Gene 885 (2023) 147720

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Copper-transporting ATPases throughout the animal evolution – From clinics to basal neuron-less animals

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ARTICLE INFO

Edited by David A. Ray

Keywords:
Copper-transporting ATPases
ATP7 genes, ATP7 proteins
Menkes disease
Wilson's disease
Animal models
Vertebrate
Invertebrate
Non-bilaterian

ABSTRACT

Copper-transporting ATPases are a group of heavy metal-transporting proteins and which can be found in all living organisms. In animals, they are generally referred to as ATP7 proteins and are involved in many different physiological processes including the maintaining of copper homeostasis and the supply of copper to cuproenzymes. A single *ATP7* gene is present in non-chordate animals while it is divided into *ATP7A* and *ATP7B* in chordates. In humans, dysfunction of ATP7 proteins can lead to severe genetic disorders, such as, Menkes disease and Wilson's disease, which are characterized by abnormal copper transport and accumulation, causing significant health complications. Therefore, there is a substantial amount of research on *ATP7* genes and ATP7 proteins in humans and mice to understand pathophysiological conditions and find potential therapeutic interventions. Copper-transporting ATPases have also been investigated in some non-mammalian vertebrates, protostomes, single-cellular eukaryotes, prokaryotes, and archaea to gain useful evolutionary insights. However, ATP7 function in many animals has been somewhat neglected, particularly in non-bilaterians. Previous reviews on this topic only broadly summarized the available information on the function and evolution of *ATP7* genes and ATP7 proteins and included only the classic vertebrate and invertebrate models. Given this, and the fact that a considerable amount of new information on this topic has been published in recent years, the present study was undertaken to provide an up-to-date, comprehensive summary of *ATP7s*/ATP7s and give new insights into their evolutionary relationships. Additionally, this work provides a framework for studying these genes and proteins in non-bilaterians. As early branching animals, they are important to understand the evolution of function of these proteins and their important role in copper homeostasis and neurotransmission.

1. Introduction

In 1993, the landmark discovery of copper-related disease genes *ATP7A* and *ATP7B*, encoding P(1B)-type copper-transporting ATPases (Arguello et al., 2007), demonstrated the existence of proteins responsible for copper transport and distribution (Bull et al., 1993; Chelly et al., 1993; Mercer et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993). Since then, many studies have investigated these genes and their protein products in humans and mice to understand pathophysiological conditions and find potential therapeutic interventions. Additionally, copper-transporting ATPases have been investigated in a few non-mammalian vertebrates, protostomes, single-cellular eukaryotes, prokaryotes, and archaea to gain useful evolutionary insights.

An earlier review elegantly summarized that copper-transporting

Abbreviations: *ATP7*/ATP7, animal copper-transporting ATPase; *ATP7A*/ATP7A, animal copper-transporting ATPase A; *ATP7B*/ATP7B, animal copper-transporting ATPase B; *SLC31A1*/CTR1, human high affinity copper uptake protein 1; *DmATP7*/DmATP7, *Drosophila* copper-transporting ATPase; *ATOX1*/ATOX1, animal copper chaperon protein; *CTR1A*, gene encoding *Drosophila* high affinity copper uptake protein 1A; *CTR1B*, gene encoding *Drosophila* high affinity copper uptake protein 1B; *CTR1C*, gene encoding *Drosophila* high affinity copper uptake protein 1C; PHM, peptidylglycine-α-hydroxylating mono-oxygenase protein; *CUA-1*/CUA-1, *Caenorhabditis* copper-transporting ATPase; CUC-1, *Caenorhabditis* copper chaperon protein; ATX1, yeast copper chaperon protein; *CHCA-1*, gene encoding *Caenorhabditis* high affinity copper uptake protein 1.

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https://doi.org/10.1016/j.gene.2023.147720
Received 17 May 2023; Received in revised form 1 August 2023; Accepted 16 August 2023
Available online 18 August 2023

ATPases were present in many different living organisms: from archaea and bacteria through fungi to humans (Gupta and Lutsenko, 2012). In prokaryotes, the original export function of these proteins, namely the maintenance of proper intracellular copper concentration, has remained the main role. However, new functions have also emerged and been retained throughout evolution. For example, the role in the pathogenesis of bacteria or the role in copper transfer through complex barriers (e.g., intestine, blood-brain barrier) (Gupta and Lutsenko, 2012). In eukaryotes, the function of copper-transporting ATPases in supplying copper to cuproenyzmes (e.g., ceruloplasmin, cytochrome *c* oxidase, superoxide dismutase, tyrosinase) has become another equally important. In parallel with the functional evolution, copper-ATPases have changed from non-trafficking plasma membrane proteins to trafficking ones (Lutsenko et al., 2007). Previous reviews broadly summarized the available information on the function and evolution of animal copper-transporting ATPases, generally referred to as ATP7 proteins, and included only the classic vertebrate and invertebrate models. Moreover, new information on *ATP7* genes and ATP7 proteins has been published in recent years. Keeping this in mind, the aim of the present study is to give a comprehensive summary of our current knowledge of *ATP7s*/ATP7s and to provide new insights into the evolutionary relationships of copper-transporting ATPases.

Our thorough searches in the available genome and/or transcriptome data of deuterostome, protostome, and non-bilaterian animals revealed candidate genes for *ATP7* homologues in many taxa that had not been previously investigated, such as placozoans, ctenophores, and poriferans. Using Hidden Markov Models, we identified sequences in all these four groups of pre-bilaterian metazoans. Then, using all-vs-all cluster-based methodologies (Fig. 1) and phylogenetic analyses (Fig. 2), we confirmed the homology of these ATP7 proteins from non-bilaterian animals. Detailed methodology of the analyses and the used sequences are included in **Supplementary File 1, 2, 3**. Our phylogenies support, for example, the idea that the *ATP7* gene was already present in the last common unicellular ancestor of animals and that *ATP7A* and *ATP7B* are products of a duplication that occurred in the common ancestors of the vertebrates. The structure of ATP7 proteins is well-known: a cytosolic N-terminal region with one to six metal-binding subdomains, 8 trans-membrane segments with important cytosolic functional domains, and a cytosolic C-terminal region (Arguello et al., 2007; Bitter et al., 2022; Gupta and Lutsenko, 2012). The alignment of the most important motifs/functional sites (e.g., DKTG, SEHPL, TGND) of copper-transporting ATPases in representative species from the Chordata, Echinodermata, Protostomia, Placozoa, Cnidaria, Ctenophore, Porifera, Tunicaraptor, and Choanoflagellata taxa revealed a high sequence conservation (Fig. 3). Detailed methodology of the analysis is presented in **Supplementary File 1**. Compared to previous studies, to the best of our knowledge, these are the most detailed analyses of ATP7 proteins to date.

In the next section, we review the available information on ATP7 genes and proteins in mammals, non-mammalian vertebrates, invertebrate deuterostomes, protostomes (arthropods, mollusks, nematodes), and non-bilaterians. To note, in most cases, *ATP7s*/ATP7s were investigated only in classic model animals in the given taxa (e.g., Arthropoda – *Drosophila melanogaster*, Nematoda – *Caenorhabditis elegans*). In addition to the conventional models, we also made an effort to collect information about ATP7 genes and proteins in non-classic model animals.

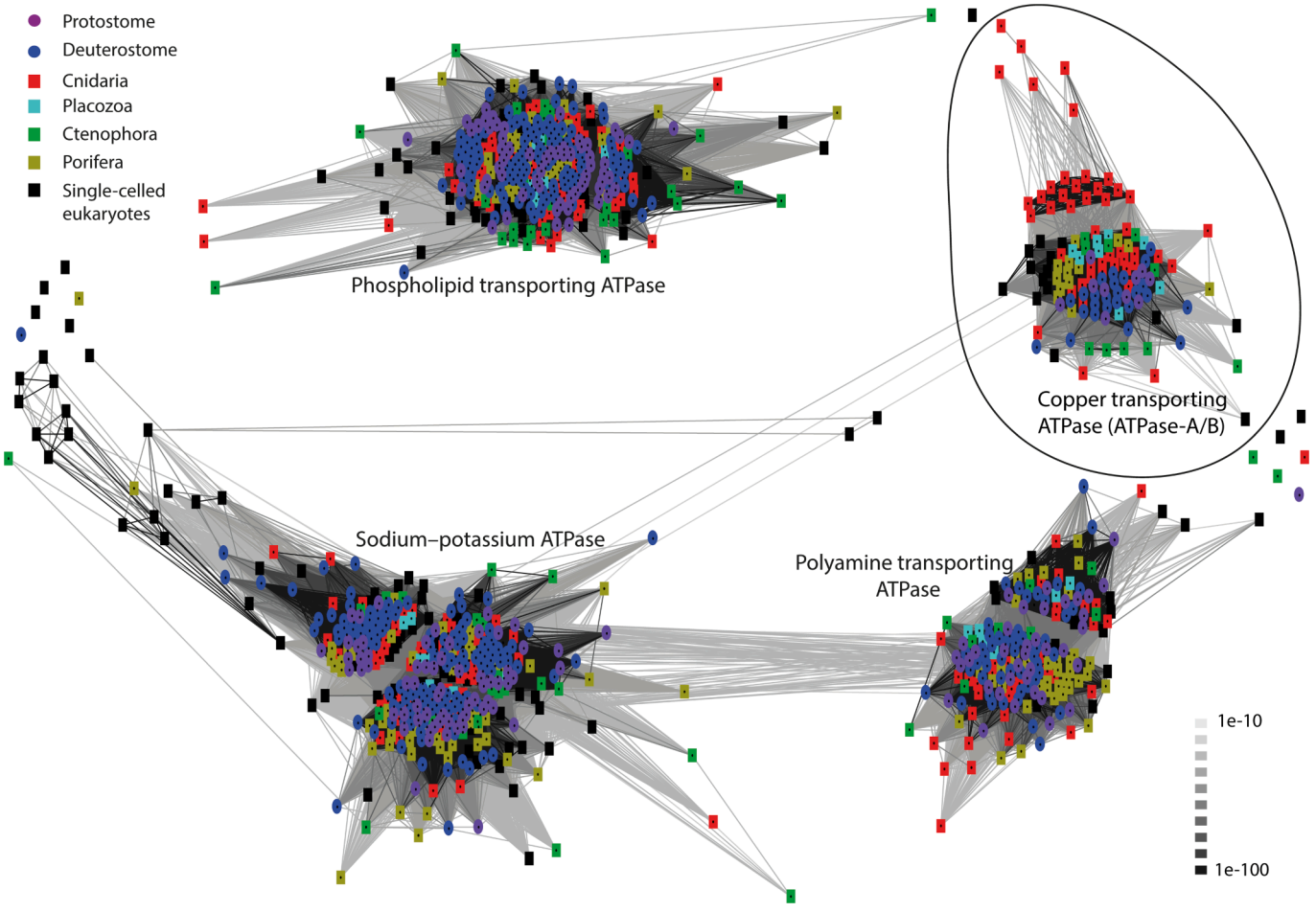

Fig. 1. Cluster map of eukaryotic P-type ATPases showing the closely related phospholipid-, calcium-, copper-, sodium-potassium-, and polyamine-transporting ATPases. The cluster containing the copper-transporting ATPases is marked with a black circle.

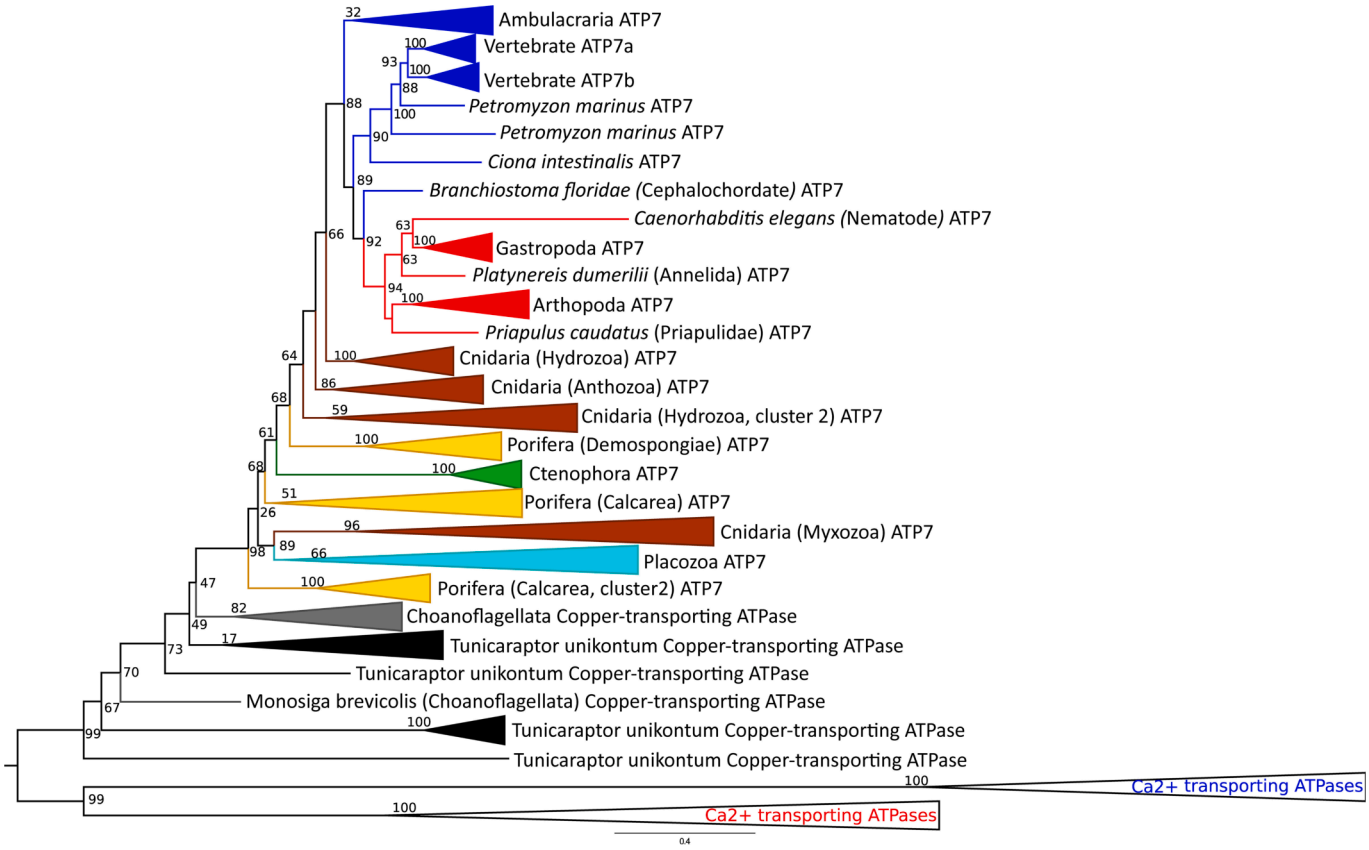

Fig. 2. Phylogenetic tree showing the occurrence and relationships of copper-transporting ATPases. The tree shows that the *ATP7A* and *ATP7B* sequences are a product of a gene duplication that occurred in the vertebrate lineage. Non-chordate deuterostomes and protostomes have only one *ATP7* gene.

	TM4-TM5	TM6-TM7	TM6-TM7	TM6-TM7	TM6-TM7
	***:	****	****:	****	***:***
H. sapiens ATP7B	ESLI**TGEA**MPVT	TVMF**DKTG**TITHGV	VGTAEAS**SEHPL**GVAVTKY	VLI**TGDN**RKTAR	AMV**GDGVNDS**PALA
H. sapiens ATP7A	ESLI**TGEA**MPVA	VVVF**DKTG**TITHGT	VGTAESN**SEHPL**GTAITKY	VLM**TGDN**SKTAR	AMV**GDG**I**NDS**PALA
C. intestinalis	ESVI**TGE**S**M**PVT	TVVF**DKTG**TITHGE	VGTAENA**SEHPL**GAAVVKR	ILL**TGDN**KKTAK	AMV**GDGVNDS**PALA
A. plancii	ESLI**TGE**S**M**PVY	TIIF**DKTG**TITYGI	VGTAEAS**SEHPL**GAAVTRH	VLL**TGDN**KKTAD	AMV**GDGVNDS**PALV
C. elegans	ESFI**TGE**S**M**PVV	TIVF**DKTG**TITEGR	SGATEAL**SEHP**I**G**NAVAAF	VLL**TGDN**SKTAE	AMV**GDGVNDS**PALA
P. caudatus	ESLV**TGEA**MPVL	AIVF**DKTG**TITHAV	ARTAEAS**SEHP**I**A**SAIRQI	ILL**TGDN**RKTAK	AMV**GDGVNDS**PALA
D. pulex	ESLI**TGE**S**M**PVL	TVVF**DKTG**TITRGF	IGIAETN**SEHP**I**A**SAITKF	ALL**TGDN**KKTAK	AMV**GDGVNDS**PALA
D. melanogaster	ESLI**TGE**S**M**PVA	TVVF**DKTG**TITHGT	VGAAEQN**SEHP**I**A**SAIVHF	VLL**TGDN**KNTAA	AMV**GDGVNDS**PALA
A. californica	ESLI**TGE**S**M**PVP	TVVF**DKTG**TVTHGV	AGTAETS**SEHP**I**A**SAILKY	ILL**TGDN**QKTAR	AMV**GDGVNDS**PALA
L. stagnalis	ESLI**TGE**S**M**PVP	VIVF**DKTG**TITHGV	AGTAETS**SEHP**I**A**SAILKY	ILL**TGDN**QKTAK	AMV**GDGVNDS**PALA
N. vectensis	ESLI**TGE**S**M**PVP	AVVF**DKTG**TLTHGK	AGTAENS**SEHPL**GVAITTY	VLL**TGDN**KKTAM	AMV**GDG**I**NDS**PALA
T. adhaerens	ESLI**TGE**P**M**SVA	VVIF**DKTG**TLTQGK	AGAAESN**SEHPL**AQAIVQH	IMM**TGDN**RRTAE	AMV**GDG**I**NDS**PALA
A. queenslandica	ESLI**TGE**S**M**PVS	AVIF**DKTG**TLTHGK	VGVAESN**SEHPL**GEAITAY	LLL**TGDN**RRTAQ	AMV**GDG**I**NDS**PALA
T. wilhelma	ESLI**TGEA**MPVP	TVVF**DKTG**TLTHGK	VGLAESN**SEHPL**GVAVTNF	VLL**TGDN**ERTAR	AMI**GDG**I**NDS**PALA
M. leydi	EAML**TGE**S**M**PVK	ICVF**DKTG**TVTLGK	AGTAESA**SEHPL**GKAITGH	FLL**TGDN**IRTAV	AMV**GDGVNDS**PALV
T. unikontum	ESMV**TGE**S**A**AVH	IVLF**DKTG**TLTVGK	VASAEAG**SEHPL**GHALTAY	AMV**TGDN**ASTAE	AMV**GDGVNDS**VALA
C. owczarzaki	----------	AIIF**DKTG**TLTHGK	LVCAESG**SEHPL**GRAIVQH	WMV**TGDN**ERTAR	AMV**GDG**I**NDS**PALA

Fig. 3. Amino acid conservation of the most important functional motifs of copper-transporting ATPases in representative species from Chordata (*Homo sapiens*, *Ciona intestinalis*), Echinodermata (*Acanthaster plancii*), Protostomia (*Ceanorhabditis elegans*, *Priapulus caudatus*, *Daphnia pulex*, *Drosophila melanogaster*, *Aplysia californica*, *Lymnaea stagnalis*) Placozoa (*Trichoplax adhaerens*), Cnidaria (*Nematostella vectensis*), Porifera (*Amphimedon queenslandica*, *Tethya wilhelma*), Ctenophore (*Mnemiopsis leydi*), Tunicaraptor (*Tunicaraptor unikontum*), and Icthyosporean (*Capsaspora owczarzaki*). TGEA(S): phosphatase domain; DTKG: phosphorylation domain; SEHPL(I): domain specific to heavy metal-transporting P-type ATPases only; TGDN: ATP-binding domain; GDGV(I)NDS: structural support.

The clinical aspects of ATP7 proteins are also presented briefly.

2. *ATP7*/ATP7 throughout the animal evolution

2.1. Mammals and clinical aspects of ATP7 proteins

As we delve into the multifaceted field of evolution, it is essential to

acknowledge that mammals, while a subset of the animal kingdom, hold a unique significance in the overall discourse. This is largely due to the occurrence of humans within this lineage, an existence that has inherently directed much of our scientific focus towards this specific group. Consequently, most mammalian research is not only relevant from an evolutionary perspective but also bears significant clinical implications. This further underscores the unique status of mammals within the broader context of evolutionary studies.

In adult mammals, *ATP7A* is expressed in most tissues but a much lower expression is found in the liver (Chelly et al., 1993; Kwok and Chan, 2019; Lenartowicz et al., 2010; Lenartowicz et al., 2015b; Vulpe et al., 1993). In contrast, *ATP7B* has a more limited expression pattern: its highest expression is in the liver but it is also expressed at lower levels in the kidney, placenta, brain, heart, lung, mammary glands, and testis (Bull et al., 1993; Michalczyk et al., 2008; Tanzi et al., 1993). Both *ATP7* genes mostly have cell-specific expression patterns and have distinct roles where co-expressed (Telianidis et al., 2013). It can be said that, in general, ATP7A and ATP7B have a dual role in cells: they provide copper to cuproenzymes at the trans-Golgi network and export excess copper from the cells via trafficking to the basolateral (ATP7A) or the apical (ATP7B) membrane (Telianidis et al., 2013). Many questions remain to be answered regarding the expression of mammalian *ATP7s*. For example, a recent study demonstrated that antibiotic treatment significantly down-regulates the expression of *ATP7A* and *SLC31A1* (which encodes high affinity copper uptake protein 1 [CTR1]; Lee et al., 2000) in the colon of mice and highlighted the potential connection between gut microbiome and copper homeostasis (Miller et al., 2019).

In humans, dysfunction of ATP7 proteins can lead to severe inherited recessive disorders, including Menkes disease and Wilson's disease, causing significant health complications. In Menkes disease, the intestinal copper absorption is limited due to the loss of ATP7A function leading to systemic copper deficiency and thus to a reduced activity of cuproenzmyes (main symptoms: "kinky" hair, hypopigmentation, growth failure, impaired neuronal development and neurodegeneration) (Kaler, 2011; Telianidis et al., 2013; Zeid et al., 2019). In Wilson's disease, the loss of ATP7B function leads to impaired biliary copper excretion resulting in systematic copper accumulation (main symptoms: vomiting, weakness, hepatitis, acute liver failure, tremors, muscle stiffness, behavioural changes) (Ala et al., 2007; Czlonkowska et al., 2018; Dev et al., 2022; Huster, 2010; Reed et al., 2018; Svetel et al., 2009).

Mice with *ATP7A* gene mutation(s), known as mottled mutants, are well-established models of Menkes disease (reviewed by (Lenartowicz et al., 2015a; Wang et al., 2019)). These models closely represent the phenotype of the disorder and are excellent for studying its pathophysiology and for testing new therapeutic interventions. Mammalian models have also been established to investigate Wilson's disease: the toxic-milk mouse, the Long-Evans Cinnamon rat, the *ATP7B* knockout mouse, and the Labrador retriever (reviewed by (Hadrian and Przybylkowski, 2021; Reed et al., 2018)). Although these models closely exhibit the hepatic phenotype of Wilson's disease patients, they do not develop a neurological deficit.

2.2. Non-mammalian vertebrates and invertebrate deuterostomes

Although orthologues to ATP7A and ATP7B proteins have been identified in many non-mammalian vertebrates and invertebrate deuterostomes (e.g., echinoderms, cephalochordates) by automated computational analysis from the genomic sequences, information on the function and structure of ATP7 proteins are limited in these species.

Until-recently, no crystal structure of eukaryotic ATP7 proteins was available, but only that of the gram-negative bacterium *Legionella pneumophila* was accessible to get insights into the molecular architecture (Gourdon et al., 2011). To address this and to better understand the unique features and physiological and pathological properties of ATP7 proteins, a recent paper described the first high-resolution cryo–electron microscopy (cryo-EM) structures of a vertebrate ATP7 protein, the E2-P_i state ATP7B of the western clawed frog (*Xenopus tropicalis*) (Bitter et al., 2022). The study highlighted shared and distinct features among ATP7 proteins and provided molecular blueprint for understanding numerous mutations associated with Wilson's disease. Building on this, a more recent paper presented the cryo-EM structure of human ATP7B in the E1 state in different forms and, by comparing the structures of the human and the frog protein, proposed the ATP-driving copper transport model of ATP7B (Yang et al., 2023). Importantly, these findings may also have therapeutic implications and confirm the importance of non-mammalian models.

Regarding expression and function, non-mammalian vertebrate ATP7 proteins have been investigated in zebrafish (*Danio rerio*), the widely used model for toxicological and biomedical research (Choi et al., 2021; Dai et al., 2014). Although both *ATP7A* and *ATP7B* genes were identified in *D. rerio* (Mendelsohn et al., 2006), most of the research was performed on *ATP7A*/ATP7A. In developing embryos, *ATP7A* is expressed in the notochord, while *ATP7B* is expressed in the liver and brain (Mendelsohn et al., 2006; Reed et al., 2018). Embryos with a mutated *ATP7A* gene (called calamity) lacked melanin pigment and had a wavy notochord (Mendelsohn et al., 2006), mimicking the phenotypes induced by copper deficiency in zebrafish (Mackenzie et al., 2004) and the phenotypes observed in Menkes disease patients and mottled mice (Lenartowicz et al., 2015a). This confirms the functional conservation of ATP7A proteins. Moreover, copper metabolism was restored by human *ATP7A* and transplantation experiments demonstrated that ATP7A functions cell autonomously, indicating important potential therapeutic implications. Interestingly, in a liver cell line of zebrafish (ZFL) and adult zebrafish specimens, *ATP7A* was shown to be highly expressed in the liver, while *ATP7B* was specifically expressed in the intestine (Leung et al., 2014). This is opposite to that in human or mammals or other fish. Functional investigations also demonstrated that ATP7B is responsible for copper export despite *ATP7B* expression level being much lower than *ATP7A* in ZFL cells (Kwok and Chan, 2019). There are still more questions to be answered, but zebrafish represent a promising model of *ATP7*/ATP7-related diseases including the development of chelators against copper-induced oxidative stress (Rakshit et al., 2018).

Not much information is available about ATP7 orthologues in invertebrate deuterostomes such as Urochordates, Cephalochordates or Ambulacrarians. However, our searches in the transcriptome and genome data demonstrated that these species have only one *ATP7* gene (Fig. 1; Fig. 2), confirming that the appearance and functional specialization of *ATP7A* and *ATP7B* have most likely happened during the gene duplication events of the chordate evolution (Fig. 2). The alignment of functional sites (Fig. 3) suggests that these ATP7 candidates are likely functional.

2.3. Arthropods

The fruit fly *D. melanogaster*, the famous invertebrate model of genetics (Jennings, 2011), has been used for many years as a model for metal-related human diseases and metal toxicity (reviewed by (Calap-Quintana et al., 2017)). As a result, among invertebrates, ATP7 has been investigated in greatest detail in this species. The orthologue of *ATP7A* and *ATP7B*, called *DmATP7*, was identified in 2004 when the copper homoeostasis was investigated in the S2 cell line (Southon et al., 2004). Of note, other *Drosophila* homologues (e.g., *ATOX1*, *CTR1A*, *CTR1B*, *CTR1C*) to proteins involved in mammalian copper homeostasis (Klomp et al., 1997; Lee et al., 2000; Zhou and Gitschier, 1997) have also been identified (Mercer and Burke, 2016; Southon et al., 2004; Zhou et al., 2003). The first functional investigation revealed that suppression of *DmATP7* by RNAi significantly increased copper accumulation, demonstrating that, in accordance with previous studies in mammalian cells, DmATP7 is essential for the efflux of excess copper (Southon et al., 2004).

Subsequent investigations further demonstrated the conservation of

function between DmATP7 and its mammalian orthologues by showing that DmATP7 function is absolutely required *in vivo* for completion of embryogenesis, early larval growth and development, and adult pigmentation, and is likely to be required for dietary copper uptake (Norgate et al., 2006). A later study also confirmed the role of DmATP7 in removing excess cellular copper and showed that copper homeostasis in *Drosophila* is maintained by complex interplay of import, storage, and behavioral avoidance (Balamurugan et al., 2007). The expression of *DmATP7* in neuronal tissues supported its assumed role in delivering copper to cuproenzymes required for neuronal function which is similar to *ATP7A* (Burke et al., 2008). Furthermore, its localization at or proximal to the basolateral membrane of midgut cells supported its role in export of copper from midgut cells (Burke et al., 2008). Gut-specific RNAi-induced silencing of *DmATP7* inhibited dietary copper absorption which resulted in an impaired neurological development during metamorphosis and increased the pre-adult mortality which is similar to Menkes patients and mottled-brindled mice (Bahadorani et al., 2010). Although the adult survivors had a reduced copper content in the head and the rest of the body, as well as being sensitized to oxidative stress, they had a normal morphology (e.g., normal cuticle pigmentation) and normal life span (Bahadorani et al., 2010). This is an important difference between mammals and *Drosophila*: a reduced copper supply during adulthood leads to early death in humans, while copper deficiency mainly causes mortality throughout the developmental stages in flies. Comparative genomic analysis demonstrated motifs involved in basolateral targeting and retention of ATP7A were conserved in DmATP7, whereas ATP7B targeting motifs were not conserved (Southon et al., 2010). Interestingly, copper dependent translocation (i.e. trans-Golgi network – plasma membrane) of DmATP7 was not observed in *Drosophila* or its two cultured cell lines but was seen in polarized mammalian cells (Southon et al., 2010).

Functional investigations in the nervous system showed that inhibition of *DmATP7* expression by cell-specific RNAi led to a decrease in mature amidated neuropeptides and the appearance of C-terminally Gly-extended neuropeptides (Sellami et al., 2012). Given that C-terminal amides in neuropeptides are produced by the sequential action of peptidylglycine-α-hydroxylating mono-oxygenase (PHM) and peptidyl-α-hydroxyglycine lyase (in vertebrates, this is implemented by the bifunctional peptidylglycine α-amidating monooxygenase) and that PHM requires copper for its normal function, the appearance of C-terminally Gly-extended neuropeptides was probably due to the loss of activity of PHM (Sellami et al., 2012). Pan-neuronal overexpression of *CTR1B* and *DmATP7* both resulted in reduced viability (copper toxicity phenotype and neural copper deficiency, respectively) and adversely affected the central nervous system (Hwang et al., 2014). In the *Drosophila* model of Huntington's disease (human huntingtin exon1-polyQ expression), overexpression of *DmATP7* significantly alleviated the phenotype, conforming the copper-facilitated protein aggregation of huntingtin (Xiao et al., 2013). Finally, a recent study demonstrated the interaction(s) between DmATP7 with the conserved oligomeric Golgi complex and that the integrity of Golgi-dependent copper homeostasis mechanisms are necessary to maintain mitochondria functional integrity and localization to synapse, resulting in the appropriate synaptic morphology, transmission, and plasticity (Hartwig et al., 2021).

2.4. Mollusks

Not much information is available about ATP7 orthologues in molluscan model species. In a paper about the neuronal transcriptome of the sea hare (*Aplysia californica*), a well-recognized model of cellular and molecular mechanisms of memory formation (reviewed by (Benjamin et al., 2021; Hawkins et al., 2006; Moroz, 2011)), 104 orthologues of 146 human genes involved in 168 neurological diseases (e.g., Parkinson's and Alzheimer's disease) were identified (Moroz et al., 2006). Importantly, a homologue for the vertebrate *ATP7* genes was also among the sequences identified. These findings suggested that the distinctive features of the simpler molluscan nervous system (reviewed by (Benjamin et al., 2021; Fodor et al., 2020b; Fodor et al., 2021; Hawkins et al., 2006; Kemenes and Benjamin, 2009; Moroz, 2011; Rivi et al., 2020; Rivi et al., 2021)) also presented opportunities to study the cellular and molecular functions of these genes and to develop relevant disease models in mollusks.

Building on the findings in *Aplysia*, many aging- and disease-relevant gene products, including the homologous sequence to vertebrate ATP7 proteins, were also identified in the neuronal transcriptome of another widely-used molluscan model species, the great pond snail (*Lymnaea stagnalis*) (Fodor et al., 2020a). Interestingly, although there is a homologue to human *SLC31A1* gene in *Lymnaea*, no homologous sequence to *ATOX1* appears to be present in this species (our own unpublished data). Also, a recent paper focusing on metal pollution has identified the homologous sequences in the mangrove oyster *Crassostrea gasar* to genes involved in copper homeostasis, further confirming the presence of *ATP7* in mollusks (Ferreira et al., 2022). Additionally, one can find further sequences on NCBI generated by automated computational analysis from genomic sequences. Based on cluster-map (Fig. 1) and phylogeny (Fig. 2) together with the conserved motifs (Fig. 3), these sequences are likely orthologues of ATP7 proteins.

Structural modelling or functional investigations have not yet been performed in any molluscan species. Taking advantage of the novel machine learning approach of AlphaFold (Jumper et al., 2021), we made an *in silico* investigation on the *Lymnaea* sequence to provide the first glimpse into the structure of molluscan ATP7 proteins (Fig. 4). Detailed methodology of the analysis is presented in **Supplementary File 1**. The predicted domain composition and organization of the *Lymnaea* ATP7 protein match well with previous studies (Barry et al., 2010; Gupta and Lutsenko, 2012; Norgate et al., 2006; Reed et al., 2018; Telianidis et al., 2013). Fig. 4**A** presents the predicted membrane topology and domain organization of the protein which show a high conservation: there are eight trans-membrane segments with the N- and C-termini (both oriented towards the cytosol). The cytosolic portion contains more functional domains: the N-terminal copper-binding domain, the actuator domain, the nucleotide binding domain, the phosphorylation domain, and the C-terminal. The functional sites of the domains also show a very high conservation (Fig. 3). The N-terminal copper-binding domain contains 5 sub-domains which support the previous idea that there is a general trend of increasing the number of copper-binding sites from lower to higher organisms (Gupta and Lutsenko, 2012). Of note, the first domain of the N-terminal also has a ferredoxin-like fold (βαββαβ) but does not contain the highly conserved copper-binding GMxCxxC motif. The predicted 3D structure of *Lymnaea* ATP7 protein (Fig. 4**B**) correlates well with recently published 3D structural and atomic models of frog and human ATP7B proteins derived from cryo-EM structure reconstruction (Bitter et al., 2022; Yang et al., 2023). Computational modelling on the surface electron potential map was also made to provide an insight into the potential copper transport pathway across the membrane in mollusks (and invertebrates in general) (Fig. 4**C**).

2.5. Nematodes

The first invertebrate *ATP7* homologue, *CUA-1*, was identified in 1997 in the extensively used invertebrate model organism of molecular and developmental biology, the nematode *C. elegans* (Sambongi et al., 1997). The functional sites of the protein exhibit high conservation (Fig. 3). Research on the functions of CUA-1 confirmed, for example, the importance of the CPC motif and that Asp phosphorylation in the DKTG motif is essential for the proper function of ATP7 proteins (Sambongi et al., 1997; Yoshimizu et al., 1998). In general, little is known about the mechanisms by which intestinal cells transport copper to maintain organismal copper homeostasis. A later study determined CUA-1 as a key intestinal copper exporter and showed that its trafficking is regulated with extraintestinal copper levels to maintain systemic copper homeostasis (Chun et al., 2017). It is worth mentioning that the copper

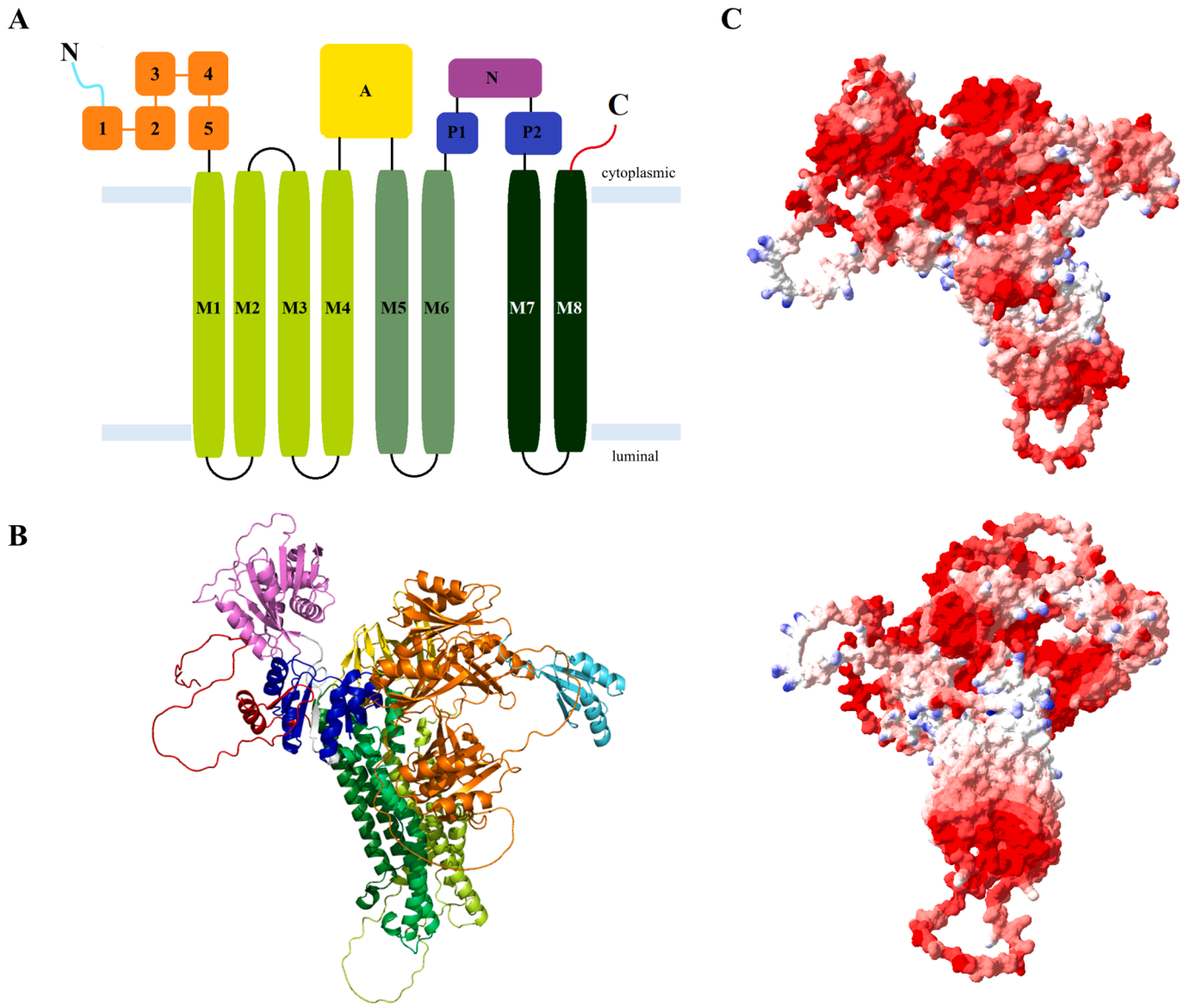

Fig. 4. Predicted structure of the ATP7 protein of *L. stagnalis*. (**A**) Predicted membrane topology and domain organization. The protein consists of eight transmembrane segments (M1-M8) with the N- and C-termini. The cytosolic portion contains the N-terminal copper-binding domain with 5 sub-domains (1–5), the actuator (A) domain (yellow), the nucleotide binding (N) domain (purple), the phosphorylation (P) domain (blue), and the C-terminal. (**B**) Predicted 3D structure of the protein. (**C**) Predicted surface electrostatic potential (red: −5 kT/e; white: neutral; blue: +5 kT/e). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chaperon protein CUC-1, the *C. elegans* homologue to yeast ATX1 and human ATOX1 (Klomp et al., 1997), has been identified and functionally investigated (Wakabayashi et al., 1998; Zhang et al., 2020). Additionally, *CHCA-1*, the homologue to mammalian *CTR1*, was also identified and functionally characterized (Yuan et al., 2018).

2.6. Non-bilaterians

As some of the most ancient animal lineages, non-bilaterians provide a unique opportunity to understand early animal evolution. Studying their genetics, development, and physiology can help us trace the origins of various biological processes and reconstruct the evolutionary history of the animal kingdom. So far, there is no analysis of the physiological role, localization, or single-cell expression pattern of ATP7 proteins in any of these species. From the four evolutionarily ancient phyla of non-bilaterian animals, *ATP7* sequences can be found in Porifera, Placozoa, and Cnidaria on NCBI, generated by automated analysis. However, to the best of our knowledge, this is the first study to address ATP7 proteins in these species. Currently, more and better-quality genome and/or transcriptome data are available for Porifera (e.g., *Amphimedon queenslandica*; (Srivastava et al., 2010)), Placozoa (e.g., *Trichoplax adhaerens*; (Wong et al., 2019)), Ctenophora (e.g., *Pleurobrachia bachei*, *Hormiphora californiensis*; (Moroz et al., 2014; Schultz et al., 2021)), and Cnidaria (e.g., *Hydra vulgaris*, *Nematostella vectensis*; (Cazet et al., 2023; Zimmermann et al., 2020)). In this study, our thorough searches for potential homologous sequences using Hidden Markov Models confirm that there are indeed homologues for *ATP7* genes in cnidarian, placozoan, and poriferan species. In addition, we demonstrate the presence of *ATP7* homologues in Ctenophores. The cluster-map (Fig. 1) and phylogeny (Fig. 2) together with the alignment of the functional sites (Fig. 3) suggest that the protein products of these sequences are bona fide ATP7 candidates in non-bilaterian animals. We hypothesize that the functions of ATP7 proteins in these species are also the maintaining of proper intracellular concentration of copper and to provide copper to

cuproenzymes. Moreover, we suppose that, similar to DmATP7, ATP7 proteins are necessary for the maturation of neuropeptides in non-bilaterians, including the neuron-less poriferans and placozoans, where a recent paper demonstrated the presence of neuropeptide-encoding genes (Yanez-Guerra et al., 2022). The presence of PHM enzyme in various non-bilaterian species, including Cnidarians, Poriferans, and Placozoans (Attenborough et al., 2012), supports this assumption.

3. Conclusions

The copper-transporting ATPases play a crucial role in copper regulation across diverse organisms, ranging from prokaryotes to eukaryotes. Over the past 30 years, many studies have investigated ATP7 proteins in humans and mice to understand pathophysiological conditions of Menkes and Wilson's diseases, exemplifying the clinical significance of these genes. In addition to the clinical aspects, copper-transporting ATPases represent an excellent object to study the evolution of large and complex proteins. For example, current research reveals that these genes underwent evolutionary transformations, leading to their functional diversity, including copper transport through complex barriers, copper supply to cuproenzymes, and their trafficking nature. Also, *ATP7*/ATP7 homologues have been identified in various non-mammalian vertebrates and invertebrate deuterostomes, opening avenues for further exploration into the functions and evolutionary trajectory of these copper-transporting ATPases. Although ATP7 proteins have also been investigated in some members of non-mammalian vertebrates and protostomes, other animals have been rather neglected. This is particularly true for non-bilaterians, a relevant group of metazoans which can provide important information about the evolution and function of genes. Here, we identified the presence of *ATP7* genes in poriferans, ctenophores, cnidarians, and placozoans, providing new evolutionary insights and the initial step into the study of these proteins in non-bilaterians. In our opinion, the function of copper-transporting ATPases in providing copper to cuproenzymes became even more important when the first animals appeared and copper-dependent enzymes diversified. It is also possible that ATP7 proteins had some relevance in the early evolution of neurotransmission, for example, by providing copper to the neuropeptide-amidating enzymes.

We agree that further vertebrate models are needed which could be utilised as *ATP7*/ATP7-related disease models. Notably, the recent structural elucidation of ATP7 protein from *Xenopus* via cryo-EM has provided critical insights into their molecular architecture, which could potentially pave the way for developing therapeutic interventions (e.g., neuroprotective compounds for the management of Wilson's disease). However, we would like to highlight that, in addition to vertebrate models, the study of invertebrates can also contribute to our understanding of the evolution of ATP7 proteins and may also have clinical implications. Future research in these models will continue to shed light on the critical role of ATP7 proteins in the maintenance of proper intracellular copper concentrations and in the support of essential biological processes across the animal kingdom.

Funding

This work was supported by the National Brain Project (#NAP2022-I-10/2022; Z.P.), the Hungarian Scientific Research Fund (#138039; Z. P.), and the BBSRC Discovery fellowship (BB/W010305/1; L.A.Y.G.).

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The graphical abstract was adopted and modified from the following paper: Jékely G & Budd GE. Animal Phylogeny: Resolving the Slugfest of Ctenophores, Sponges and Acoels? *Curr Biol* 31 (2021) R202-R204. https://doi.org/10.1016/j.cub.2020.12.026.

CRediT authorship contribution statement

István Fodor: Conceptualization, Investigation, Writing – original draft, Data curation, Visualization. **Luis Alfonso Yañez-Guerra:** Investigation, Writing – review & editing, Data curation, Visualization, Funding acquisition. **Bence Kiss:** Investigation, Writing – review & editing, Data curation, Visualization. **Gergely Büki:** Writing – review & editing. **Zsolt Pirger:** Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors thank Dr. Gábor Markó (neurologist; St. György Hospital, Székesfehérvár, Hungary) for one of his posts on Twitter that inspired the idea of this work. Also, the authors thank Prof. Robert J. Walker (University of Southampton; the UK) for checking the English of the manuscript. Open access funding was provided by Eötvös Loránd Research Network (ELKH).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2023.147720.

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