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Developmental and genomic insight into the origin of the tardigrade body plan

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Abstract

Tardigrada is an ancient lineage of miniaturized animals. As an outgroup of the well-studied Arthropoda and Onychophora, studies of tardigrades hold the potential to reveal important insights into body plan evolution in Panarthropoda. Previous studies have revealed interesting facets of tardigrade development and genomics that suggest that a highly compact body plan is a derived condition of this lineage, rather than it representing an ancestral state of Panarthropoda. This conclusion was based on studies of several species from Eutardigrada. We review these studies and expand on them by analyzing the publicly available genome and transcriptome assemblies of *Echiniscus testudo*, a representative of Heterotardigrada. These new analyses allow us to phylogenetically reconstruct important features of genome evolution in Tardigrada. We use available data from tardigrades to interrogate several recent models of body plan evolution in Panarthropoda. Although anterior segments of panarthropods are highly diverse in terms of anatomy and development, both within individuals and between species, we conclude that a simple one-to-one alignment of anterior segments across Panarthropoda is the best available model of segmental homology. In addition to providing important insight into body plan diversification within Panarthropoda, we speculate that studies of tardigrades may reveal generalizable pathways to miniaturization.

KEYWORDS
body plan evolution, Panarthropoda, Tardigrada

1 | INTRODUCTION

Tardigrada is a cosmopolitan lineage of miniaturized bilaterian animals (Gross et al., 2019; Schmidt-Rhaesa, 2001). Tardigrades have diversified to inhabit a plethora of environments, including environments that are typically hostile to life. For example, tardigrades can be found in the frigid environments of Antarctica and Arctic glaciers, and radioactive springs (Nelson et al., 2018). Some species are so resilient that they can survive exposure to the vacuum of outer space, even given the extremes in pressure, temperature, and radiation in this environment

(Jönsson et al., 2008). The ability of tardigrades to survive in extreme environments is made possible by mysterious physiological mechanisms that researchers are actively seeking to characterize. Recent advances have been made in understanding the molecular underpinnings that regulate the transition into the tun state, a state in tardigrades where they are almost completely desiccated, metabolically quiescent, and resilient to environmental extremes in temperature, pressure, and radiation (Boothby et al., 2017; Hashimoto et al., 2016; Hibshman et al., 2020; Nguyen et al., 2022; Tanaka et al., 2022). Interest in understanding the biology of tardigrades has been the impetus for genome and transcriptome sequencing projects (Boothby et al., 2017; Hashimoto et al., 2016; Kamilari et al., 2019; Yoshida et al., 2017), and the development of new research tools for tardigrade studies, such as RNA interference, transgenics, and CRISPR/Cas9 genome editing technology (Goldstein, 2022; Kumagai et al., 2022; Tanaka et al., 2023; Tenlen et al., 2013). These recent advances establish tardigrades as enticing models for interrogating many important questions related to evolution and development.

Molecular clock estimates suggest that crown group Tardigrada has a Cambrian origin, although there is a large span of uncertainty in this estimate (Howard et al., 2022). Tardigrada is composed of two lineages, Heterotardigrada, which includes both marine and limnoterrestrial species, and Eutardigrada, which includes mostly limnoterrestrial species. Over 1400 tardigrade species have been described (Degma & Guidetti, 2007, 2023; Guidetti & Bertolani, 2005), with potentially thousands of species yet to be discovered (Bartels et al., 2016). Although Tardigrada is an ancient and diverse lineage, prominent aspects of the tardigrade body plan are strictly conserved. The tardigrade body plan consists of a simple head and four trunk segments that each have a pair of lobopodal legs and a ganglion (Figure 1a,a'). A simple brain composed of cell-body-rich lobes and dorsal neuropil is located in the head (Mayer, Kauschke, et al., 2013; Mayer, Martin, et al., 2013; Persson et al., 2012; Persson et al., 2014; Schulze et al., 2014; Smith & Jockusch, 2014b; Smith et al., 2017; Zantke et al., 2008). The dorsal neuropil is contiguous with inner brain connectives that extend from both sides of the dorsal neuropil ventroposteriorly to connect the brain to the ganglion of trunk segment one. Outer connectives also extend from trunk segment one dorsoanteriorly around the outer brain lobe to the dorsal neuropil. Each trunk segment has a ganglion, and a pair of ventral nerve cords connects trunk ganglia. The buccopharyngeal apparatus consists of a buccal tube that connects the mouth to a triradiate pharynx and a pair of piercing tooth-like stylets that aid in feeding (Eibye-Jacobson, 2001; Guidetti et al., 2015). Although the features discussed here define the tardigrade body plan, they do vary quite extensively in shape and relative size across tardigrade species.

The somatic musculature of tardigrades consists of single muscle fibers, sometimes branched, extending through the hemocoel to two or more epidermal attachment points (Figure 1a,a') (Gross & Mayer, 2019; Halberg et al., 2009; Schmidt-Rhaesa & Kulessa, 2007; Smith & Jockusch, 2014b). Segmentally reiterated leg muscles are the only segmental features of the tardigrade muscle system (Gross & Mayer, 2019). Muscle anatomy in tardigrades is diverse and phylogenetically informative (Marchioro et al., 2013; Persson et al., 2019). Nevertheless, the presence of seven ventromedian muscle attachment points is characteristic of nearly all

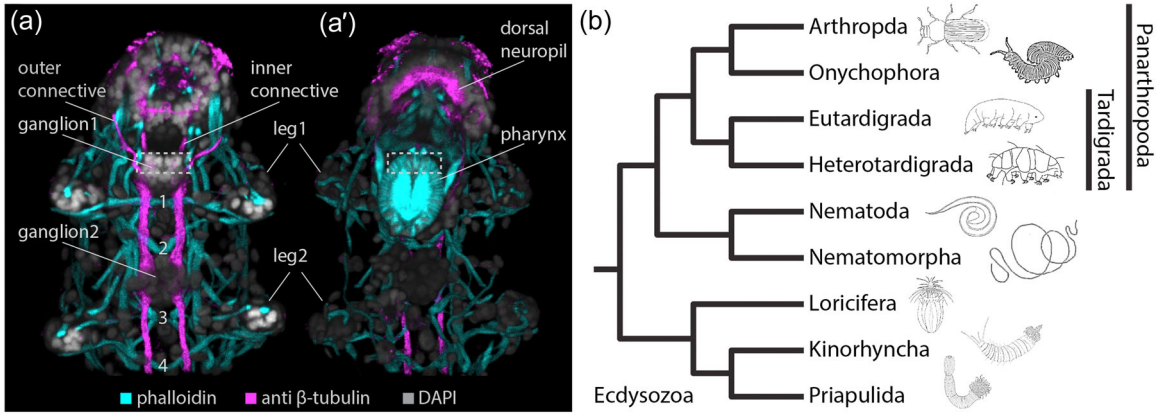

FIGURE 1 The body plan of Tardigrada and the phylogeny of Ecdysozoa. (a) Ventral view of *Hypsibius exemplaris* specimen. Ventral muscle attachment sites are numbered. (a') Dorsal view of *H. exemplaris* specimen. (a, a') Phalloidin stains muscles. Anti β-tubulin stains the nervous system. DAPI stains nuclei. Dashed box marks the position identified as the endomesodermal interface in Strausfeld et al. (2022). (b) Phylogeny of Ecdysozoa based on Howard et al. (2022). [Color figure can be viewed at wileyonlinelibrary.com]

tardigrade species analyzed to date, and represents the ancestral condition for this lineage (Marchioro et al., 2013; Persson et al., 2019).

Understanding the origin of animal body plans requires phylogenetic context (Hejnol & Lowe, 2015). Tardigrada is a member of Ecdysozoa (Figure 1b), a lineage of molting animals (Edgecombe et al., 2011; Giribet & Edgecombe, 2017). Morphological analyses recover Tardigrada, Arthropoda, and Onychophora as a monophyletic group within Ecdysozoa referred to as Panarthropoda. All possible interrelationships of these lineages have found support in morphological analyses (Caron & Aria, 2017; Howard et al., 2020; Legg et al., 2013; Nielsen et al., 1996; Peterson & Eernisse, 2001; Smith & Ortega-Hernández, 2014; Waggoner, 1996; Wu et al., 2023; Yang et al., 2016). Analyses of mitochondrial sequences, phylogenomic analyses, investigations of the phylogenetic distribution of microRNA molecules, and presence/absence of orthologous genes have all recovered Tardigrada as the sister-group of an Arthropoda + Onychophora lineage (Campbell et al., 2011; Howard et al., 2022; Rota-Stabelli et al., 2010; Yoshida et al., 2017). By contrast, some molecular analyses have recovered Tardigrada as the sister-group of Nematoda (Borner et al., 2014; Hejnol et al., 2009; Laumer et al., 2015; Yoshida et al., 2017). However, this grouping is most likely due to long branch attraction (Campbell et al., 2011). Here, we adopt the view that Tardigrada is an outgroup of Arthropoda + Onychophora within a monophyletic Panarthropoda (Figure 1b), while acknowledging that the placement of Tardigrada within Ecdysozoa may not be satisfactorily resolved (Giribet & Edgecombe, 2017).

As a member of Panarthropoda, and the probable outgroup of the other panarthropod lineages, studies of tardigrades are critical for resolving the origin of characteristics of incredible significance in Panarthropoda—segmentation and ventral appendages. Studies of tardigrades may lend even broader insight into body plan diversification within Bilateria. In the present work, we review and expand upon previous research to gain a clearer view of the origin of the tardigrade body plan and its implications for our general understanding of body plan evolution.

2 | TARDIGRADE EMBRYOGENESIS

Embryogenesis has been studied in two species of eutardigrade using modern microscopy techniques, *Hypsibius exemplaris* and *Thulinia stephaniae*. In these species, embryos exhibit holoblastic radial cleavage (Gabriel et al., 2007; Hejnol & Schnabel, 2006). Roughly synchronous stereotyped cleavage is characteristic of embryogenesis in *H. exemplaris*, with some cell divisions being invariably unequal (Gabriel et al., 2007). In *T. stephaniae*, cleavage is equal, asynchronous, and irregular with descendants of the same blastomere positioned in distinct locations in different embryos (Hejnol & Schnabel, 2005, 2006). Furthermore, blastomere fate determination appears highly regulative and indeterminate based on results of cell ablation experiments in *T. stephaniae* (Hejnol & Schnabel, 2005). The earliest cells to internalize in *H. exemplaris* and *T. stephaniae* are thought to represent primordial germ cells (Gabriel et al., 2007; Hejnol & Schnabel, 2005, 2006). Two presumptive primordial germ cells internalize in *T. stephaniae*, while four internalize in *H. exemplaris*. Orthologs of the germ-line markers *piwi* and *vasa* are expressed in the four presumptive primordial germ cells in *H. exemplaris* (Heikes et al., 2023). Endomesodermal cells enter through the blastopore during gastrulation in *H. exemplaris*, with the blastopore closing by epiboly (Gabriel et al., 2007). After gastrulation, *H. exemplaris* embryos progress to the epithelium stage, in which they appear as a ball of endomesodermal cells surrounded by column-shaped ectodermal epithelial cells (Gabriel et al., 2007). By contrast, during *T. stephaniae* gastrulation, germ cells, and endomesodermal cells migrate into one pore, while ectodermal cells migrate through a separate pore (Hejnol & Schnabel, 2005). After gastrulation in pre-elongated embryos, mesodermal bands become visible between the ectodermal and endoderm germ layers in *T. stephaniae* embryos. In both species, gastrulation is followed by elongation, characterized by development of a comma-shaped embryo, and then segmentation (Gabriel et al., 2007; Hejnol & Schnabel, 2005, 2006). Cell rearrangement and growth underlie the elongation process, but posterior growth is not seen during tardigrade development. The appearance of mesodermal somites is the first sign of segmentation in *T. stephaniae* (Hejnol & Schnabel, 2005, 2006). By contrast, the first sign of segmentation in *H. exemplaris* is the appearance of endomesodermal pouches located in the trunk (Gabriel et al., 2007). The endomesodermal pouches do not exhibit distinct endodermal and mesodermal cell layers at this stage in *H. exemplaris*. It is unclear precisely when mesodermal cells differentiate in *H. exemplaris*. Shortly after the appearance of endomesodermal pouches, *engrailed* is expressed in four stripes in the developing trunk (Gabriel & Goldstein, 2007). Next, epidermal segmental furrows develop between the head and the trunk, and between all trunk segments in *H. exemplaris* embryos. The furrows between trunk segments develop immediately posterior to the stripes

of *engrailed* expression. In both species, segmental trunk ganglia and legs appear later (Gabriel et al., 2007; Gross et al., 2017; Hejnol & Schnabel, 2005, 2006). In *H. exemplaris*, the central nervous system develops in anteroposterior (AP) order (Gross & Mayer, 2015). The intriguing differences in how *H. exemplaris* and *T. stephaniae* develop, despite both being eutardigrades, highlight the importance of embryological studies of additional tardigrade species, particularly heterotardigrade species, to illuminate the evolution of developmental diversity in Tardigrada.

3 | RESOLVING THE LOSS OF HOX GENES IN TARDIGRADA

Hox genes are a group of paralogous genes that encode transcription factors that regulate development of regionalized patterns along the AP axis of bilaterian animals (Angelini & Kaufman, 2005a; Hughes & Kaufman, 2002). Hox genes have been analyzed in the genomes or transcriptomes of four tardigrade species—*H. exemplaris*, *Paramacrobiotus richtersi*, *Milnesium tardigradum*, and *Ramazzottius varieornatus* (Smith et al., 2016; Yoshida et al., 2017). In each species, orthologs of the Hox genes *labial* (*lab*), *Hox3*, *Deformed* (*Dfd*), *fushi tarazu* (*ftz*), and *Abdominal-B* (*Abd-B*) were identified. However, *proboscipedia* (*pb*), *Sex combs reduced* (*Scr*), *Antennapedia* (*Antp*), and a UbdA motif-encoding Hox gene, represented by both *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) in other panarthropods, were not detected. These missing Hox genes should be present in tardigrade genomes based on their conservation in other panarthropods and outgroups of Panarthropoda (Smith et al., 2016). Based on these studies, it appears that several Hox genes have been lost in Tardigrada.

Previous analyses of tardigrade Hox genes were restricted to species from Eutardigrada. Data from Heterotardigrada, which includes all other tardigrade species, is required to help resolve the ancestral complement of Hox genes for crown group Tardigrada, and determine where in tardigrade phylogeny specific Hox orthologs were lost. For this purpose, we analyzed the recently published genome and transcriptome assemblies for the heterotardigrade species *Echinisus testudo* (Murai et al., 2021). The genome assembly of this species was derived from a single *E. testudo* specimen. Coverage for the genome assembly is 85X and the BUSCO completeness score for this assembly is 92.7% compared to the eukaryotic data set. N50 is 6,674. 98.6% of the transcript sequences closely matched sequences in the genome assembly, and all predicted protein sequences from the genome assembly matched translated sequences from the transcriptome assembly. Taken together, these results indicate that the genome assembly is nearly complete, though it is fragmented. We identified several highly similar candidate sequences for most candidate genes that we searched for in the *E. testudo* gene predictions (Supporting Information: Table S1). In terms of nucleotide identity, these distinct sequences were more similar than what is typical for paralogs, but more different than what is typical for alleles. We do not speculate on the nature of these similar sequences, but note that Murai et al. (2021) suggested that the draft genome might include duplicate assemblies. For phylogenetic analyses, we only include one sequence from each cluster of highly similar sequences. Therefore, for each candidate gene, we only determine whether *at least one* ortholog is present in *E. testudo* assemblies.

We identified several candidate Hox orthologs in *E. testudo* by reciprocal BLAST search. These candidates clustered with Hox orthologs that are also found in eutardigrades in our phylogenetic analyses of predicted homeodomain sequences, based on evolutionary distance (Figure 2a). *E. testudo* candidates were nested within monophyletic Hox ortholog groups in the majority rule consensus tree that resulted from analyses of an untrimmed Hox protein matrix (Figure 2b). Additionally, these sequences encode amino acids that are diagnostic for Hox orthologs (Figure 2c) (Janssen et al., 2014). Taken together, we conclude that *E. testudo* retains the same suite of Hox genes that are found in the eutardigrades that have been studied. Only *Abd-B* and *lab* were recovered as monophyletic with strong support in our analyses (Figure 2a,b), but this is not surprising given the few potential synapomorphies that unite Hox orthologs (Figure 2c). Our results indicate that the last common ancestor of Tardigrada had already lost orthologs of *pb*, *Scr*, *Antp*, and at least one UbdA motif-encoding Hox gene. This raises the question of what their ancestral functions were and why they became dispensable in the ancient ancestors of Tardigrada (see below).

We identified an unusual Hox-like sequence in the transcriptome assembly that we refer to as Et-HD. This sequence clustered in the Ftz orthology group in our homeodomain analyses (Figure 2a). It did not cluster with any Hox orthology group in our analyses of untrimmed Hox sequences (Figure 2b). This sequence does not encode an HX domain and encodes amino acid insertions in the homeodomain, according to our analysis (Figure 2c, ftz alignment). Therefore, it is unlikely to represent a functional Hox gene. This sequence was not a close match to any gene predictions from the genome assembly. However, we did identify a similar sequence in close

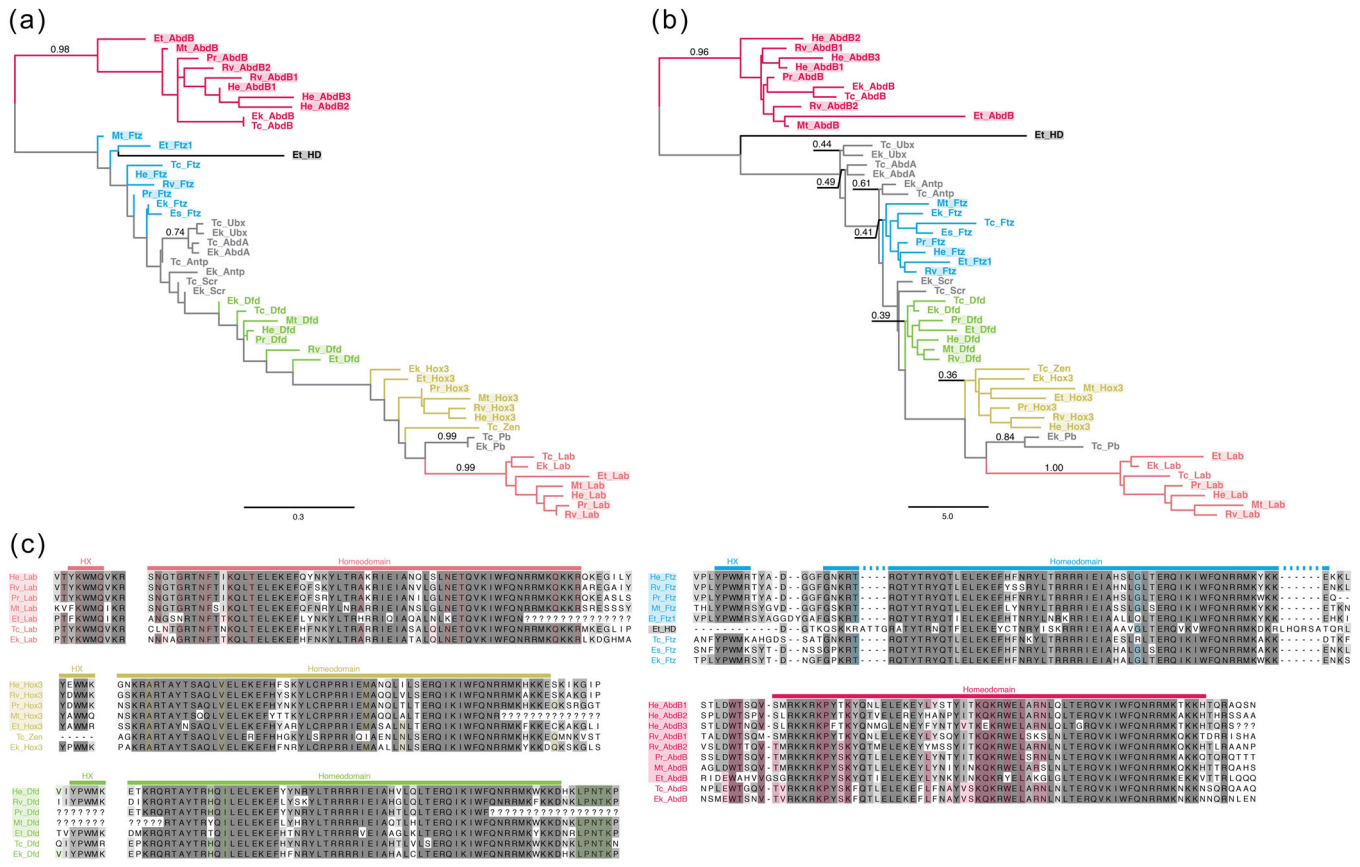

FIGURE 2 Hox gene phylogenies and homeodomain alignments. Tardigrade sequences are in colored boxes. (a) Phylogeny based on Hox gene homeodomain alignment. Q. insect + G model was used for analyses. (b) Phylogeny based on alignment of untrimmed Hox sequences. Q. insect +R + F model was used for analyses. (a, b) Majority rule consensus phylogenies based on three maximum likelihood trees each. Bootstrap support values are provided as percentages out of 500 replicates. Branch support values are only shown for Hox orthologs that were recovered as monophyletic. The trees are unrooted. (c) HX and Homeodomain alignments. Diagnostic amino acids are shaded in color based on (Janssen et al., 2014). Ek, *Euperipatoides kanangrensis*; Es, *Endeis spinosa*; Et, *Echiniscus testudo*; He, *Hypsibius exemplaris*; HX, Hexapeptide; Mt, *Milnesium tardigradum*; Pr, *Paramacrobiotus richtersi*; Rv, *Ramazzottius varieornatus*; Tc, *Tribolium castaneum*. [Color figure can be viewed at wileyonlinelibrary.com]

proximity to a *ftz* sequence on scaffold jcf7180001734878 (identity = 93.51%; query coverage = 91%). Its close proximity to *ftz* in the genome may indicate that it represents a recent duplication of *ftz*, although we cannot completely rule out the possibility that this sequence represents a different Hox ortholog. The other candidate Hox genes were located on independent scaffolds in the *E. testudo* genome assembly (Supporting Information: Table S1).

4 | THE EVOLUTION OF THE COMPACT TARDIGRADE BODY PLAN

Based on the expression patterns of Hox genes and other homeobox genes in *H. exemplaris*, it has been suggested that the head and first three trunk segments of a tardigrade are directly homologous to the anteriormost four head segments of an arthropod or onychophoran (Smith & Goldstein, 2017; Smith et al., 2016, 2018). The anterior region of the fourth trunk segment was suggested to align to the fifth segment of an arthropod or an onychophoran based on expression of *ftz*, and the posterior region was suggested to align to the posteriormost segment(s) of arthropods and onychophorans based on expression of *Abd-B* (Smith & Goldstein, 2017; Smith et al., 2016). In other words, these studies suggested that the tardigrade body plan is constructed of segments that are primarily homologous to the anteriormost segments of other panarthropods, while a small posterior region of tardigrades is directly homologous to the posteriormost region of other panarthropods. The Hox genes *Antp*, *Ubx*, and *abd-A*, genes that are missing in tardigrades (see above), are expressed in the segments located between these regions in other panarthropods (Angelini & Kaufman, 2005a; Hughes &

Kaufman, 2002; Janssen et al., 2014). These segments make up a relatively large portion of the body axis in most other panarthropods, for example, the thorax and most of the abdomen of an insect. A trunk region in which orthologs of *Antp*, *Ubx*/*Abd-A* are expressed is found in outgroups of Panarthropoda (Fröbius et al., 2008). Therefore, a trunk region that expresses these genes most likely represents an ancestral state of Panarthropoda. Taken together, available data support the conclusion that the compact body plan of Tardigrada evolved by the loss of a trunk region.

The loss of *Antp* and *Ubx*/*abdA* is unlikely to be the cause of the loss of a trunk region in tardigrades. Rather, these genes may have become dispensable after the segments that they patterned were no longer produced during development in ancient ancestors of Tardigrada (Smith et al., 2016). The absence of *pb* and *Scr* is more difficult to explain because segments in which these genes are predicted to be expressed are conserved in tardigrades (Smith et al., 2016, 2018). As with other Hox genes, *pb* and *Scr* regulate segment identity specification in arthropods (Angelini & Kaufman, 2005a; Hughes & Kaufman, 2002; Smith & Jockusch, 2014a), and presumably onychophorans (Eriksson et al., 2010; Janssen et al., 2014), and may have played this role in the ancient ancestors of Panarthropoda. If so, then the loss of these genes may indicate that trunk segments were more heteronomous in stem group ancestors of Tardigrada than modern tardigrades.

Although the loss of a trunk region explains the absence of some Hox genes in Tardigrada, the question of how tardigrades lost this region still remains. One clue may come from how the AP axis develops in tardigrades. During elongation in tardigrades, all regions of the AP axis appear simultaneously (Gabriel et al., 2007; Hejnol & Schnabel, 2005). By contrast, in many bilaterians, the anterior region of the body axis develops first. Later, the rest of the body axis develops sequentially through posterior growth (Gonzalez et al., 2017). Despite the diversity of morphogenetic processes that underlie posterior growth in bilaterians (Mayer et al., 2010), similar gene regulatory networks regulate this process (Fritzenwanker et al., 2019; McGregor et al., 2009). Posterior growth most likely represents an ancestral mode of AP axis development in Bilateria (Fritzenwanker et al., 2019; Gold et al., 2015). Posterior growth is a common mode of development in Arthropoda and Onychophora (Mayer et al., 2010; Williams & Nagy, 2017). The midtrunk segments that are missing in tardigrades develop by posterior growth in many other panarthropods. Therefore, the evolution of the compact body plan of Tardigrada may be explained in part by reduction and eventual loss of posterior growth (Smith et al., 2016).

Placing tardigrades into a paleontological context provides independent support for the model of body plan evolution presented above. All three extant panarthropod lineages are thought to have evolved from lobopodian ancestors. Lobopodians have an extensive Cambrian fossil record and typically exhibit many more segments than a tardigrade. Several phylogenetic analyses have recovered Tardigrada as nested within lineages that include lobopodians, that is, Tardigrada is resolved as more closely related to some lobopodians than others in these studies (Caron & Aria, 2017; Howard et al., 2020; Kihm et al., 2023; Smith & Ortega-Hernández, 2014; Yang et al., 2016). Although these phylogenetic studies disagree on the exact relationship of tardigrades to lobopodians, their recovered topologies all suggest that the limited segment number characteristic of Tardigrada is a derived state of this lineage, as predicted by the model based on analyses of AP axis patterning genes. Taken together, these results support a model in which tardigrades have lost a contiguous series of intermediate trunk segments relative to ancient lobopodians (Kihm et al., 2023).

5 | COMPARING MODELS OF TARDIGRADE SEGMENT HOMOLOGY

The model of segment homology discussed above supports a one-to-one alignment of the anteriormost segments across Panarthropoda, with the tardigrade head aligning to the protocerebral segment of arthropods and the frontal appendage segment of onychophorans (Figure 3a, hypothesis 1) (Smith & Goldstein, 2017; Smith et al., 2018). Anatomical studies of extant and extinct panarthropods support the one-to-one model of segment alignment (Martin et al., 2022; Mayer, Kauschke, et al., 2013; Ortega-Hernández et al., 2017; Park et al., 2018). However, recently two new models of panarthropod segment homology suggest that the tardigrade head is homologous to more than a single segment of arthropods or onychophorans. One of these models is based on new data from a study of the Cambrian lobopodian *Cardiodictyon catenulum* (Strausfeld et al., 2022). Strausfeld et al. (2022) recovered *C. catenulum* as a stem group panarthropod in their phylogenetic analysis. To aid in comparisons between models, when we refer to the protocerebral segment below, we are referring to both the proso- and protocerebral regions of Strausfeld et al. (2022). The head of *C. catenulum* includes three appendage pairs and their associated nervous system domains, which are referred to in AP order as ce1, ce2, and ce3. The ce1 and ce2 regions of *C. catenulum* align to the protocerebral

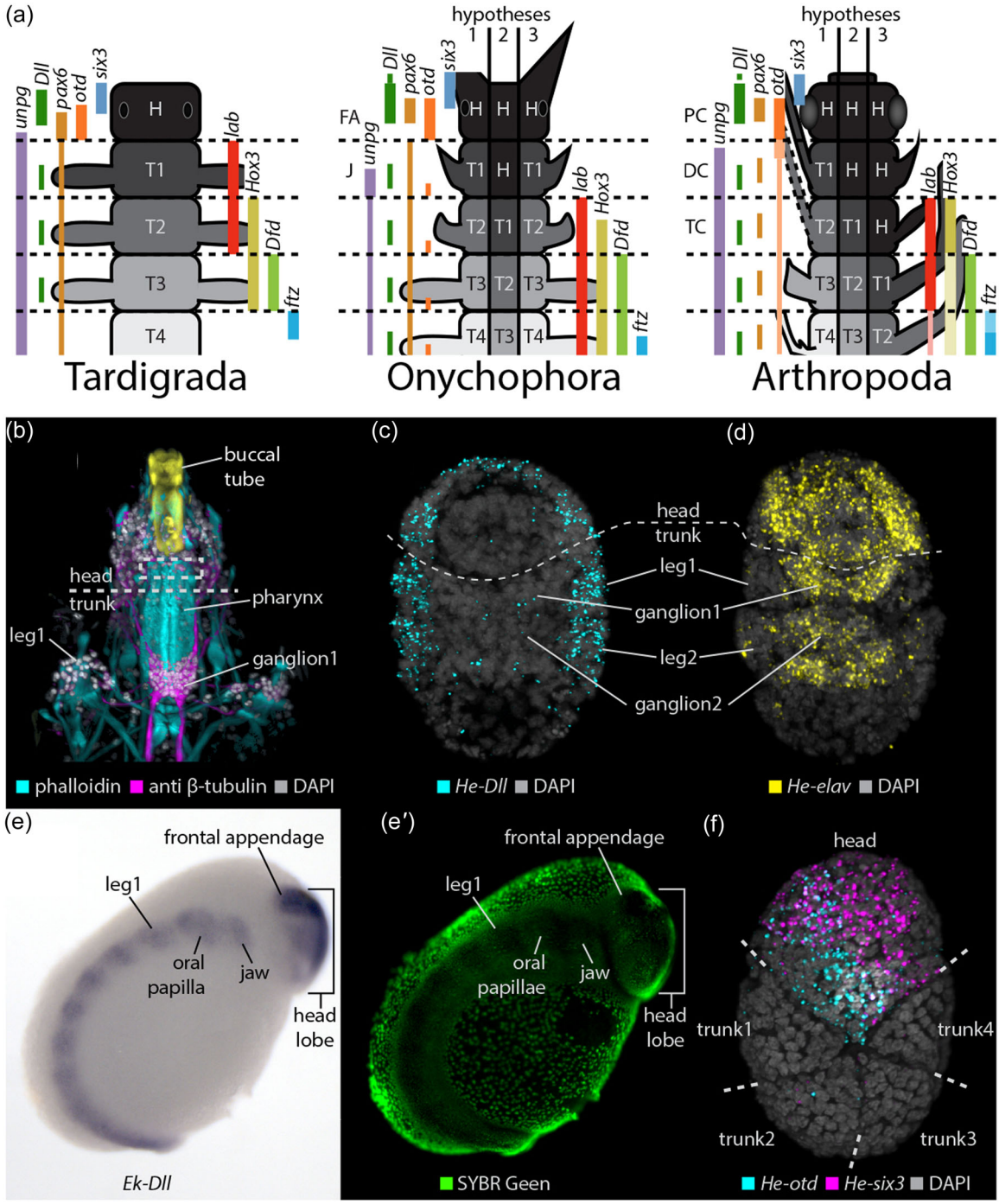

FIGURE 3 (See caption on next page).

segment of arthropods and frontal appendage segment of onychophorans according to this new model. The ce3 region aligns to the deutocerebral segment of arthropods and the jaw segment of onychophorans. By contrast, the head of tardigrades aligns with the ce1, ce2, and ce3 regions of *C. catenulum*, according to this model.

In contrast to the one-to-one model, the model of Strausfeld et al. (2022) suggests that the tardigrade head is homologous to the first two segments of an arthropod or onychophoran, the proto- and deutocerebral segments, and the frontal appendage and jaw segments, respectively (Figure 3a, hypothesis 2). This alignment depends on the homology of the position identified as the endomesodermal interface. According to the model of Strausfeld et al. (2022), the endomesodermal interface of the tardigrade *H. exemplaris* is positioned near the junction between the buccal tube and the pharynx. By

contrast, a region of the gut that is positioned posterior to the buccal tube, pharynx, and a region referred to as the stomodeum was identified as the endomesodermal interface in *C. catenulum*. If instead, the interface between the buccal tube and the pharynx is used to align segments between *H. exemplaris* and *C. catenulum*, which likely represents a homologous position of the gut between these species, then the head of *H. exemplaris* aligns to only the ce1 domain of *C. catenulum*, rather than ce1–ce3. By extension, this alternate alignment supports homology of the tardigrade head to the protocerebral segment of arthropods and the frontal appendage segment of onychophorans, in support of the one-to-one model.

The model of Strausfeld et al. (2022) also depends on the conservation of the position of the endomesodermal interface relative to the position of segments. However, within tardigrades, the proposed endomesodermal interface exhibits positional variation relative to the segments. In *H. exemplaris*, the interface between the buccal tube and pharynx is positioned near the boundary between the head and first trunk segment, in close alignment to the anterior edge of the first trunk ganglion (Figure 1a,a'; Strausfeld et al., 2022). In species of the genus *Milnesium*, this position is within the head, far removed from the first trunk ganglion (Figure 3b) (Schmidt-Rhaesa & Kulessa, 2007). In *Hypsibius* sp. and *Halobiotus crispae*, the buccal tube meets the pharynx within the head, and the entire buccopharyngeal apparatus, or at least a majority of it, are located within the head (Halberg et al., 2009; Schmidt-Rhaesa & Kulessa, 2007). The position of the proposed interface evolves independently of the position of segments in Tardigrada. Likewise, the position of the stomodeum, which denotes the endomesodermal interface of arthropods and onychophorans in this model, is hypothesized to have migrated posteriorly relative to segmental anatomy in the arthropod and onychophoran lineages, a migration that is recapitulated during embryogenesis of these animals (Ortega-Hernández et al., 2017). Therefore, even if the endomesodermal interface represented a homologous position of the gut across panarthropods, the evolutionary and developmental flexibility of its position relative to the position of segments precludes its utility for aligning segments across Panarthropoda.

Furthermore, *Distal-less* (*Dll*) expression, which was used to support the model of Strausfeld et al. (2022), provides stronger support for the one-to-one model. In all panarthropod phyla examined, strong *Dll* expression marks a single anterior region of the developing central nervous system. It marks the brain of *H. exemplaris* (Figure 3c,d), the head lobes of the onychophoran *Euperipatoides kanangrensis* (Figure 3e,e'), and the protocerebrum of arthropods (Lemons et al., 2010; Pechmann et al., 2011). These expression domains support the one-to-one model (Figure 3a).

A second new alignment of panarthropod segments is based on differences in the segmentation process in pregnathal versus the postgnathal segments in arthropods (Lev & Chipman, 2021; Lev et al., 2022). The pregnathal segments in arthropods are the proto-, deuto-, and tritocerebral segments, while the remaining segments are referred to as postgnathal segments. The architects of this new model noted several important aspects of segmentation that differ in development of the pregnathal segments compared to the postgnathal segments (Lev & Chipman, 2021; Lev et al., 2022). For example, pair-rule genes do not regulate development of

FIGURE 3 Comparison of segment alignment hypotheses. (a) Segment alignment models. Horizontal dashed lines represent segment boundaries. Gene expression patterns are modified from a model in Smith et al. (2018). Thin lines denote expression domains that are less useful for aligning segments between panarthropod lineages because they label structures found in many or all segments or they are reduced in expression relative to the primary expression domain. Tardigrade expression patterns are based on studies of *Hypsibius exemplaris* and onychophorans expression patterns are based on studies of *Euperipatoides kanangrensis*. Arthropod expression patterns are based on studies of a diversity of species. The left side of the arthropod anatomical model represents a mandibulate. A dashed line outlines the second antenna because it is only found in crustaceans. The right side of the arthropod anatomical model represents a chelicerate. In the arthropod model, lower opacity gene expression colors represent expression domains that are found in a subset arthropod species that have been investigated. Hypothesis 1 = one-to-one model. Hypothesis 2 = Strausfeld et al. (2022). Hypothesis 3 = Lev et al. (2022). DC, deutocerebral segment of Arthropoda; FA, frontal appendage segment of Onychophora; H, tardigrade head or homologous segment; J, jaw segment of Onychophora; PC, protocerebral segment of Arthropoda; T1–T4, tardigrade trunk segment 1–4 or homologous segment; TC, tritocerebral segment of Arthropoda. (b) Ventral view of a *Milnesium* n. sp. specimen. Phalloidin stains muscles. Anti β-tubulin stains the nervous system. DAPI stains nuclei. The buccal tube is false colored yellow. The image represents a reanalysis of data originally collected for Smith et al. (2017). Dashed box marks the position identified as the endomesodermal interface in Strausfeld et al. (2022). (c) Expression of *Dll* in a limb bud stage *H. exemplaris* embryo revealed by HCR in situ. (d) Expression of *elav* in a limb bud stage *H. exemplaris* embryo revealed by HCR in situ. *Elav* labels the nervous system (Smith et al., 2018). (e) *Dll* expression in a prelimb outgrowth *E. kanangrensis* embryo revealed by in situ hybridization. (e') SYBER green stains nuclei. (f) Expression of *six3* and *otd* in an ectodermal segmentation stage *H. exemplaris* embryo as revealed by HCR in situ. [Color figure can be viewed at wileyonlinelibrary.com]

pregnathal segments, but play important roles in regulating segmentation in the postgnathal segments. There are also interesting differences in expression or function of segment polarity genes. Stripes of *hedgehog* expression form by stripe splitting in the pregnathal segments, unlike in the postgnathal segments. *Engrailed* is expressed after *hedgehog* expression in the pregnathal region, while these genes are first expressed nearly simultaneously in the other segments. Additionally, Hox genes specify segment identity in all other segments, but of the pregnathal segments, only tritocerebral identity is specified by a Hox gene. Lev et al. (2022) suggested that these differences reflect an independent origin of the pregnathal segments. In this new model, the pregnathal segments evolved from the splitting of a single segment in ancestors of Arthropoda (Lev & Chipman, 2021; Lev et al., 2022). This ancestral single segment is homologous to the head of a tardigrade and the frontal appendage segment of an onychophoran in this model. Therefore, this model disagrees with the one-to-one model in terms of the alignment of arthropod segments to segments of both tardigrades and onychophorans, while remaining consistent with the one-to-one model in terms of the alignment of tardigrade segments to onychophorans segments (Figure 3a, hypothesis 3).

We favor the one-to-one model for several reasons. First, the model of Lev et al. (2022) requires extensive anterior shifts in the expression domains of AP axis patterning genes to accommodate the segmental alignments it suggests (Figure 3a). For example, this model predicts that the frontal appendages of onychophorans are directly homologous to the deutocerebral appendages of arthropods, either chelicerae or antennae. However, *six3* is expressed in the frontal appendages of onychophorans, while *six3* is not expressed in deutocerebral appendages in arthropods (Eriksson et al., 2013; Steinmetz et al., 2010). Instead, *six3* expression is restricted to the anterior part of the protocerebral segment in arthropods. Furthermore, the expression domains of *six3* and *otd* are localized to the anteriormost segment during the early stages of segmentation across Panarthropoda (Figure 3a,f) (Steinmetz et al., 2010). Second, although Hox genes are not expressed in deutocerebral appendages in arthropods, a Hox code does specify the identity of deutocerebral appendages in Arthropoda; the absence of Hox gene expression equals deutocerebral appendage identity. If Hox gene function is abolished in insects, all ventral appendages develop into antennae (Brown et al., 2002). Conversely, if the function of antennal selector genes is disrupted, the antennae develop into legs (Angelini et al., 2009; Setton et al., 2017; Shippy et al., 2009; Smith et al., 2014). Similar disruptions result in transformation of chelicerae to legs in spiders (Sharma et al., 2015). We interpret this ease of homeosis between postgnathal appendage types and deutocerebral appendages as evidence of their serial homology. By extension, we view the deutocerebral segment as serially homologous to the postgnathal segments. Furthermore, the absence of Hox expression is also not strong evidence of lack of serial homology of the deutocerebral segment to postgnathal segments. In onychophorans, Hox genes are not expressed in the jaw segment even though the jaw segment is clearly serially homologous to the more posterior segments based on matching expression patterns of segment polarity genes (Eriksson et al., 2009, 2010; Franke & Mayer, 2014; Hogvall et al., 2014; Janssen & Budd, 2013; Janssen et al., 2014). Additionally, the segment polarity network is not completely conserved across Arthropoda, given that *wg* is unlikely to play a canonical segment polarity function in spiders (Damen, 2002; Janssen, Gouar, et al., 2010, 2021). Therefore, homologous segments can be produced by different developmental mechanisms.

The hypothesis of Lev et al. (2022) is based on the proposition that differences in gene expression and function between pre-gnathal and postgnathal segments indicate that different character identity networks (ChINs) regulate development in these two groups of segments (see Wagner, 2007). Although the ChIN is a useful concept for understanding the nature of homology, utilizing this concept for distinguishing between different hypotheses of homology is difficult. There is no clear way to identify a ChIN independent of a preconception of what characters are homologous. If the segment polarity network that operates in postgnathal segments in arthropods represents a ChIN, then the postgnathal segments must not be serially homologous to pregnathal segments. By contrast, if the pregnathal segments are serially homologous to the postgnathal segments, then the segment polarity network that operates in postgnathal segments of arthropods must not represent a ChIN. Instead, in the ChIN framework, the differences identified between the networks operating in the pre-gnathal and postgnathal segments could represent differences in character state between these segment groups, rather than character identity. Furthermore, the expression patterns of AP axis patterning genes that support the one-to-one model reflect a highly conserved gene regulatory network that patterns the AP axis across Bilateria (Hejnol & Lowe, 2015). In our view, this gene regulatory network should take priority over any other network for aligning regions of the AP axis between panarthropod lineages.

Distinguishing between the one-to-one model and the model of Lev et al. (2022) primarily depends on whether more weight is given to similarities in expression patterns of AP axis patterning genes among panarthropods (one-to-one model) or the differences in how pre- and postgnathal segments develop within arthropods (model of Lev et al., 2022). This problem represents an interesting case where conclusions about segment homology differ even though there is very little disagreement regarding the developmental underpinnings of these models. Further considerations of the evolutionary and developmental implications of the different models of segment homology will hopefully lead to a clear consensus in the field.

6 | LEG PATTERNING

In arthropods, the leg gap genes, *Dll*, *dachshund* (*dac*), *homothorax* (*hth*), and *extradenticle* (*exd*) are expressed in regionalized patterns across the proximodistal axes of appendages (Angelini & Kaufman, 2005b). They are referred to as leg gap genes because the loss of function of these genes leads to the reduced growth or deletion of the region of the proximodistal axis where they are normally expressed (Angelini & Kaufman, 2005b; Angelini et al., 2012; Bruce & Patel, 2020; Sharma et al., 2013, 2015). During development, *Dll* plays a role in outgrowth, and is expressed in the distal tip of appendages. *Dac* is expressed in the intermediate region of appendages. *Hth* and *exd* are co-expressed in the proximal-most region of appendages. The leg gap genes are expressed in similar regionalized patterns in the unjointed appendages of onychophorans (Janssen, Eriksson, et al., 2010).

Genome and transcriptome analyses have revealed orthologs of *Dll*, *exd*, and *hth* in *H. exemplaris* and *R. varieornatus* (Game & Smith, 2020). However, a *dac* ortholog could not be identified in the genomes of these species. Expression patterns of orthologs of *Dll*, *exd*, and *hth* were previously characterized in *H. exemplaris* embryos (Game & Smith, 2020). Orthologs of *Hth* and *exd* were broadly expressed across the first three leg-pairs. *Dll* was expressed across the entire developing limb bud in all four tardigrade leg-pairs. Regionalized expression patterns of leg gap genes have been identified in developing limbs from species across Bilateria (Pueyo & Couso, 2005; Tarazona et al., 2019). Therefore, tardigrades most likely evolved from an ancestor in which the leg gap genes were expressed in regionalized patterns in developing legs. In this view, the absence of *dac* and the unregionalized expression patterns of the remaining leg gap genes in *H. exemplaris* represent derived states, potentially related to miniaturization and the accompanying secondary simplification of tardigrade legs (Game & Smith, 2020).

We investigated leg gap genes in the genome of *E. testudo*. We identified an ortholog of *Dll* in the transcriptome assembly of *E. testudo* (Supporting Information: Table S1). We could not identify a *Dll* ortholog in the *E. testudo* gene prediction data set in our initial reciprocal BLAST search analysis. However, the *E. testudo Dll* transcript sequence matches a small scaffold of only 1841 nt in length in the genome assembly (jcf7180001566155; identities = 1507/1548) and matches the sequence of the predicted gene from this scaffold (g24451.t1, identities = 823/825), which is missing the *Dll* homeobox. We identified orthologs of *exd*, and *hth* in *E. testudo* genome and transcriptome data sets (Supporting Information: Table S1). We could not identify an ortholog of *dac* in *E. testudo*. The absence of *dac* in the genome assemblies of both eutardigrade and heterotardigrade species suggests that this gene was lost in the tardigrade lineage before the emergence of crown group Tardigrada. Interestingly, some lobopodians had relatively longer legs than are common in Tardigrada, presenting the possibility that the loss of *dac* in Tardigrada is related to reduction in relative leg length in this lineage (Kihm et al., 2023).

7 | WNT SIGNALING

Canonical Wnt (cWnt) signaling regulates several important developmental processes in Arthropoda, including posterior growth and segment polarity (McGregor et al., 2009). Expression patterns of Wnt ligand-coding genes suggest that these roles are conserved in the onychophoran *E. kanangrensis* (Hogvall et al., 2014). However, among other differences compared to arthropods, some Wnt ligand-coding genes are first expressed in Hox-like regionalized patterns before segmentation in *E. kanangrensis* (Hogvall et al., 2014). Several Wnt-ligand coding genes are also expressed in Hox-like regionalized patterns during development in the tardigrade *H. exemplaris* (Chavarria et al., 2021). Such regionalized expression patterns of Wnt ligand-coding genes may reflect ancestral functions of these genes that are retained in tardigrades and onychophorans, but that have been lost in the arthropod lineage. Unlike in other panarthropods, Wnt ligand-coding genes are not expressed in stripes during the segmentation stage in *H. exemplaris* (Chavarria et al., 2021). Therefore, these genes do not appear to play roles in regulating segment polarity during the segmentation stage, but could regulate segment polarity at later stages, for example, during leg development.

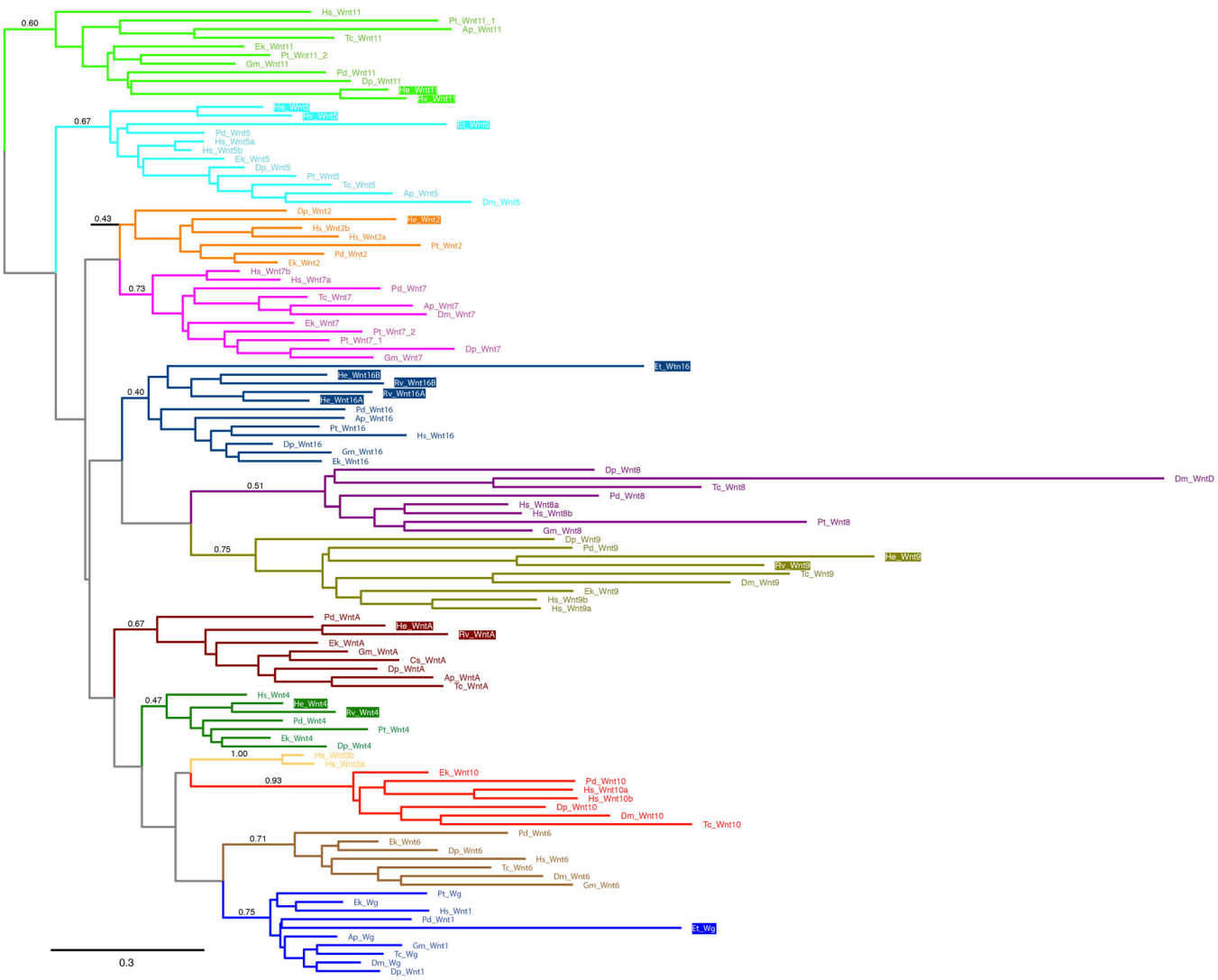

FIGURE 4 Majority rule consensus Wnt phylogeny. The consensus is based on three maximum likelihood trees. Tardigrade sequences are in colored boxes. Bootstrap support values are provided as percentages out of 500 replicates. LG + G + I was the model used for analyses. The tree is unrooted. Ap, *Acyrthosiphon pisum*; Cs, *Cupiennius salei*; Dp, *Daphnia pulex*; Dm, *Drosophila melanogaster*; Ek, *Euperipatoides kanangrensis*; Et, *Echiniscus testudo*; Gm, *Glomeris marginata*; He, *Hypsibius exemplaris*; Hs, *Homo sapiens*; Pd, *Platynereis dumerilii*; Pt, *Parasteatoda tepidariorum*; Rv, *Ramazzottius varieornatus*; Tc, *Tribolium castaneum*. [Color figure can be viewed at wileyonlinelibrary.com]

Chavarria et al. (2021) analyzed the genomes of *H. exemplaris* and *R. varieornatus* to characterize components of the cWnt signaling pathway in Tardigrada. They identified most intracellular components associated with the cWnt signaling pathway, including a full complement of Frizzled membrane-bound receptors. However, they could not identify an ortholog of the coreceptor Arrow (Arr), also referred to as LRP5/6, in either species. Furthermore, they could not identify several orthologs of Wnt ligand-coding genes that were predicted to be present, including an ortholog of *wingless* (*wg*), based on comparative studies (Janssen, Gouar, et al., 2010). Here we analyzed genome and transcriptome assemblies of *E. testudo* to identify cWnt signaling genes (Supporting Information: Table S1). We were only able to identify three Wnt orthologs in *E. testudo*. Interestingly, one of these appears to represent an ortholog of *wg* (Figure 4), which suggests that this gene was present in the last common ancestor of Tardigrada, and was lost in a eutardigrade ancestor of *H. exemplaris* and *R. varieornatus*. We could only identify two distinct sequences that encode both the Frizzled domain and the seven-(pass)-transmembrane domain that are characteristic of Frizzled orthologs. These sequences did not cluster with any Frizzled ortholog group with strong support (Supporting Information: Figure S1). The protein

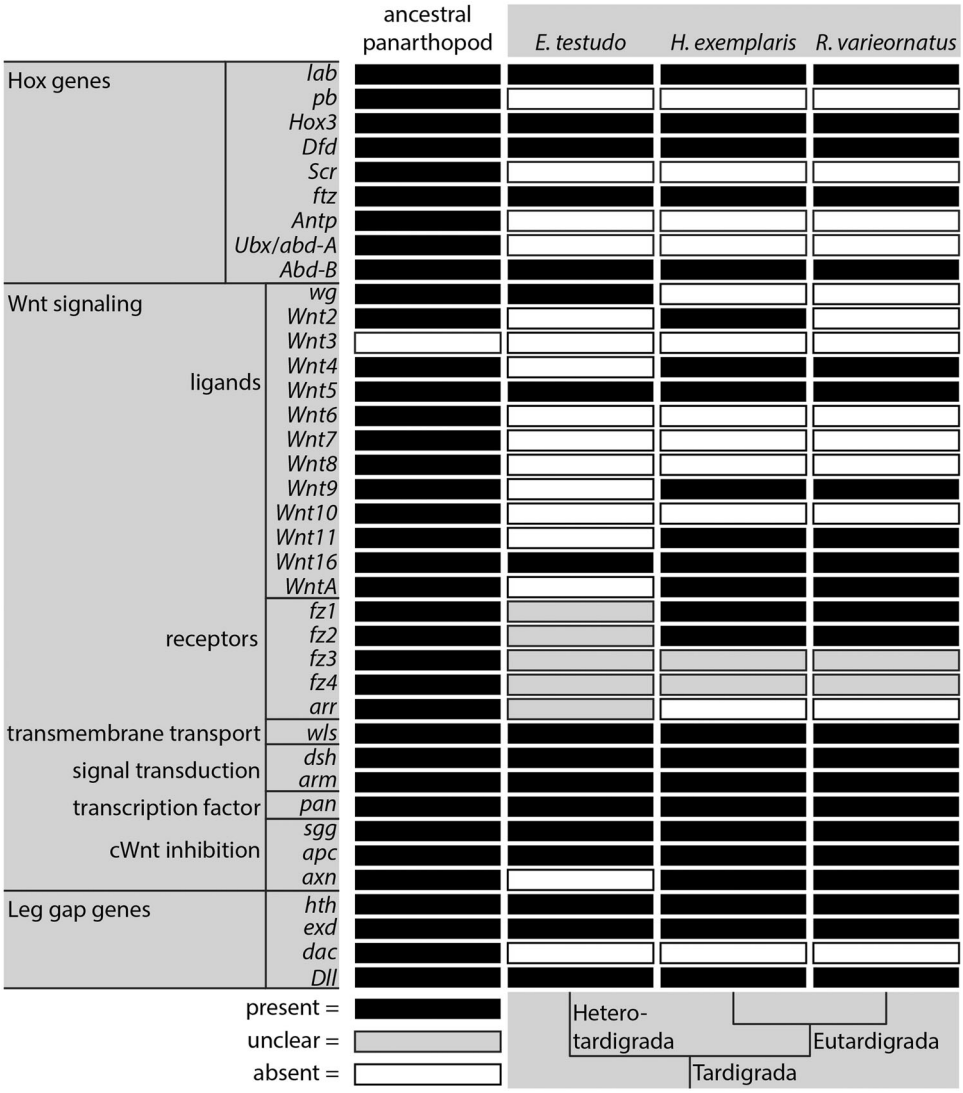

FIGURE 5 Summary of tardigrade genome and transcriptome analyses. Only species with publicly available genome assemblies are summarized. For tardigrades, black shading indicates that at least one ortholog was identified in a genome/transcriptome assembly. White shading indicates that an ortholog was not identified in a genome/transcriptome assembly. Gray shading indicates low support for the presence of an ortholog in a genome/transcriptome assembly. Genes that are denoted as present in the ancestral panarthropod are found in onychophorans or arthropods and outgroups of Panarthropoda. The orthology of Fz orthologs in *Echiniscus testudo* is unclear based on our phylogenetic analyses. Eutardigrades have four Fz orthologs, but two are poorly resolved. We identified a candidate ortholog of *arr* in *E. testudo*, but the predicted protein structure for this gene was unusual compared to known orthologs.

structure of the best candidate Arr coding sequence was unusual compared to typical orthologs of this protein (Supporting Information: Figure S2). We identified candidate orthologs of *armadillo* (*arm*), also referred to as *β-catenin*, *dishevelled* (*dsh*), *pangolin* (*pan*), and *wntless* (*wls*). We also identified candidate orthologs of *shaggy* (*sgg*) and *adenomatous polyposis coli tumor suppressor* (*apc*), inhibitors of cWnt signaling as components of the β-catenin destruction complex (Stamos & Weis, 2013). We could not identify an *axin* (*axn*) ortholog in *E. testudo*, an additional β-catenin destruction complex component.

8 | CONCLUSION

Tardigrades have lost many transcription factors and signaling molecules that are typically highly conserved in bilaterian animals, and that typically regulate important developmental processes. Our analyses suggest that many of these losses are ancestral features of Tardigrada (Figure 5). Both eutardigrades and heterotardigrades are also missing the internal components of the Toll pathway (Mapalo et al., 2020), which plays important roles in regulating development broadly across Metazoa (Anthoney et al., 2018). The explanation for these losses

may reside in the minute size of these animals. The loss of many highly conserved developmental genes may be associated with the evolution of a miniaturized body plan in the stem tardigrade lineage (Chavarria et al., 2021; Game & Smith, 2020; Smith et al., 2016). Like many other minute animals, Tardigrada is thought to have a meiofaunal origin (Giere, 2008). The wide distribution of meiofaunal animals has raised the question of whether Ecdysozoa or Spiralia have meiofaunal origins or whether highly miniaturized meiofaunal lifestyles evolved several times independently in these lineages (Worsaae et al., 2023). As with tardigrades, other lineages with meiofaunal origins, such as Rotifera, Nematoda, and Platyhelminthes are missing several Wnt ligand-coding genes or Hox genes (Aboobaker & Blaxter, 2003; Chavarria et al., 2021; Fröbius & Funch, 2017; Liu et al., 2018; Riddiford & Olson, 2011). We hypothesize that these independent losses represent a macroevolutionary trend in genome evolution related to secondary miniaturization and the anatomical simplification that accompanies this process. This macroevolutionary trend may be explained by limited evolutionary pathways to miniaturization and predictable consequences of this process (Chavarria et al., 2021). In this view, miniaturization that accompanies a meiofaunal lifestyle evolved independently several times in both Spiralia and Ecdysozoa. As the field of evolutionary developmental biology continues to advance, we will gain a better picture of the developmental mechanisms that were active in ancient ancestors of animal phyla, and a better understanding of how these mechanisms were modified to produce the incredible diversity of animal body plans.

9 | METHODS

9.1 | Identifying candidate genes

We used reciprocal BLAST search analyses to identify genes of interests from a recently published *E. testudo* transcriptome assembly and gene predictions from a genome assembly (Murai et al., 2021). We used CD-Search to confirm that candidate genes encoded predicted conserved protein domains (Marchler-Bauer & Bryant, 2004). We used ORFinder (Rombel et al., 2002) or Augustus (Stanke & Morgenstern, 2005) for protein translations. For phylogenetic analyses, amino acid sequences were aligned with MUSCLE (Edgar, 2004). Alignments were trimmed with Gblocks (Castresana, 2000). Maximum likelihood phylogenetic analyses were performed in PhyML with automatic model selection by SMS using BIC (Guindon et al., 2010; Lefort et al., 2017). At least three maximum likelihood analyses were performed on each alignment. Resulting trees were visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Majority rule consensus trees with a required clade frequency of 0.5 were produced in Mesquite (Maddison & Maddison, 2018). For Hox genes, we used Jalview to make visual representations of protein alignments (Waterhouse et al., 2009). Diagnostic amino acid residues for specific Hox orthologs were identified based on Janssen et al. (2014).

9.2 | In situ hybridization, immunohistochemistry, and imaging

Anti β-tubulin and phalloidin stainings, and microsocopy for these stainings were performed as in Smith et al. (2017). Hybridization chain reaction (HCR) in situ was based on a previously published protocol (Smith, 2018) with modifications for HCR based on protocols provided by the manufacturer (Molecular Instruments). A detailed HCR in situ protocol is provided as a supplement (Supplemental Information: Protocol S1). Tardigrade specimens were mounted in DAPI Fluoromount-G (SouthernBiotech). HCR in situ data was collected on an Olympus FV1000 Fluoview confocal microscope using a UPlanSApo 100×/1.40 oil objective. *E. peripatoides* in situ hybridization and imaging were performed as in Janssen, Eriksson, et al. (2010).

AUTHOR CONTRIBUTIONS

The first draft of the manuscript was written by Frank W. Smith, Mandy Game, Raul A. Chavarria, and Taylor R. Harrison. All authors made significant contributions to the revised manuscript. Frank W. Smith performed genomic and phylogenetic analyses. Frank W. Smith and Mandy Game prepared figures. Mandy Game and Marc A. Mapalo performed HCR in situ on *H. exemplaris* embryos. Ralf Janssen perform in situ hybridization on *E. kanangrensis* embryos. Frank W. Smith performed antibody and phalloidin staining on *Milnesium* n. sp. specimens. All authors approved the final draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The *E. testudo* transcriptome and genome sequence data are publicly available under NCBI BioProject accession number PRJNA669587 (Murai et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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