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REVIEW ARTICLE

The importance of eukaryotic ferritins in iron handling and cytoprotection

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Ferritins, the main intracellular iron storage proteins, have been studied for over 60 years, mainly focusing on the mammalian ones. This allowed the elucidation of the structure of these proteins and the mechanisms regulating their iron incorporation and mineralization. However, ferritin is present in most, although not all, eukaryotic cells, comprising monocellular and multicellular invertebrates and vertebrates. The aim of this review is to provide an update on the general properties of ferritins that are common to various eukaryotic phyla (except plants), and to give an overview on the structure, function and regulation of ferritins. An update on the animal models that were used to characterize H, L and mitochondrial ferritins is also provided. The data show that ferritin structure is highly conserved among different phyla. It exerts an important cytoprotective function against oxidative damage and plays a role in innate immunity, where it also contributes to prevent parenchymal tissue from the cytotoxicity of pro-inflammatory agonists released by the activation of the immune response activation. Less clear are the properties of the secretory ferritins expressed by insects and molluscs, which may be important for understanding the role played by serum ferritin in mammals.

Key words: cytoprotection, ferritin, iron metabolism, oxidative damage.

INTRODUCTION

Ferritin is a ubiquitous and highly symmetrical protein, characterized by a distinct brown colour and a remarkably high stability to temperature and extreme pH values. These properties facilitate its recognition, purification and crystallization, allowing it to be among the first proteins to be identified and named [1]. Important milestones in ferritin research were achieved by discovering that: (a) its expression is iron-dependent, (b) it is present in serum at concentrations related to body iron stores, (c) mammalian ferritins are formed by two subunit types, (d) small dodecameric ferritin DNA-binding proteins from starved cells (DPSs) are expressed by bacteria, and (e) mitochondria harbour a specific form of ferritin. However, the most important accomplishment has probably been the resolution of its unique 3D structure. Although this protein is ancient, the interest in it has never really declined and it keeps attracting the attention of many researchers working in different fields. New structural and functional properties of ferritin are constantly identified in different organisms and/or organs, and recently its versatile structure has been exploited in a number of nanotechnological applications [2,3]. Reviews on ferritins are periodically published [4–16], showing the continuing interest in this molecule. The aim of the present review is to give an overview of the ferritins expressed in various eukaryotes (except plants) including mammals, stressing similarities and surprising differences in structure and cellular localization among different phyla. It also provides an update on the different cellular and animal models that were used to characterize the structure, regulation, and biological and biochemical functions of ferritin in iron handling and beyond.

GENERAL PROPERTIES OF EUKARYOTIC FERRITINS

Ferritin genes in eukaryotes

Ferritin has been identified in many species of different phyla, so it is often stated that it is ubiquitous in all organisms, with the notable exception of yeasts [9]. However, the recent explosion of genomes, transcriptomes and proteomes allows verification of this assertion. Besides yeasts, other ferritin-less organisms include stramenopiles, a eukaryotic lineage that comprises unicellular algae, macroalgae and plant parasites [17]. Ferritin has not been reported in most of the ancient centric diatoms, although they show a mineral iron phase resembling a ferritin core [17]. In similar pennate diatoms, ferritin expression was found to confer a proliferative advantage, with a high number of cell divisions in the fertilization occurring in the oceans even in the absence of added iron [18]. This finding stimulated the transcriptome analysis of 47 diatom species: ferritin was undetectable in most centric diatoms, but present in all of the other classes analysed. The ferroxidase centre, necessary for iron incorporation, is conserved in all of the ferritins and an ancient duplication event led to two distinct paralogues that differed by a few residues at the C-terminus. A phylogenic analysis suggested a vertical rather than a lateral inheritance of these genes [19].

Most organisms contain more than one functional ferritin gene (including bacteria that can have three or more). Only a minority has just one ferritin gene, among which are *Aplysia* sp. [20], several shrimps and shellfish, and bivalves. Ferritin paralogues may have distinctive properties that are classified as: the H- (or M-) type carrying the residues for a functional ferroxidase centre, and the L-type with inactivated ferroxidase centre due to the substitution of key residues. The cytosolic

Abbreviations: Aβ, amyloid β-peptide; ER, endoplasmic reticulum; FTH, ferritin H-chain; FTHL17, ferritin-heavy-polypeptide-like-17; FTL, ferritin L-chain; FtMt, mitochondrial ferritin; HDAC, histone deacetylase; HIF, hypoxia-inducible factor; HK5, high-molecular-mass kininogen domain 5; HKa, high-molecular-mass kininogen; HLH-29, helix-loop-helix 29; IDE, iron-dependent enhancer; IRE, iron-responsive element; IRP, iron-regulatory protein; KO, knockout; NCOA4, nuclear receptor co-activator 4; NF-κB, nuclear factor κB; NF-Y, nuclear factor Y; ROS, reactive oxygen species; TfR1, transferrin receptor-1; tg, transgenic; TIM, T-cell immunoglobulin domain and mucin domain; WSSV, white spot syndrome virus.

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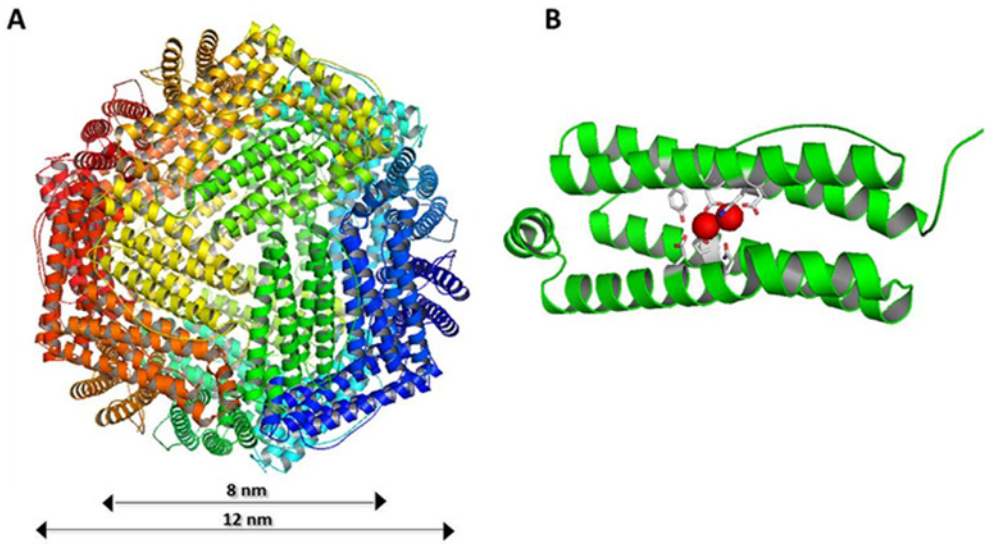

Figure 1 Structural properties of typical cytosolic eukaryotic ferritin

(**A**) Representation of the ferritin shell viewed down one of the eight 3-fold axes showed the hydrophilic pore where iron transits to the cavity. (**B**) Representation of the ferritin fold comprising the four-helix bundle and the fifth C-terminal helix. The iron-binding site of the ferroxidase centre of the bullfrog M-chain is shown. The ligand residues and iron atoms (red spheres) are highlighted.

ferritins, of 170–180 residues, are the best characterized and in mammals these are encoded by two genes, *FTH* and *FTL*, with four exons and similar structures. Moreover human and mouse genomes contain more than 40 non-interrupted ferritin-like sequences, probably pseudogenes arising from retrotransposition [21]. Spliced transcript variants are rare and their biological validity has not been determined [22]. On the contrary, secretory ferritins, predominantly expressed in insects and molluscs, but also present in mammals (e.g. serum ferritin), are normally generated as precursors of 200–240 residues with a leader sequence that is cleaved in the mature protein. Moreover, plants, mammals and insects (*Drosophila* sp.) have another type of ferritin characterized by an N-terminal extension for mitochondrial or plastid export, which is cleaved after maturation [23]. Recently, a novel functional ferritin gene has been identified on chromosome X in humans and mice, named ferritin-heavy-polypeptide-like-17 (*FTHL17*). It is transiently expressed at the embryonic stage and the protein product assembles in ferritin shells that partially accumulate in the nucleus [24].

Structures of eukaryotic ferritins

The highly symmetrical structure of ferritin greatly facilitated its crystallization; indeed horse spleen ferritin was one of the first proteins obtained in a crystal form. The hard work of Pauline Harrison and collaborators succeeded in resolving the crystallographic structure of this ferritin in 1984 [25]. It disclosed that ferritin is an almost spherical shell, or nanocage, composed of 24 subunits that assemble to form a dodecahedron 12 nm in diameter, with a large hollow cavity 8 nm across (Figure 1). Each subunit is folded into a four-helix bundle of similar length, with a long loop between helices B and C, and a fifth short C-terminal helix E. This basic structure is characteristic, unique and conserved in all ferritin types. The next success was the resolution of the structure of the recombinant human ferritin H-chain, which disclosed the structure of the ferroxidase centre common to most ferritins [26]. This was followed by 3D structures of many prokaryotic ferritins from different species and a few eukaryotic ones, listed in Table 1. They include ferritin paralogues from humans, mice, horses, bullfrogs, the insect *Trichoplusia ni* and the dipennate diatoms *Pseudo-nitzschia multiseries*. Plant ferritin structures have been discussed elsewhere [27]. In some eukaryotes the ferritins are generated by the self-assembly of two subunit types (see below). Once assembled in the 24-mer structure, the ferritins define a large cavity for iron storage which is separated from the solution by a 2-nm-thick protein shell. This is pierced by two types of channels: those on the 4-fold axes are made of the C-terminal helices of four subunits; they are tight and hydrophobic and probably permeable to oxygen, but not to ions. Those on the 3-fold axes are hydrophilic, permeable to iron and other ions, and lined by carboxy groups forming binding sites for a variety of metal ions [28,29].

The metal ions inside the four-helix bundle of H-chains identify the ferroxidase centre, which is composed of two metal-binding sites at a close distance, named A and B; in the crystals these are often occupied by non-redox metals, although iron is generally absent because it forms a labile complex. Iron was found after soaking bullfrog M-ferritin [30] and diatom ferritins [31] in Fe(II) solutions. Crystallographic studies of mammalian ferritins revealed that the pockets at the 2-fold intersubunit are available to bind organic molecules, including protoporphyrin [32], anaesthetics [33] and, more recently, fatty acids [34]. Of interest, the ferritin-bound long-chain fatty acids, such as arachidonate, project the carboxylate into the cavity, contributing to ferrihydrite mineralization and accelerating iron uptake. It remains to be assessed how these molecules can pass through the shell to reach the binding site.

The chemistry of iron incorporation into eukaryotic ferritins

Natural ferritins purify as iron-containing proteins and the iron is readily extracted by reducing agents. The apoferritin so obtained reacts with Fe(II) ions in the presence of dioxygen to oxidize and incorporate it into a mineral core similar to that of natural ferritins. This reaction, thought also to occur *in vivo,* has been studied by many groups for over 40 years and is rather well

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Table 1 Representative 3D structures of eukaryotic ferritins

1 Å = 0.1 nm.

Species	Ferritin	PDB ID	Resolution (Å)	Comments	Reference
Pennate diatoms (*Pseudo-nitzschia multiseries*)	H-chain	3E6R	2.4	Structure of recombinant mature ferritin. Analysed also after soaking in Fe(II) solution	[18]
Insect (*Trichoplusia ni*)	H- and L-chains	1Z6O	1.91	The only structure of ferritin heteropolymer	[54]
Amphibian, bullfrog (*Rana catesbeiana*)	M-chain	1MFR	2.8	The structure of a well-characterized ferritin with an active ferroxidase site. Analysed also after soaking in Fe(II) solution and subjected to various mutageneses	[175]
Amphibian, bullfrog (*R. catesbeiana*)	L-chain	1RCI	2	Structure of a ferroxidase-less L-chain	[176]
Mammal, mouse (*Mus musculus*)	H-chain	3WNW	2.24	The only structure of mouse H-ferritin	None
Mammal, mouse (*M. musculus*)	L-chain	1LB3	1.21	The ferritin structure with the highest resolution. One of the three ferroxidase-less structures to be solved	[177]
Mammal, horse (*Equus caballus*)	L-chain	1AEW	1.95	The first ferritin type to be crystallized and its structure resolved	[178]
Human (*Homo sapiens*)	H-chain	2FHA	1.9	The first recombinant ferritin to be produced and crystallized that showed the presence of a ferroxidase centre	[178]
Human (*H. sapiens*)	L-chain	2FG8	2.5	A recombinant ferroxidase-less ferritin	[179]
Human (*H. sapiens*)	Mitochondrial	1RO3	1.7	The recombinant mature FtMt that shows a high level of similarity to the H-chain	[180]

characterized. Natural horse spleen ferritin, recombinant human H and L, bullfrog M and diatom ferritins are the eukaryotic models studied more carefully, e.g. detailed studies of soaking crystals for minutes to hours in a Fe(II) solution under aerobic conditions were conducted for frog M-ferritin [30] and diatom ferritins [31], to characterize the structure and dynamics of the ferroxidase centre. In the most widely accepted model, the basic process of ferritin iron uptake involves the entry of Fe(II) atoms into the cavity via hydrophilic 3-fold channels, a process that is facilitated by an electrostatic gradient attracting metal cations [35] and the funnel-shaped 3-fold channels with conserved carboxy groups that act as transient iron-binding sites [36]. Fe(II) transit is fast, <3 ms, and follows a facilitated diffusion rate [37]. Once internalized, Fe(II) migrates to the ferroxidase centre of the H subunits, which are 2 nm apart, probably after a path involving cavity-exposed threonine, histidine and tyrosine residues [38,39]. In the ferroxidase centre, the Fe(II) atoms occupy the two co-ordination sites A and B, and encounter dioxygen (or hydrogen peroxide) to be oxidized in a diferric–peroxo complex that can be monitored for its absorbance at 650 nm. This complex rapidly decays, leaving the two Fe(III) atoms linked by a μ-oxo-/hydroxo-bridge and hydrogen peroxide. The oxidized iron then moves to the nucleation centre where, in a slower reaction, it is hydrolysed with the release of protons and mineralized as ferrihydrite [29,40,41].

The reaction of Fe(II) oxidation produces hydrogen peroxide, which acts in place of dioxygen to oxidize Fe(II) [42], so a major property of ferritin ferroxidase activity is the capacity to consume both reagents of the Fenton reaction, reduce the production of toxic hydroxyl radicals and have a general antioxidant activity [43]. The path from the ferroxidase centre to the cavity has been analysed by a paramagnetic nuclear magnetic resonance (NMR) study indicating that, although moving to the cavity, the diferric–peroxo complexes interact to form multimeric Fe(III) entities before reaching the nucleation sites to form the iron core [44]. In an alternative model, the iron core formation does not need the aggregation of small clusters [16]. Based on the characterization of the prokaryotic ferritin from *Pyrococcus furiosus*, it was suggested that in mammalian ferritin the ferroxidase centre is also a stable prosthetic group that acts by oxidizing Fe(II) and transferring electrons to oxygen [16,45]. Iron oxidation occurs directly on the mineral surface with simultaneous nucleation in a reaction that most probably does not occur *in vivo* [46]. Probably biologically relevant are oxoanions, in particular phosphate, that increase the rate of iron core formation [47]. When iron increments are above the saturation of the ferroxidase centre, 48 Fe atoms per H homopolymer, the incoming Fe(II) ions increase turnover at the catalytic site [48]. This mechanism implies a low stability of the di-iron complex, unlike bacterioferritins and enzymes with dioxygen activation such as ribonucleotide reductase and methane mono-oxygenase, in which the di-iron complex is stable and acts as a cofactor rather than a substrate, as in mammalian H-ferritins [46]. According to this, the ferroxidase centre of bullfrog M-ferritin was modified to introduce the iron-co-ordinating residues found in the enzymes. However, the iron co-ordinated at the site maintained the properties of a substrate rather than a cofactor, indicating that electrostatic alterations, steric changes and hydrophobicity of the cofactor site associated with its second sphere environment make important contributions to the activation of O_2 by binuclear iron enzymes [49].

In addition, L-ferritin can react with Fe(II) to form an iron core. However, this reaction needs a spontaneous Fe(II) autoxidation occurring at pH >6.5 and with high iron increments, leading to the formation of iron cores more ordered than in the ferritin H-chain (FTH) [50]. It is thought that this does not occur *in vivo* and that the L-chain participates in the biological reaction only when associated with the H-chain in heteropolymers (see below). In fact, the ferritin L-chain (FTL) has a more efficient iron nucleation site that co-operates with the ferroxidase activity of H-subunits to improve ferritin iron incorporation [51]. Indeed, mammalian H/L-heteropolymers are thought to be more efficient than the corresponding homopolymers in iron incorporation. It has been observed that the shape of the mineralized iron core is affected by the presence and proportion of L-chains, consistent with the hypothesis that they facilitate iron nucleation [52]. A recent study indicated a new function of FTL, showing that the electrons released during iron oxidation were transported across the ferritin cage, specifically through L-chains [53].

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Ferritin self-assembly and heteropolymers

A major property of ferritins is the capacity to self-assemble into the 24-mer shell, both *in vivo* and *in vitro*, leaving no free subunits in solution. Moreover, ferritins from mammals, plants [8], insects [54] and fish [55] were shown to form heteropolymers made of two or more different subunit types. Of particular interest is the structure of the secreted ferritin from the insect *T. ni* which is made up of 12 heterodimers of H- and L-chains arranged with tetrahedral symmetry, compared with vertebrate ferritins made of a single subunit type arranged with octahedral symmetry [54]. This structure explains why the expression of both subunit types is necessary to form functional ferritins in *Drosophila* sp. [56]. In mammalian cells the H- and L-chains assemble in the proportion that is dictated by their relative level of expression. However, the exogenous ferritin subunits expressed in transiently transfected COS7 did not co-assemble with the endogenous ones [57], although those expressed after stable transfection did [58]. This suggested that, *in vivo*, the formation of heteropolymers is time dependent due to the slow turnover of the endogenous ferritins which cannot associate with the fast, newly synthesized, ferritin chains. *In vitro* ferritin assembly occurs spontaneously and, when H- and L-subunits are present, the formation of hetero- over homo-polymers is strongly preferred. This allows the production of heteropolymers of the desired H/L proportion [59,60], but the mechanism of the co-assembly is difficult to clarify. That subunit dimers are the first intermediates in the self-assembly pathway has been suggested before [61–63], and recently confirmed using a new technique in which the interaction for the subunit dimers was engineered to make it copper-dependent. In the absence of copper, the subunits folded into monomers that were incompetent in assembly. The presence of Cu(II) promoted the formation of the dimers and the assembly in ferritin shells [64]. It remains to be clarified whether, in the formation of heteropolymers, the intermediates are subunit heterodimers, as in the insect ferritins, or homodimers. Whatever the mechanism, the easy formation of assembled/disassembled structures of ferritin has been exploited in nanotechnology and material sciences [2,65], and heteropolymers can be exploited in nanotechnology for the introduction of different functions in one molecule.

Regulation, homoeostasis and degradation of eukaryotic ferritins

Most, if not all, eukaryotic ferritins are regulated by iron. In mammals and higher eukaryotes, most of the iron-dependent regulation occurs at a post-transcriptional level and involves the binding of iron-regulatory proteins 1 and 2 (IRP-1/2) to iron-responsive elements (IREs) located at the 5′-UTR of the transcripts. This mechanism, elucidated almost 20 years ago, has been carefully described in excellent reviews [66], and it will not be considered here any further. In nematode worms, such as *Caenorhabditis elegans*, the regulation occurs at a transcriptional level and is discussed below. In many species, ferritin is also induced by oxidative stress or during infection, e.g. in turtles ferritin responds to oxygen deprivation and oxidative stress, being transcriptionally regulated via the activation of the nuclear factor κB (NF-κB) signal transduction pathway [67].

Cytosolic ferritin acts as an iron buffer. Although it is known that iron must be recycled when needed, the biological mechanism through which it is released from ferritin remains more elusive than its uptake. The current understanding of this process has recently been reviewed [68]. Ferritin iron is readily released on incubation with reducing agents, even bulky ones that cannot penetrate the shell [69], suggesting that the same may also occur *in vivo*. In fact it may take place under specific conditions, e.g. *Bacillus cereus* expresses a surface protein named IlsA which binds ferritin and facilitates its iron release in the presence of siderophores. This mechanism of iron acquisition is important for the proliferation and pathogenicity of *B. cereus* [70]. Fast iron release without disruption of the ferritin shell occurs only after iron reduction, which implies the formation of free radicals, and it was proposed to happen only under conditions of oxidative damage [71]. The ferritin shell can be degraded by the proteasomal or autophagic machinery. Studies indicate that autophagy is the major pathway of iron recycling, particularly under iron-depleted conditions, whereas, in iron-replete cells, the lysosomal targeting of ferritin did not involve autophagy, a mechanism absent from several cancer-derived cells [72].

Some insights into ferritin autophagy were recently revealed. In a study of quantitative proteomics aimed to identify autophagosome-enriched proteins in human cells, the protein nuclear receptor co-activator 4 (NCOA4) was found to be highly enriched in autophagosomes, and in associated proteins recruiting cargo–receptor complexes into the autophagosome. NCOA4 bound ferritin and was required to deliver it to lysosomes. Thus, NCOA4 acted as a cargo receptor for the autophagic turnover of ferritin (ferritinophagy), which is critical for iron homoeostasis [73]. Similar results were obtained by another group using a new screen for autophagy substrates. NCOA4 was found to co-localize with autolysosomes and bind directly to ferritin. Moreover, NCOA4-deficient mice showed accumulation of iron in the spleen [74]. NCOA4 has various functions and it was recently shown to act as a regulator of DNA replication origins, helping to prevent inappropriate DNA synthesis and replication stress [75]. Thus, a major actor in the mechanism of iron degradation, particularly under conditions of iron deprivation, has been identified. However, the chemistry of iron core dissolution, iron reduction and transfer back to the cytosol is still obscure and an interesting matter for future research.

FERRITIN IN EUKARYOTES

Ferritins in invertebrates

Diatoms

Diatoms are a major group of unicellular algae among the most common types of phytoplankton. Their high photosynthesis activity, which contributes strongly to global oxygen production, requires high levels of iron. Ocean iron studies showed that, after fertilization, a phytoplankton bloom occurs transiently, dominated by pennate diatoms. This was attributed to the finding that these organisms express ferritin, at variance with other members of the phytoplanktons. Ferritin expression, closely tied to photosynthetic competence, was induced by iron and enhanced iron storage, allowing the diatoms to undergo several cell divisions even in the absence of added iron [18]. The mature ferritin from one of these pennate diatoms, *P. multiseries*, was produced in a recombinant form and its 3D structure and iron-binding site are discussed above.

Ticks

Ticks are the second most important disease vectors after mosquitoes. They consume an enormous quantity of blood relative to their body size, so it is assumed that ferritin is important to detoxify the excess iron. In fact, ticks have two ferritin genes, both

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with a ferroxidase centre: *FER1* for cytosolic ferritin and *FER2* for secretory ferritin. *FER2* was also found to be a good antigen for an anti-tick vaccine [76]. The two ferritins were differently expressed according to the organs and developmental stage analysed, e.g. only *FER2* was detected in ovaries and eggs. Experimental down-regulation of *FER2* with RNAi diminished post-blood meal body weight (leading to high mortality and decreased fecundity), and the presence of abnormalities in digestive cells. Together these results indicate that the iron storage and protective functions of ferritin are crucial for successful blood feeding and reproduction of hard ticks [77]. Moreover, it has been shown that ferritins are essential antioxidant molecules to protect hard ticks from iron-mediated oxidative stress during blood feeding [78].

Insects

Insect ferritin is mainly secretory, and the crystallographic studies described above showed it to be composed of H- and L-subunit types in a 1:1 ratio. There is little information on the mechanism of assembly of these two subunits and of the *in vitro* functional properties of these ferritins, because they have not yet been produced in abundant recombinant forms. Most studies on these ferritins have been carried out in *Drosophila melanogaster*. Single-particle transmission electron microscopy confirmed that its ferritin has a 3D structure very similar to that of the ferritin of *T. ni*, and that it also contains small amounts of zinc and manganese. Ferritin iron loading varied in the different species, and the level of bioavailable iron depended on the levels of ferritin expression [79]. Specific ablation of ferritin in the *Drosophila* midgut resulted in a local iron accumulation, accompanied by systemic iron deficiency and reduced survival. In addition, the specific inactivation of ferritin in many non-intestinal tissues caused local iron accumulation with severe tissue damage and cell loss, showing an essential role for the secretory ferritins in dietary iron absorption and tissue iron detoxification [80]. Ferritins have also been studied in malaria mosquitoes, the females of which, similar to ticks, live on iron-rich blood meals. They have secretory ferritins composed of H- and L-chains, both regulated by iron levels; in conditions of its excess, iron associated with ferritin is secreted by the cells [81].

Shellfish

The interest for ferritins in shellfish was stimulated by reports describing their involvement in the innate defence against viruses and pathogens infecting cultivated species [82]. The oyster *Crassotrea gigas* has four distinct ferritin genes, two for cytosolic and two for secretory ferritins [83], which had distinct expression patterns in the tissues and during developmental stages, indicating functional differences [84]. Of interest, one of its secretory ferritins is unusually long and shows a similarity with the ferritin gene of the snail *Lymnea stagnalis*, identified long ago [85]. Four ferritin genes are also in another oyster type, in which one of the secretory ferritins was induced by iron and bacterial infection, and showed antibacterial activity [86,87]. It has also been shown that some shrimps and shellfish express a single ferritin type, similar to H-ferritin, which is up-regulated by immuno challenges [87–89] and infection with white spot syndrome virus (WSSV), one of the most devastating viral pathogens in shrimp farming [90,91]. Ferritin down-regulation with RNAi increased virus replication and shrimp mortality after infection, whereas injection with a recombinant ferritin reduced virus replication and shrimp mortality [92].

Worms

The worm *C. elegans* is an interesting model for iron homoeostasis, knowing in particular that its ferritins are regulated by hypoxia and insulin signalling, although with mechanisms that differ from mammalian ones. *C. elegans* has two ferritin genes for the cytosolic H type (*ftn-1* and *ftn-2*). They are transcriptionally regulated via an iron-dependent enhancer (IDE) located in their promoters, with a mechanism that has recently been reviewed [93]. Basal expression of *ftn-1* and *ftn-2* is mediated by the intestinal GATA transcription factor ELT-2 which binds GATA sites located in ferritin IDEs. Moreover, the hypoxia-inducible factor-1 (HIF-1) represses the expression of these genes by binding inside the IDE during iron deficiency [94]. Upstream of the HIF-1-binding element in the ferritin promoter there is another regulatory element that is recognized by helix–loop–helix 29 (HLH-29), a transcription factor involved in the regulation of growth and lifespan [95]. In addition, *ftn-1* is regulated by DAF-16, a transcription factor activated by nutrient deprivation that modulates genes for stress resistance, metabolism and immunity [96]. It was also shown that the expression of *ftn-2* is necessary for the full protective response of *C. elegans* against bacterial pathogens, both Gram-negative and Gram-positive [82]. This indicates that ferritin's role in the innate immune response originated early.

Ferritin in vertebrates

Fish

Ferritin in fish has been analysed in a few species, most of which expressed one or two ferritin genes, both with ferroxidase activity corresponding to the M- and H-type. The two subunits of cold-adapted Antarctic fish formed heteropolymers in the liver but not in the spleen, which contained only M-homopolymers [55]. Ferritin was highly expressed in the liver of all of the fish analysed, which protects against oxidative stress and microbial infection [97]. The recombinant ferritin from a sole fish inhibited the growth of six different species of fish pathogens; however, this effect was completely abolished when the ferroxidase site of ferritin was inactivated by site-directed mutagenesis [98]. The cold water in which icefish live is sufficiently rich in oxygen that they do not need haemoglobin in their blood for respiration; nevertheless these fishes express H- and M-ferritins in most tissues [99]. The low iron trafficking in these fish suggests that ferritins have a minor role in iron storage, although they may be important in immunity.

Birds

Chickens were one of the first organisms in which a ferritin gene was identified and cloned, after the human and tadpole ones [100]. It is of the H-type with the same structure as human ferritins and an IRE in the mRNA. Chicken liver ferritin was found to purify together with coated vesicles [101]. Chicken erythrocyte ferritin was made up of only an H-chain, contained iron, and presented the same properties as a previously identified microtubule-associated protein named syncolin [102]. Moreover, ferritin was found in the nuclei of mature erythrocytes [102]. More attention to chicken nuclear ferritin has been paid by the laboratory of Linsenmayer, who first identified ferritin in the nuclei of chicken corneal epithelial cells [103], and found it to have a protective role against oxidative damage to DNA [104]. The translocation to the nucleus was found to be mediated by a ferritin-like protein that was named ferritoid [105]. This protein of

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273 residues contained a functional nuclear localization sequence and was regulated by development and iron concentration, similar to ferritin. However, it was expressed only in the cornea and not in the liver (erythrocytes were not analysed). Ferritoid bound to ferritin H-chain to form a complex that was good at entering the nucleus and binding DNA [106]. It is interesting that this activity was shown to be dependent on the phosphorylation of serine residues of the C-terminal part [107]. This gene is present in most bird genomes and is annotated as ferritin light-chain. Despite the low sequence identity with the human L-chain, it maintains ferritin-like properties, such as the predicted folding in a four-helix bundle. However, it presents extensions in the N-terminus (~20 amino acids) and C-terminus (~70 amino acids), and some residue substitutions of the ferroxidase activity centre, which is probably inactive.

It remains to be established whether this ferritin-like subunit is able to form functional heteropolymers with H-subunits, because the structure of the complexes has not yet been detected. In most organs, except the cornea (and perhaps the erythrocytes), chicken ferritin seems to be composed only of an H-chain, an observation confirmed by the purification of liver ferritin [108]. Probably more interesting is the involvement of ferritin in the magneto-sensitive properties of birds. The magnetic sensors have been localized in the avian hair cells, and recently it was shown that these cells contain an iron-rich organelle, which consists of ordered aggregates of ferritin [109]. To verify this hypothesis, the low-field paramagnetic susceptibility of ferritin was studied. The results suggest that ferritin corpuscles in avian ears may function as intracellular magnetic oscillators which might generate cellular electric potential to be sensed by the animal [110].

Ferritin in mammals

Mammals have four differentially regulated ferritin genes for cytosolic H- and L-chain, mitochondrial ferritin and *FTHL17*. The ubiquitously expressed cytosolic H- and L-ferritins are regulated at a post-transcriptional level by the IRE/IRP machinery. A few groups have also studied the transcriptional regulation of FTH and FTL in humans and mice and found that FTH is induced by inflammatory cytokines activating NF-κB with the binding site located some 5 kb upstream of the *FTH* gene [111]. The protein p53 down-regulates FTH expression after association with nuclear factor Y (NF-Y) and its recruitment on an FTH promoter [112]. FTH expression is induced by histone deacetylase (HDAC) inhibitors through a transcriptional mechanism that involves Sp1- and NF-Y-binding sites located near the transcriptional start site of the FTH promoter. It is interesting that HDAC inhibitors were found to regulate ferritin by increasing NF-Y binding to the FTH promoter without changes in histone acetylation, with a novel mechanism of action of HDAC inhibitors [113], similar to that of p53. More recently, it was shown that FTH expression is regulated by an miRNA [114] and that FTH expression acts on the expression of some miRNAs and a variety of genes in K562 cells [115]. Less attention has been given to the *FTL* gene, although it was found that its promoter contains a Maf-recognition element (MARE) and an antioxidant-responsive element (ARE) that responded strongly to oxidative stress and haemin, a finding that may explain why serum ferritin, composed of L-chain, is up-regulated by inflammatory conditions [116].

Mitochondrial ferritin lacks IREs and is expressed only in a few cell types with high metabolic activity. It was found primarily in the testis, heart, kidney and brain [117]. Study of its regulation is problematic, because it is undetectable in cultured cells. The 5′-end of the gene is within a GpG island that is strongly methylated in all five cell lines analysed that do not express it, although the GpG island is hypomethylated in germ cells that express it. Treatments with demethylating agents, such as 5-aza-2′-deoxycytidine, produced some induction of mitochondrial ferritin (FtMt) [118]. Lastly, *FTHL17* lacks IREs and is transiently expressed in spermatogonia and germ cells but its regulation has not been analysed yet [119].

Ferritin receptors

It has been known for a long time that FTH can be taken up by cells, be incorporated [120] and, in some cases, affect cell proliferation [121], but the identity of the ferritin receptors remained elusive until 2005 when, during the characterization of the expression of the T-cell immunoglobulin domain and mucin domain (TIM) proteins, it was shown that mouse TIM-2 is expressed on various cells and binds FTH, but not FTL. Thus, TIM-2 is the mouse FTH receptor, involved in FTH cellular uptake and delivery to endosomes for lysosomal degradation [122]. However, the TIM-2 homologue has not been found in humans. The human FTH receptor was found to be transferrin receptor-1 (TfR1) using expression cloning [123]. On binding to TfR1, FTH is delivered to endosomes and lysosomes for degradation and iron recycling. The dual function of TfR1 in binding the two major iron proteins, i.e. ferritin and transferrin, is intriguing. On the other hand, the identification of an FTL receptor needed a more sophisticated approach, based on the observation that *TfR1* gene deletion in mice is embryonically lethal but does not inhibit organogenesis, suggesting other mechanisms for internalizing iron. They were characterized by producing chimaeric mice with fluorescently tagged *TfR1*-null cells and untagged wild-type cells. The observations revealed that some kidney cells were capable of internalizing ferritin through the expression of a novel receptor, named Scara5, which is able to bind and take up FTL [123]. This receptor was found to be expressed in mouse and human retinas and it could transfer, via retinal blood, the FTL injected intravenously into mice, suggesting that it might be implicated in retinopathy and be a possible therapeutic target [124].

Ferritin functions

Ferritins undoubtedly have a rigid structure that contrasts with the need for flexibility for their functions [9]. Their major and fundamental function is to oxidise and incorporate iron and keep it in a non-toxic form. This simple task has a large number of implications, the most important being to inhibit oxidative damage. Important examples come from studies on cardiac protection. The oxidative damage caused by heart ischaemia/reperfusion is protected by ischaemic pre-conditioning procedures, which involve the induction of ferritin by a transient ‘iron signal’. It is interesting that pre-treatments of rat heart with proteasomal and lysosomal protease inhibitors, which reduce ferritin breakdown and iron recycling, also suppressed the ‘iron signal’ [125]. Further insight into the role of FTH in cardiac protection came from the observation that diabetic hearts respond poorly to ischaemic pre-conditioning. Part of this effect was attributed to the high basal level of ferritin in diabetic hearts, and to the rapid and extensive loss of ferritin levels during prolonged ischaemia in diabetic hearts [126]. Other studies confirmed that ferritin has a cardioprotective role, e.g. the protective effect of the drug metformin on adult mouse cardiomyocytes (HL-1 cell line) against doxorubicin toxicity was attributed to the capacity of the drug to induce ferritin expression, and the effect was reduced by experimental FTH down-regulation with

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siRNAs or by NF-κB inhibitors [127]. Doxorubicin toxicity in HL-1 cells was associated with increased free iron pools, inhibition of mitochondrial complex I activity and loss of mitochondrial membrane potential, the ensuing cytochrome *c* release and the activation of apoptotic signals. The induction of FTH by metformin prevented these events [128], confirming the previously shown protective role of FTH in apoptosis [129]. Also, the tumour necrosis factor (TNF) protection against serum-starvation-mediated apoptosis of hepatocellular carcinoma cells involves the activation of the NF-κB signalling pathway and consequently the reactive oxygen species (ROS) suppression by FTH [130].

A novel role for circulating ferritin on angiogenesis has been proposed by Torti's group [131]. They showed that ferritin binds with a high affinity (K_d = 13 nM) to cleaved high-molecular-mass kininogen (HKa), which is an endogenous inhibitor of angiogenesis, and that ferritin antagonized the anti-angiogenic effects of HKa, enhancing the migration, assembly and survival of HKa-treated endothelial cells. *In vivo*, ferritin opposed HKa's anti-angiogenic effects in a human prostate cancer xenograft, restoring tumour-dependent vessel growth. Ferritin bound a subdomain of HKa that is critical for its anti-angiogenic activity [131]. Ferritin, both FTH and FTL, reduced binding of HKa to endothelial cells, restored the association of the urokinase-type plasminogen activator receptor (uPAR) with $\alpha5\beta1$ integrin, promoted adhesion and survival of the cells, and restored adhesion signalling pathways mediated by extracellular-signal-regulated kinase (ERK), Akt, focal adhesion kinase (FAK) and paxillin [132]. The interaction of ferritin with high-molecular-mass kininogen domain 5 (HK5) was found to involve a histidine/glycine/lysine-rich region within HK5, which is an intrinsically unstructured protein, and the interaction with ferritin was mediated by metal ions such as Co(II), Cd(II) and Fe(II), independent of the iron core of ferritin [133].

A role for FTL has been found in the regulation of γ-secretase activity, which is involved in the production of amyloid β peptide (Aβ) in the brain. FTL was found to physically interact with PEN-2, a component of the γ-secretase complex. FTL overexpression increased the protein levels of PEN-2 and promoted γ-secretase activity, which leads to an enhanced production of Aβ. The opposite was observed when FTL was down-regulated. The finding that iron supplementation increased γ-secretase activity via FTL induction poses a novel link between iron and Aβ generation in Alzheimer's disease [134].

Table 2 provides a summary of the proposed functions of vertebrate ferritins, and Figure 2 shows the alignment of representative cytosolic and non-cytosolic ferritins.

ANIMAL MODELS FOR THE STUDY OF FERRITIN

Mouse models of ferritin H-chain

FTH is essential for embryogenesis and its inactivation is embryonically lethal in mice [135]. However, the heterozygous $FTH^{+/-}$ mice are healthy with elevated L-ferritin levels, particularly in serum [136]. Detailed studies in the brain of these mice showed that H-ferritin deficiency was accompanied by signs of oxidative stress and alterations of iron transport proteins similar to those found in Parkinson's disease [137], and also by an imbalance of the levels of neurotransmitters such as glutamate and γ-aminobutyric acid (GABA) in different areas of the brain [138]. The protective role of H-ferritin in the brain was confirmed by the evidence that FTH down-regulation with specific siRNAs made mouse models of human gliomas more sensitive to chemotherapy [139]. No disabling mutation of the gene has been observed so far [140], thus confirming its importance. The generation of a mouse strain with a floxed *FTH* gene allowed the production of mice with the conditional inactivation of *FTH* at different stages of differentiation and in different organs.

FTH-flox/Mx-Cre mice

The first ferritin deletion was obtained by crossing FTH-flox with Mx-Cre mice, which resulted in a strong reduction of ferritin in the liver, spleen and bone marrow of adult animals. These mice lost their cellular iron stores but did not show any visible disadvantage and survived up to 2 years. However, when mice were fed with an iron-rich diet they had severe liver damage. Similarly the embryonic fibroblasts from these mice died soon after iron supplementation, presenting a major increase in cytoplasmic free iron, ROS and mitochondrial depolarization. That ferritin H-chain plays a major role in preventing iron-mediated cell and tissue damage was also demonstrated in the context of infectious diseases, such as severe forms of malaria. High FTH expression reduced the susceptibility to *Plasmodium* infection and tissue damage, whereas low FTH levels resulted in iron cytotoxicity, programmed cell death and major disease severity, as observed in humans and mice [141]. The capacity of FTH to dictate the outcome of malaria infection and provide a metabolic adaptation to tissue iron overload relies on preventing the unregulated generation of ROS and inhibiting oxidative stress-mediated sustained activation of c-Jun N-terminal kinase (JNK), which leads to programmed cell death [142]. This cytoprotective mechanism might be observed in other types of infections and/or pathological conditions, because tissue iron overload characterizes a variety of disorders [143].

The FTH-flox/Mx-Cre mice revealed that FTH deficiency in bone marrow reduced the number of mature B-cells and peripheral T-cells in all lymphoid organs, and increased cellular free iron, ROS and mitochondrial depolarization. This also occurred after B-cell-specific FTH deletion, which caused a reduction in mature B-cells and an increase in bone marrow B-cell proliferation. Also, T-cell-specific FTH deletion caused T-cell loss, showing that FTH is required for B- and T-cell survival by reducing the labile iron pool, because it was suggested that natural B- and T-cell maturation was influenced by intracellular iron levels and possibly deregulated in iron excess or deprivation [144].

FTH-flox/villin-Cre mice

Next, FTH-flox/villin-Cre mice were generated to delete intestinal ferritin-H. These mice showed increased body iron stores and transferrin saturation and a 2-fold increase in intestinal iron absorption, despite up-regulated liver hepcidin. The data indicated that duodenal ferritin is involved in the so-called 'mucosal block', i.e. the capacity to limit and regulate iron efflux from intestinal cells [145]. It is interesting that a similar phenotype with suppression of intestinal FTH was observed with the conditional deletion of the *Mbd5* gene, which encodes a member of the methyl-CpG-binding domain family and is involved in the regulation of FTH expression. Histone H4 acetylation of the FTH promoter was reduced in the intestine of these mice, suggesting a role for histone acetyltransferase in *Mbd5*-induced FTH transcription [146].

FTH-flox/$PT^{-/-}$ mice

Then followed the generation of mice with a conditional deletion of FTH in the renal proximal tubule ($FtH^{PT-/-}$), which

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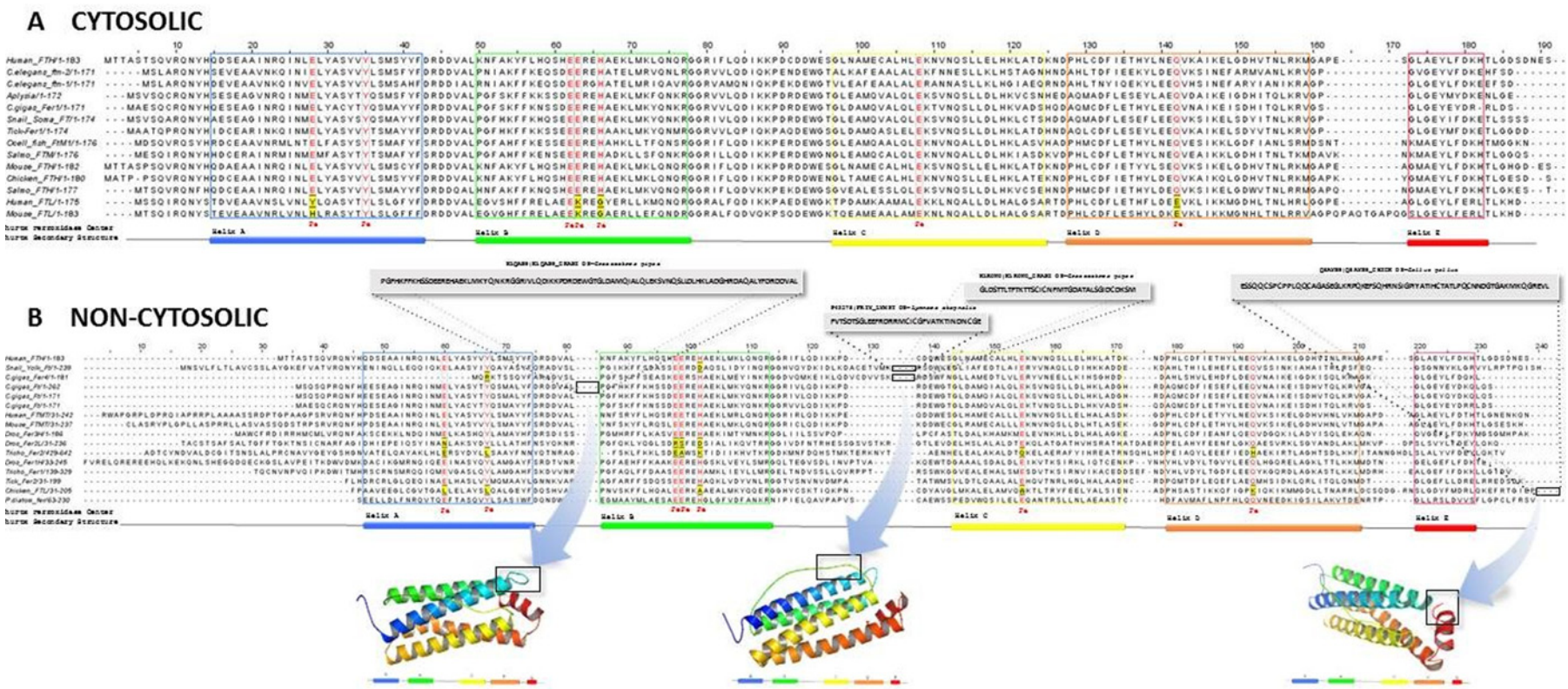

Figure 2 Amino acid sequence alignment of representative eukaryotic ferritins

The α helices (A–E) and ferroxidase iron-binding residues are shown below the sequences. The α helices are indicated in different colours and their boundaries are boxed. Amino acids highlighted in red are the conserved residues of the ferroxidase centre and those on a yellow background are the substituted ones. (**A**) Alignment of cytosolic ferritins, which shows the high conservation of the subunit sizes, minor length differences at the N- and C-termini and only one evident insertion in the D–E loop typical of the mouse ferritins. The residues of the ferroxidase centre are conserved in all but the human and mouse FTL. The listed ferritin species, type and UniProt entry numbers from the top are: *Homo sapiens* FTH (P02794), *Caenorhabditis elegans ftn-1* (Q9TYS3), *C. elegans ftn-2* (O16453), *Aplysia californica* ferritin (A5GZU9), *Crassostrea gigas* Fer1 (Q70MM3), *Lymnaea stagnalis* soma ferritin (P42577), *Haemaphysalis longicornis* Fer1 (Q6WNX1), *Chionodraco rastrospinosus* FtM1 (R4ZCU0), *Salmo salar* ferritin M-chain (P49947), *Mus musculus* FTH (P09528), *Gallus gallus* FTH (P08267), *S. salar* FTH (P49946), *H. sapiens* FTL (P02792), *M. musculus* FTL (P29391). (**B**) Alignment of representative non-cytosolic ferritins showing the predicted processed and mature sequences. Human FTH (cytosolic) at the top is used as a reference. It points out that the sequences forming the α-helices are not interrupted, whereas insertions are located in the turns/loops between the helices and at the protein termini. The most remarkable insertions are represented above and below the sequences on a grey background. One of the two secretory ferritins from the shellfish *C. gigas* of 262 amino acids has an insertion 91 residues long between the A and B helices, which is expected to form a structure exposed on the outer surface. The other secretory ferritin from *C. gigas* and the yolk ferritin from the snail *L. stagnalis* have an insertion in the loop between helices B and C of 36 and 34 residues, respectively, which are expected to form structures that protrude outside the protein. An unusually long C-terminal extension of 68 residues is found in the chicken L-chain, also known as ferritoid, which is expected to protrude inside the cavity, perhaps interfering with ferritin iron incorporation. The listed ferritin species, type and UniProt entry numbers from the top are: *H. sapiens* FTH (P02794), *L. stagnalis* yolk ferritin (P42578), *C. gigas* Fer4 (K1R0W0), *C. gigas* ferritin (K1QAG9), *C. gigas* ferritin (K1QHW8), *C. gigas* ferritin (Q70MM3), *H. sapiens* FTMT (Q8N4E7), *M. musculus* FTMT (Q9D5H4), *Drosophila melanogaster* sp. Fer3H (Q9VYH1), *Drosophila melanogaster* Fer2L (A0A0B4KHF0), *Trichoplusia ni* Fer2 (Q52SA8), *D. melanogaster* Fer1H (A0A0B4KI27), *T. ni* Fer1 (Q52SA9), *H. longicornis* Fer2 (M5AYG7), *G. gallus* FTL (Q8AYG9) *Pseudo-nitzschia multiseries* fer (B6DMH6).

Table 2 Functions attributed to representative ferritins

FTM, ferritin M-chain; Cyt, cytosolic; Mit, mitochondrial; Nuc, nuclear; Sec, secretory; H, ferritin of the H-type with ferroxidase centre; L, ferritin of the L-type without functional ferroxidase centre.

Species	Name	Type	Function	Reference(s)
Tick (*Haemaphysalis longicornis)*	Fer2	Sec-H	Vaccine antigen	[76]
Tick	Fer2, fer2	Sec-H and Cyt-H	Protective for feeding and antioxidant	[78]
Drosophila sp.	Fer1HCH, Fer2LCH	Sec-L and Sec-H	Essential for dietary iron absorption and detoxification	[80]
C. elegans	Ftn-2	Cyt-H	Necessary for the full protective response against bacterial pathogen	[82]
Shrimp (*Litopenaeus vannamei)*	Ferritin	Cyt-H	Protection against WSSV infection	[92]
Fish: (*Scophthalmus maximus* and *Cynoglossus semilaevis*)	FTM and FTH	Cyt-H	Protection against microbial infection	[97,98]
Chicken corneal epithelial cells	FTH	Nuc-H	Protection of DNA against oxidative damage	[181]
Birds	FTH	Cyt-H	Putative magnetic sensor	[110]
Human	FTH	Cyt-H	Cardiac protection	[125]
Human/Mouse	FTH and FTL	Sec-H / Sec-L	Angiogenesis	[132]
Human	FTL	Cyt-L	Regulation of γ-secretase activity	[134]
Mouse	FTH	Cyt-H	In intestine, regulation of iron absorption	[182]
Mouse	FTH	Cyt-H	Protection in acute kidney injury	[147]
Mouse	FTH	Cyt-H	Required for T- and B-cells	[144]
Mouse	FTH	Cyt-H	Confers tolerance to malaria	[142]
Mouse	FTH	Cyt-H	Implicated in the development of leukaemia/lymphoma,	[160]
Mouse	FTL	Cyt-L	Not essential: KO animals have a minor phenotype	[166]
Mouse	FTMT	Mit-H	Protection against cardiac toxicity	[172]

showed significant mortality, worse structural and functional renal injury, and increased levels of apoptosis in rhabdomyolysis and cisplatin-induced acute kidney injury. The mice also had increased urinary levels of the iron acceptor proteins neutrophil gelatinase-associated lipocalin, haemopexin and transferrin. The data showed that FTH has a protective role in acute kidney injury and a critical role in proximal tubule iron trafficking [147]. Moreover, after injury, these mice exhibited a marked increase in pro-inflammatory macrophages, with an abnormally high level of inflammatory chemokines and fibrosis, allowing the conclusion that FTH has a critical role during kidney injury in mediating the cross-talk between tubular macrophages and epithelial cells [148]. Analyses of differentiated podocytes (the epithelial cells covering the outer surface of the glomerular tuft in the kidney) showed that they express high levels of FTH, which contributes to their elevated resistance to oxidative damage [149].

FTH-flox/Emx1-Cre mice

Mice with a forebrain-specific inactivation of the *FTH* gene were produced to study the role of FTH in the brain. It is interesting that these mice did not show modifications of brain iron content, but after 2 weeks they showed an accumulation of cerebrospinal fluid in the lateral ventricles and subarachnoid space, the origin of which remained unclear [150]. In fact, there is substantial evidence for a major role for ferritin in the brain, which is summarized in various reviews [4,151–153].

FTH-transgenic mice

For better investigation of the role of FTH in different organs, various laboratories developed transgenic mice (tg-mice), e.g. the salutary effect of FTH in the brain was demonstrated by generating a tg-mouse with FTH under the control of a tyrosine hydroxylase promoter specific for dopaminergic neurons. These animals displayed lower iron accumulation and oxidative-stress-mediated neuronal death, and were protected against the development of Parkinson's disease [154]. Although brain iron overload may be favoured by the capacity of immune cells to buffer iron [155] and infiltrate the brain [156], preliminary experiments suggest that the levels of FTH in both compartments are crucial to counteract iron cytotoxicity, because higher or lower expression in one may dictate the proper functioning of the other (Raffaella Gozzelino et al., unpublished work). In another transgenic model, the Tet-OFF system was used to induce a 6–10-fold increase in FTH expression in muscle and kidney, which caused a local severe iron depletion [157]. A recent study reports that two lines of FTH-tg-mice expressing human FTH in almost all brain cells, including neurons, some glial cells and ependymal cells, were viable with normal blood indices of iron status [158]. However, the body size of these animals was reduced when compared with controls and presented, at 3–5 weeks of age, a temporary loss of coat hair on the trunk, but not on the head or face. The temporary hairless phenotype was associated with epidermal hyperplasia with hyperkeratosis, dilated hair follicles, bent hair shafts and keratinous debris [158].

It is of interest that this transient hairless phenotype is similar to that of the Mask mice characterized by the deletion of a functional part of the *TMPSS6* gene, which causes hepcidin overexpression and severe systemic iron deficiency [159]. Another study showed that ubiquitous tg-FTH expression caused aggressive radiation-induced thymic lymphoma/leukaemia, with earlier onset after treatment. The proliferative activity of the tg-lymphoma cells was higher and associated with the differential expression of some leukaemia/lymphoma-related genes. Moreover, apoptosis was augmented in bone marrow, but not in the thymus of treated tg-mice [160]. This indicates that FTH may be implicated in the development of leukaemia/lymphoma, in agreement with its abnormal expression described in earlier studies.

Mouse models of ferritin L-chain

The role of L-ferritin is rather enigmatic, because it is not as ubiquitous as FTH, being present only in mammals, fish and molluscs. *In vitro* studies showed that it facilitates iron nucleation and therefore co-operates with the H-chain in improving ferritin's iron incorporation capability [161]. The L-chain-rich ferritins from the liver or spleen are typically more iron-loaded than the L-chain-poor ferritins of the heart or brain [9]. It was suggested that the presence of heteropolymers with two subunits confers an

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advantage, allowing modulation of the iron storage capacity of the total ferritin without modification of the ferroxidase activity. However, the experimental up-regulation or down-regulation of L-chains in cells did not alter cellular iron homoeostasis [162]. In fact individuals with mutations in the 5′-UTR of the L-chain transcript had serum and tissue L-ferritin levels 2–5-fold higher than normal, but no alteration in iron homoeostasis [163]. Similarly an individual with L-chain haploinsufficiency due to a disabling mutation of one allele showed hypoferritinaemia and decreased tissue L-ferritin, but no signs of altered iron metabolism [164]. More importantly, an individual homozygous for a disabling mutation of the L-chain has recently been described [165]. Despite the absence of L-ferritin, the individual did not show evident signs of iron deregulation, but some neurological problems possibly associated with the mutation [165].

An L-chain-knockout (KO) mouse has recently been described and it is of interest that this animal did not have alterations in serum transferrin, liver iron and other parameters of iron status [166], but showed fertility problems and possibly some movement disorders, points that should be studied in the future. In fact, neuroferritinopathy is a dominant genetic disorder associated with mutations in the fourth exon of the ferritin L-gene. The insertion of one or two nucleotides results in frameshifts, which cause dramatic alterations of the C-terminus of the protein involved in the formation of 4-fold interactions [153]. The mutated chains act in a dominant-negative way by altering ferritin permeability and reducing the capacity to incorporate and detoxify iron [59]. The iron excess forms iron deposits in the brain and triggers oxidative damage, which is the probable cause of neurodegeneration [167].

Mouse models of mitochondrial ferritin

Mitochondrial ferritin is located in a strategic position where the abundant iron needed for haem and iron–sulfur cluster biosynthesis provides a probable and easy contact with ROS produced by the mitochondrial respiratory chain. The iron availability may be controlled by the local presence of a functional ferritin which has an important protective role against toxic free radical formation. This notion is supported by several studies on transfected cultured human cells, showing that expression of FtMt reduced the damage caused by experimental oxidative stress and protected the mitochondria [43]. FtMt is highly expressed in the testis, heart and some neurons, all cell types with a high metabolic activity [117,168]. However, it was also found that the expression of FtMt in sideroblasts of sideroblastic anaemia patients preceded the mitochondrial iron accumulation typical of the disorder, suggesting that it may be the cause of, rather than the response to, local iron overload [169]. A protective role for FtMt in the brain has been shown in cultured primary neuronal cells [170]. More importantly, FtMt-KO mice did not exhibit any evident phenotype, being viable and fertile, and no significant defects were observed after treatment with agents stimulating sideroblast formation [171]. We have also generated a FtMt-KO mouse strain that confirmed the absence of an evident phenotype. Then we subjected mice to doxorubicin treatments, an anti-tumour drug inducing a well-characterized cardiotoxicity, and showed that FtMt-KO mice were more sensitive to the drug. These animals presented an enhanced mortality and altered heart morphology with fibril disorganization and severe mitochondrial damage, characterized by biochemical indices of oxidative stress and increased autophagy. Even untreated mice showed signs of mitochondrial damage [172], confirming the antioxidant role for FtMt *in vivo*, at least in organs in which it is highly expressed. Finally, as the highest expression of FtMt is in the testis, we are now exploring whether this may play a role in male fertility. Preliminary data indicate that the litter sizes of FtMt-KO males are significantly smaller compared with those of controls and FtMt-KO females. In fact the number of FtMt-KO spermatozoa is reduced (Federica Maccarinelli et al., unpublished work), also showing a probable protective role of FtMt in this cell type.

CONCLUDING REMARKS

Recently, the interest in ferritin has spread from mammals to different organisms in which it was generally found to have a crucial role in iron metabolism and protection. Although very important, ferritin does not appear to have a vital role for many organisms, in fact many of monocellular species can survive nicely without it. Of interest in this context is the finding that phytoplankton species with ferritin have an advantage over the ferritin-less ones under conditions of iron starvation. The exploration of ferritin in various eukaryotes opens some interesting questions, e.g. why is the presence of multiple ferritin chains so common in many species, including plankton, ticks, flies, worms and up to mammals? It should be mentioned that most of the protective functions attributed to ferritins are linked to the ferroxidase activity of the H-chain, which acts by removing Fe(II) and consuming hydrogen peroxide, the two major substrates of the poisonous Fenton reaction. In addition, the conservation of the ferroxidase-less L-chain is enigmatic, considering that animals without this protein can survive perfectly well. Only a few activities have been attributed to it and the recent evidence of neurological problems in the only individual with L-ferritin inactivation described so far and in KO mice point to a role in neurodegeneration that should be researched further.

Another problem is posed by secretory ferritins. They are present in insects and shellfish, and their characterization may contribute to an explanation of the role of mammalian serum ferritin (so useful for the diagnosis of anaemia). Studies on *Drosophila* sp. and mosquitoes showed that these secretory ferritins accumulate in the membranes and the finding in ticks that these are good antigens for vaccines confirms their localization on plasma membranes. However, there are few data demonstrating that these ferritins are secreted and can transport iron from one cell to another. In fact, storing iron outside the cell poses serious problems on its recycling, unless the secretory ferritins are trapped in endosomes and directed to lysosomes. Indeed data on flies indicate that part of secretory ferritins co-localizes with lysosome markers. Another interesting problem is how the secretory ferritins in the endoplasmic reticulum (ER) or Golgi apparatus have access to iron. Major iron enzymes in the ER are the lysine and proline hydroxylases necessary for collagen formation and stabilization. However, we are not aware of any studies on defining how they acquire iron.

Besides the canonical cytosolic H-chains, some eukaryotes have exoteric ferritin chains with long extensions at the N- or C-termini or insertions in the loops or turns (see Figure 2). Plant ferritins have a long N-terminal extension that participates in iron uptake and oxidation [173], and it is possible that similar roles can be attributed to the longer subunits in the invertebrates. It is of interest that the bird L-chain, named ferritoid by Linsenmayer and shown to facilitate ferritin nuclear localization, is also characterized by a long C-terminal extension. The L-type ferritins do not seem to have any particular function when alone, and there is no example of a natural L-chain homopolymer. Therefore, they are expected to co-assemble with H-chains and modulate their activity in some way.

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In conclusion, the interest in ferritin for iron storage has apparently been declining in recent times, whereas its function in protecting from oxidative damage has proved important in most, if not all, of the organisms tested, and its role in innate immunity has been assessed in invertebrates. This supports the data that show that ferritin is crucial to immunity, although also playing a more complex role in mammals [174]. The picture that is emerging is that the ferroxidase activity of H-ferritins is fundamental to controlling the reactivity of intracellular free iron, an interesting parallelism with hepcidin, which in vertebrates controls the availability of systemic iron.

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