and from duodenum to ileum [201,202], although the physiologic significance of these observations is unclear. Knockout of duodenal Hfe in mice does not lead to iron overload [205]. Expression is prominent in gastric epithelial cells, tissue macrophages, and blood monocytes and

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granulocytes [202,203]. Using immunohistochemical technique, staining for HFE in human liver was positive in the basolateral plasma membranes of bile ductular epithelium and sinusoidal lining cells [206]. In another study, liver immunostaining for HFE was most prominent in human Kupffer cells [207]. In rat liver, there was high expression of HFE mRNA, predominantly in hepatocytes, using quantitative real-time polymerase chain reaction [208]. These results differ from those of two previous studies [206,207]. A possible cause of the discrepancy could be non-specific immunostaining of non-HFE MHC class 1 molecules. HFE is expressed on apical plasma membranes of the syncytiotrophoblast, an iron transport tissue in the placenta [204].

Duodenal expression of HFE and TFR2 (but not TFRC) in wild-type mice and humans was restricted to small intestinal crypt cells where the respective proteins are co-localized [209]. HFE and TFRC are also co-localized in 293 and HeLa cells [195]. In human Caco-2 cells, HFE and TFR2 co-localized to a vesicular compartment that had marked signal enhancement on exposure to iron-saturated transferrin ligand, indicating that HFE preferentially interacts with TFR2 in an early endosomal transport pathway for TF:Fe [209]. In HuH7 hepatoma-derived cells, normal HFE, TFR2, and hemojuvelin form a membrane-associated complex that functions to regulate hepcidin [210].

In cells from HFE p.C282Y homozygotes with hemochromatosis, p.C282Y is not present at cell surfaces, has a diffuse cytoplasmic localization, does not co-localize with β2M and TFRC, and is retained in the endoplasmic reticulum [50]. Cell surface-associated HFE signal is reduced in gastric epithelial cells, monocytes, and macrophages [203]. The cellular distribution of TFR2 in small intestinal crypt cells from p.C282Y homozygotes is also altered [209]. p.C282Y is retained in the endoplasmic reticulum and middle Golgi compartment, fails to undergo late Golgi processing, and is subject to accelerated degradation [201].

6. Mice with *Hfe* knockouts

6.1. Discovery of murine *Hfe*

Hashimoto and colleagues isolated the mouse ortholog of human *HFE* and designated the mouse gene as “MR2” [211], now widely known as *Hfe*. Compared with human HFE, mouse Hfe has a predicted amino acid sequence similarity of ~66% and is analogously expressed in various tissues. Eight amino acid residues between mouse Hfe α1 and α2 domains that are not present in human HFE are due to a coding sequence from the intron [211]. Whereas human *HFE* is telomeric to the MHC on chromosome 6p, mouse *Hfe* has been translocated from a site telomeric to the MHC on chromosome 17 to chromosome 13, along with other genes [211]. Soon after human *HFE* was reported [13], structural details of mouse *Hfe* were described [212].

6.2. *Hfe* knockouts

Zhu and colleagues produced a targeted knockout of all six *Hfe* exons in the mouse [213]. The mRNA transcript of 1.9 kb, present in multiple tissues from $Hfe^{+/+}$ mice, was present in reduced amounts in $Hfe^{+/-}$ mice, and was not detectable in the livers, kidneys, and spleens

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of *Hfe*$^{-/-}$ mice. Thus, the knockout produced a null allele. On a standard diet, *Hfe*$^{-/-}$ mice had elevated TS and increased liver iron, predominantly in hepatocytes. Iron measures in heterozygous *Hfe*$^{+/-}$ mice were normal at age 10 weeks. Iron-related traits of *Hfe*$^{-/-}$ mice were inherited in an autosomal recessive pattern. This mouse *Hfe* knockout model simulates genetic and biochemical abnormalities of *HFE* hemochromatosis [213]. Iron-related characteristics of different *Hfe*$^{-/-}$ mouse strains vary [214]. Hepatic gene expression profiles differ according to strain and age [215]. The pattern of hepatic iron loading inheritance in *Hfe*$^{-/-}$ mice is polygenic [216].

HFE interacts with TFRC in the α1–α2 groove [14]. Bahram and colleagues created a knockout mouse by deleting the second and third *Hfe* exons (corresponding to α1 and α2 domains of Hfe) [217]. Mice homozygous for this deletion had increased duodenal iron absorption, elevated plasma iron and TS levels, and iron overload, predominantly in hepatocytes [217].

Levy and colleagues used targeted mutagenesis to produce two mutations in *Hfe*. The first deleted a large portion of the coding sequence, generating a null allele. The second introduced a missense mutation (C282Y) [218]. Homozygosity for both mutations caused iron loading, although effects of the null mutation were more severe. Mice heterozygous for either mutation accumulated more iron than normal controls. Thus, the murine *Hfe* C282Y mutation does not result in a null allele [218].

6.3. Tissue-specific *Hfe* knockouts

Mice with deletion of *Hfe* in crypt and villous enterocytes had normal plasma iron and TS values, normal unbound iron-binding capacity, normal liver and spleen iron concentrations, and normal hepcidin mRNA expression [205]. These observations demonstrate that small intestinal Hfe is not necessary for the physiologic control of systemic iron homeostasis [205]. Mice with tissue-specific *Hfe* knockout in macrophages had normal plasma iron measures and normal iron concentrations in liver and spleen [219]. This is consistent with observations in wild-type mice subjected to macrophage depletion which have normal hepatic iron levels and hepcidin responses to iron challenges [220,221]. Mice with tissue-specific *Hfe* knockout in hepatocytes developed iron phenotypes similar to those of *Hfe*$^{-/-}$ mice, including elevated serum iron and TS values, severe hepatic iron accumulation, and reduced splenic iron content [219]. These findings indicate that Hfe must be expressed in hepatocytes to prevent iron overload because it is important for appropriate hepcidin mRNA expression [219].

6.4. *Hfe* knockins

Levy et al. used a targeting vector to introduce *HFE* C282Y into murine *Hfe* codon 282 and a second vector to construct a *Hfe* null allele [218]. Mice heterozygous for either mutant allele developed more iron loading than wild-type control mice. Mice homozygous for the null allele developed massive iron overload. Mice homozygous for the C282Y knockin had an iron phenotype intermediate between that of null homozygotes and wild-type mice. This indicates that C282Y caused hepatic iron accumulation without total loss of function. Mice homozygous for each *Hfe* mutation had less non-heme splenic iron than wild-type mice

[218]. Relative resistance of the spleen to iron loading also occurs in *HFE* hemochromatosis [4,222].

Ajioka et al. performed additional studies of *Hfe* mutant (both C282Y knockins and *Hfe* knockouts) and wild-type mice [223]. In *Hfe* mutant mice and wild-type mice, down-regulation of iron absorption occurred with dietary iron loading and with age, although the *Hfe* mutant mice continued to absorb more iron than wild-type mice. Iron absorption increased in response to reduced iron stores and accelerated erythropoiesis to a similar degree in *Hfe* mutant and wild-type mice. These results suggest that mouse Hfe plays a minor role in down-regulation of iron absorption but does not influence its up-regulation [223].

Tomatsu et al. generated knockin mice homozygous for *Hfe* H67D (corresponding to human H63D), homozygous for *Hfe* C294Y (corresponding to human C282Y), and *Hfe* C294Y/H67D compound heterozygotes. Hepatic iron loading was significantly greater in all three groups of mice with *Hfe* mutations than in control wild-type mice. Iron loading was most severe in C294Y homozygotes, less severe in C294Y/H67D compound heterozygotes, and even less in H67D homozygotes. TS was increased only in C294Y homozygotes. Tomatsu et al. concluded that *Hfe* H67D in a homozygous configuration or in compound heterozygosity with C294Y results in partial loss of Hfe function and increased hepatic iron loading [224].

Schmidt et al. introduced mutations into a ubiquitously expressed *Tfr1* transgene or the endogenous *Tfr1* locus to promote or prevent Hfe/Tfr1 interaction [225]. In one mouse model, Hfe constitutively interacted with Tfr1. In two other models, most or all Hfe was free of Tfr1. Under conditions favoring constitutive Hfe/Tfr1 interaction, mice developed iron overload attributed to inappropriately low expression of hepcidin. Mice with a mutation that interferes with Hfe/Tfr1 interaction developed iron deficiency associated with inappropriately high hepcidin expression. High-level expression of a liver-specific *Hfe* transgene in *Hfe*$^{-/-}$ mice was also associated with increased hepcidin production and iron deficiency. These results suggest that Hfe induces hepcidin expression when it is not complexed with Tfr1 [225].

6.5. Iron absorption

In a study of iron uptake by duodenal enterocytes of *Hfe*–/– mice, Herrmann et al. examined ferric reductase duodenal cytochrome b (Dcytb) mRNA and speculated that Dcytb may be important in iron uptake[226]. In persons with *HFE* hemochromatosis, *CYBRD1* (*DYCTB*) expression was increased in some reports [227,228] and not in others [229–231]. In *Hfe*$^{-/-}$ mice, DMT mRNA transcripts containing an iron-responsive element are greatly increased, despite the existence of iron overload [232]. Immunoreactive DMT is up-regulated in *Hfe*$^{-/-}$ mice [233]. Absorption of both ferrous and ferric iron by *Hfe*$^{-/-}$ mice is greater than that of *Hfe*$^{+/+}$ mice [233]. In *HFE* hemochromatosis, DMT mRNA levels are also increased [234].

Down-regulation of iron absorption occurred in *Hfe*$^{-/-}$ mice with dietary iron loading and with age, although to a lesser extent than in wild-type mice [223]. Up-regulation of iron absorption consequent to phlebotomy-induced iron deficiency or phenylhydrazine-induced hemolysis was similar in *Hfe*$^{-/-}$ and wild-type mice [223]. Levels of liver hepcidin mRNA

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are higher in *Hfe*$^{+/+}$ than *Hfe*$^{-/-}$ mice [235]. In *Hfe*$^{-/-}$ mice, an iron challenge down-regulated hepcidin production and decreased hepatic Tfr2 levels [235]. Iron overload was abrogated in *Hfe*$^{-/-}$ mice with constitutive overexpression of the gene that encodes hepcidin (*Hamp*) [236]. The normal relationship between body iron stores and liver hepcidin mRNA levels is altered in *Hfe*$^{-/-}$ mice, such that liver hepcidin expression is relatively decreased despite iron overload [199]. These observations substantiate that hepcidin produced in the liver is a central controller of iron absorption [16].

6.6. Iron uptake by hepatocytes

Chua et al. studied hepatocyte iron uptake in *Hfe*$^{-/-}$ mice and in iron-loaded and non-iron-loaded wild-type mice [237]. Tfr1-mediated iron and Tf uptake of hepatocytes were significantly greater in *Hfe* knockout mice than in wild-type mice with similar iron levels and Tfr1 expression. This indicates that Tfr1-mediated hepatocyte iron uptake is regulated by Hfe. Hepatocyte iron uptake was much greater via the Tfr1-independent pathway than the Tfr1 pathway but the former was not regulated by Hfe [237]. Tfr2 levels are higher in livers of *Hfe*$^{-/-}$ than *Hfe*$^{+/+}$ mice, indicating that loss of Hfe function does not interfere with iron-responsive regulation of Tfr2 [238]. Diferric transferrin up-regulated hepatocyte Tfr2 protein expression but not iron uptake, suggesting that Tfr2 has a limited role in the Tfr1-independent pathway [237]. Mice with hepatocyte-specific knockout of *Hfe* also develop hepatic iron overload [219]. In *Hfe*$^{-/-}$ mice, hepatocyte export of iron via ferroportin is decreased [239].

6.7. Iron and erythropoiesis

Hfe is expressed by splenic erythroid cells and in vitro splenic erythroid colonies of phlebotomized wild-type mice [240]. These results suggest but do not prove that Hfe is not a sensor for hepcidin in erythroid cells [240]. *Hfe*$^{-/-}$ mice down-regulate hepcidin expression to the same extent as wild-type mice in response to both phlebotomy and erythropoietin injections but recover more rapidly from phlebotomy- or phenylhydrazine-induced anemia than wild-type mice with iron overload of similar severity [240]. Erythroid cell uptake of iron per Tfr1 is greater in *Hfe*$^{-/-}$ than wild-type mice, suggesting that Hfe interferes with erythroid Tf-Fe uptake [240]. Iron absorption increased similarly in response to hypoxia in *Hfe*$^{-/-}$ and *Hfe*$^{+/+}$ mice [241]. Thus, at least two independent mechanisms regulate iron absorption, only one of which requires Hfe [241].

7. *HFE* mutations and iron overload in other mammals

Black rhinoceroses (*Diceros bicornis*) develop iron overload [91,242,243]. To determine whether the *HFE* gene of black rhinoceroses has undergone mutation as an adaptive mechanism to improve iron absorption from iron-poor diets, Beutler et al. sequenced the entire *HFE* coding region of four species of rhinoceros (two browsing and two grazing species). Although *HFE* was well conserved across the species, numerous nucleotide differences were found between rhinoceros and human or mouse, some of which changed deduced amino acids. Only one allele, p.S88T in the black rhinoceros, was a candidate that might adversely affect HFE function. p.S88T occurs in a highly conserved region involved in the interaction of HFE and TFRC [244]. Bottle-nosed dolphins (*Tursiops truncatus*) also

develop iron overload [245,246] but sequencing dolphin *hfe* did not reveal deleterious mutations [247]. Red deer with iron storage disease (*Cervus elaphus elaphus*) did not have pathogenic *HFE* mutations [248].

Acknowledgments

This review and the corresponding Gene Wiki article were written as part of the Cardiac Gene Wiki Review series - a series resulting from a collaboration between the journal GENE, the Gene Wiki Initiative, and the BD2K initiative. The Cardiac Gene Wiki Initiative is supported by National Institutes of Health (GM089820). Additional support for Gene Wiki Reviews is provided by Elsevier, the publisher of GENE. This work was supported in part by Southern Iron Disorders Center. The corresponding Gene Wiki entry for this review can be found here:<<https://en.wikipedia.org/wiki/HFE_%28gene%29>>.

Abbreviations

BLAST	Basic Local Alignment Search Tool
β2M	beta-2 microglobulin
chromosome 6p	short arm of chromosome 6
DMT	divalent metal-ion transporter
HEIRS Study	Hemochromatosis and Iron Overload Screening Study
HFE	hemochromatosis gene (human)
Hfe	hemochromatosis gene (mouse)
HLA	human leukocyte antigen
MHC	major histocompatibility complex
OMIM	Online Mendelian Inheritance in Man
PARP1	poly (ADP-ribose) polymerase 1
PCT	porphyria cutanea tarda
sHFE	soluble HFE
TF	transferrin (human)
Tf	transferrin (mouse)
TF-Fe	iron-loaded transferrin (human)
Tf-Fe	iron-loaded transferrin (mouse)
TFRC	transferrin receptor (human)
Tfr1	transferrin receptor (mouse)
TFR2	transferrin receptor-2 (human)
Tfr2	transferrin receptor-2 (mouse)

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TS	transferrin saturation
SNP	single nucleotide polymorphism
URO-D	uroporphyrinogen decarboxylase

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Highlights

- *HFE,* the hemochromatosis gene, is linked to the major histocompatibility complex on chromosome 6p
- *HFE* encodes HFE, an extracellular protein that binds beta-2 microglobulin
- HFE is a positive upstream regulator of hepcidin.
- Common *HFE* mutations account for most hemochromatosis cases
- Iron phenotypes of mice homozygous for *Hfe* knockouts are similar to those of *HFE* hemochromatosis

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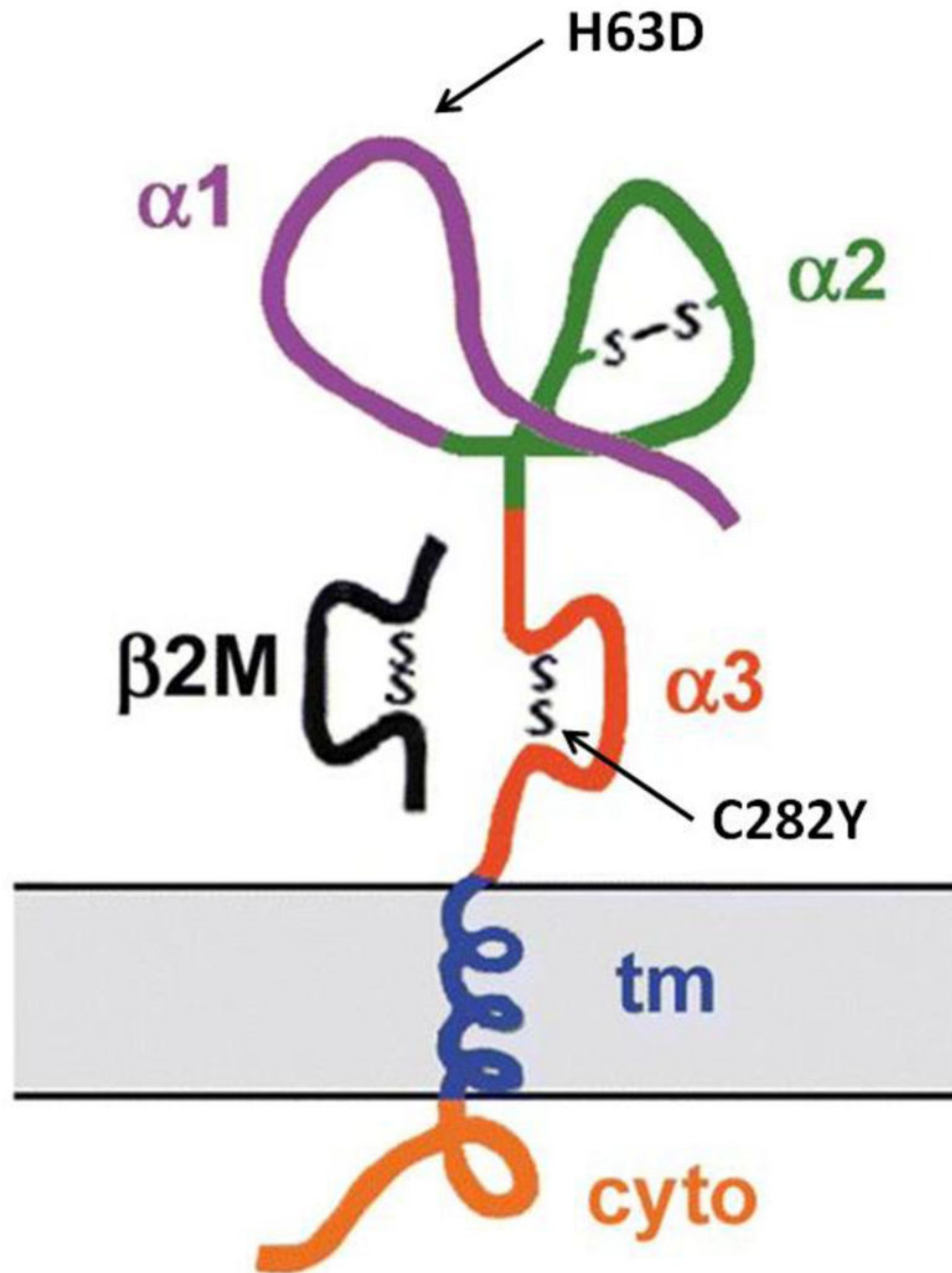

Figure 1.

HFE protein in association with beta-2 microglobulin (β2M) at the cell surface. The three extracellular domains of HFE are designated α1, α2, and α3. β2M is shown associated with the α3 domain. Abbreviations: cyto, cytoplasmic tail; tm, transmembrane domain. Adapted from R.E. Fleming, W. S. Sly, Mechanisms of iron accumulation in hereditary hemochromatosis. Annu Rev Physiol 64 (2002) 663–680. Used with permission.

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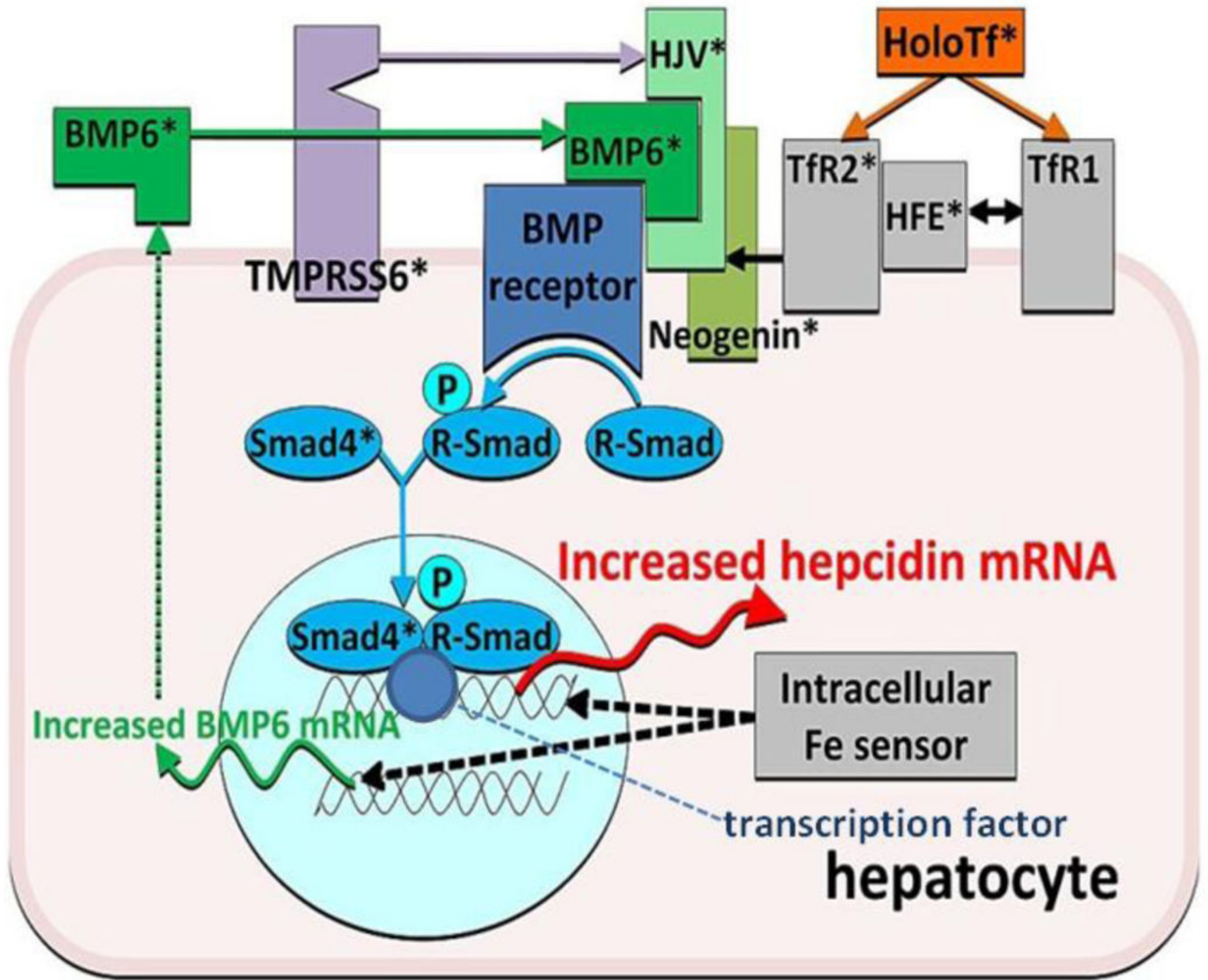

Figure 2.

A model of regulation of hepcidin transcription by iron. Iron as holotransferrin is shown in orange, iron sensors and associated molecule in gray, bone morphogenic protein (BMP) receptor and its transduction pathway in shades of blue, the ligands and co-receptors of the BMP receptor in shades of green, and the negative regulator protease in purple. *Molecules the ablation of which caused iron dysregulation. Adapted from T. Ganz, Hepcidin and iron regulation, 10 years later, Blood 117 (2011) 4425–4433. Used with permission.

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Table 1.

Mutations of the hemochromatosis gene (*HFE*)[1, 2]

Exon	cDNA alteration	Protein alteration	Phenotype[3]	Reference
5'UTR	c.−20G→A	fs	1	[78]
2	88C→T	L30L	0	[79]
2	[4]128G→A + 187C→G	G43D + H63D	1	[80]
2	138T→G	L46W	1	[81]
2	c.del149–170	L50fs	0	[82,83]
2	128G→A	G43D	1	[84]
2	157G→A	V53M	0	[85]
2	175G→A	V59M	0	[85]
2	187C→G	H63D	1	[13]
2,4	[4]187C→G + 845G→C	H63D + C282Y	1	[86,87]
2	189T→C	H63H	0	[85]
2	193A→T	S65C	1	[88]
2	196C→T	R66C	1	[79]
2	199C→T	R67C	1	[83]
2	c.del203	V68fs	2	[89]
2	211C→T	R74X	2	[90]
2	277G→C	G93R	2	[91]
2	277del	G93fs	2	[92]
2	314T→C	I105T	1	[91]
2	340G→A	E114K	1	[83]
	IVS2(+4)T→C	—	0	[90]
3	381A→C	Q127H	1	[85]
3	385G→A	D129N	0	[81]
3	414C→G	Y138X	2	[81]
3	471del	A158fs	2	[93]
3	478del	P160fs	2	[94]
3	502G→C	E168Q	1	[95]
3	502G→T	E168X	2	[96]
3, 2	[4]502G→C + 187C→G	E168Q + H63D	1	[97]
3	506G→A	W169X	2	[96]
3	527C→T	A176V	1	[98]
3	548T→C	L183P	2	[99]
	IVS3(+1)G→T	(null allele)	2	[100]
	IVS3(+21)T→C	G43D	1	[79]
	IVS3(+21)T→C	—	0	[79]

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Exon	cDNA alteration	Protein alteration	Phenotype[3]	Reference
	IVS3(–48)C→G	—	0	[101]
4	c.del616–48C→T	—	0	[102]
4	636G→C	V212V	0	[103]
4	671G→A	R224G	1	[79]
4	676C→G	R226G	1	[104]
4	689A→T	Y230F	2	[81]
4	c.del691–693	Y231X	2	[105]
4	696C→T	P232P	0	[79]
4	697C→T	Q233X	2	[106]
4	c.dup794	W267fs	2	[107]
4	724G→A	D242D	0	[102]
4	747G→A	K249K	0	[102]
4	814G→T	V272L	0	[108]
4	829G→A	E277K	0	[103]
4	845G→A	C282Y	2	[13]
4	845G→C	C282S	2	[109]
4	[4]845G→A + 842C→A	C282Y + T281K	1	[110]
4	847C→T	G283X	2	[111]
4	848A→C	Q283P	2	[112]
4	867C→G	L289L	0	[79]
4	884T→A	V295E	1	[102]
4	884T→C	V295A	?	[89]
4	867G→C	L289L	0	[79]
	IVS4(+37)A→G	—	0	[85]
	IVS4(+48)G→A	—	0	[113]
	IVS4(+109)A→G	—	0	[85]
	IVS4(–44)T→C	—	?	[114]
	IVS4(–50)A→G	—	?	[115]
	IVS4(+115)T→C	—	0	[85]
	942T→C	A314A	0	[98]
5	989G→T	R330M	2	[85]
	IVS5(+1)G→A	—	1	[116]
	IVS5(–47)G→A	—	?	[114]
6	c.1022–1034del13	H341X	2	[83]
—	[5]*HFE*del	—	1 or 2	[117]

[1]Modified from C.Q. Edwards, J.C. Barton, Hemochromatosis in: J.P. Greer, D.A. Arber, B. Glader, A.F. List, R.T. Means Jr., F. Paraskevas, and G.M. Rodgers (Eds.), Wintrobe's Clinical Hematology, Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia, 2014, pp. 662–81. Permission to publish requested from publisher.

[2]Most alleles were identified in persons with hemochromatosis phenotypes or their family members. *HFE* Y231X was identified in a hemochromatosis cell line.

[3]Phenotype: 0 = none known; 1 = probably weak effect on iron homeostasis; 2 = probably strong effect on iron homeostasis.

[4]Complex allele with two mutations in *cis*.

[5]An *Alu*-mediated recombination caused loss of the complete *HFE* gene sequence. Homozygosity for the corresponding chromosome 6p is a common cause of hemochromatosis in Sardinia [118].

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