Title: Ferritins in *Chordata*: potential evolutionary trajectory marked by discrete selective pressures.

Subtitle: History and reclassification of ferritins in chordates and geological events’ influence on their evolution and radiation

Keywords: ferritins, *Chordata,* evolution, phylogenetics, adaptation

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**Abstract**

Ferritins (FTs) are iron storage proteins that are involved in managing iron-oxygen balance. In our work, we present a hypothesis on the putative effect of geological changes that have affected the evolution and radiation of ferritin proteins. Based on sequence analysis and phylogeny reconstruction, we hypothesize that two significant factors have been involved in the evolution of ferritin proteins: fluctuations of atmospheric oxygen concentrations, altering redox potential, and changing availability of water rich in bioavailable ferric ions.

Fish, ancient amphibians, reptiles, and placental mammals developed the broadest repertoire of singular FTs, attributable embryonic growth in aquatic environments containing low oxygen levels and abundant forms of soluble iron. In contrast, oviparous land vertebrates, like reptiles and birds, that have developed in high oxygen levels and limited levels of environmental Fe2+ exhibit a lower diversity of singular FTs, but display a broad repertoire of subfamilies, particularly notable in early reptiles.

**Introduction**

Ferritins (FTs) are iron storage cage-forming oligomeric proteins that are involved in the transportation and managing iron-oxygen balance in all organisms. FTs belong to the Ferritin family (Pfam: PF00210). These proteins undergo oligomerization to form cages, able to store a large quantity of iron ions, up to 4500 atoms per cage, in bioavailable, nontoxic, and soluble Fe2O3(H2O)n ferric oxide forms [1,2]. TheFT cage is most often present as a 24 ferritin dimeric subunit form; albeit, in cardiac and skeletal muscles, a larger form that is composed of 36 subunits has been described [3,4,5]. FT cages may consist of ferritin units of either identical or mixed type.

Among all *Animalia*, *Vertebrata* display the broadest diversification of FT types. This phenomenon is most likely associated with whole genome duplication (WGD), occurring during the earliest period of evolutionary history, allowing for a rapid expansion of new taxons and species [6]. Traditionally, three types of FT have been described, based upon their relative molecular mass: H (heavy), M (medium), or L (light), a classification that is independent of other biological properties. FT tissue localizations have been described for only few *Mammalia* species and one *Amphibia* (*Rana catesbeiana*)[7]. For instance, in frogs, the FTM transcripts were detected in the liver, while transportive FTH and FTL were expressed in the blood cells [8]. Depending upon the tissue type, different types of the ferritin monomers will assemble into FT cages. In the tissues where iron is retained for long periods, such as spleen and liver, [9,10], the FTL type is predominant. In metabolically active tissues, such as heart [10], teeth [11] and brain (especially in the neurons and the oligodendrocytes), FTH is predominant [12,13]. The oligodendrocytes (OLG) are the most abundant glial cells in the central nervous system (CNS) responsible for the myelinization process, nerve bundles protection, and axon sheath development. They are also the source of the redox-active ferrous ions necessary for the brain's crucial metabolic processes [14]. Therefore, the OLG has the highest iron concentration among all CNS cells [15]. On the other hand, accumulation of the redox-active ions leads to the development of multiple pathologies, emphasizing the need for preserving the precise homeostasis provided by FT [16]. Destabilization of this delicate balance might lead to different neurological symptoms and diseases [17].

The production of FT occurs at the cytosolic ribosomes [18], and a portion of FT, synthesized on the ER–bounded polyribosomes, is secreted into the blood plasma and extracellular fluids [19,20]. A unique variant of the 'heavy' FTH type is present in the mitochondria [21]. The function of the heavy variant resembles that of the other FTs: iron sequestration (supply of Fe for the heme biosynthesis), with putative mitochondrial protection against the iron-dependent reactive oxygen species (ROS) production [22]. Iron-loaded ferritin is transported in mammalian blood in the form of a cage bound to apolipoprotein B through the heme moiety (ApoB–FT; [23]. Consequently, the FT ability to bind heme is evolutionarily conserved and appears to be crucial in iron transportation in the organism. FT are reabsorbed from blood by V-set Ig-type receptors and HAVCR (Hepatitis A virus cellular receptor) known also as TIM (T-cell immunoglobulin and mucin domains) [24,25], transferrin receptor-1 (TfR1) [26] or ferritoid, a FT-like protein [27].

Structural studies of the ferritin cages, formed by either FTL, or FTH-type-subunits, show that homo-FTH (PDB ID: 4OYN) displays a higher efficiency of iron binding and uptake compared to homo-FTL (PDB ID: 5LG8)[28,29]. Iron ions (II) are transported and loaded into the FT cage through inter-subunit pores formed by C‑terminal regions of oligomerizing FT subunits [30]. A conserved residue found on FL subunits, Leu134 [31], is required for proper pore functioning, a process associated with subunit unfolding upon iron uptake. In mixed FTH-FTL cages, the FTLs are responsible for the nucleation and mineralization of the iron core, a process that improves ferroxidase activity [32,33]. FTH scarry on ferroxidase activity, converting Fe2+ into partly crystalline or amorphic structures of Fe2+ and Fe3+ oxides, along with hydroxides mineral form of ferrihydrite, magnetite, hematite in the ferritin shell [34,35]. In human FTH, the ferroxidation centre involves Glu27, Glu61, Glu62, His65, Gln141 [32,36]. Corresponding residues in FTL are involved in the cage stabilization by forming salt bridges, what makes FTL-based structure more stable [37]. The FTH cage incorporates more iron than the FTL cage by its nucleation to ferrihydrite on its inner surface within the ferritin complex [38,39]. Iron oxidation facilitates further iron incorporation and therefore is essential for the regulation of cellular iron levels. Oxidationis also responsible for detoxification of the bioactive ferrous form into the relatively inert ferric form [40]. FTs act as redox-balance buffering proteins in a finely-tuned cyclic conversion Fe2+-Fe3+ by various ferroxidase centres, with relatively slow rates of oxidation [41]. Small, although targeted, differences in amino acid residues between FTs lead to profound changes in electrochemical and biochemical properties.

In addition to ferroxidation, FTH cages possess UV-protection ability [42,43], probably due to the accumulated iron oxides [44]. Stress-related expression of the FTH gene seems to be conserved among the mammals and birds; exposure to environmental stressors such as UV radiation redox stress [42,45,46], infrared radiation [47] or heat [48], are associated with elevated levels of FTH transcripts. Increased levels of FTH lead to the repression of HIF (hypoxia-induced factor), a process that regulates the oxygen-sensing pathway in the cells [49]. Some studies in fish indicate a putative role of ferritins as a protective factor and part of the immunity mechanism against bacterial infection, due to competition for iron between pathogen and host [50].

FT is also involved in the magnetic field sensing observed in the fish and birds. In rainbow trout (*Oncorhynchus mykiss*), FT genes are overexpressed upon changes in the magnetic field [51]. In birds, FTH cages are abundantly present in the magnetosomes and are involved in the magnetic field sensing [52,53]. Some authors postulate that ferritins could also be responsible for the magnetodetection and navigation in the mammalian brain [54].

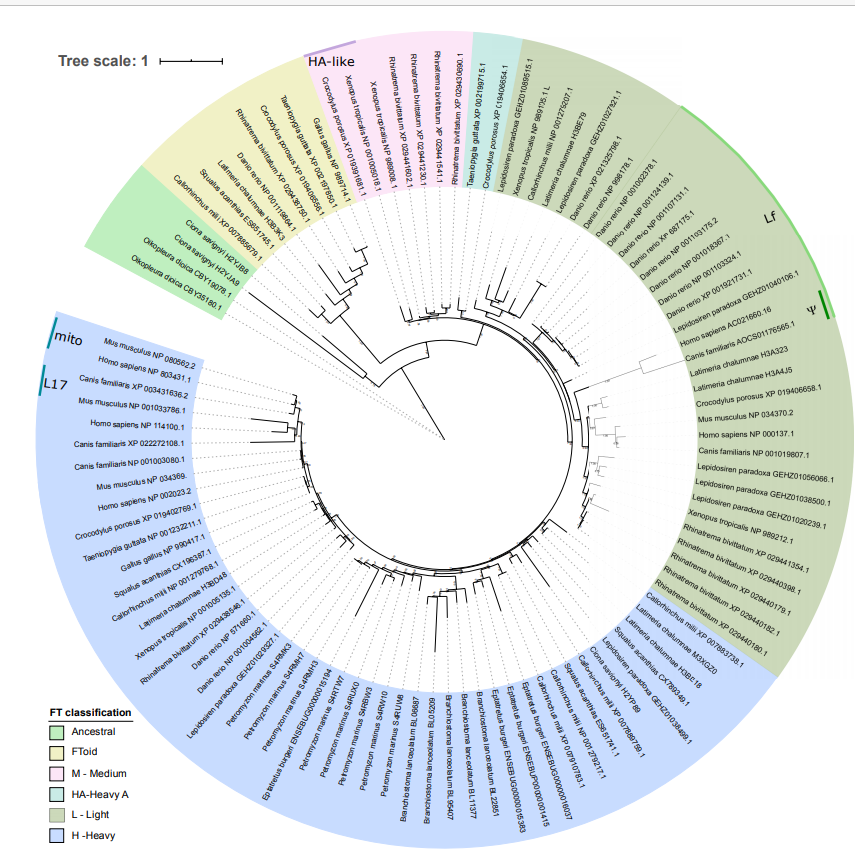
Previous studies of the ferritin sequences showed the conservation of residues forming the ferroxidase active site [1]. Authors speculated that multiple FTs originated from FTH, derived from an ancestral and ubiquitous rubrerythrin-like protein [2]. A mixed sequence-structure based approach supported the positioning of FTs among rubrerythrins [55]. These previous studies, albeit, did not seek to address the overall evolution of FT subfamilies.

Evolutionary relationships between vertebrate FTs and their non-vertebrate homologs were first presented by Lee et al. [56]. The authors hypothesized that the ancestral vertebrate FT originated from a single-chain type, similar to the one modelled for the extant basal chordates *(Tunicata, Myxyni*, *Branhiostoma*). For early vertebrates, they reported that duplication of the single FT chain occurred independently of invertebrates, resulting in lineage-specific duplications. Lee and co-workers [56] suggested that duplication might lead to the functional convergence. As an example, in both *Drosophila* and *Mammalia*, functional FTs are expressed in testicular mitochondria. In contrast, as amphibians need to adapt to the different aquatic and terrestrial environments during their life cycles, the presence of the divergent types of L/M FT chains reflects their iron demand. Hence, authors hypothesize that the differences between paralogous chains of teleosts FT and mammalian FTL evolved due to the adaptation of tetrapods to the terrestrial life, a process associated with significantly different requirements for iron uptake and storage.

In this paper, we classify vertebrate FTs into six subfamilies based upon their phylogeny, taxonomic distribution, and other traits such as ferroxidative ability. Our study clarifies the evolutionary relationships between vertebrate FTs. It allows us to speculate about the evolution of their functions in the context of vertebrate adaptations to different geological events, resulting in oxygen concentration changes in the atmosphere and iron availability during embryo development. The detailed information regarding phylogenetic analyses is provided in supporting information.

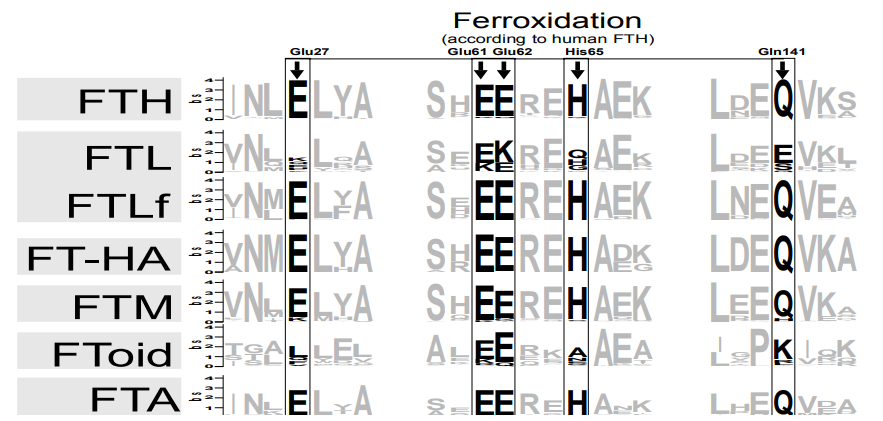
***Chordata* have biochemically diverse ferritin isoforms divisible for six FT subfamilies**

A phylogenetic analysis of the 101 sequences from the 18 model chordates shows that vertebrate FTs form at least six subfamilies, unequally distributed among taxa (Fig. 1, Supplementary File 4-5). These subfamilies roughly correspond to previously described types of ferritins. Using this analysis, we are able to distinguish ancestral forms of FT named: FTA (ancestral), FToid (ferritoid), FT-HA (heavy chain A), FTM (medium), FTL (light) and FTH (heavy). There are limited number of published data, regarding this super – clades and they are not recovered using other tree reconstruction methods, therefore the monophyly of FTL and FTH subfamilies seems uncertain (Supplementary File 2). Although its position on the tree varies depending on the method used, the FToid subfamily forms a well-distinguished clade in all tested approaches.



**Figure 1.** Maximum likelihood tree of 101 ferritins from model chordates. Subfamilies (legend) and specific variants (depicted around the perimeter). Representatives of *Tunicata* were selected as an outgroup and used to root the tree.

Among all animals, *Vertebrata* possesses the broadest spectrum of FT subfamilies and isoforms. As a highly organized and mobile group, this subphylum developed highly specialized iron transport systems as an adaptive response to environmental changes and diverse ecological niches. Due to evolutionary adaptations to various oxygen and iron content in the surrounding environment among Earth history (Fig. 3), the evolution of FTs is likely to have been substantially influenced by these conditions. We show that vertebrate FTs may be divided into the six subfamilies, and the vertebrates' FT were duplicated and derived from the ancient *Chordata*, like *Tunicata*, as suggested by Lee et al. [56], not from FTH, as it was thought until now in some earlier reports [1]. We also found that the ferroxidation active site is mostly conserved across FT representatives. Notable exceptions include FTL and FToid that lack conserved residues, corresponding to Glu27 and Gln141 in human FTL.



**Figure 2.** Alignment of six FT-subfamily sequence logo with an indication of amino acids potentially involved in iron ferroxidation (above alignment).

**Calm Precambrian oceans' life and origin of ancient chordates' ferritins**

*Tunicata* are considered to be 'living fossils', and represent the oldest chordate group, having evolved at the end of Precambrian, c.a. 541 My ago in anoxic oceans that were saturated with a high level of iron ions. The appearance of *Tunicata* coincides with the end of the Ediacarian-Cambrian hyperactive magnetic field changes (Fig. 3)[57]. During the Cambrian Explosion, atmospheric oxygen level increased to over 20%, albeit strong UV radiation through a weak ozone layer have led to generation of the reactive oxygen species (ROS), which might be a reason for the extinction of many early animals. Genomes of many tunicates possess two to three copies of FT genes, which encoded proteins form a separate clade, the ancestral FT (FTA), with putative ferroxidation ability. In the subsequent Cambrian epoch (530 Mya ago) the *Cephalochordata* appeared, with a different newly diverged FTHs instead of the ancestral ones, albeit still able to conduct ferroxidation and accumulate UV protective iron oxides. The presence of FTH in all *Vertebrata* and *Cephalochordata* analysed suggests, with the separation of these lineages, that the divergence of FTA into FTH occurred concomitantly.

**Cornea, jaws and nervous system evolution in *Vertebrata* were concurrent with diversification of modern ferritin subfamilies during Ordovician-Silurian Great Extinction.**

Similar to their Precambrian ancestors, early evolved vertebrate and jawed fish *Chondrichthyes*, *Coelacanthimorpha*, *Actinopterygii* and *Dipnoi* were subjected to significant environmental pressures during Ordovician-Silurian Great Extinction event. The depletion of Earth's magnetic field, low oxygen levels, high levels of UV irradiation, cooling of the climate, and increased release of high amounts of metal ions from the ocean's depth during tectonic events [58,59] were major selective pressures.

During this time, as an eye-protective structure called the cornea evolved in vertebrates. The subfamily of FT, forming a distant clade in all analyses, is FToid, localized in cornea cell nucleus, participates in UV and oxidative stress protection [60,45]. The first description of FToid was done on bird cornea [27]. FToid subfamily members have not retained the amino acid residues described as responsible for ferroxidative properties of FTH in human and horse. If FToid corneal localization is conserved among other vertebrates one might speculate its evolution was shaped by the eye protection against harmful ROS. It might suggest, that cornea, as an eye protective structure, occurred early in evolution of *Chordata*, in the ancestor of jawed fish. FToid subfamily members are present in most of vertebrate lineages.

Simultaneous occurrence of magnetic field disturbance could be a factor, revealing another useful property of FTs, a magnetoperception, ability to sense the magnetic field, observed in modern *Actinopterygii* [51] and birds [52,53]. Simultaneously to the jaw, which evolved from the first gill arch and primarily might be necessary for oxygen uptake support [61,62], external pharyngeal gills, nerve bundles, innervating them, and oligodentrocytes, evolved. Diversification of vertebrates co-occurred with the origin of several subfamilies of ferritins. FTL subfamily is represented in almost all vertebrates (excluding birds), gradually was losing its putative ferroxidative ability in favour to nucleate iron [32,33], with the exception of *Actinopterygii* and *Dipnoi* (hence named FTLf-ferroxidative, Tab.1), whereas FTH subfamily, which appeared already in *Cephalochordata*, is broadly distributed among all jawed vertebrates and parallel lineage *Cyclostomata*.

***Vertebrata* on land were equipped with a large arsenal of ferritins**

Devonian Earth was unfriendly for early *Amphibia*, due to harmful UV exposure [63], low level of waters in the ocean, and limited water availability on land. For these reasons most of their life was still carried out in aquatic environments. It is possible that, similarly to fish, early amphibians possessed numerous FT belonging to diverse subfamilies. Modern amphibians (*Lissamphibia*), which appeared during the drying end of the Permian, might gradually reduce their ferritin repertoire FTs, a fish-specific ferroxidative variant of FTL, leaving only four ones: FTL (transporting one), FTM (liver-storage one), FToid (cornea specific) and FTH (another transporting)[8]. Regarding the complexity of *Lissamphibia* development, their FTs displayed functional and tissue specialization. Currently, due to lack of experimental data, it is hard to estimate which FT subfamily is particularly dominant in every developmental stage and tissue under variable iron availability and oxygen conditions, albeit their ratio is changing during development from FTH in tadpole to FTL ones in adult form [64]. Among FTL and FTH present in *Lissamphibia*, is unknown which FT is responsible for long distance-iron transport, since known characterized members are focused in particular tissue or cellular compartment, like frog FTM, which is attributed to liver [8]. Basing on our phylogenetic analysis and due to lack of some extinct fish taxa, we may only surmise that FTM members appeared early in the ancestor of jawed fish.

**The reptilian epoch: Novel ferritins emerge**

Around 305 million years ago, the Carboniferous rainforest collapsed, and early reptiles evolved a calciferous eggshell and amnion structures as protective measures that improved embryonic survival. Imbalance of water management, high oxygen content in the atmosphere (~30%) and limited iron availability on dry land were selective pressure’s factors. These changes coincide with the evolution of new FT-HA subfamily of unknown function, and with limited taxonomic distribution, in reptiles and birds. FT-HA subfamily members retained amino acid residues required for ferroxidase activity (supplementary file 2). One might speculate that this subfamily was involved in managing iron oxides and intracellular high oxygen content. Sequenced reptiles’ genomes possess ferritins belonging to all modern subfamilies FTH, FTL, FTM, FT-HA, and FToid. The latter was lost in sequenced representatives of snakes, and FTM is present only in their few taxons of lizards among *Bifurcata* (supplementary file 2).

**Changes in the ferritin arsenal in the first mammals, and speciation of the FTH subfamily**

After the Great Extinction in the late Permian, *Mammalia* evolved with its broad spectrum of FTH variants. Early oviparous mammals, like monotremes (i.e., platypus and echidna), possess a reptilian set of FTs, which underwent a graduate reduction to single FTL and FTH isoforms in marsupials. Viviparous mammals completely lost the FTM subfamily. Mammalian FTL is unable to ferroxidize [32,33], albeit mammals maintained a pseudogene resembling fish-specific FTLf variant as a remnant of FTL evolution (note in Fig. 1, -pseudogene subclass). FTH subfamily members have experimentally validated ferroxidative ability [32,36].

Contrary to reptilian-like platypus or marsupials, the evolution of the mammalian placenta [65] was associated with a high degree of anatomical changes in the reproductive system and resulted in increased delivery of nutrients, such as iron and oxygen, to the fetus, a process that could require novel FTH variants. Examples of novel sequences, which appearance coincide with these changes include the FTHmito variant expressed in the brain [66,67], heart [68,69], and testicular mitochondria [70,68], involved in iron supply to sex hormone steroidogenesis [70]. Iron delivery by FT is abundantly demanded also by Sertoli and Leydig cells for the spermatogenesis and protection of the testicular cells [71,72]. A second, newly evolved placentalian FT-HL17 variant was reported in mouse spermatogonia and in early embryonic cell [73]. Because FT-HL17 displays reduced stability, it appears to primarily transport, rather than store, iron. The placenta constitutes a barrier for Fe delivered by typical FTH [74,75]; the bulk of FT is accumulated in syncytiotrophoblast subsequently taken up by the fetus, by the process of ferritinophagy [76].

**Jurassic extinction and modern bird radiation with reduced but specialized ferritin selection**

After Triassic-Jurassic Extinction, birds were subjected to significant evolutionary pressures. Analyses of sequenced avian genomes showed that they completely lost FTL, and retained FTH as the main (and only) FT isoform able to transport iron [77,78]. Similar to the mammalian form, FTH also plays a protective role against UV radiation [42]. In some taxons, *Neognathae*, for instance inperching birds (*Passeriformes*), penguins (*Sphenisciformes*), pelican order (*Pelecaniformes*), and in diurnal birds such as falcons and caracaras (*Falconiformes*), the ancient reptilian FT-HA isoform was retained. Both subfamilies FTH and FT-HA potentially ferroxidize iron. There is no data for FT distribution in avian embryo, although Richards [79] observed dynamic changes of FT-like fraction during development. Similar to fish, early amphibians and reptile ancestors, all birds possess a potentially cornea-specific nuclear FToid [27]. Birds also have magnetosomes in labyrinthine domains [52], which, at least in pigeon hair cells [53], are formed with FTH since this taxon possesses only the FTH subfamily.

**Further perspectives**

Considering the available experimental and sequence data, we hypothesize that FT diversity reflects the need for new ways to manage iron and redox balance, through gradual ferroxidation. Immunohistological studies of embryonic development in chordate representatives would clarify the distribution of particular FTs in tissues and organs. Furthermore, to test the redox-tuning hypothesis, biochemical and biophysical data of each FT subfamily, like iron capacity, ferroxidation activity under alter redox potential or oxygen levels, and differential pulse voltammetry, should supplement our limited knowledge of the functions of FTs.

**Conclusions**

We propose a reclassification and an updated nomenclature for the ferritins in chordates, summarized in Table 1.

**Table 1.** A simplified summary of FT subfamily distribution and their ferroxidative ability (+/-) among various chordate taxa with their timescale of evolution. FTA (ancestral), FToid (ferritoid), FTM (medium), FT-HA (heavy like), FTL (light) and FTH (heavy).

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|  | Mya | FTA | FToid | FTM | FT-HA | FTH | FTL | |  |
|  | F- | F+ |  |
| *Tunicata* (outgroup) | 514-636.1[80] | F+ |  |  |  |  |  |  |  |
| *Cephalochordata* | 534-566[81] |  |  |  |  | F+ |  |  |  |
| *Cyclostomata* | 358.5-636.1[80] |  |  |  |  | F+ |  |  |  |
| *Chondrichthyes* | 433.4-443.8[82] |  | F- |  |  | F+ | F- |  |  |
| *Coelacanthimorpha* | 407-419[83] |  | F- |  |  | F+ | F- |  |  |
| *Actinopterygii* | 383-425[84] |  | F- |  |  | F+ | F- | F+ |  |
| *Dipnoi* | 380-400[85] |  |  |  |  | F+ | F- | F+ |  |
| *Amphibia* | 358.9-372.2[86] |  | F- | F+ |  | F+ | F- |  |  |
| *Reptilia* | 310-320[87] |  | F- | F+ | F+ | F+ | F- |  |  |
| *Mammalia* | 164.9-201.5[80] |  |  |  |  | F+ | F- | F+ |  |
| *Aves* | 168.3-170.3[88] |  | F- |  | F+ | F+ |  |  |  |
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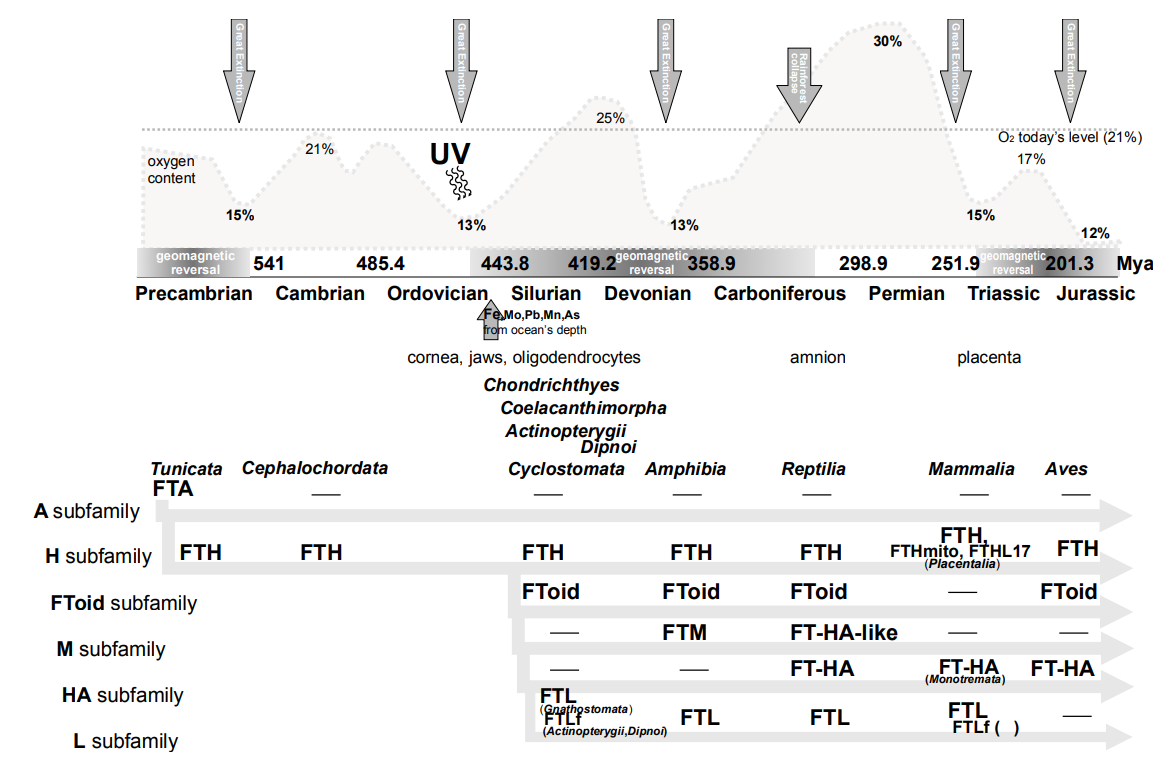
*Chordata* radiation was shaped by geological events, resulting in the great extinction due to exposure to UV, changes in the water availability, atmospheric oxygen concentration; these events triggered redox imbalance. To deal with these environmental challenges, chordates evolved protective measures, such as FTs variants.

The extended FTs repertoire might have been an answer for a need of anti-UVB protection and to cope with a decreased oxygen level in the Precambrian and the Great Ediacaran Extinction [89].

A second expansion of FTs likely happened in the ancestor of *Gnathostomata*, leading to the formation of FTL and FToid subfamilies. This process might be connected to the Ordovician-Silurian Great Extinction with its significant environmental pressures, like rapidly falling oxygen level [90], redox imbalance, the necessity for UVB protection, and a declining magnetic field. The appearance of the cornea coincides with FToid evolution. Simultaneously, jaws evolved and the nervous system complexity increased together with oligodendrocytes.

Expansion of ferritins in vertebrates correlates with two rounds (three for *Actinopterygii*) of whole genome duplication, ~500 Mya [91,92]. For non-vertebrates, WGD occurred only in limited taxons, later in their evolution [91].

We observed a correlation between amount of FT isoforms and oxygen level and water dissolved Fe2+/3+ availability, especially during early embryonic development of each vertebrate group. Regarding these conditions, *Amniota* evolved the broadest repertoire of FTs, probably for subtle redox tuning and improved iron assimilation. The appearance of the new FTHmito and FTHL17 variants in *Placentalia* coincides with the placenta creation and aquatic fetus development. Reptiles and birds lack easily accessible iron from water, and reproduce *via* oviparity in dry and oxygenated conditions. They might therefore present a reduced number of FT isoforms as a response to increased redox stress in favour of expanded amount of FT subfamilies for reptiles.



**Figure 3.** An outline of the chronologic timeline of geological events, vertebrate evolution, newly evolved anatomical structures and vertebrate FT subfamilies. Geological events are described in text. Oxygen level was overlaid according to published data [90].

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