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Structural analysis of the transferrin receptor multifaceted ligand(s) interface

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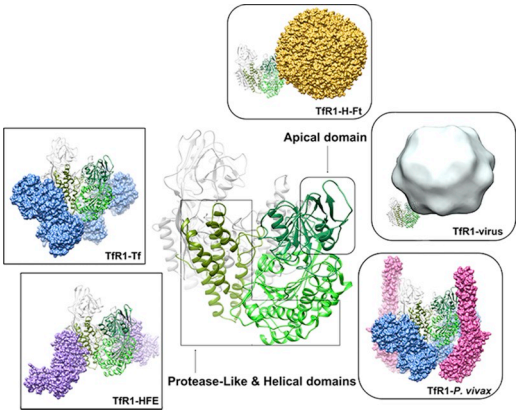
HIGHLIGHTS

- Multiple ligand binding and ligand binding modes govern the TfR1 cellular functions.
- Sequence conservation at TfR1/Tf binding sites preserves their role in iron-uptake.
- Sequence variability at TfR1 apical domain explains its ligand binding promiscuity.

GRAPHICAL ABSTRACT



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ABSTRACT

The transferrin receptor 1 (TfR1) is one of the key regulators of iron homeostasis for most higher organisms. It mediates cellular iron import through a constitutive clathrin-dependent endocytosis mechanism and by recruiting iron- regulator proteins as transferrin, Hereditary Hemochromatosis factor (HFE) and serum ferritin in response to cellular demand. The receptor is also opportunistically exploited by several viruses and the malaria parasite as a preferential door for cell invasion. In this review, we analyze the structural information available for TfR1 and all its functional complexes to figure out how structural signals in a single receptor can guide the recognition of multiple ligands and how the conservation of key residues in TfR1 might have a role in iron uptake and cell infection.

1. Introduction

Transferrin receptor 1 (TfR1), also identified as Cluster of Differentiation CD71, is a promiscuous and ubiquitously expressed "heavy duty" carrier that is capable of recognition and internalization of plasma iron-carrier proteins, such as transferrin (Tf) and ferritin (Ft), in order to guarantee adequate iron supply to the cell. A clathrin-mediated endocytosis mechanism is at the basis of the TfR1 capability of mediating cell entry of its molecular ligands [1]. Being one of the primary gatekeepers of iron metabolism and homeostasis, its critical

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role requires a tight molecular control to maintain the correct balance of iron in the body. This level of control is achieved by combining the regulation of TfR1 gene expression to a fine tune of its activity through binding to multiple ligands [2,3].

The best understood mechanism of iron uptake mediated by TfR1 involves the internalization of Tf-iron complex (Fe-Tf) that occurs through a receptor-mediated endocytosis mechanism *via* a clathrin dependent pathway [2]. Endosomal acidification triggers a cascade of molecular and structural events at the TfR1 dimer interface that ultimately serves in assisting iron release from Tf [4–6]. Once passed through the endosomal membrane, iron can be both used for metabolic functioning, *i.e.* the synthesis of heme and non-heme iron-containing proteins, and stored at high concentration within the iron storage protein ferritin. Endosomes containing TfR1-Tf complex are deviated from the lysosomal pathway, avoiding the degradation of Tf and the receptor [7] and favoring their recycling to the cell surface. A pH dependent mechanism also regulates the apo-Tf release: the slightly basic pH of the extracellular environment favors its dissociation and apo-Tf is released to the bloodstream [1]. Although several evidences show TfR1 to be constitutively internalized into the cell either in its ligand-free state or bound to Fe-Tf, a few number of evidences also supports the possibility that the specific binding to the ligand triggers its internalization [8,9].

The transmembrane type I hemochromatosis protein, HFE, also binds the TfR1 on sites that only partially overlap with the ones contacted by Tf, but that are sufficient to reduce the Fe-Tf binding affinity. Unlike Tf, once translocated into the endosomes, the acidic pH of the environment favors the HFE release [10]. The Tf-common contacting area over the helical domain allows simultaneous binding and formation of a ternary HFE/TfR1/Fe-Tf complex only if the two ligands bind the two opposite polymer chains of the receptor [11]. The HFE/Fe-Tf competition for cellular entry through TfR1 provides a molecular dynamic tool to keep the intracellular iron concentration under a tight control [3,12].

The role of TfR1 in regulating iron homeostasis in mammalian cells through the binding and internalization of multiple ligands is also accomplished by its capability to interact with the human ferritin (Ft) [13], present in serum as well as inside the cell. Mainly produced by macrophages and hepatic cells through a non-canonical secretory pathway, the physiological role of serum Ft remains uncertain, but increased levels in the blood are associated to inflammation states or cancer [14–16]. Only recently the structural explanation for a not competitive binding mechanism observed for iron-loaded-Tf and Ft to TfR1 has been provided, showing that the two TfR1 iron-carrier ligands bind different epitopes of the ectodomain of the receptor [17]. A clathrin-mediated endocytosis also leads to Ft cellular internalization by TfR1, but differently from Tf, Ft might dissociate in the endosomes allowing its translocation to lysosomes and its subsequent degradation that eventually allows iron delivering to cells [13]. However, given the only recent availability of structural information about the system, many aspects of Ft internalization by TfR1 still require some elucidations.

In order to allow the cell to finely tune iron storage, uptake and release, thus maintaining its optimal intracellular levels and avoiding the excess to induce detrimental reactions, multiple levels of regulation control TfR1 expression in a tissue- or cellular stage-specific manner.

TfR1 is expressed at low levels in virtually all cell types, whereas high expression occurs in highly differentiated and in iron-avid rapidly dividing cells, as well as immature erythroid cells and placental tissue. However, many examples of nonproliferating cells, such as reticulocytes, hepatocytes, endothelial cells of the blood-brain barrier and others [7], also show high expression of transferrin receptors.

Distinct stimuli at post-transcriptional level respond to changes in intracellular iron: in case of cellular iron deprivation, iron regulatory proteins (IRPs) bind specific iron responsive elements (IREs) in the 3′ untranslated region of transferrin receptor mRNA and protect the TfR1 transcript from nuclease degradation, with the consequent increase in TfR1 level and enhanced iron uptake. When levels of intracellular iron rise, IRP binding is reduced, resulting in TfR1 mRNA degradation [2]. IRE/IRP-independent mechanisms at the transcriptional level are described for cells and tissues with specific iron requirements, such as erythroid cells, the most avid consumers of iron in the organism, that constantly maintain high transferrin receptor levels, or macrophages that pair elevated iron levels to an increase (rather than decrease) of TfR1 mRNA and protein levels [7].

Cancer cells show abnormal TfR1 overexpression levels because of their uncontrolled escalation of proliferation rate that, to be sustained, needs the internalization of increased amount of iron. Many metastatic cancers overexpress up to a hundred times more TfR1 than normal cells [18–24]. Indeed, several cell growth-related transcription factors can specifically induce TfR1 expression in proliferating cells by binding the 5′ flanking region of the TfR1 gene [25]. Therefore, numerous TfR1-based strategies have been developed for anticancer treatment being TfR1 exploitable either as an alternative drug target to deplete malignant cells of iron, or as a selective route of access to tumor cells for the internalization of modified iron-carrier proteins, such as Tf or Ft, chemically conjugated or filled up with cytotoxic drugs [26–30].

A curious aspect of the TfR1 capability of multiple ligand binding is that this portal to the cell is also hijacked by pathological ligands, such as select viruses and one of the most common malaria parasites, to invade host cells and start infection [31–39]. Pathogens recognize epitopes on the host-encoded TfR1 receptor through proteins that are exposed to their surface, thus triggering their internalization [40]. Many physiological aspects of TfR1 make it vulnerable for pathogen infections [3], among those its ubiquitous and abundant expression and its exposure to the cell-membrane surface, together with its constitutive endocytosis guided recycling back to the cell surface that allows multiple rounds of infection to occur [41].

Therefore, TfR1 emerges as a multifunctional receptor whose functions have important implications in the regulation of iron metabolism, in the control of specific protein internalization through clathrin-mediated endocytosis, in favoring virus infection and in its potential exploitation as target in anticancer treatment.

In this review, all structural information available for TfR1 and its functional complexes are examined to understand how structural signals in a single receptor can guide multiple ligand recognition and how the conservation of key residues in TfR1 might have a role in iron uptake and cell infection.

2. Structural insight into the multiple ligand recognition and ligand binding modes of TfR1

The human TfR1 is a homodimeric type II transmembrane glycoprotein of about 170 kDa. It is composed of: *i)* a small N-terminal cytoplasmic domain (residues 1–67) containing the endocytosis motif YTRF, the signal for internalization and recycling back to the cell surface through endosomes [42,43], and post-translational modifications such as phosphorylation at Ser24 [44] and acylation at Cys62 [45] which contributes to the regulation of the endocytosis mechanism [46]; *ii)* a single-pass transmembrane region (residues 68 to 88) that anchors the receptor to the membrane guiding its proper insertion [47,48]; *iii)* a large extracellular domain (residues 89–760) which is separated from the membrane by a stalk (residues 89–120) of about 30 Å where two intermolecular disulfide bonds on Cys89 and Cys98 covalently linking the monomers are located [49–51]. N- and O- glycosylations respectively at Asn251, 317, 727 and at Thr104 were proved to be essential to guarantee the normal functions of the receptor, their absence showing impaired transferrin-binding activity and increased susceptibility to proteolytic cleavage [52–54] (Fig. 1).

The ectodomain of the receptor (residues 121–760), that keeps a dimeric oligomerization even in the absence of the stalk region, constitutes a large extracellular portion responsible for binding of ligands;

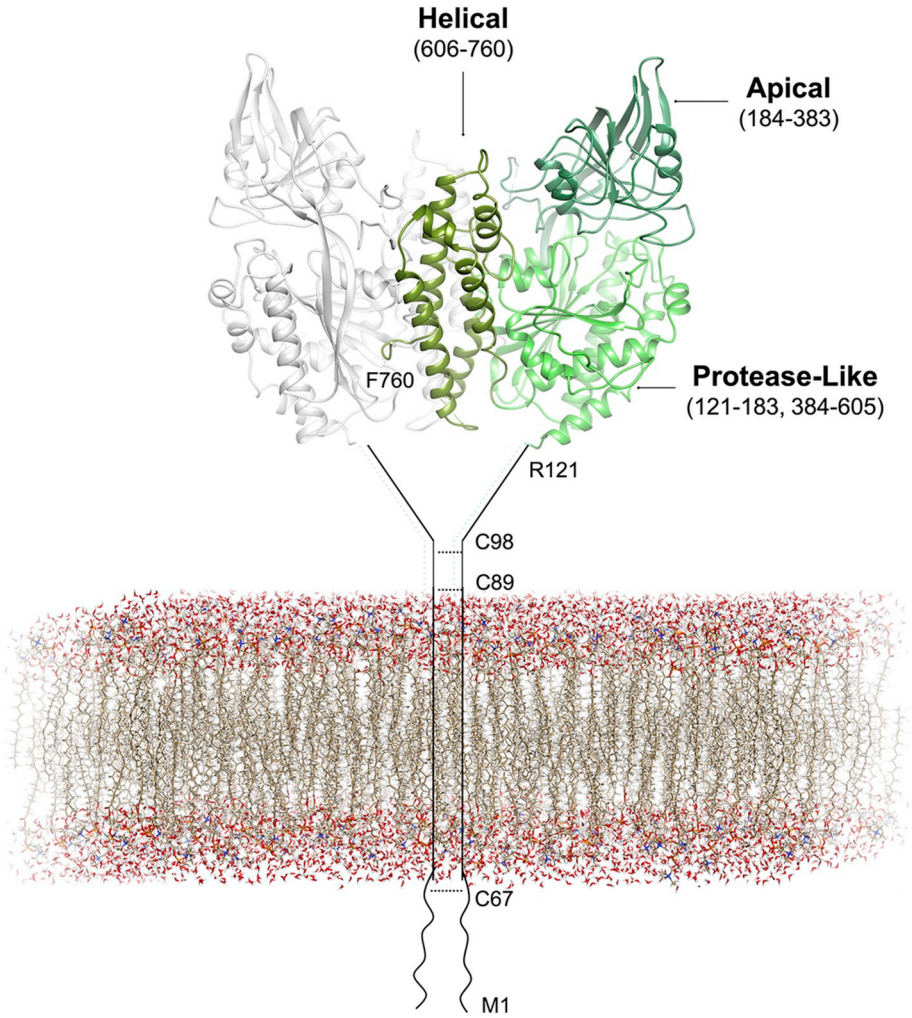


Fig. 1. Overall structure of the human Transferrin Receptor 1. The ectodomain (residues 121–760, ribbon representation) is a dimer (PDB code 3KAS [39]); one monomer is colored in light grey, while the other one is in shades of green according to the specific domain: the protease-like is in light green, the apical in emerald, the helical in olive. The stalk region (residues 89–120) contains two cysteines (C98 and C89) forming disulfur bond. The transmembrane (residues 67–88) and the cytoplasmic (residues 1–67) regions are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

each subunit is capable to provide one binding site for one molecule of ligand.

The crystal structure of the human TfR1 ectodomain revealed the overall butterfly-like shape of the dimer, each subunit being organized in three domains: the protease-like domain (residues 121–183, 384–605), which is in contact to the cell membrane at its N-terminus, an apical domain (residues 184–383), and a helical domain (residues 606–760) that comprises the dimer contact regions (Fig. 1) [51].

In the light of the available structural data, obtained by x-ray crystallography and single-particle cryo-electron microscopy, each domain of a monomer is physiologically responsible for the recognition of a specific TfR1 functional ligand, namely transferrin (Tf), HFE and ferritin (Ft) (Fig. 2).

Tf binds TfR1 by contacting residues located at the helical and the protease-like domains (Table 1), burying an area of about 1330 $Å^2$ (Fig. 2A). Each unit of TfR1 binds one Tf molecule thus forming an overall complex with a 2:1 = ligand:receptor stoichiometry, where two Tf molecules sandwich one homodimeric receptor [4,55,56]. The N- and C-lobes that compose the Tf structure are both involved in receptor binding: the N-lobe is mostly involved in interaction with the protease-like domain, while the C-lobe establishes extensive contacts with the TfR1 helical portion.

The helical domain of the receptor also constitutes the binding site for the HFE ligand (Table 1), forming, similarly to Tf, a 2:1 = HFE:TfR1 stoichiometry complex, with two HFE molecules contacting opposite chains of the TfR1 dimer, burying an area of about 1000 $Å^2$ per monomer (Fig. 2B) [10].

Several residues over the helical domain are contacted by both Tf and HFE (Table 1), representing the structural reason for the observed competitive binding between the two ligands to the receptor [11,13]. However, the interactions established by the two proteins lead to different structural organizations of the final complex: helices αIII-1 and αIII-3 of the TfR1 helical domain form an antiparallel interaction with the α1 and α2 helices of HFE, while helix α-1 and the strand β-2 of the C-lobe of Tf contact the same helices perpendicularly [4,10]. Although different, the Tf and HFE binding to both side of the receptor induces a similar effect on the TfR1 structure: a rotation along the TfR1 dimer interface, which results in a translation of the protease-like and apical domain that allows four TfR1 histidines (His475 in each protease-like domain and His684 in each helical domain) to approach each other. This cluster of histidines might constitute the pH responsive epicenter that regulates iron release from Tf/TfR1 complex and the HFE release from TfR1 in endosomes and apo-Tf release once the apo-Tf/TfR1 complex returns to the cell surface. A proton shuttle, possibly starting from the external and solvent exposed R680, is believed to be responsible for the pH sensitivity of the histidine cluster buried in the TfR1 dimer interface [4,10].

For several years the physiological role of the apical domain of TfR1 remained unknown. In 2010, when TfR1 has been also found to work also as a specific receptor for the H-chain human ferritin (H-Ft), the observation of a not competitive binding between Tf or HFE and H-Ft led to hypothesize the existence of alternative binding sites over the receptor for the different ligands [13]. Only recently these observations found a structural explanation thanks to the reconstruction of a single particle cryo-electron microscopy (cryo-EM) electron density map of a

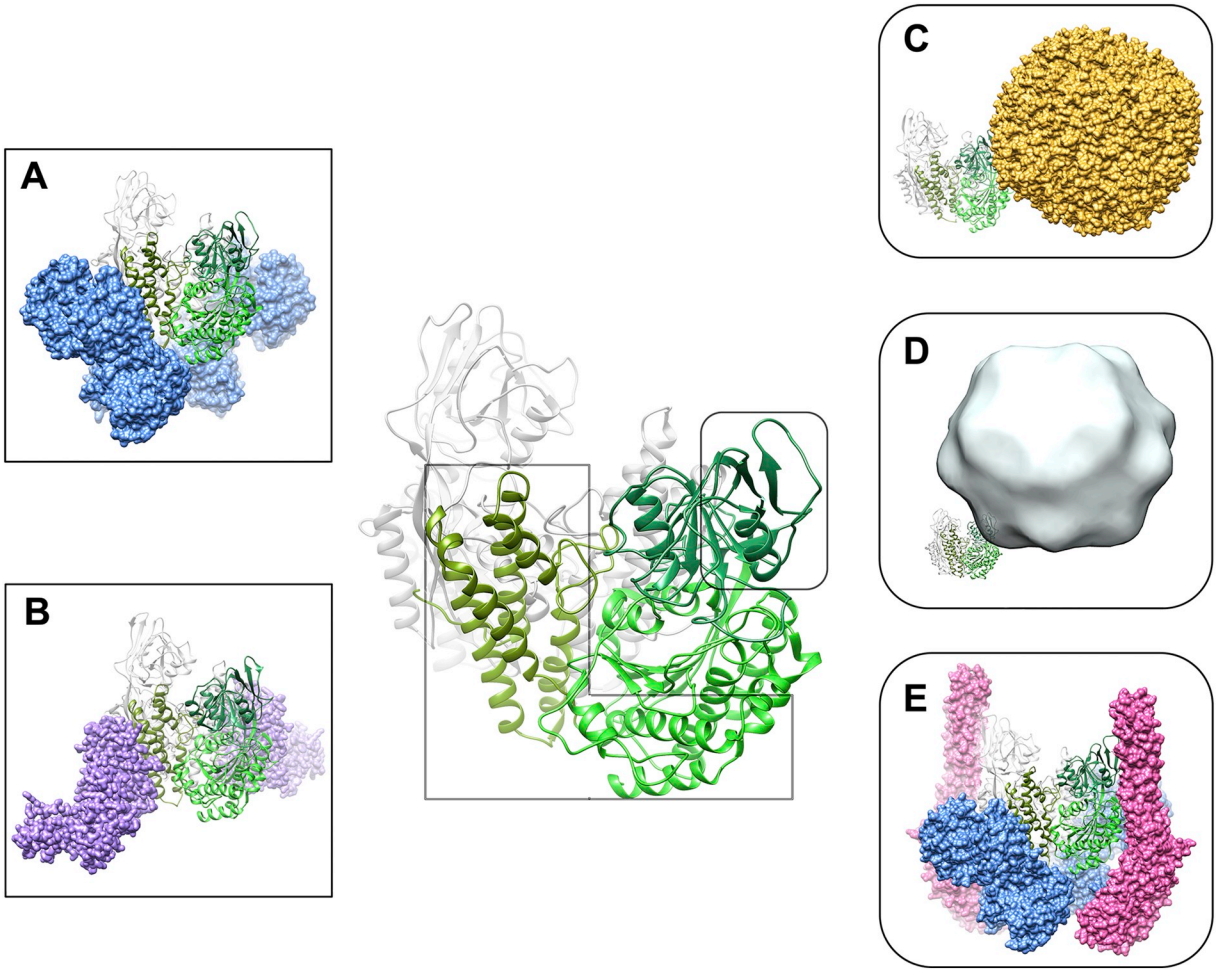


Fig. 2. Multiple ligands and ligand binding modes: the structures of TfR1 and its functional and infectious complexes. TfR1 is shown in ribbon representation (image center). A L-shaped squared box highlights helical and protease-like domains and a rounded box indicates the apical region. On the left, the structures of TfR1 in complex with its helical and protease-like ligands are represented (panel A, Tf, light blue surface, PDB 1SUV [4,55]; panel B, HFE, violet surface, PDB 1DE4 [10]). On the right, the structures of TfR1 bound to its apical interacting partners are shown (panel C, H-chain ferritin (H-Ft), yellow surface, PDB 6H5I [17]; panel D, canine parvovirus capsid, gray surface, PDB 2NSU [35]; panel E, *Pv*RBP2v from *P.vivax*, pink surface, with Tf, PDB 6D03 [33]. TfR1 domains are colored according to Fig. 1. Figures were produced using UCSF Chimera. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1:1 stoichiometry complex of the human TfR1/H-Ft (Fig. 2C) [17]. The resulting structural model clearly shows the TfR1 apical domain to be the specific binding site for the apo H-Ft (Table 2), the interaction covering an overall area of about 1900 Å^2. The local low resolution of the TfR1 electron density hampered the observation of a structural reorganization of the receptor induced by H-Ft binding, that in this model is limited to the side chain of amino acids directly contacting H-Ft, one of the regions endowed with a local resolution that allowed model building. The 1:1 stoichiometry of the complex observed in the single particle reconstruction represents only one possible scenario of the actual physiological interaction. The 2D-classes generated during the cryo-EM data analysis showed the complex to explore other stoichiometry possibilities. Among those, the binding of multiple TfR1s to the same H-Ft was observed, thus supporting the possibility that in physiological conditions more than one receptor is required to mediate the internalization of one H-Ft molecule [16]. Surface plasmon resonance (SPR) binding assays performed on TfR1 and H-Ft reveals a complex scenario difficult to interpret, but that is likely due to the multivalent nature of both ligands [17,57].

The physiological significance of this interaction is still not completely clarified, since undefined is the role of serum Ft [14,15].

An intriguing aspect highlighted by the analysis of the structure of the TfR1/H-Ft complex is that several residues on the apical domain which are crucial for the interaction with H-Ft are also contacted by pathogen proteins exposed on the capsid surface of several viruses and on the external merozoite apical region of one of the most common malaria parasite, *Plasmodium vivax* (Fig. 2E), allowing their internalization in the host cell [3,17,31,34–40,58].

The crystal structure of the human TfR1 in complex with the glycoprotein 1 (GP1) of the New World Clade B Machupo arenaviruses (MACV GP1) shows that the most of the contacts established are located on the αII-2 helix and the βII-2 strand of the TfR1 apical domain (Table 2), the latter secondary structural element being also previously identified as a critical determinant of virus-TfR1 host specificity [31,38,59]. No relevant conformational changes within the TfR1-bound structure have been highlighted in this work. Fig. 2D reports the structure of TfR1 bound to the canine parvovirus capsid as an example of the interaction involving the TfR1 apical domain and viral particles [35].

More recently, the single-particle cryo-EM structure of a ternary complex formed between TfR1, the *P. vivax* recitulocyte-binding protein 2b (*Pv*RBP2b) and Tf has been determined [33]. *Pv*RBP2b forms a stable complex with TfR1 only if iron-loaded Tf is also bound to the receptor; several contacts are indeed established with the N-terminal region of Tf. Although a few number of interacting residues were identified between *Pv*RBP2b and the protease-like domain of TfR1 (Table 1), most of the

Table 1
Summary of the interactions between TfR1 and its protease-like and helical domain ligands.

Human TfR1 domain	Human TfR1 (residues)	Human Tf	Human HFE	*Pv*RBP2b (*P. vivax*)
Protease-Like	R121	D166		
Protease-Like	Y123	P145, D166, F167		
Protease-Like	D125	P142		
Protease-Like	D126	K148		
Protease-Like	E149			R359
Protease-Like	K574			E556
Protease-Like	E578			K563
Helical	S616		L22, H74,	
Helical	L619		L22, H74, V78, W81	
Helical	V622	V360	V78, W81	
Helical	R623	N361, V363	L22, W81	
Helical	N626	N361	W81	
Helical	R629	N361, G617	T82, E85, N86, E146	
Helical	Q640	L353	E146, H150, R153	
Helical	W641		H150, I152	
Helical	Y643	E357	V78, T82	
Helical	S644		H150	
Helical	R646	S359	H74, M75,	
Helical	D648		Q156	
Helical	F650	E367	G71, H74	
Helical	R651	D356, S368	Q156	
Helical	S654		Q67	
Helical	T657		L63, Q67	
Helical	T658		Q64	
Helical	G661	Y68		
Helical	N662	Y71, A73		
Helical	A663	L72		
Helical	E664	R50, A73, N75		
Helical	D667	P74		
Helical	D757	H349		
Helical	N758	H349		

interactions are established with its apical domain. If compared to the structure of TfR1 bound to the sole iron-loaded Tf [4], binding of *Pv*RBP2b induces a small conformational change which is confined to the apical region of TfR1 and that mainly involves loops rearrangement at the upper part of the domain. It is reasonable to hypothesize that the structural rearrangements induced by binding to iron-loaded Tf, that mutually reorganizes the position of each domain of the receptor, stabilize the interactions with *Pv*RBP2b more than the ones formed with the unbound TfR1; afterwards, the *Pv*RBP2b binding further relocates the apical domain.

Therefore, while binding of diverse proteins that interact with the helical domain leads to structural rearrangements that involve the relative relocation of the three main components of the TfR1 ectodomain, without affecting the individual conformation of each subunit [4,10,17,31,55], no structural changes within the TfR1 structure have been described for the apical domain binders [17,31], unless a simultaneous binding of a helical binding ligand occurs [33]. Nevertheless, we cannot exclude that binding of a physiological ligand of the apical domain, such as H-Ft, could induce TfR1 structural rearrangements.

Notably, a comparative analysis of residues of the TfR1 apical domain interacting with H-Ft, GP1 (MACV) and *Pv*RBP2b revealed that Y211, L212 and N215 on the βII-2 strand and the following βII-2/βII-3 loop of TfR1 are contacted by all the apical domain ligands, thus shedding light on the possibility for these amino acids to work as molecular signals that allow a ligand to bind the apical domain (Table 2).

The structural information here summarized show TfR1 as a molecular platform whose surface provides diversified binding sites

Table 2
Summary of interactions between TfR1 and ligands of the apical domain.

Human TfR1 (residues on apical domain)	Human H-Ft	GP1 (MACV)	*Pv*RBP2b (*P. vivax*)
S195	K119, D123		
Q197	E116		
S199	D15		
I201	A18	F226	
D204		D114	
R208	T5, R79		N527, E530, D531
L209	Q14, R79, F81	S97, F98	
V210	Q14, E17, A18, N21	R111, F226, Y228	
Y211	F81, Q83	R111, S113, I115, V117	S541
L212	A18, A19, R22	P223, F226	Y542
V213			Y604
E214			K600
N215	R22, E116	E171	D603
A293		Y122	
E294		M119, Y122, K169	K600
E343	K86	Y122	
K344	N25	M119, D123, K169	Y604
N348	Q83, K87	D114, S116	
S370		D114	
K371		S116	Y538
K374	Q14, D15		

specifically exploited by multiple physio/pathological ligands to enter cells. A strong contribution to the regulation of the binding capability of the receptor toward its physiological partners is the presence or the absence of overlapping binding sites among ligands that hampers or allows their simultaneous egress. Mutations at those sites were proved to decrease or abolish binding capability and cellular internalization through TfR1 [17,38,60]. This ligand-mediated control mechanism might have a functional role in regulating iron incorporation or specific protein internalization in response to precise cellular signals. However, other sites on TfR1 are contacted in a substrate-specific manner. The exclusive contacts might play a specific functional role, for example the interaction between D757 and N758 on the C-terminus of the receptor with the crucial H349 on the N-terminal cap of Tf α-1 is involved in iron release [4] (Table 2).

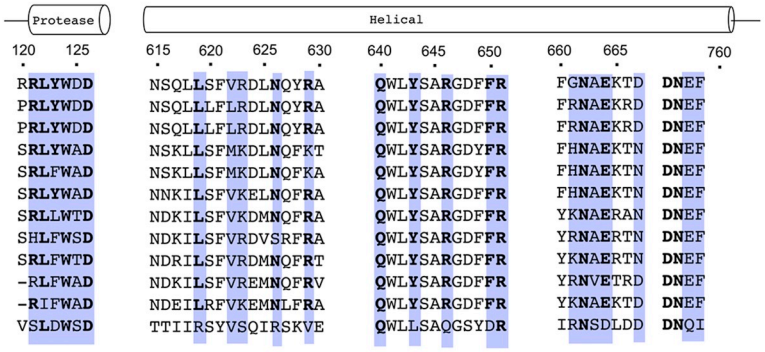
Notably, residues in all TfR1 ligands involved in interaction with the receptor are generally conserved among species, also among GP1s from different clade B arenavirus subfamilies (MACV, JUNV, GTOV, AMAV and TCRV), in which case few variations were observed to be compensated by variation in the host-receptor, and among natural occurring polymorphisms onto the *Pv*RBP2b sequence.

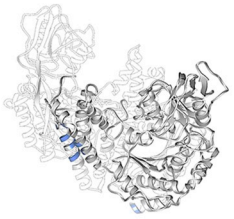
3. Conservation of key residues in TfR1

TfR1 is ubiquitously expressed in all vertebrates [61]. Given the critical physiological role in regulating homeostasis of iron, an element that possesses both toxic and beneficial properties for cellular health, TfR1 is expected to be highly conserved among species, especially in regions contacting the physiological iron-regulatory proteins Tf, HFE and H-Ft. As a matter of fact, sequence alignment analysis performed on different vertebrates (*i.e. Homo sapiens* (Human), *Macaca mulatta* (Rhesus macaque), *Macaca fascicularis* (Cynomolgus monkey), *Mus musculus* (mouse), *Rattus novergicus* (rat), *Cricetulus griseus* (hamster), *Equus caballus* (horse), *Feline catus* (cat), *Canis familiaris* (dog), *Suus scrofa* (pig), *Bovine taurus* (cow), *Danio rerio* (zebrafish) [60,61]) revealed a strong interspecies conservation in TfR1s, showing > 76% sequence homology with respect to the human variant. The only exception is represented by TfR1 from *D. rerio* that shares only 44% identity with *H.sapiens*. Indeed, zebrafish, as well as many other aquatic

A

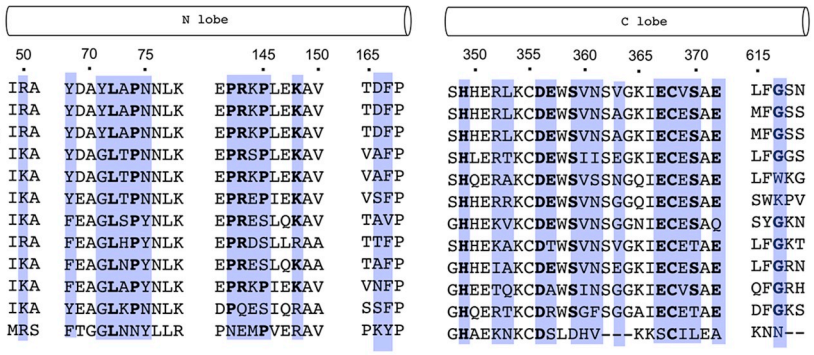
TfR1

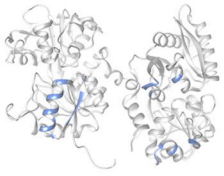




B

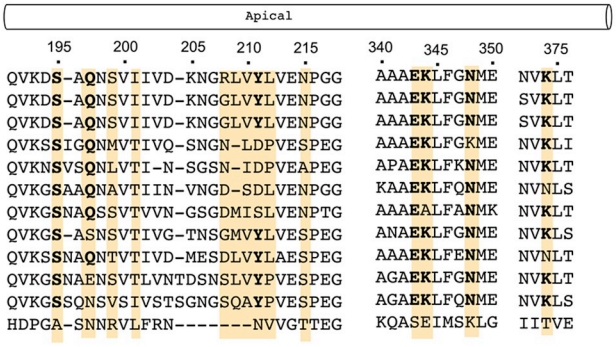
Transferrin

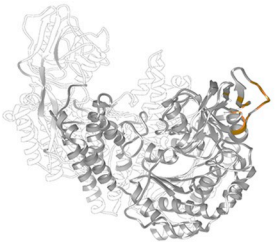




C

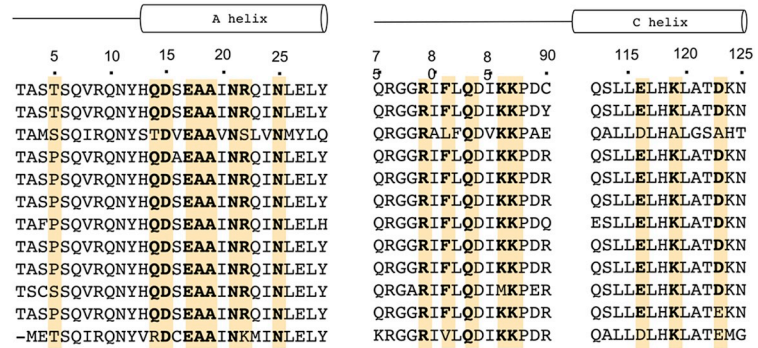
TfR1





D

H-Ferritin



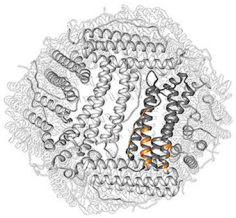


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Fig. 3. Conservation of residues involved in complexes with physiological iron-binding ligands. Cylinders, showing secondary structure of the respective region, and numbers are referred to the human proteins. Identical residues in most of the species, conserved with respect to human, are in bold. The coloring for Tf or H-Ft (H-Ferritin in the figure) follows Fig. 2, light blue lines for Tf/TfR complex and yellow lines for H-Ft/TfR complex. A. *(Left)* Conservation of TfR1 amino acids involved in complex with Tf, highlighted by light blue lines. (*Right*) Position of these residues in one monomer of TfR1, PDB 3KAS. B. (*Left*) Conservation of Tf amino acids involved in complex with TfR1, highlighted by light blue lines. (*Right*) Position of these in human Tf, PDB 1D3K. C *(Left)* Conservation of TfR1 amino acids involved in complex with H-Ft, highlighted by yellow lines. (*Right*) Position of these residues in one monomer of TfR1, PDB 3KAS. D (*Left*) conservation of H-Ft amino acids involved in complex with TfR1, highlighted by yellow lines. (*Right*) Position of these in one monomer (over 24) of human H-Ft, PDB 3AJO [82]. All alignments were performed using ClustalW2 [83]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vertebrates, shows peculiar features regarding TfR1 expression, since this receptor is present in two copies (*tfr1a* and *tfr1b*) whose combined functions are similar to the human one, with *tfr1a* responsible for iron uptake by erythrocytes [62].

However, wide areas of non-homogeneity among TfR1 ecto- subdomains are found throughout the analyzed species, as also previously noticed [13]. For this reason, in order to understand how conservation of key residues in TfR1 might have a role in controlling binding to multiple iron regulating proteins, we focused our analysis on amino acid sequences of TfR1 orthologs belonging to the two ligand-binding regions: *a)* the protease-like and the helical domains (the Tf and HFE binding epitopes); *b)* the apical domain (the H-Ft binding epitope). Furthermore, to figure out how specific ligands of TfR1 might have co-evolved with their physiological receptor, sequence analysis has been extended to the iron-carrier TfR1 ligands (Tf and H-Ft) coming from the same species, focusing the study on regions directly involved in the interaction with the receptor.

The elements of human TfR1 that contact Tf comprise about 23 amino acids (R121-D126, L619, V622-R623, N626, R629, Q640, Y643, R646, F650-R651, G661-E664, D667, D757-N758 in human TfR1 numbering (Table 1) [4,55]; they are highlighted in blue in Fig. 3A). These regions are highly conserved among species (> 78%, excluding zebrafish): notably, L122, W124, Q640, R651, N662, D757-N758 are perfectly conserved, including *D. rerio*. Thanks to this tight conservation of TfR1 amino acids that bind Tf, some extent of promiscuity among species is permitted, such that the human TfR1 was found to be able to bind with high affinity rabbit [63–65], rat [63] and pig [65] Tfs and with medium affinity the equine one [64]. Conversely, human TfR1 is not able to bind bovine [64,65] and chicken [64–67] Tfs.

Two recurrent motifs of TfR1 are particularly important for Tf binding: the sequence LYWD (residues 122–125) in the protease-like domain and the RGD sequence (residues 646–648) in the helical domain. The first segment binds the N-lobe of Tf [4,55]: sequence analysis shows that while Y123 is often replaced by a phenylalanine or a leucine residue, L122 and W124 are always conserved, thus supporting the relevance of the LYWD segment for the interaction. Its importance is also highlighted by the observation that bovine TfR1, lacking L122 and Y123, does not bind human Tf [65]. Similarly, the RGD segment represents a fundamental structural element for Tf binding [4,55,68]. Indeed, R646G mutation has been found to completely tune down the interaction with Tf [60]. Even if G647 and D648 are not directly involved in Tf binding, their presence strongly influences the formation of the TfR1/Tf complex, since their mutation severely hampers its formation [68]. This tripeptide is also fundamental for the interaction with HFE, the sole TfR1 ligand that only recognizes its helical domain. Notably, the RGD segment is present in all the vertebrates, amphibians and reptiles included, with the exceptions of fishes (*e.g.* in zebrafish R646 is replaced by a glutamine and D648 by a serine). This exception might be ascribed to the absence of an ortholog of the human HFE in fishes, thus underlining the effect of ligand/receptor co-evolution to the sequence variability of this segment [61,62,69].

Following these observations, it is interesting to note that the maintenance of TfR1 sequences is not only limited to the direct ligand-interacting residues, but it also involves residues that do not directly participate to the binding, as indicated by the example of G647-D648 above discussed. Other two similar examples are represented by residues W641 and F760 at the helical domain. Although these two amino acids are not directly involved in Tf binding, their conservation among species reflects their indirect role in tuning binding specificity toward Tf. Indeed, their mutations into alanines were shown to markedly decrease TfR1 binding affinity for Tf (56-fold and 16-fold reduction with respect to wild type human TfR1, respectively [60,70]). Altogether these evidences show that TfR1 binding specificity toward Tf is also controlled by residues not directly participating to the interactions with the ligand.

The residues on human Tf that are involved in interactions with the human TfR1 are R50, Y68, Y71-N75, P142-P145, K148, D166-F167, H349, R352-L353, D356-E357, S359-N361, V363, E367-S370, E372, G617 (Fig. 3B) [4,55]. As well as in the case of the counterpart residues of TfR1, an excellent degree of homology can be observed among all the orthologous Tfs examined.

A specific Tf motif that was shown to be crucial for the binding to the protease-like domain of TfR1 is the PRKP segment at the N-lobe (residues 142–145): it represents a molecular passe-partout that allows binding of any non-human Tfs to the human TfR1 [4,65,69,71]. Indeed, this motif, highly conserved in primates, rodents and pig allows their Tfs to bind human TfR1 [63]. Conversely, bovine Tf, where the PRKP segment is substituted by the PQES one, results in a Tf variant uncapable to bind the human receptor [64,65].

Among residues of Tf that are involved in iron binding and release, H349 has emerged as a pH-sensitive switch responsible for iron release in the acidic environment of the endosomes [4]. Consistently with its crucial functional role in Tf, H349 is perfectly conserved across all the species here analyzed, even in *D. rerio*. It has been proposed that this residue has been selected during evolution to act as the driving force in triggering iron uptake by cells through Tf/TfR1 pathway [70,72]. Indeed, mutation at this site into alanine abolished iron release from TfR1 [55,70].

From what discussed until now, the Tf/TfR1 mediated regulation of iron metabolism is extremely shared in all vertebrates, thus highlighting its vital importance [2,73].

We found a different scenario in terms of sequence conservation for the human TfR1 apical domain epitope contacting the H-chain Ft (14 TfR1 amino acids involved: S195, Q197, S199, I201, R208-L212, N215, K374, E343-K344, N348 in human numbering (Table 2), emphasized in yellow in Fig. 3C). Ferritin has been extensively characterized over the years, but its internalization in cells *via* human TfR1 has been discovered only in 2010 [13], and few months ago the H-Ft/TfR1 interacting regions have been elucidated [17]. Therefore, data regarding inter-species H-Ft and TfR1 interactions are missing.

Differently from the Tf-binding region, the apical part of the receptor is not well conserved among vertebrates: few residues have been maintained during evolution, and only three of them (E343, K344, N348) are critical for H-Ft binding (Table 2).

A high number of differences are found between human and rodents TfR1s. Among those, *M. musculus* shares only 29% homology with the correspondent 14 human TfR1 interacting residues, that in the R208-L212 sub-region is reduced to a 0% similarity, showing a totally different sequence. In the light of this observation, it is reasonable to hypothesize that *M. musculus* H-Ft, that shares 92% sequence homology with the human variant (Fig. 3C), might not be able to bind its own TfR1 receptor. This hypothesis is consistent with the fact that H-Ft

internalization in mouse is mediated by another receptor, *i.e.* TIM-2 (T cell immunoglobulin and mucin domain protein-2), not expressed in humans [13,74,75]. However, it is still not clear if TIM-2 works as a unique receptor suitable for mouse H-Ft or if mouse TfR1 plays a similar role to its human counterpart. The analysis here described supports the hypothesis that a human-like H-Ft internalization in mice might not exist, as already proposed [13]. Proving this hypothesis will be of great interest in the context of iron-delivery mechanisms and it would also provide an important validation of the usage of mice as animal models for human Ft-based drug delivery studies in the fields of nanotechnology and nanomedicine.

The differences between human and murine TfR1s in R208-L212 segment, that result in a hampered murine H-Ft internalization *via* murine TfR1, have also other implications: the existence of some viruses that are able to infect only rodents or only humans, but not both of them. Indeed, in humans the very same H-Ft route of access to cells is mimicked also by a plethora of viruses and one of the most common malaria parasite [36,37]. In particular, the segment R208-L212 determines the efficiency of viral infection through TfR1 [31,38]. The New World Arenaviruses are all able to invade humans, macaques and cats through TfR1 binding [3,38], while are ineffective in entry mouse, rat and hamster cells because of the huge differences in 208–212 sequence homology. Notably, introducing the R208-L212 human segment in murine TfR1 allows its conversion to an efficient New World Arenavirus receptor [38]. *Vice versa*, Mouse Mammary Tumor Virus (MMTV) recognizes specific epitopes on murine TfR1 and it is consequently able to infect only mice and rats, but not humans, cats, dogs or hamsters [32,76].

Specific residues of this segment finely tune species selectivity between viruses and TfR1 orthologs, shaped by evolution pressures [31,32,38,59]. For example, the above-mentioned arenaviruses are not able to infect dogs (probably because of the presence of an aspartate instead of the feline glycine or the human arginine in position 208) [3,38], while Canine Parvovirus (CPV) is able to bind only canine TfR1 [77–80]; conversely, Feline Panleukopenia virus (FPV) and Mink Enteritis Virus (MEV) infect only TfR1 of cats [3,77,79,80].

Moreover, it is interesting to note that the presence of an arginine at position 208, typical of human TfR1 and substituted with a glycine both in *M. mulatta* and *M. fascicularis*, might represent a defense mechanism of human TfR1 against arenaviruses and a molecular barrier to overcome by arenaviruses to start infection. In fact, a single R208G mutation of human TfR1 has been shown to induce not only enhanced infectivity of MACV, JUNV and GTOV, but even a total new capability of Tacaribe virus (TCRV) in binding human TfR1, a virus that normally does not infect humans [3,31]. As a lateral note, a L212V single nucleotide polymorphism (SNP) that is present in Asian populations has been found to exert a similar protection mechanism against arenavirus entry [32].

The sequences of New World arenavirus GP1s are different one from each other, and they also considerably diverge from H-Ft contacting residues; nonetheless, TfR1 is the receptor for all of them. The R208-L212 motif of apical domain has been already characterized as the substrate for the adaption required by pathogens to invade new species [32]. Conversely, this very part of human TfR1 might have evolved in order to block viral entry while preserving its house-keeping iron uptake properties [32]. This assumption might be linked with the irregular pattern of conservation of the apical domain among different species, that contrasts with the strictly preserved helical and protease-like ones, in which little differences between the ligand sequences (for example, human Tf and bovine Tf) account for completely different affinities. Hence, we suggest that the inter-species variability of TfR1 apical domain might match its flexibility in binding multiple structurally different ligands.

As far as H-chain Fts are concerned, H-Fts are ubiquitously expressed in all the kingdoms of the life [81]. In vertebrates analyzed in this work, H-Ft is highly conserved (> 92%), especially in the human TfR1-contacting residues (> 94%; *D. rerio* 70%), here listed: T5, Q14-D15, E17-A19, N21, R22, N25, R79, F81, Q83, K86-K87, E116, K119, D123 (human H-Ft numbering; Table 2; Fig. 3D). Unlike Tf, none of these residues are iron ligands [81]. The strongly inter-species conservation of H-Fts finds an explanation in the critical role played by this protein in the cytoplasm, where it works as a molecular storage of iron protecting the cell against oxidative damage, that might overcome its physiological role in serum that could have evolved only in a more recent phase.

It is interesting to notice that the H-chain Ft orthologue, the L-chain Ferritin, that differs from H-Ft only for 7 amino acids over the total contacts needed for human TfR1 recognition (S5, T14, S22, L81, D116, A119, A123), it is not able to bind the receptor [13,17].

4. Conclusions

In the light of the analysis of all the structural information available and of the sequence homology analysis here performed, we conclude that the physiological significance of the H-Ft route of access to cells *via* human TfR1 for iron import seems to be redundant with respect to the well-conserved Tf mechanism, as already noted [13]. This pathway might not be exploited in some species, such as rodents, that have evolved different internalization mechanisms based on different receptors, such as TIM2, not expressed in humans.

Species-specificity has also an effect on the capability of viruses and parasites of entering cells for invasion through TfR1. From the analysis here reported, it emerges that changes due to SNPs within TfR1 apical domain might protect a specific host from viruses or parasites entry, at the expense of H-Ft uptake.

Altogether, these observations support the leading role in iron-uptake from plasma played by Tf/TfR1 pathway. Iron-loaded Ft in serum is low and Ft/TfR1 pathway for iron uptake is exploited only when the binding capacity of Tf is exceeded. Nevertheless, since the role of serum Ft is still under debate, we cannot exclude that Ft/TfR1 internalization pathway might mediated alternative cellular processes different from iron internalization [2,73].

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