VERSITA

Central European Journal of Biology
DOI: 10.2478/s11535-007-0007-0
Review article
CEJB 2(1) 2007 34–55

Transferrin receptor 2 is emerging as a major player in the control of iron metabolism

Alessia Calzolari, Isabella Oliviero, Ugo Testa*

Department of Hematology, Oncology and Molecular Medicine,
Istituto Superiore di Sanità,
00161 Rome, Italy

Received 18 October 2006; accepted 15 January 2007

Abstract: Our knowledge of mammalian iron metabolism has advanced dramatically over recent years. Iron is an essential element for virtually all living organisms. Its intestinal absorption and accurate cellular regulation is strictly required to ensure the coordinated synthesis of the numerous iron-containing proteins involved in key metabolic processes, while avoiding the uptake of excess iron that can lead to organ damage. A range of different proteins exist to ensure this fine control within the various tissues of the body. Among these proteins, transferrin receptor (TFR2) seems to play a key role in the regulation of iron homeostasis. Disabling mutations in TFR2 are responsible for type 3 hereditary hemochromatosis (Type 3 HH). This review describes the biological properties of this membrane receptor, with a particular emphasis paid to the structure, function and cellular localization. Although much information has been garnered on TFR2, further efforts are needed to elucidate its function in the context of the iron regulatory network.

Keywords: Transferrin, iron, cell signaling, membrane receptors

1 Introduction: a brief outline of iron metabolism

Iron (Fe) is an essential, but potentially dangerous, metal for all living organisms. Its precise cellular regulation is necessary to ensure the synthesis of numerous iron-containing proteins required for metabolic processes, including those involved in oxygen transport and storage, DNA synthesis and electron transport. Its precise regulation is also vital in order to avoid the build-up of potentially toxic levels of iron. In humans, iron deficiency results in anaemia, while excess iron leads to organ damage as a result of non-transferrin-

* E-mail: u.testa@iss.it

bound iron (NTBI) accumulation. Particularly, under conditions of iron overload, the iron-binding capacity of plasma transferrin can be exceeded, resulting in the appearance of NTBI. NTBI is rapidly cleared from plasma and taken up mostly by the liver [1], but when in great excess it may lead to tissue damage [2]. Iron accumulation may also be the consequence of excessive transferrin-Fe uptake, as well as the inability to export iron (reviewed in [3]). In recent years, the identification of a number of novel proteins has begun to clarify the mechanisms of iron uptake, storage and metabolic regulation.

Multiple physiological processes are used to maintain body iron homeostasis, for example iron absorption, metabolic iron use, iron storage and iron export. Specialized tissues play a key role in these various steps of iron metabolism.

Since there are no significant physiological mechanisms to regulate iron loss, the tight regulation of intestinal iron absorption represents the main method to control iron body content. The mechanism of dietary iron absorption has, in part, been elucidated during recent years. Absorption mainly occurs in the duodenum, where iron can be taken up as heme and non-heme iron. Intestinal heme transport and absorption involves the recently investigated heme-carrier protein 1 (HCP1), which is responsible for heme uptake in the gut [3, 4]. In addition to HCP1, additional proteins act in other tissues as heme transporters; thus in developing erythroid precursors ABCB6 (ATP-binding cassette, subfamily B, member 6) acts as a mitochondrial heme transporter [5], while FLCVR (feline leukemic virus receptor, subgroup C) acts as a heme transporter in the liver and in the cytoplasm of erythroblasts [3]. Other molecules, such as ABCG2 (also known as BCRP, breast cancer resistance protein) are involved in the efflux of heme from cells [3]. Non-heme iron is absorbed through the action of the divalent metal transporter (DMT1, also known as NRAMP2) [6] and Dcytb, a ferrireductase present at the apical membrane that donates electrons for reduction of ferric iron [Fe(III)] to the ferrous state [Fe(II)] [7]. Heme internalized into intestinal cells is degraded by heme oxygenase and the released iron, together with dietary absorbed ionic iron, enters the intracellular non-heme iron pool. This subsequent release of iron from intestinal cells to general circulation is mediated by two proteins: ferroportin-1, an iron exporter [8, 9] and hephaestin, a ferroxidase [10]. Intestinal iron absorption is influenced by dietary factors and is controlled by the hormone hepcidin, a small peptide able to bind and regulate the expression of ferroportin-1 [11].

Iron exported from intestinal cells circulates bound to transferrin (Tf), an abundant protein synthesized by the liver that binds to iron atoms with a high affinity. Tf keeps iron soluble, preventing Fe(III) from precipitating, and transports iron between the various cellular compartments.

Between one and two milligrams of iron are absorbed by humans each day. However, approximately 25 mg are required on a daily basis to sustain the hemoglobinization of newly produced erythroid cells. To negate this apparent iron shortfall, iron recycling of that already present in the body is employed in order to ensure sufficient iron levels. This recycling process involves the mobilization of iron stored in cells of the reticuloendothelial system, namely tissue macrophages, such as spleen and bone marrow macrophages, which phagocytose senescent erythrocytes to scavenge their iron from hemoglobin and return

it into circulation. Additional iron may be obtained through the mobilization of cellular iron stores, mainly from hepatocytes [12].

Tf-mediated uptake is the primary mechanism of iron uptake for the majority of cells. This process is particularly pronounced in erythroid cells that need large amounts of iron to sustain the high rates of heme synthesis, incorporated into hemoglobin. This process of cellular iron uptake is known as the "transferrin cycle". The binding of iron-saturated transferrin, holotransferrin (holoTf), to its receptor, transferrin receptor 1 (TFR1), triggers clathrin-mediated invagination of the cell membrane, with subsequent endocytosis of the holoTf-TFR1 complex into endosomes. The pH inside the endosomes is rapidly lowered through the action of proton pumps, resulting in conformational changes of Tf and TFR1, and the release of iron from holoTf with the formation of apoTf. Iron is then transported across the endosomal membrane into the cytoplasm by DMT1. Since Tf carries ferric iron, whereas DMT1 is selective for ferrous iron, Fe(III) must be reduced to Fe(II) through the action of the recently identified ferrireductase, Steap3 [13]. The apoTf-TFR1 complex is then returned back to the cell surface and apoTf is released, becoming available to be re-charged with iron and to start the cycle anew. Iron released in the cytosol can be used for cellular metabolism, and the formation of essential iron-containing proteins present in the cytosol and mitochondria.

The portion of cellular iron that is not needed for immediate utilization is stored by ferritin, an ubiquitous and multimeric protein [14]. Ferritin consists of an apoprotein shell of 24 light (L) and heavy (H) chain subunits, surrounding a core of about 4500 iron atoms. The ratio of H and L subunits varies in different tissues and in response to various stimuli (e.g. inflammation or infection) [14].

The maintenance of cellular iron homeostasis requires coordinated expression of the various genes involved in iron uptake, storage and export. This complex regulation is in large part accomplished through the interaction of iron regulatory proteins (IRPs) with the iron regulatory elements (IREs), conserved hairpin structures found in the untranslated regions (UTRs) of some mRNAs encoding iron-related proteins. Single IREs are located in the 5'-UTR of H and L ferritin chains, mithocondrial aconitase and ferroportin-1; the binding of IRPs to these IREs determines inhibition of the translation process [15]. In contrast, multiple IREs are located in the 3'-UTR of TFR1 mRNA; in this case the interaction of IRPs with these IREs leads to the stabilization of the mRNA [16]. Mammalian cells contain two IRPs, IRP1 and IRP2, whose activity is regulated by the cellular labile iron pool. When iron levels are high, a cubane [4Fe-4S] cluster assembles in IRP1, inhibiting IRE binding activity and converting IRP1 to the enzymatic protein aconitase. When cellular iron stores are low, IRP1 activity is stimulated and it binds to IRE targets. In contrast, IRP2 does not contain a Fe-S cluster and its activity is regulated through the control of its degradation rate according to iron levels; in iron-depleted cells IRP2 accumulates while it is degraded when iron levels are high [17]. Specifically IRP2 is regulated in iron-replete cells by oxidation-induced ubiquitination and subsequent degradation by the proteasome, with concomitant loss of its IRE binding activity [18]. The binding of heme to IRP2 protein causes its oxidation and its subsequent recognition by the heme-

oxidized IRP2 ubiquitin ligase-1 (HOIL-1) [19, 20]. The Cys201 residue of IRP2 binds ferric heme, while His204 is a ferrous heme binding site, with both residues required for the heme-mediated ubiquitination and degradation of IRP2.

An alternative iron uptake system, particularly active in epithelial cells, is represented by neutrophil gelatinase-associated lipocalin (NGAL/24p3) and its receptor 24p3R. 24p3R mediates the ability of the holo-form of 24p3 to donate iron to cells and prevent apoptosis, while the internalization of apo-24p3 by cells leads to iron efflux that results in cell death [21, 22].

Liver cells play a key role in the control of iron metabolism. This is related to the capacity of hepatocytes to synthesize hepcidin, a small peptide (20 to 25 amino acids for the two most frequent isoforms) that plays an essential role in the control of iron homeostasis [23]. Hepcidin promotes internalization and degradation of the iron exporter ferroportin-1, and through this mechanism decreases both intestinal iron absorption and macrophage iron release [23]. Mice lacking hepcidin expression and humans with mutations in the hepcidin gene have been found to develop severe iron overload at an early age (juvenile hemochromatosis) [24, 25]. Hepcidin expression by hepatic cells is regulated by another protein involved in the control of iron metabolism, hemojuvelin (HJV). Importantly, mutations of the HJV gene cause a form of juvenile hemochromatosis phenotypically indistinguishable from hemochromatosis due to hepcidin gene mutations. HJV is a bone morphogenetic protein (BMP) co-receptor that through its signalling regulates hepcidin expression by liver cells [26].

In 1999, a new protein involved in iron metabolism was identified, a second transferrin receptor called TFR2 [27, 28]. This protein seems to play an important regulatory role in iron metabolism, but the mechanism through which it exerts this function remains largely unknown. This review analyzes the main biological properties of this receptor, compared to those of the TFR1, and outlines the areas that need to be investigated to gain an insight into the understanding of the exact role played by TFR2 in iron metabolism.

2 TFR2 structure

The human TFR2 gene was cloned independently by two groups. In 1998 Glöckner *et al.* sequenced a large region of chromosome 7q22 which contains TFR2 and other important genes such as erythropoietin [27]. One year later, Kawabata *et al.* fortuitously cloned the gene during an attempt to identify new transcription factors [28]. Two transcripts resulting from alternative splicing or promoter usage have been identified, TFR2-α and TFR2-β. The α-form, which is 2.9 Kb long has been cloned from a cDNA library of TF-1 and consists of 18 exons. The β-form, cloned from a cDNA library of HL60, is 2.5 kb in length, it lacks exons 1-3 of the alpha form and its first exon (exon 4 of the α-form) has an additional 142 nucleotides at the 5'-end [28]. Both TFR2 transcription products contain no typical iron responsive elements (IRE) in their 3'- or 5'- untranslated regions. TFR2-α encodes an 801 amino acid type II transmembrane protein, consisting of cytoplasmic (residues 1-80), transmembrane (residues 81-104) and extracellular (residues 105-801)

domains. The C-terminus extracellular domain is 45% identical and 66% similar with that of TFR1 and contains two cysteines (residues 108 and 111), a finding that suggests a homodimeric configuration of the protein. Conversely, the N-terminal cytoplasmic regions of TFR1 and TFR2 show no similarity. In addition, TFR2-α contains a YQRV motif (residues 23-26) in the intracellular domain which may act as an internalization signal, similar to the YTRF motif in TFR1 [29–31]. Furthermore, four putative N-glycosylation sites (residues 240, 339, 540 and 754) occur in the TFR2-α protein, which is known to be glycosylated [28, 32]. Importantly, TFR2-α possesses a RGD sequence in the extracellular domain that is identical to a TFR1 sequence that has been shown to be essential for transferrin binding [33]. In TFR1 this sequence is located within a helix of its helical domain [34], which is responsible for interacting with the hemochromatosis protein, HFE, in the co-crystal structure [35, 36]. By creating point mutations the RGD sequence of TFR2-α has been demonstrated to be crucial for Tf-binding [37].

In contrast, TFR2-β protein lacks the N-terminal portion of TFR2-α, including the cytoplasmic, transmembrane and part of the extracellular domain [28], suggesting that it may be a soluble, intracellular form of TFR2. Thus far, the existence of TFR2-β protein has not been demonstrated, and as such only TFR2-α will be discussed in this review.

3 Mutations

Hereditary hemochromatosis (HH) is a genetic disorder that results from the disruption of the mechanisms that regulate iron absorption at the duodenal level, leading to progressive parenchymal iron overload and organ damage. HH is classically associated with a Cys282Tyr (C282Y) polymorphism of the HFE gene [38]. Non-HFE HH is a definition applied to all the hemochromatosis disorders that are not related to HFE mutations. The genetic classification of HH is reported in Table 1.

Type	Gene/Protein	Inheritance	Phenotype	Severity
1	HFE/HFE	AR	Parenchymal Fe overload	Highly variable
2A	HJV/Hemojuvelin	AR	Parenchymal Fe overload	Variable
2B	HAMP/Hepcidin	AR	Parenchymal Fe overload	Severe
3	TFR2/Transferrin receptor 2	AR	Parenchymal Fe overload	Severe
4	SCL40A1/Ferroportin1	AD	Reticulo-endothelial Fe overload	Variable

AR, autosomal recessive; AD, autosomal dominant.

Table 1 Genetic classification of hemochromatosis.

Type 3 HH, due to TFR2 mutation, is a rare recessive form of iron overload characterized by increased serum iron parameters due to increased duodenal iron absorption. The disorder was first characterized in two unrelated Sicilian families, where several members were found to be homozygous for a nonsense mutation (Y250X) [39]. As a result of this observation, many patients affected by non-HFE hemochromatosis were screened for the presence of Y250X TFR2 mutations. This analysis did not reveal a single positive

case, only polymorphic changes [40–43]. However, as listed in Table 2 other rare TFR2 inactivating mutations have been subsequently characterized both in Italy [44–47] and elsewhere [48–52], and appear throughout the entire sequence of the gene. These mutational data support the hypothesis that TFR2 mutations are rare and most of them are isolated. Moreover, iron overload severity is variable and genotype-phenotype correlation is not always apparent, because of the limited number of patients with the same mutation and the limited phenotypic data available. However, it seems that iron overload in type 3 HH is more rapid and severe than that usually reported in HFE-related HH, which usually develops in the fourth to fifth decade of life [51, 53–55]. According to the distribution of the reported mutations along the gene it is possible to speculate that the different effects of the mutations on the encoded protein may influence the phenotype severity (reviewed in [56]). As shown in Figure 1, most mutations affects both TFR2 forms, but some of them, such as E60X and R105X, affect only the α-form. The mutation M172K, which occurs in exon 4, causes a missense in the α-protein and also affects the putative initiation codon of the β-variant, negating its translation. Although the role of the alternate transcripts has not yet been established, it is interesting to note that patients who maintain the β-form intact (E60X and R105X patients) have a less severe phenotype. Indeed, the most severe phenotypes have been observed in patients carrying Y250X and M172K homozygous mutations.

4 Expression pattern and tissue distribution

TFR1 and TFR2 exhibit a different pattern of tissue distribution and expression. TFR1 is expressed in virtually all tissues, except mature erythrocytes. Despite this ubiquity some particular cell types, such as erythroid and rapidly proliferating cells, express high levels of TFR1 [57]. The highest levels are observed in developing erythroid cells, where elevated rates of iron uptake are required to sustain high rates of heme and hemoglobin synthesis [58]. On the contrary, TFR2 expression is tissue specific. TFR2 mRNA has been found to be highly expressed in the liver and, to a lesser extent, in spleen, lung, muscle, prostate and peripheral mononuclear cells [28, 59]. In addition, high levels of the TFR2 transcript have been detected in HepG2 (human hepatoma), K562 and HEL-R (both human erythroleukemic) cell lines. The human lymphoid cell lines Raji and MOLT-16; and the human myeloid cell lines U937, NB-4, HL-60, KCL22 and KG-1 express low or undetectable levels of TFR2 mRNA [59]. In contrast, TFR1 mRNA expression has been detected in all the lymphoid, myeloid and erythroid cell lines tested; the highest being observed in OCI-M1 erythroid cells. TFR2 mRNA was also detected in normal, early erythroid precursors and in primary myeloid leukemic blasts, especially derived from FAB M6 subtype [28, 59, 60]. At the level of protein synthesis, expression of TFR2 in normal erythroid cells was initially demonstrated by an immunohistochemical analysis using a polyclonal antibody [59]; however, it was subsequently shown by Western blot that TFR2 protein was not expressed at any stage of normal erythroid differentiation [61]. Studies on the tissue distribution of TFR2 protein using a large panel of monoclonal antibodies and

DNA variation	Protein change	Type	Exon	Country	References
Mutations					
84-88insC*	E60X	Frameshift	2	Southern Italy	Roetto et al. 2001
515T→A*	M172K	Missense	4	Central Italy Central Italy	Roetto et al. 2001 Majore et al. 2006
750C→G*	Y250X	Nonsense	6	Sicily Sicily Sicily	Camaschella et al. 2000 Roetto et al. 2001 Piperno et al. 2004
1780-1791del*	AVAQ594-597del	Deletion	16	Northern Italy Japan	Girelli et al. 2002 Hattori et al. 2003
1186C→T†	R396X	Nonsense	9	Scotland	Lee et al. 2006
2374G→A†	G792R	Missense	18	Scotland	Lee et al. 2006
2069A→C*	Q690P	Missense	17	Portugal	Mattman et al. 2002
313C→T*	R105X	Nonsense	2	Northern France	Le Gac et al. 2004
1469T→G*	L490R	Missense	11	Japan	Koyama et al. 2005
1665delC*	V561X	Deletion	14	Japan	Koyama et al. 2005
Not described*	Q317X	Nonsense	8	Southern Italy	Pietrangelo et al. 2005
Polymorphisms					
1391G→A	R455Q	Missense	10	Asia Scotland	Hofmann et al. 2002 Lee et al. 2006
64G→T	V22I	Missense	2	Northern Italy	Biasiotto et al. 2003
714C→G	I238M	Missense	5	Asia Japan	Lee et al. 2001 Koyama et al. 2005 Mattman et al. 2002
224C→T	A75V		2		Mattman et al. 2002
1127C→A	A376D		9		Mattman et al. 2002
2255G→A	R752H		18		Mattman et al. 2002
1770C→T	D590D		15		Lee et al. 2001
1851C→T	A617A		17		Lee et al. 2001
2355C→T	3'-UTR				Lee et al. 2001

* Mutations detected in the homozygous condition ;
† Mutations detected in the heterozygous condition in the same patient.

Table 2 Variations of the TFR2 gene reported in type 3 hemochromatosis.

immunohistochemical techniques revealed major staining in cells that play a prominent role in the regulation of iron homeostasis. Indeed, high levels of expression were found in hepatocytes, a major iron-storage site, and in the enterocytes of the crypts and villi, localized in the portion of the gastrointestinal tract involved in iron absorption [62]. These authors also investigated the subcellular localization of TFR2, showing that the receptor was clustered in selected areas of the cell membrane. A subsequent study reported that within the duodenal epithelium, TFR2 was selectively expressed in crypt cells [63] with a pattern quite distinct from the distribution of TFR1 along the entire villous axis [64]. These authors also showed a co-localization of TFR2 and HFE in crypt areas of human and mouse duodenum, although an earlier study had demonstrated that soluble, purified ectodomains of HFE and TFR2 did not interact *in vitro* [36]. This last finding contrasts a recent study by Goswami *et al.*, in which TFR2-HFE interaction was assessed in crude membrane fractions of transfected AML12 mouse hepatocytes [65]. The hepatic expression of TFR2 was recently confirmed by sensitive real-time RT-PCR at the mRNA level, and by immunoblot analysis at the protein level [66]. In this study, the authors analysed

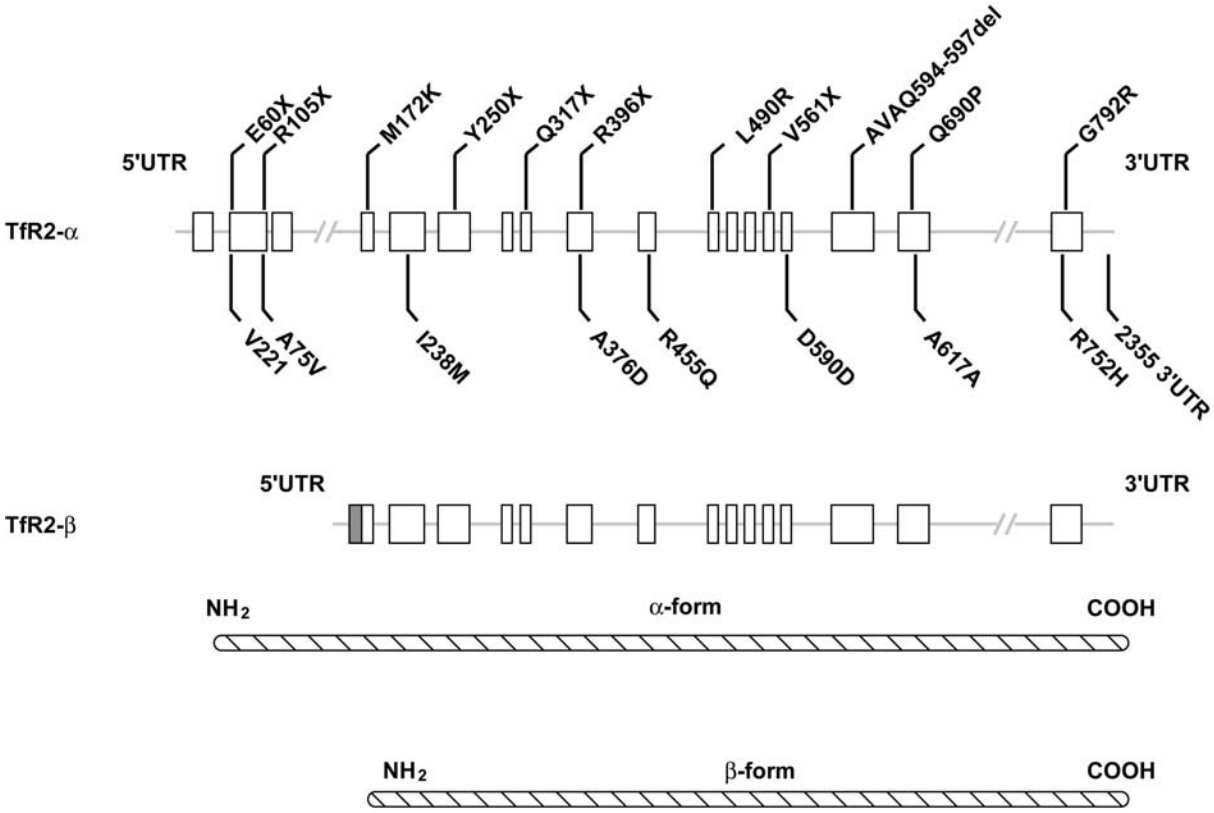

Fig. 1 Schematic representation of TFR2 mutations.
Two transcripts, possibly resulting from alternative splicing (as suggested by Kawabata *et al.* (1999)), are shown. Compared to TFR2-α transcript, which contains 18 exons (white boxes), the β-transcript lacks exons 1-3 and has an additional 142 nucleotides at the 5'-end (grey box). The causal mutations are reported above the gene sequence, while the polymorphic changes are illustrated below the gene. TFR2 α-protein and β-protein are also shown.

the cellular and subcellular localization of TFR2 in rat liver by immunofluorescence microscopy, showing that the protein was localized to the hepatocytes with no detectable staining of Kupffer cells; while at the subcellular level TFR2 immunoreactivity was found at the basolateral membrane domain of hepatocytes [66]. It is important to note that in normal hepatocytes the expression of TFR2 is much higher than that of TFR1.

5 Receptor internalization

Receptor-mediated endocytosis is the process by which integral membrane proteins are selectively internalized from the plasma membrane to the endocytic pathway. During the process of receptor-mediated endocytosis, membrane proteins are concentrated in specialized regions of the plasma membrane, known as clathrin-coated pits. Invagination of coated pits into the cytoplasm generates transport vesicles that deliver their content

to the endosomal compartment [67]. Some proteins then recycle back to the cell surface, while others are sorted and sent to different destinations.

Receptor-mediated endocytosis of transferrin via TFR1 is a clathrin-dependent endocytic pathway that has been the subject of recent and detailed analysis [68]. This is a highly regulated process involving many proteins; particularly Rab proteins, members of a large GTPase family with homology to ras. Rab proteins are localized to the cytoplasmic faces of all organelles involved in intracellular transport, and are associated with vesicle membranes in their activated GTP-bound form. Rab4 and Rab5 proteins co-localize with TFR1 in early endosomes [69, 70]. Others, such as Rab7, are localized in late endosomes and do not co-localize with TFR1 [71]. Rab11 also co-localizes with TFR1 in early endosomes and it is functionally important for Tf recycling [72]. Finally, Rab22a controls the transport of TFR1 from sorting to recycling endosomes [73].

The endocytosis of TFR1 requires an internalization signal present on the cytoplasmic tail of the receptor and represented by the tetrapeptide sequence YTRF (Tyr-Gln-Arg-Phe, residues 20 through 23) [29]. The most critical residues in the transferrin receptor internalization signal appear to be the two aromatic amino acids.

It is important to note that TFR1 is constitutively clustered in coated pits and undergoes rapid internalization even in the absence of the ligand. Thus in HeLa cells, only 20-30% of TFR1 is on the cell surface, with about 50-60% residing in the endosomes and the remaining 20% in the Golgi complex and the biosynthetic pathway [74]. The intracellular pool of TFR1 results from continual internalization of unoccupied receptors [75]. These observations indicate that, in addition to the main route of receptor recycling, 5 to 15% of the surface TFR1 that enters the cell is transported through the Golgi complex [76, 77].

Only a limited amount of information is available on TFR2 internalization. The presence of the internalization motif in the cytoplasmic tail (YTRF for TFR1 and YQRV for TFR2) would convey the two TFRs to common cellular compartments where the internalization of the plasma membrane occurs. In this context, a recent study indicated that Tf delivered through both receptors was initially detected at the plasma membrane and tubulovesicular endosomes; however at later stages of the endocytic pathway Tf internalized through TFR2 is delivered to multivesicular bodies (MVB), while Tf endocytosed through TFR1 is not observed in this compartment [78]. These results suggest that TFR2 internalization does not follow the classic clathrin-mediated endocytic route, and this may be due to the localization of TFR2 and TFR1 to different regions of the cell membrane. Indeed, we recently showed that TFR2 constitutively localizes in lipid rafts microdomains of the plasma membrane [79]. Rafts consist of a dynamic assembly of cholesterol and glycosphyngolipids that form islands of liquid-ordered domains which float in the fluid disordered bilayer of largely unsaturated lipids of the surrounding membrane [80]. Certain proteins are preferentially partitioned into the ordered raft domains; some of these are targeted to rafts by hydrophobic modifications, which pack well into an ordered lipid environment [81–84]. One morphologically identifiable raft structure is the caveola [84]. Caveolae are flask-shaped membrane invaginations that are formed from

lipid rafts by polymerisation of their scaffolding proteins, caveolins. Given their ability to recruit or exclude specific lipids and proteins, lipid rafts have been implicated in the regulation of various physiological processes, such as lipid sorting, protein trafficking, cell polarization [85] and signal transduction [80, 86, 87].

Recent work has established a role for caveolae and lipid rafts in non-classic (clathrin-independent) endocytic pathways [88]. These internalization routes are not well characterized, but the observation that they are sensitive to cholesterol depletion [88], and that some rafts-associated proteins internalize through non-clathrin pathways [89, 90], have led to the idea that these alternative routes are raft-dependent. One important non-clathrin internalization route requires caveolin-1 expression. For instance, albumin binding to its receptor, gp60, triggers caveolae endocytosis via a Gi-coupled Src kinase-mediated pathway [91]. Albumin uptake requires caveolin-1/gp60 interaction, and it is inhibited in caveolin-1 null cells [91, 92]. Moreover, some non-enveloped viruses, such as Simian virus 40 (SV40), use caveolae as portals for entry into the cell [93]. Once internalized, the caveolar vesicles appear to deliver the viruses to newly discovered organelles called caveosomes [93], which are distinguished from the early endosomes by their neutral pH and by the high expression of caveolin-1 [94]. From these structures the viruses are delivered to ER, however it is important to point out that raft-dependent internalization also occurs in the absence of caveolin. For instance, caveolin-independent routes have been shown to mediate the internalization of interleukin-2 receptor-β (IL2Rβ) [95]; and can direct some GPI-anchored proteins to the Rab11-positive recycling endosomes [96], where they can traffic back to the plasma membrane. Indeed, recent data has shown that caveolin-expressing vesicles can interact with early endosomes in a Rab5-dependent process [97], suggesting that clathrin-independent endocytic pathways can crosstalk with components of the classic endosomal system.

Very little is known about TFR2 intracellular trafficking. Tf internalized through TFR2 seems to be directed to different intracellular compartments with respect to the classic Tf-TFR1 internalization pathway [78]. At odds with TFR1, which is rapidly endocytosed and is largely distributed in intracellular compartments [74, 98], TFR2 has been found to localize mainly at the level of the plasma membrane, and to a lesser extent (<10% of the total protein) in Rab5-expressing intracellular organelles [79]. This is in line with a previous study reporting the localization of receptors endocytosed via lipid rafts in rab5$^+$ endosomes [97]. This data would seem to indicate that further investigations are necessary to carefully characterize the intracellular trafficking of TFR2, and its possible involvement in non-clathrin-mediated endocytosis.

6 Receptor exocytosis

An important similarity between TFR1 and TFR2 is that both receptors are actively exocytosed from the cells through the exosomal pathway [79, 99]. Exosomes are defined as the intraluminal vesicles (ILVs) of multivesicular bodies (MVBs) following their secretion into the extracellular milieu. The ILVs originate during endosomes formation and matu-

ration through the inward budding and scission of vesicles from the limiting membrane into the endosomal lumen. During this complex process, transmembrane and peripheral membrane proteins are incorporated into the invaginating membrane, maintaining the same topological orientation as the plasma membrane, while cytosolic components are engulfed and enclosed into the small 50-90 nm vesicles [100]. Exosomes derived from different cell types include common sets of molecules, but also display a set of cell-specific components [100]. A large variety of proteins, including transmembrane and associated peripheral membrane proteins, cytosolic proteins and chaperons have been detected in exosomal vesicles. One of the molecules typically observed in exosomes is TFR1 [101]. The heat-shock cognate (Hsc70) protein interacts with TFR1, and this interaction regulates TFR1 release via exosomes [102].

It is also important to note that many raft-associated proteins are sorted in exosomes, including the tetraspanins CD9, CD63 and CD81 [101]; GPI-anchored proteins such as CD55, CD58 and CD59 [103]; the Src kinase Lyn [104, 105]; and the acylated proteins flotillin-1 and stomatin [105]. The release of raft proteins in these vesicles reflects the presence in exosomal membrane of lipid raft-like domains [105].

The biological functions of exosomes remain largely unknown. However, it was proposed that these peculiar subcellular structures, through their capacity to bind target cells, are likely to be part of a complex network of intercellular communications and to modulate selected cellular activities [106]. Indeed, an "exosomal-type" of communication has been demonstrated in the immune system and may be exploited by cells to interact with each other locally or at a distance. Furthermore, the presence of exosomes in blood, other biological fluids and tissues strongly suggests their participation in physiological and/or pathological processes [106].

7 Biological function

In most cells, TFR1 mediates the major pathway of cellular iron uptake through the binding and endocytosis of iron-loaded transferrin. TFR2 shares many similarities with TFR1; both receptors are type II transmembrane glycoproteins and both have quite similar primary structures [28]. Since its cloning, TFR2 has been hypothesized to mediate a second mechanism of cellular iron uptake, as inferred by the flow cytometric analyses of Tf binding to TFR2 transfected into a model of Chinese hamster ovary cells lacking TFR1 [28]. In addition, TFR2 has been found to support cell growth of TFR1-deficient Chinese hamster ovary cells both *in vitro* and *in vivo* [32], demonstrating that TFR2 is functional in Tf-mediated iron uptake. However, kinetic analysis of Tf binding to the soluble TFR2 ectodomain showed that the affinity at pH 7.5 of TFR2 for diferric Tf (K_d approximately 27 nM) is about 25-fold lower than that of TFR1 (K_d approximately 1 nM) [32, 36, 107]. Furthermore, TFR2 binding to Tf is less specific when compared with TFR1, since TFR2 is able to interact with both human and bovine Tf [37]. The different ligand affinities and specificities of the two TFRs may be related to their different roles in iron metabolism. Indeed, murine TFR2 does not fully compensate for the function of

TFR1, since TFR1-knockout mice die *in utero* because of severe anemia and neurological abnormalities [108]. Since TFR2, in contrast with TFR1, is expressed in a very restricted number of cells, it is not surprising that it cannot compensate for the absence of TFR1. The exact role of TFR2 in iron metabolism remained unclear until the identification of TFR2 mutations in patients with HFE-unrelated hemochromatosis [39] (now called type 3 HH), leading to a phenotype of hepatic iron loading. Furthermore, in humans homozygous for the Y250X (the first reported TFR2 mutation), and in mice with a targeted mutation of TFR2 (Y245X, orthologous to the human Y250X), the liver accumulates iron despite an absence of membrane-bound TFR2 and a reduction of TFR1 [109], suggesting that the uptake of Tf-bound iron for use by the hepatocytes is not the principal function of TFR2. Accordingly, it was suggested that TFR2 could play a regulatory role as an iron-sensor molecule, probably through the identification of Fe-Tf in the environment. The hypothesis that TFR2 might sense body iron levels by sensing the levels of Tf in serum is compatible with its low affinity for Tf, and with the finding that the TfR2 protein is stabilized *in vitro* by the addition of holoTf in a time- and dose-dependent manner, with a response that seems to be hepatocyte-specific [110, 111]. Many investigators have hypothesized that the interaction of TFR2 with iron-loaded Tf may be the first step of a complex, and as yet unknown, regulatory pathway that leads to the control of hepcidin synthesis by the liver. Indeed, several pieces of data support a TFR2/hepcidin link: (i) both genes are predominantly expressed in the liver [23, 28, 109]; (ii) serum Tf is closely correlated with hepcidin expression in the liver *in vivo* [112]; (iii) the expression of both hepcidin mRNA and TFR2 protein are up-regulated by iron loading in wild-type mice [113]; (iv) urinary hepcidin is low or absent in patients with TFR2 hemochromatosis [114]. Furthermore, homozygous TFR2 knockout mice develop significant iron overload, with no upregulation of liver hepcidin mRNA or pro-hepcidin protein, suggesting that hepatic hepcidin expression is deregulated in the absence of TFR2 [115]. A recent study in a TFR2 (Y245X) mutant mouse model showed the upregulation of Fe duodenal uptake (Dcytb, DMT1) and transport (ferroportin) genes, with increased duodenal iron absorption, increased liver iron uptake and decreased liver hepcidin expression, despite systemic iron loading [116]. Since mutations of TFR2, hemojuvelin and HFE lead to different types of primary iron overload, sharing some common phenotypical features, it has been suggested that these proteins may be functionally linked [117, 118]. In this liver-specific iron-sensing pathway hepcidin appears to be the central regulator of body iron levels downstream of HFE, TFR2 and hemojuvelin. To date, published data about TFR2-HFE interaction remains controversial. Indeed, the co-localization of TFR2 and HFE has been demonstrated in crypt cells of mouse and human duodenum [63], although a previous study failed to show any direct interaction between the purified soluble ectodomains of TFR2 and HFE [36]. However, Goswami *et al.* recently assessed TFR2-HFE interaction in crude membrane fractions of transfected AML12 mouse hepatocytes [65]. These authors suggested that HFE may sense Tf saturation and switch from a TFR1 to TFR2 complex. Indeed under low or basal serum iron conditions, HFE and TFR1 exist as a complex at the plasma membrane, while with increased serum Tf

saturation, HFE binding to TFR1 is competed by Fe_2-Tf. The net result of this is the dissociation of HFE from TFR1 and the binding of HFE to TFR2. Thus, HFE acts as an iron sensor which interacts with TFR2 to convey a downstream signal transduction cascade, leading to the modulation of hepcidin production. According to this model, in HFE- and TFR2-related hemochromatosis this sensor mechanism would not be functional and hepcidin production would not be stimulated despite high levels of diferric Tf, thus resulting in parenchymal iron overload [65]. Furthermore, a functional interaction of TFR2 with hemojuvelin in the context of the liver-specific pathway controlling hepcidin production is currently under discussion [119]. To test this hypothesis, Merle *et al.* have recently investigated the cellular and subcellular localization of these proteins in the liver, showing that both TFR2 and hemojuvelin localize at the level of the basolateral membrane domain of hepatocytes [66], the same localization previously reported for prohepcidin [120]. In line with this immunoreactivity pattern, the authors speculate that hemojuvelin and TFR2 can functionally interact, leading to a cascade signalling hepcidin localized in the same region of hepatocyte membrane. The hypothesis that TFR2 could function as a signalling receptor is in accordance with our own recent finding that it is located at the level of lipid raft microdomains, where it promotes cell signalling along the MAPK pathway, following activation with holoTf [79]. Interestingly, hemojuvelin is a GPI-anchor protein type [121, 122], and therefore it could localize in lipid rafts, where it could functionally interact with TFR2.

8 Conclusions

Given all the available data concerning the biological properties and function of TFR2, we propose a model for serum iron sensing in which TFR2 plays a pivotal role through its signalling activity. In this model the signalling of TFR2 is dependent on the level of Tf-iron saturation, i.e. when Tf-iron saturation is high, TFR2-mediated signalling is particularly active, whereas in conditions of low Tf-iron saturation, TFR2 signalling is low. The cell signalling cascade activated by TFR2 thus induces hepcidin mRNA transcription. This hypothesis suggests that the biological role of TFR2 is to sense the Tf saturation of serum, and to convey to liver cells a signal whose intensity depends on the whole-body iron status. Downstream induction of hepcidin production contributes to systemic iron homeostasis.

To prove this hypothesis, three major points remain to be demonstrated: (i) TFR2-induced cell signalling is dependent on the coefficient of iron saturation of Tf; (ii) the signalling cascade activated by TFR2 leads to the transcription of the hepcidin gene (or alternatively to a stabilization of its mRNA); (iii) in TFR2-related hereditary hemochromatosis, gene mutations result in defective signal transduction, and /or in the disruption of the receptor lipid raft association, leading to the dysregulation of hepcidin expression.

Since HFE, hemojuvelin and TFR2 knockouts give rise to a highly similar phenotype and, particularly, to an abrogation of the iron-dependent regulation of hepcidin, the

exact relationship between these three proteins needs to be carefully investigated. HJV acts as a major regulator of hepcidin production, interacting with BMP ligands and BMP type I and II receptors, resulting in signalling through a SMAD pathway and induction of hepcidin expression [26]. Furthermore, it has been recently shown that HFE and TFR2 are associated with HJV in a stable protein complex that can be isolated by co-immunoprecipitation or Ni-affinity chromatography (Andrews N.C., ASH Meeting abstract n. 267, Orlando 2006). Taking into account the new data, we propose a model for the HFE, TFR2 and HJV inter-connected control of iron homeostasis (Figure 2). Thus HJV interacts with BMP receptors to form an active complex and TFR2 and HFE act as an iron sensor complex at the plasma membrane. When serum Tf saturation increases, the TFR2-HFE complex associates with HJV and amplifies BMP signalling, leading to hepcidin synthesis. In this context, TFR2 could serve to recruit HFE to this complex.

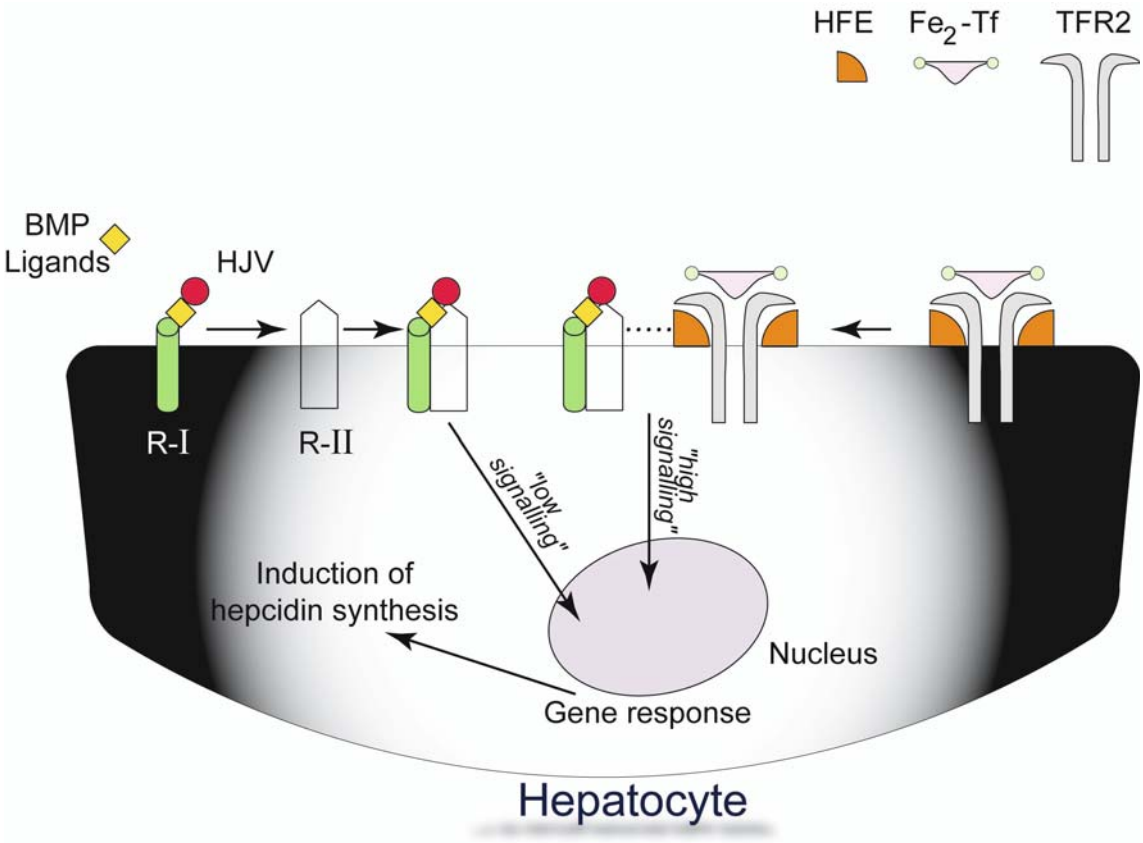

Fig. 2 Schematic diagram of the role of HJV, HFE and TFR2 in the signalling pathway upstream of hepcidin production in hepatic cells.
From one side, HJV interacts with BMP ligands and BMP type I (R-I) and type II (R-II) receptors to generate an active signalling complex. From the other side, HFE and TFR2 act as an iron sensor complex at the plasma membrane. When diferric transferrin (Fe_2-Tf) levels are high, the HFE-TFR2 complex associates with HJV and amplifies BMP signalling, leading to hepcidin production. Downstream induction of hepcidin synthesis contributes to whole-body iron homeostasis.

Therefore, the actual challenge is to dissect the molecular pathways involved in the transcriptional response of hepcidin to body iron content, and to understand how this response is mediated by HFE, hemojuvelin and TFR2 inter-connected activities.

References

[1] J.P. Liuzzi, F. Aydemir, H. Nam et al.: "Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells", *Proc. Natl. Acad. Sci. USA*, Vol. 103, (2006), pp. 13612–13617.

[2] G.J. Anderson: "Non-transferrin-bound iron and cellular toxicity", *J. Gastroenterol. Hepatol.*, Vol. 14, (1999), pp. 105–108.

[3] G.O. Latunde-Dada, R.J. Simpson and A.T. McKie: "Recent advances in mammalian heme transport", *TRENDS Biochem. Sci.*, Vol. 31, (2006), pp. 182–188.

[4] M. Shayeghi: "Identification of an intestinal heme transporter", *Cell*, Vol. 122, (2005), pp. 789–801.

[5] P.C. Krishnamurthy, G. Du, D. Sun et al.: "Identification of a mammalian/mitochondrial porphyrin transporter", *Nature*, Vol. 443, (2006), pp. 586–589.

[6] H. Gunshin, B. MacKenzie, U.V. Berger et al.: "Cloning and characterization of a mammalian proton-coupled metal-ion transporter", *Nature*, Vol. 388, (1997), pp. 482-488.

[7] A.T. McKie, D. Barrow, G.O. Latunde-Dada et al.: "An iron-regulated ferric reductase associated with the absorption of dietary iron", *Science*, Vol. 291, (2001), pp. 1755–1759.

[8] A. Donovan, A. Brownlie, Y. Zhou et al.: "Positional cloning of zebrafish ferroportin 1 identifies a conserved vertebrate iron exporter", *Nature*, Vol. 403, (2000), pp. 776–781.

[9] A. Donovan, C.A. Lima, J.L. Pinkus et al.: "The iron exporter ferroportin/Slc40a 1 is essential for iron homeostasis", *Cell Metab.*, Vol. 1, (2005), pp. 191–200.

[10] C.D. Vulpe, Y.M. Kuo, T.L. Murphy et al.: "Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse", *Nat. Genet.*, Vol. 21, (1999), pp. 195–199.

[11] E. Nemeth, M.S. Tuttle, J. Powelson et al.: "Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization", *Science*, Vol. 306, (2004), pp. 2090–2093.

[12] U. Testa: "Proteins of iron metabolism", *CRC press*, (2001), pp. 1–608.

[13] R.S. Ohgami, D.R. Campagna, E.L. Greer et al.: "Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells", *Nat. Genet.*, Vol. 37, (2005), pp. 1264–1269.

[14] F.M. Torti and S.V. Torti: "Regulation of ferritin genes and protein", *Blood*, Vol. 99, (2002), pp. 3505–3516.

[15] M. Muckenthaler, N.K. Gray and M.W. Hentze: "IRP1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex e IF4F", *Mol. Cell*, Vol. 2, (1998), pp. 383–388.

[16] M.W. Hentze and L.C. Kuhn: "Molecular control of vertrebate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress", *Proc. Natl. Acad. Sci. USA*, Vol. 93, (1996), pp. 8175-8182.

[17] M.W. Hentze, M.U. Muckentales and N.C. Andrew: "Balancing acts: molecular control of mammalian iron metabolism", *Cell*, Vol. 117, (2004), pp. 285–297.

[18] K. Iwai, S.K. Drake, N.B. Wehr et al.: "Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: implications for degradation of oxidized proteins", *Proc. Natl. Acad. Sci. USA*, Vol. 95, (1998), pp. 4924–4928.

[19] K. Yamanaka, H. Ishikawa, Y. Megumi et al.: "Identification of the ubiquitin-protein ligase that recognizes oxidized IRP2", *Nat. Cell Biol.*, Vol. 5, (2003), pp. 336–340.

[20] H. Ishikawa, M. Kato, H. Hori et al.: "Involvement of heme regulatory motif in heme-mediated ubiquitination and degradation of IRP2", *Mol. Cell*, Vol. 19, (2005), pp. 171–181.

[21] J. Yang, D. Goetz, J.Y. Li et al.: "An iron delivery pathway mediated by lipocalin", *Mol. Cell.*, Vol. 10, (2002), pp. 1045–1056.

[22] L.R. Devireddy, C. Gazin, X. Zhu et al.: "A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake", *Cell*, Vol. 123, (2005), pp. 1293–1305.

[23] A. Hugman: "Hepcidin: an important new regulator of iron homeostasis", *Clin. Lab. Haem.*, Vol. 28, (2006), pp. 75–83.

[24] G. Nicolas, M. Bennoun, I. Devaux et al.: "Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice", *Proc. Natl. Acad. Sci. USA*, Vol. 98, (2001), pp. 8780–8785.

[25] A. Roetto, G. Papanikolaou, M. Politou et al.: "Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis", *Nat. Genet.*, Vol. 33, (2002), pp. 21–22.

[26] J.L. Babitt, F.W. Huang, D.M. Wrighting et al.: "Bone morphogenetic protein signalling by hemojuvelin regulates hepcidin expression", *Nat. Genet.*, Vol. 38, (2006), pp. 531–539.

[27] G. Glockner, S. Scherer, S. Schattevoy et al.: "Large-scale sequencing of two regions in human chromosome 7q22: analysis of 650 Kb of genomic sequence around the EPO and CUTL1 loci reveals 17 genes", *Genome Res.*, Vol. 8, (1998), pp. 1060–1073.

[28] H. Kawabata, R. Yang, T. Hirama et al.: "Molecular cloning of transferrin receptor 2 : a new member of the transferrin receptor-like family", *J. Biol. Chem.*, Vol. 274, (1999), pp. 20826–20832.

[29] J.F. Collawn, M. Stangel, L.A. Kuhn et al.: "Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis", *Cell*, Vol. 83, (1990), pp. 1061–1072.

[30] S.Q. Jing, T. Spencer, K. Miller et al.: "Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalisation", *J. Cell Biol.*, Vol. 110, (1990), pp. 283–294.

[31] A.O. Johnson, R.N. Ghosh, K.W. Dunn et al.: "Transferrin receptor containing the SDYQRL motif of TGN38 causes a reorganization of the recycling compartment but is not targeted to the TGN", *J. Cell Biol.*, Vol. 135, (1996), pp. 1749–1762.

[32] H. Kawabata, R.S. Germain, P.T. Vuong et al.: "Transferrin receptor 2-α supports cell growth both in iron-chelated cultured cells and in vivo", *J. Biol. Chem.*, Vol. 275, (2000), pp. 16618–16625.

[33] V. Dubljevic, A. Sali and J.W. Goding: "A conserved RGD (Arg-Gly-Asp) motif in the transferrin receptor is required for binding to transferrin", *Biochem. J.*, Vol. 341, (1999), pp. 11–14.

[34] C.M. Lawrence, S. Ray, M. Babyonyshev et al.: "Crystal structure of the ectodomain of human transferrin receptor", *Science*, Vol. 286, (1999), pp. 779–782.

[35] M.J. Bennett, J.A. Lebrn and P.J. Bjorkman: "Crystal structure of the hereditary hemochromatosis protein HFE complexed with transferrin receptor", *Nature*, Vol. 403, (2000), pp. 46–53.

[36] A.P. West Jr, M.J. Bennett, V.M. Sellers et al.: "Comparison of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the hereditary hemochromatosis protein HFE", *J. Biol. Chem.*, Vol. 275, (2000), pp. 38135–38138.

[37] H. Kawabata, X. Tong, T. Kawanami et al.: "Analysis for binding of the transferrin family of proteins to the transferrin receptor 2", *Brit. J. Haematol.*, Vol. 127, (2004), pp. 464–473.

[38] J.N. Feder, A. Gnirke, W. Thomas et al.: "A novel MHC class I-like gene is mutated in patients with hereditary hemochromatosis", *Nat. Genet.*, Vol. 13, (1996), pp. 399–408.

[39] C. Camaschella, A. Roetto, A. Calì et al.: "The gene TfR2 is mutated in a new type of hemochromatosis mapping to 7q22", *Nat. Genet.*, Vol. 25, (2000), pp. 14–15.

[40] E.H. Barton, P.A. West, C.A. Rivers et al.: "Transferrin receptor-2 (TFR2) mutation Y250X in Alabama Caucasian and African American subjects with and without primary iron overload", *Blood Cells Mol. Dis.*, Vol. 27, (2001), pp. 279–284.

[41] P. Aguilar-Martinez, C. Esculia Coste, M. Bismuth et al.: "Transferrin receptor 2 gene and non C282Y homozygous patients with hemochromatosis", *Blood Cells Mol. Dis.*, Vol. 27, (2001), pp. 290–293.

[42] P.L. Lee, C. Halloran, C. West et al.: "Mutation analysis of the transferrin receptor 2 gene in patients with iron overload", *Blood Cells Mol. Dis.*, Vol. 27, (2001), pp. 285–289.

[43] W.K. Hoffman, X.J. Tong, R.S. Ajioka et al.: "Mutation analysis of transferrin-receptor 2 in patients with atypical hemochromatosis", *Blood*, Vol. 100, (2002), pp. 1099–1100.

[44] A. Roetto, A. Totaro, A. Piperno et al.: "New mutations inactivating transferrin receptor 2 in hemochromatosis type 3", *Blood*, Vol. 97, (2001), pp. 2555–2560.

[45] D. Girelli, C. Bozzini, A. Roetto et al.: "Clinical and pathologic findings in hemochromatosis type 3 due to a novel mutation in transferrin receptor 2 gene", *Gastroenterology*, Vol. 122, (2002), pp. 1295–1302.

[46] S. Majore, F. Milano, F. Binni et al.: "Homozygous p.M172K mutation of the TFR2 gene in an Italian family with type 3 hereditary hemochromatosis and early onset iron overload", *Haematologica*, Vol. 91, Suppl. 8, (2006), ECR33.

[47] A. Pietrangelo, A. Caleffi, J. Henrion et al.: "Juvenile hemochromatosis associated with pathogenic mutations of adult hemochromatosis genes", *Gastroenterology*, Vol. 128, (2005), pp. 470–479.

[48] A. Hattori, S. Wakusawa, H. Hayashi et al.: "AVAQ594-597 deletion of the TfR2 gene in a Japanese family with hemochromatosis", *Hepatol. Res.*, Vol. 26, (2003), pp. 154–156.

[49] P. Lee and JC. Barton: "Hemochromatosis and severe iron overload associated with compound heterozygosity for TFR2 R455Q and two novel mutations TFR2 R396X and G792R", *Acta Haematol.*, Vol. 115, (2006), pp. 102–105.

[50] A. Mattman, D. Huntsman, G. Lockitch et al.: "Transferrin receptor 2 (TfR2) and HFE mutational analysis in non-C282Y iron overload: identification of a novel TfR2 mutation", *Blood*, Vol. 100, (2002), pp. 1075–1077.

[51] G. Le Gac, F. Mons, S. Jacolot et al.: "Early onset hereditary hemochromatosis resulting from a novel TFR2 gene nonsense mutation (R105X) in two siblings of north French descent", *Br. J. Haematol.*, Vol. 125, (2004), pp. 674–678.

[52] C. Koyama, S. Wakusawa, H. Hayashi et al.: "Two novel mutations, L490R and V561X, of the transferrin receptor 2 gene in Japanese patients with hemochromatosis", *Haematologica*, Vol. 90, (2005), pp. 302–306.

[53] R.S. Ajioka and J.P. Kushner: "Hereditary hemochromatosis", *Semin. Hematol.*, Vol. 39, (2002), pp. 235–241.

[54] A. Piperno, A. Roetto, R. Mariani et al.: "Homogygosity for transferrin receptor-2 Y250X mutation induces early iron overload", *Haematologica*, Vol. 89, (2004), pp. 359–360.

[55] A. Pietrangelo: "Hereditary hemochromatosis - A new look at an old disease", *N. Engl. J. Med.*, Vol. 350, (2004), pp. 2383–2397.

[56] A. Roetto and C. Camaschella: "New insights into iron homeostasis through the study of non-HFE hemochromatosis", *Best Pract. Res. Clin. Haematol.*, Vol. 18, (2005), pp. 235–250.

[57] U. Testa, E. Pelosi and C. Peschle: "The transferrin receptor", *Crit. Rev. Oncog.*, Vol. 4, (1993), pp. 241–276.

[58] N.M. Sposi, L. Cianetti, E. Tritarelli et al.: "Mechanisms of differential transferrin receptor expression in normal hematopoiesis", *Eur. J. Biochem.*, Vol. 267, (2000), pp. 6762–6774.

[59] H. Kawabata, T. Nakamaki, P. Ikonomi et al.: "Expression of transferrin receptor 2 in normal and neoplastic hematopoietic cells", *Blood*, Vol. 98, (2001), pp. 2714–2719.

[60] P. Kollia, M. Samara, K. Stamatopoulos et al.: "Molecular evidence for transferrin

receptor 2 expression in all FAB subtypes of acute myeloid leukemia", *Leuk. Res.*, Vol. 27, (2003), pp. 1101–1103.

[61] A. Calzolari, S. Deaglio, N.M. Sposi et al.: "Transferrin receptor 2 is not expressed in normal erythroid cells", *Biochem. J.*, Vol. 381 (2004), pp. 629–634.

[62] S. Deaglio, A. Capobianco, A. Calì et al.: "Structural, functional, and tissue distribution analysis of human transferrin receptor-2 by murine monoclonal antibodies and a polyclonal antiserum", *Blood*, Vol. 100, (2002), pp. 3782–3789.

[63] W.J.H. Griffiths and T.M. Cox: "Co-localization of the mammalian hemochromatosis gene product (HFE) and a newly identified transferrin receptor (TfR2) in intestinal tissue and cells", *J. Histochem. Cytochem.*, Vol. 51, (2003), pp. 613–623.

[64] M. Lombard, A.B. Bomford, R.J. Polson et al.: "Differential expression of transferrin receptor in duodenal mucosa in iron overload. Evidence for a site-specific defect in genetic hemochromatosis", *Gastroenterology*, Vol. 98, (1990), pp. 976–984.

[65] T. Goswami and N.C. Andrews: "Hereditary hemochromatosis protein, HFE, interaction with transferrin receptor 2 suggests a molecular mechanism for mammalian iron sensing", *J. Biol. Chem.*, Vol. 281, (2006), pp. 28494–28498.

[66] U. Merle, F. Theilig, E. Fein et al.: "Localization of the iron-regulatory proteins hemojuvelin and transferrin receptor 2 to the basolateral membrane domain of hepatocytes", *Histochem. Cell Biol.*, 2006 Aug 25; [Epub ahead of print].

[67] F.R. Maxfield and T.E. McGraw: "Endocytic recycling", *Nat. Rev. Mol. Cell Biol.*, Vol. 5, (2004), pp. 121–132.

[68] I.S. Trowbridge, J.F. Collawn and C.R. Hopkins: "Signal-dependent membrane protein trafficking in the endocytic pathway", *Annu. Rev. Cell Biol.*, Vol. 9, (1993), pp. 129–161.

[69] P. Van der Sluijs, M. Hull, A. Zahraoui et al.: "The small GTP-binding protein rab4 is associated with early endosomes", *Proc. Natl. Acad. Sci. USA*, Vol. 88, (1991), pp. 6313–6317.

[70] C. Bucci, R.G. Parton, I.H. Mather et al.: "The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway", *Cell*, Vol. 70, (1992), pp. 715–728.

[71] Y. Feng, B. Press and A. Wandinger-Ness: "Rab7: an important regulator of late endocytic membrane traffic", *J. Cell Biol.*, Vol. 131, (1995), pp. 1435–1452.

[72] A.J. Lindsay and M.W. McCaffrey: "Rab11-FIP2 functions in transferrin recycling and associates with endosomal membranes via its COOH-terminal domain", *J. Biol. Chem.*, Vol. 277, (2002), pp. 27193–27199.

[73] J.C. Magadán, M.A. Barbieri, R. Mesa et al.: "Rab22a regulates the sorting of transferrin to recycling endosomes", *Mol. Cell Biol.*, Vol. 26, (2006), pp. 2595–2614.

[74] J.E. Lamb, F. Ray, J.H. Ward et al.: "Internalization and subcellular localization of transferrin and transferrin receptors in HeLa cells", *J. Biol. Chem.*, Vol. 258, (1983), pp. 8751–8758.

[75] R.S. Ajioka and J. Kaplan: "Intracellular pools of transferrin receptors result from constitutive internalization of unoccupied receptors", *Proc. Natl. Acad. Sci. USA*, Vol. 83, (1986), pp. 6445–6449.

[76] C.R. Hopkins: "Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells", *Cell*, Vol. 35, (1983), pp. 321–330.

[77] C.R. Hopkins and I.S. Trowbridge: "Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells", *J. Cell Biol.*, Vol. 97, (1983), pp. 508–521.

[78] A.D. Robb, M. Ericsson and M. Wessling-Resnick: "Transferrin receptor 2 mediates a biphasic pattern of transferrin uptake associated with ligand delivery to multivesicular bodies", *Am. J. Physiol. Cell Physiol.*, Vol. 287, (2004), pp. C1769–C1775.

[79] A. Calzolari, C. Raggi, S. Deaglio et al.: "TfR2 localizes in lipid raft domains and is released in exosomes to activate signal transduction along the MAPK pathway", *J. Cell Sci.*, Vol. 119, (2006), pp. 4486–4498.

[80] K. Simons and D. Toomre: "Lipid rafts and signal transduction", *Nat. Rev. Mol. Cell Biol.*, Vol. 1, (2000), pp. 31–39.

[81] S. Chatterjee and S. Mayor: "The GPI-anchor and protein sorting", *Cell. Mol. Life Sci.*, Vol. 58, (2001), pp. 1969–1987.

[82] M.D. Resh: "Fatty acylation of proteins: new insight into membrane targeting of myristoylated and palmitoylated proteins", *Biochim. Biophys. Acta*, Vol. 1451, (1999), pp. 1–16.

[83] L. Rajendran, M. Masilamani, S. Solomon et al.: "Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells", *Proc. Natl. Acad. Sci. USA*, Vol. 100, (2003), pp. 8241–8246.

[84] T.V. Kurzchalia and R.G. Parton: "Membrane microdomains and caveolae", *Curr. Opin. Cell Biol.*, Vol. 11, (1999), pp. 424–431.

[85] R. Jacob, M. Heine, J. Eikemeyer et al.: "Annexin II is required for apical transport in polarized epithelial cells", *J. Biol. Chem.*, Vol. 279, (2004), pp. 3680–3684.

[86] J.F. Hancock: "Ras proteins: different signals from different locations", *Nat. Rev. Mol. Cell Biol.*, Vol. 4, (2003), pp. 373–384.

[87] R.G. Parton and J.F. Hancock: "Lipid rafts and plasma membrane microorganization: insights from Ras", *Trends Cell Biol.*, Vol. 14, (2004), pp. 141–147.

[88] R.G. Parton and A.A. Richards: "Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms", *Traffic*, Vol. 4, (2003), pp. 724–738.

[89] B. Nichols: "Caveosomes and endocytosis of lipid rafts", *J. Cell Sci.*, Vol. 116, (2003), pp. 4707–4714.

[90] S.B. Sato, K. Ishii, A. Makino et al.: "Distribution and transport of cholesterol-rich membrane domains monitored by a membrane-impermeant fluorescent polyethylene glycol-derivatized cholesterol", *J. Biol. Chem.*, (2004), Vol. 279, pp. 23790–23796.

[91] R.D. Minshall, C. Tiruppathi, S.M. Vogel et al.: "Endothelial cell-surface-gp60 activates vesicle formation and trafficking via Gi-coupled Src kinase signaling pathway", *J. Cell. Biol.*, Vol. 150, (2000), pp. 1057–1070.

[92] B. Razani, J.A. Engelman, X.B. Wang et al.: "Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities", *J. Biol. Chem.*, Vol. 276, (2001), pp. 38121–38138.

[93] L. Pelkmans and A. Helenius: "Endocytosis via caveolae", *Traffic*, Vol. 3, (2002), pp. 311–320.

[94] L. Pelkmans, J. Kartenbeck and A. Helenius: "Caveolar endocytosis of Simian virus 40 reveals a new two-step vesicular-transport pathway to the ER", *Nat. Cell Biol.*, Vol. 3, (2001), pp. 473–483.

[95] C. Lamaze, A. Dujeancourt, T. Baba et al.: "Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway", *Mol. Cell.*, Vol. 7, (2001), pp. 661–671.

[96] S. Sabharanjak, P. Sharma, R.G. Parton et al.: "GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway", *Dev. Cell.*, Vol. 2, (2002), pp. 411–423.

[97] L. Pelkmans, T. Burli, M. Zerial et al.: "Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic", *Cell*, Vol. 118, (2004), pp. 767–780.

[98] C. Watts: "Rapid endocytosis of the transferrin receptor in the absence of bound transferrin", *J. Cell Biol.*, Vol. 100, (1985), pp. 633–637.

[99] R.M. Johnstone, M. Adam, J.R. Hammond et al.: "Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)", *J. Biol. Chem.*, Vol. 262, (1987), pp. 9412–9420.

[100] G. Van Niel, I. Porto-Carreiro, S. Simoes et al.: "Exosomes: a common pathway for a specialized function", *J. Biochem.*, Vol. 140, (2006), pp. 13–21.

[101] C. Thery, L. Zitvogel and S. Amigorena: "Exosomes: composition, biogenesis and function", *Nat. Rev. Immunol.*, Vol. 2, (2002), pp. 569–579.

[102] C. Geminard, A. De Gassart, L. Blanc et al.: "Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TfR for sorting into exosomes", *Traffic*, Vol. 5, (2004), pp. 181–193.

[103] H. Rabesandratana, J.P. Toutant, H. Reggio et al.: "Decay-accelerating factor (CD55) and the membrane inhibitor of reactive lysis (CD59) are released within exosomes during in vitro maturation of reticulocytes", *Blood*, Vol. 91, (1998), pp. 2573–2580.

[104] A. Savina, M. Vidal and M.I. Colombo: "The exosome pathway in K562 cells is regulated by Rab11", *J. Cell Sci.*, Vol. 115, (2002), pp. 2505–2515.

[105] A. De Gassart, A. Geminard, B. Fevrier et al.: "Lipid raft-associated protein sorting in exosomes", *Blood*, Vol. 102, (2003), pp. 4336–4344.

[106] R.M. Johnstone: "Exosomes biological significance: a concise review", *Blood Cell. Mol. Dis.*, Vol. 36, (2006), pp. 315–321.

[107] H. Tsunoo and H.H. Sussman: "Placental transferrin receptor: evaluation of the presence of endogenous ligand on specific binding", *J. Biol. Chem.*, Vol. 258, (1983), pp. 4118–4122.

[108] J.E. Levy, O. Jin, Y. Fuiiwara et al.: "Transferrin receptor is necessary for development of erythrocytes and the nervous system", *Nat. Genet.*, Vol. 21, (1999), pp. 396–399.

[109] R.E. Fleming, M.C. Migas, C.C. Holden et al.: "Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis", *Proc. Natl. Acad. Sci USA*, Vol. 97, (2000), pp. 2214–2219.

[110] M.B. Johnson and C.A. Enns: "Regulation of transferrin receptor 2 by transferrin: diferric transferrin regulates transferrin receptor 2 protein stability", *Blood*, Vol. 104, (2004), pp. 4287–4293.

[111] A. Robb and M. Wessling-Resnick: "Regulation of transferrin receptor 2 protein levels by transferrin", *Blood*, Vol. 104, (2004), pp. 4294–4299.

[112] S.G. Gehrke, H. Kulaksiz, T. Hermann et al.: "Expression of hepcidin in hereditary hemochromatosis: evidence for a regulation in response to serum transferrin saturation and non-transferrin-bound iron", *Blood*, Vol. 102, (2003), pp. 371–376.

[113] H. Kawabata, R.E. Fleming, G. Dorina et al.: "Expression of hepcidin is down regulated in TfR2 mutant mice manifesting a phenotype of hereditary hemochromatosis", *Blood*, Vol. 105, (2005), pp. 376–381.

[114] E. Nemeth, A. Roetto, G. Garozzo et al.: "Hepcidin is decreased in TfR2 hemochromatosis", *Blood*, Vol. 105, (2005), pp. 1803–1806.

[115] D.F. Wallace, L. Summerville, P.E. Lusby et al.: "First phenotypic description of transferrin receptor 2 knockout mouse, and the role of hepcidin", *Gut*, Vol. 54, (2005), pp. 980–986.

[116] S.F. Drake, E.H. Morgan, C.E. Herbison et al.: "Iron absorption and hepatic iron uptake are increased in a transferrin receptor 2 (Y245X) mutant mouse model of hemochromatosis type 3", *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2006 Aug 24; [Epub ahead of print].

[117] G. Le Gac and C. Férec: "The molecular genetics of hemochromatosis", *Eur. J. Hum. Genet.*, Vol. 13, (2005), pp. 1172–1185.

[118] A. Pietrangelo: "Hereditary hemochromatosis", *Biochim. Biophys. Acta*, Vol. 1763, (2006), pp. 700–710.

[119] L. Lin, Y.P. Goldberg and T. Ganz: "Competitive regulation of hepcidin mRNA by soluble and cell-associated hemojuvelin", *Blood*, Vol. 106, (2005), pp. 2884–2889.

[120] H. Kulaksiz, S.G. Gehrke, A. Janetzko et al: "Pro-hepcidin: expression and cell-specific localization in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia", *Gut*, Vol. 53, (2004), pp. 735–743.

[121] G. Papanikolau, M.E. Samuels, E.H. Ludwig et al.: "Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis", *Nat. Genet.*, Vol. 36, (2004), pp. 77–82.

[122] V. Niederkofler, R. Salie, M. Sigrist et al.: "Repulsive guidance molecule (RGM) gene function is required for neural tube closure but not retinal topography in the mouse visual system", *J. Neurosci.*, Vol. 24, (2004), pp. 808–818.