Molecular evidence for a single origin of ultrafiltration-based excretory organs

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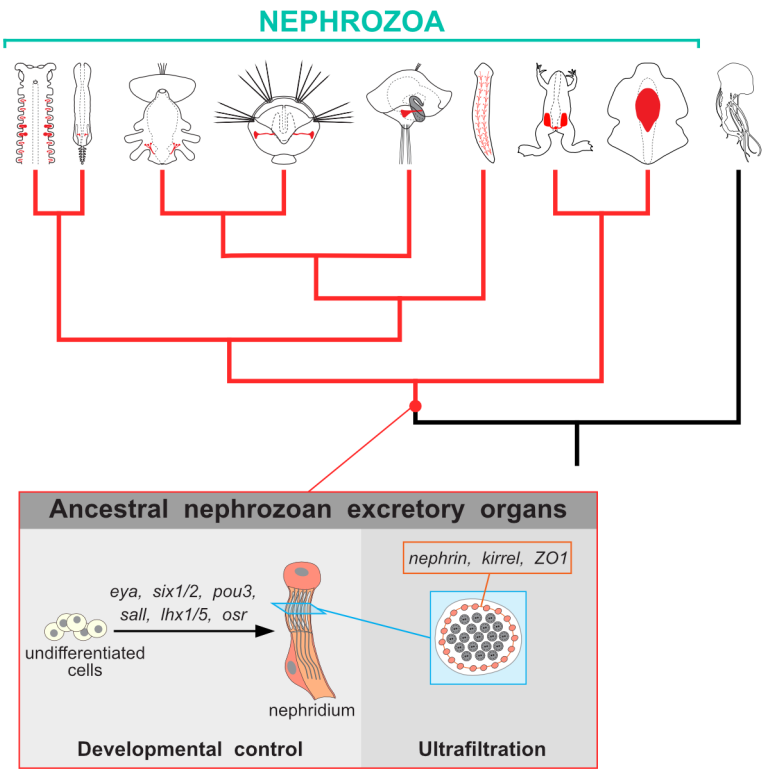


Summary

Excretion is an essential physiological process, carried out by all living organisms regardless of their size or complexity[1-3]. Both protostomes (e.g. flies, flatworms) and deuterostomes (e.g. humans, sea urchins) possess specialized excretory organs serving that purpose. Those organs exhibit an astonishing diversity, ranging from units composed of just few distinct cells (e.g. protonephridia) to complex structures, built by millions of cells of multiple types with divergent morphology and function (e.g. vertebrate kidneys)[4, 5]. Although some molecular similarities between the development of kidneys of vertebrates and the regeneration of the protonephridia of flatworms have been reported[6, 7], the molecular underpinnings of the development of excretory organs has never been systematically studied in a comparative context[4]. Here we show that a set of transcription factors (*eya, six1/2, pou3, sall, lhx1/5, osr*) and structural proteins (*nephrin, kirre, zo1*) is expressed in the excretory organs of a phoronid, brachiopod, annelid, onychophoran, priapulid and hemichordate that represent major protostome lineages and non-vertebrate deuterostomes. We demonstrate that the molecular similarity observed in the vertebrate kidney and flatworm protonephridia[6, 7] is also seen in the developing excretory organs of those animals. Our results show that all types of ultrafiltration-based excretory organs are patterned by a conserved set of developmental genes, an observation that supports their homology. We propose that the last common ancestor of protostomes and deuterostomes already possessed an ultrafiltration-based organ that later gave rise to the vast diversity of extant excretory organs, including both, proto- and metanephridia.

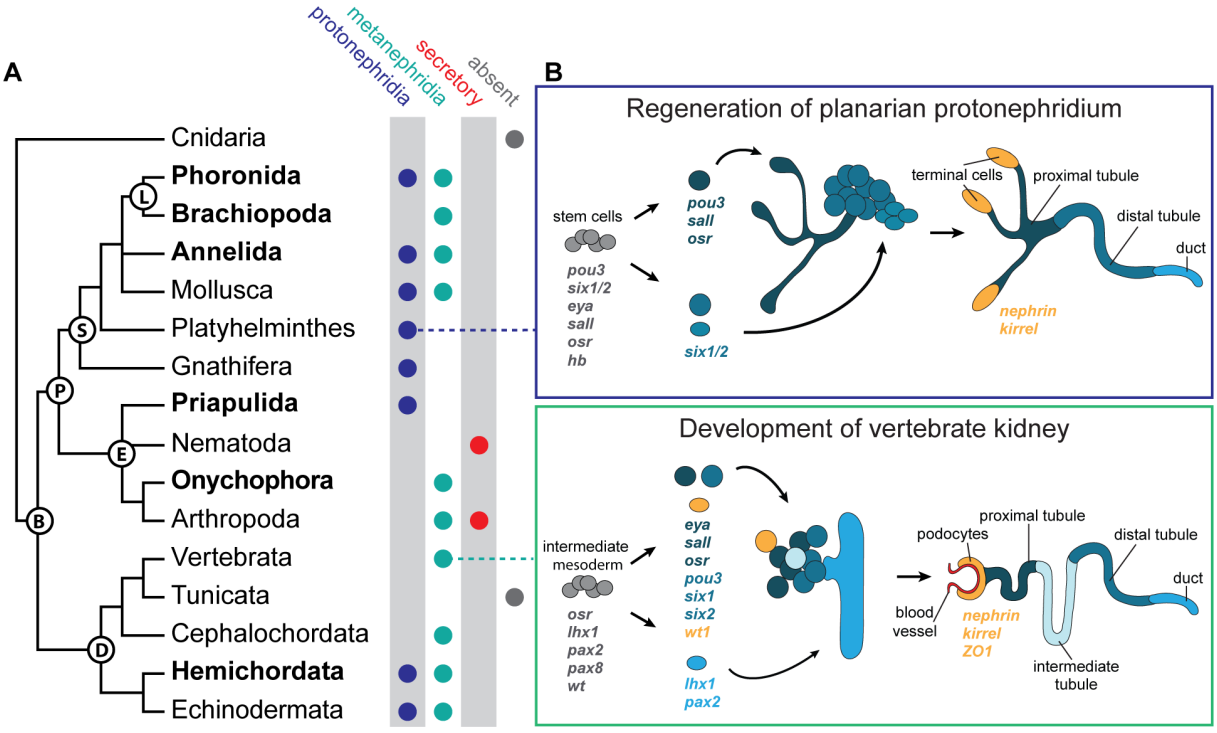
Results and discussion

Excretory organs are thought to be one of the key evolutionary innovations of the emergence of complex body plans[8, 9], and are believed to facilitate the conquest of new habitats, such as freshwater and terrestrial environments[3, 10]. Specialized excretory organs are a common

feature of protostomes and deuterostomes (Figure 1A), sometimes united in the clade Nephrozoa [8, 9, 11] (however see [12, 13] and references therein for the recent discussion on the topic whether Nephrozoa is not simply synonymous with Bilateria). The diverse excretory organs can be grouped into secretory organs (e.g. Malpighian tubules of insects), in which primary urine is produced by the means of active, transcellular transport[3, 4, 10], and organs that are based on the principle of ultrafiltration (UF). The latter is a pressure-driven physiological process in which the body fluid (e.g., blood) of the organism is filtered through an extracellular filter to produce primary urine[3, 4, 10, 14-17]. UF-based excretory organs include human kidneys, but also protonephridia and metanephridia, present in numerous invertebrates[3, 4, 14-17]. Their extracellular filters share ultrastructural and molecular properties, even between distantly related animals with divergent morphologies of their excretory organs[7, 15], which might suggest a common evolutionary origin of UF[3, 14-17]. Two main hypotheses on the evolution of the UF-based excretory organs have been proposed based on their comparative morphology[3]. In the first[16, 17], all the UF-based excretory organs are homologous and differences in their architecture depend solely on the animal body size (protonephridia in small animals, metanephridia in larger ones). The second hypothesis proposes that protonephridia represent the ancestral organs that were replaced by the metanephridia several times independently, as a consequence of the development of the secondary body cavities (i.e. the coelom)[14].

Despite the importance of excretory organs for animal evolution, relatively little is known about the molecular basis of their development: the developmental genetic interactions of UF-based excretory organs have been so far described only for the vertebrate kidney (Figure 1B)[4]. Interestingly, some of the transcription factors (TFs) (*eya*, *six1/2*, *pou3*, *sall*, *osr*) involved in the development of the vertebrate kidney are also expressed during the regeneration of the flatworm protonephridia (Figure 1B)[6]. These similarities might suggest homology between

kidneys and protonephridia[6]. However, even though sets of conserved regulatory molecules might imply putative common ancestry of a structure between species[18, 19], such inference becomes problematic when the homology is based on findings in only two distantly related species[20]. Evolutionary comparisons require broader sampling of multiple intermediate evolutionary lineages that could bridge the evolutionary distance[20, 21]. Therefore, a comparison of gene expression during the development of multiple, divergent UF-based excretory organs is desired to test homology of the molecular underpinnings of their development. Here, we studied the expression of *eya, six1/2, pou3*, *sall*, *hb* and *osr,* genes known to be involved in the regeneration of planarian protonephridia[6], as well as *lhx1/5*, a homologue of the vertebrate gene *lim1*, indispensable for the formation of nephric tubules in vertebrates[22, 23]. Although all of those transcription factors have multiple functions during development of our study species, we aimed to systematically test, whether their unique combined expression profiles, specific for vertebrate kidneys and planarian protonephridia, can be detected specifically in the UF-based excretory organs of other protostomes and deuterostomes.



Molecular development of spiralian nephridia

In order to study the expression of our candidate genes during nephridial development in two closely related species, but with divergent morphology of their excretory organs, we first investigated the phoronid *Phoronopsis harmeri* and the brachiopod *Terebratalia transversa*. Phoronids and brachiopods (together lophophorates) belong to the large animal group called Spiralia (Figure 1A), which also includes flatworms, molluscs and annelids[8, 11].

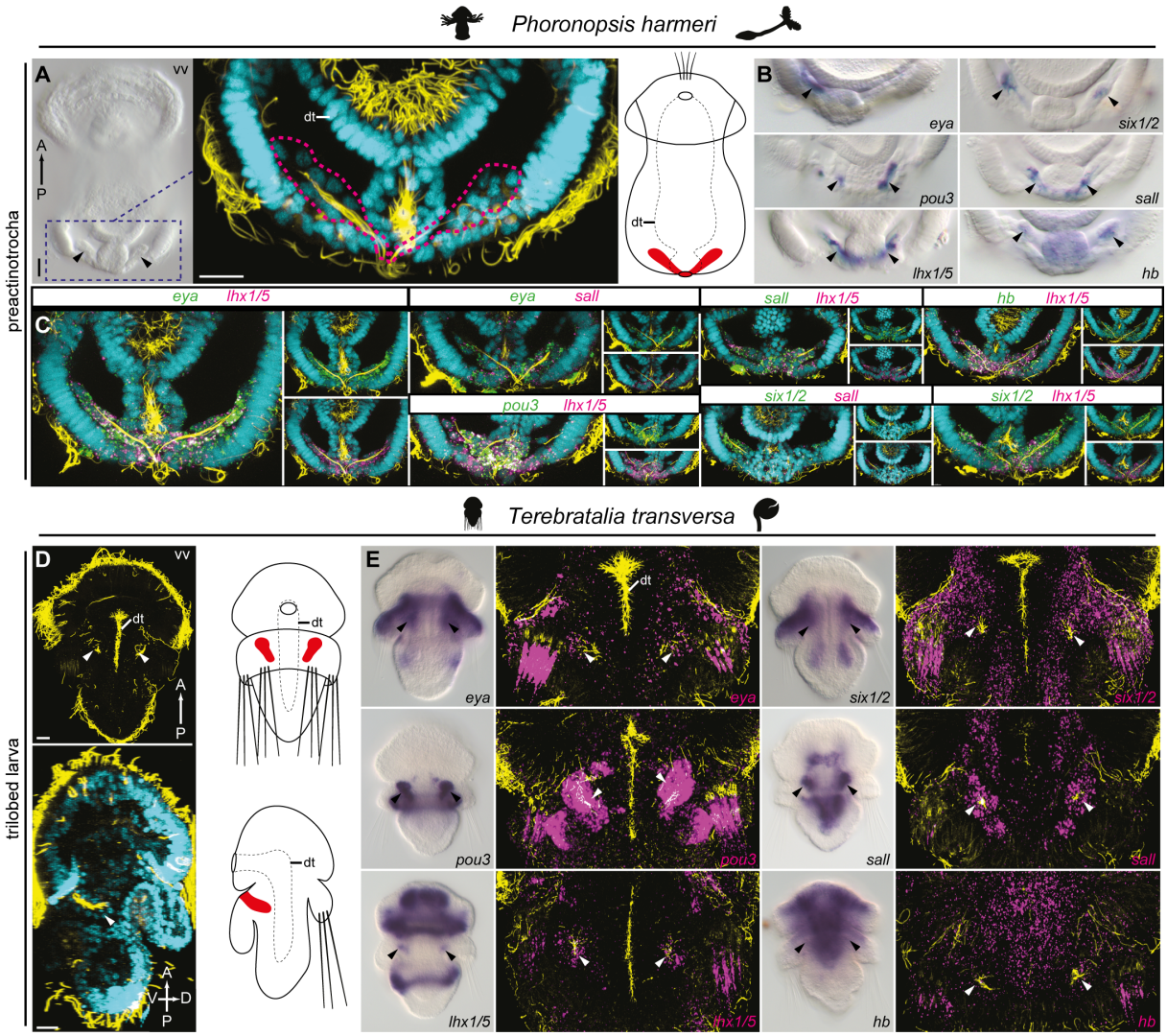
Phoronids develop through a long-lived actinotrocha larva, which possess a pair of ciliated protonephridia (Figures 2A, S1A, B)[24, 25]. During metamorphosis the terminal portion of the larval protonephridia responsible for filtration is lost, while the nephridioduct merges with the newly developing ciliated funnel, giving rise to the definitive adult metanephridium[24, 25] that is associated with the podocyte-lined filtration sites in the coelom [26]. We detected expression of all of our candidate genes in the developing protonephridia (Figures 2B, C, S1A, B), with the exception of *osr*, which instead was exclusively expressed in the sphincters of the digestive tract (Figure S2). Double *in situ* hybridization showed that most of the TFs are co-expressed in the identical cells of the protonephridial rudiments at the pre-tentacular larval stage (Figure 2C), similar to what was demonstrated for the flatworm progenitor cells, from which protonephridia regenerate[6].

In contrast to phoronids, brachiopods develop through a rather short-lived larval stage[27]. Those larvae possess a pair of simple ciliated structures interpreted as larval nephridial rudiments (Figure 2D)[27], which give rise to the adult metanephridia shortly after metamorphosis (Figure S1C)[28, 29]. We detected expression of *eya, six1/2, pou3*, *sall*, *lhx1/5* and *hb* in those larval nephridial rudiments (Figure 2E), which persisted (with the exception of *hb*) in the juvenile metanephridia after metamorphosis (Figure S1D). Out of those TFs, *pou3* and *sall* seem to be broadly expressed in entire nephridial rudiments in both larvae and juveniles (Figures 2E, S1D), while expression of *eya*, *six1/2* and *lhx1/5* is restricted to the more median

domain in larvae (Figure 2E) and to the proximal nephrostome of the juvenile metanephridium (Figure S1D), indicating molecular compartmentalization of the brachiopod nephridium. As in phoronids, we did not detect expression of *osr* in the nephridial tissue; instead, the gene was expressed in the anterior digestive tract in both larvae and juveniles (Figures S1D, S2B). These data suggest that the same developmental regulatory program used for patterning of protonephridia (in both the planarians[6] and phoronids), seems to be deployed for the brachiopod metanephridia, despite their different morphology.

While most spiralians excrete either by simple protonephridia or typical metanephridia (Figure 1A)[3, 14], some of them evolved more divergent types of UF-based excretory organs. To test whether these more aberrant nephridial types also express the same genes, we studied the annelid *Owenia fusiformis*. As a larva, *O. fusiformis* possesses distinct excretory organs (Figure 3A) that differ greatly from the excretory organs of other spiralians, and which, based on their morphology and ultrastructure, were described as "deuterostome-like nephridia"[30]. In contrast to the previous reports[30, 31] that suggested loss of larval protonephridia during metamorphosis, we found that those ciliated larval organs contribute to the adult metanephridia as a pair of ciliated dorsal ducts (Figure S3A), while structures interpreted previously as metanephridial rudiments[32] are non-ciliated (Figure S3A) and probably represent prospective tube-secreting glands[33, 34]. *Eya*, *six1/2*, *sall* and *hb* were all expressed in the protonephridia of both early and advanced larvae (Figure 3B), while *pou3* expression was detected in the nephridial duct only at the late larval stage (Figure 3B). Out of the four paralogues of *lhx1/5* present in the transcriptome of *O. fusiformis,* one was detected in the developing nephridia (Figure 3B), while neither of the two *osr* paralogues was expressed in the nephridial tissues (Figure S2C). Instead, they were expressed in the sphincters of digestive tract (similar to phoronids and brachiopods) as well as in the epidermis of the adult worm rudiment (Figure S2C). Altogether these results show that even peculiar, lineage-specific spiralian UF-based

excretory organs deploy the same conserved set of molecular developmental TFs during their development.



Molecular development of UF-based organs in Ecdysozoa

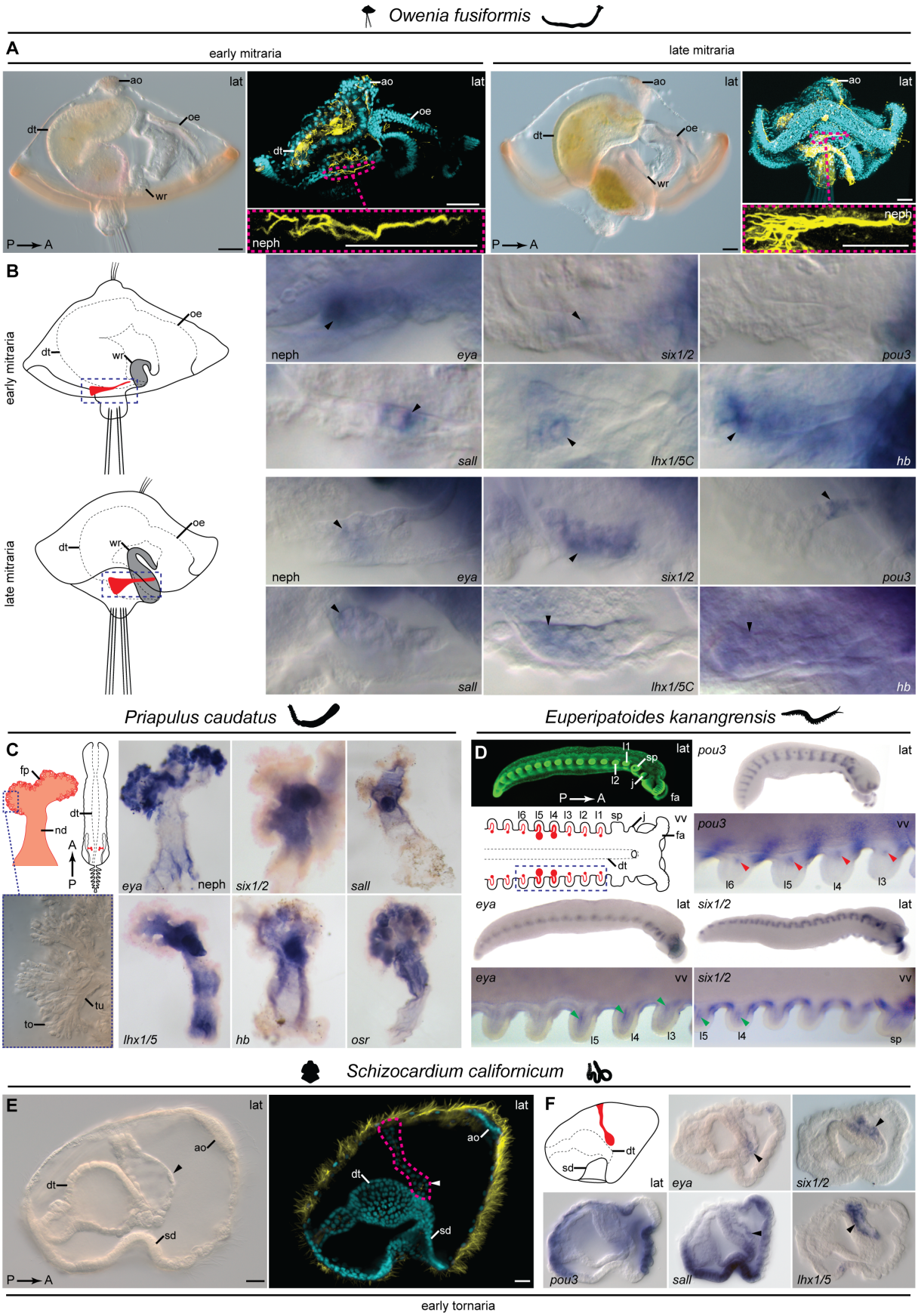
Since we showed that the expression of the developmental nephridial genes is highly conserved among various spiralian species, we examined whether a similar conservation is present in members of the second major protostome group, the Ecdysozoa. Two of the well-studied invertebrate model systems – the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster* – belong to Ecdysozoa (Figure 1A); however, they both lack UF-based excretory organs and instead use presumably derived secretion-based excretory systems[3, 4] that develop without expressing the aforementioned conserved set of nephridial TFs[35, 36]. Therefore, in

order to reconstruct the ancestral molecular patterning of nephridiogenesis of the Ecdysozoa, it is essential to study species with UF-based excretory organs. Here, we investigated the expression of these conserved genes in two species that deploy UF for excretion, the priapulid *Priapulus caudatus* and the onychophoran *Euperipatoides kanangrensis*.

In the case of *P. caudatus,* we studied gene expression in dissected adult protonephridia, which are part of the posteriorly positioned urogenital system and include the nephroduct as well as the filtering part, composed of fine tubules and terminal organs with ultrafiltration sites (Figure 3C)[37, 38]. *In situ* hybridization of *eya*, *six1/2*, *sall*, *lhx1/5*, *hb* and *osr* in the adult protonephridium (Figure 3C) show that these genes are expressed in different portions of the organ – e.g., *eya* is mainly expressed in the terminal filtering portion, while expression of *six1/2*, *hb* and *lhx1/5* is restricted to the nephroduct (Figure 3C).

Directly developing onychophorans (Figure S3B) possess serial, ciliated metanephridia in each trunk segment (Figure 3D), which develop directly from the mesodermal rudiment at the base of each leg and, unlike the metanephridia of spiralians, are not preceded by any larval organs[39]. Expression of some of the candidate nephridial genes has been already investigated in onychophorans and showed that *osr* and *lhx1/5* are indeed expressed in the metanephridial rudiments[40, 41], while *hunchback* does not seem to be directly involved in nephridiogenesis[42] (however it is expressed in the limb mesoderm, which might include some progenitor cells giving rise to the metanephridia[39]). Three of the remaining TFs (*eya*, *six1/2* and *pou3*) are expressed in the developing metanephridia associated with the walking appendages (Figures 4D, S3C). In the advanced embryos, *pou3* is expressed more proximally, likely in the filtering portion of the nephridium (the so-called sacculus), while *eya* and *six1/2* mark the more distal, nephridial canal (Figure 3D). Signal from the probes against each gene is the strongest in the leg segments 4 and 5 (Figures 3D, S3C), which corresponds to the presence of larger nephridia in those segments[39].

These results show that the conserved set of TFs expressed during the development of spiralian proto- and metanephridia is also likely to be involved in the formation and maintenance of corresponding UF-based excretory organs in Ecdysozoa. Moreover it shows that onychophoran metanephridia, which are not developing from any protonephridial rudiments [39] and therefore cannot be simply regarded as ontogenetically homologous to protonephridia, express the same set of regulatory genes during their development.


Owenia fusiformis
A
early mitraria
late mitraria
ao
lat
oe
dt
wr
P → A
neph
B
early mitraria
late mitraria
eya
six1/2
pou3
sall
lhx1/5C
hb
Priapulus caudatus
Euperipatoides kanangrensis
C
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Schizocardium californicum
E
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F
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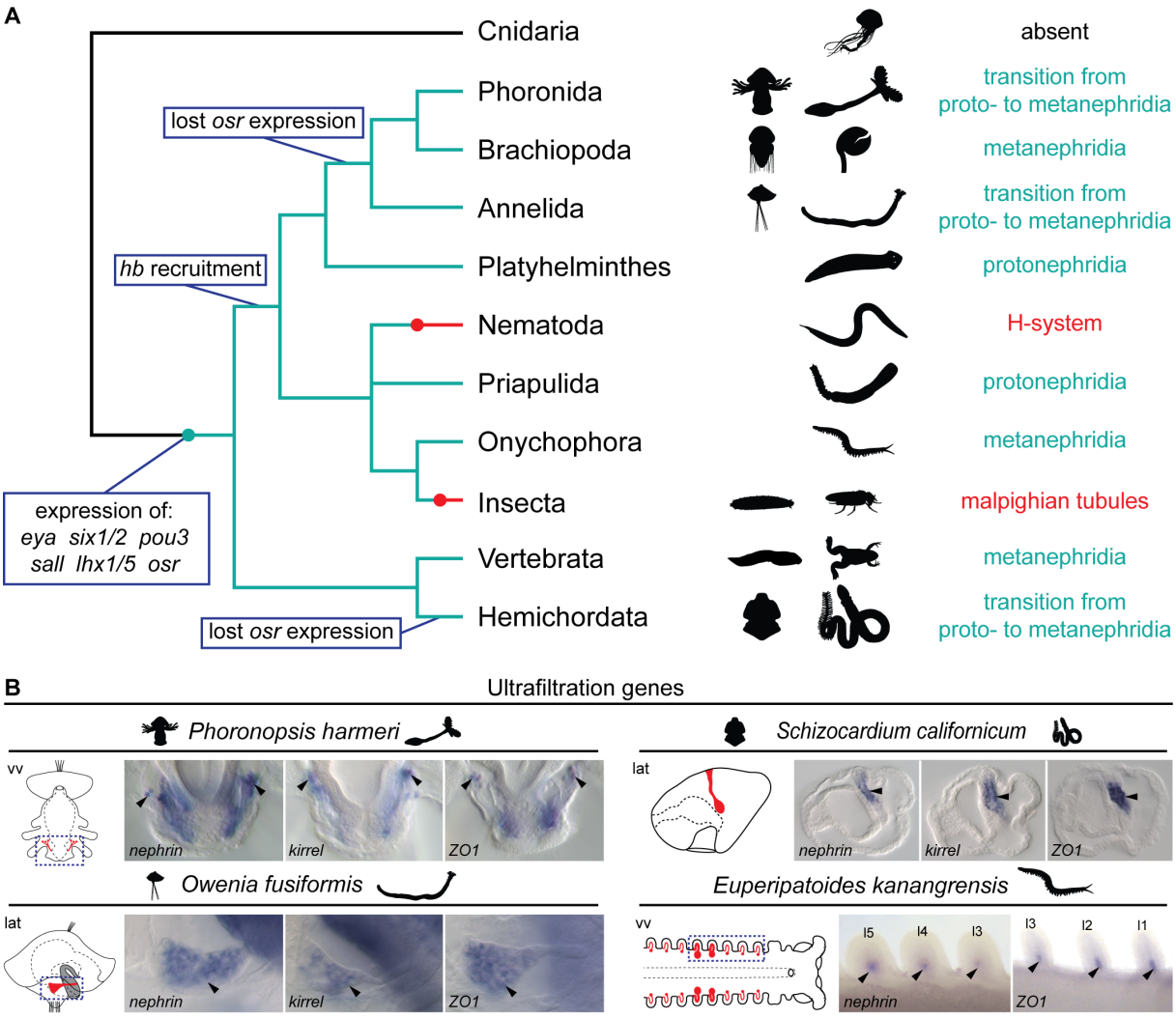
Molecular development of nephridia in non-vertebrate deuterostomes

The genetic control of vertebrate kidney development is well described, however vertebrate kidneys represent evolutionarily derived metanephridial system[4]. To investigate gene expression during the development of deuterostomes with less derived excretory organs, we tested the expression of the conserved TFs in the indirect developing hemichordate *Schizocardium californicum*. Excretion in hemichordates is performed through an anterior coelomic compartment that initially functions as a protonephridium in the larva, and later gives rise to the adult metanephridial system with a podocyte-lined ultrafiltration site[43-46]. In *S. californicum* the larval protonephridium appears in early larvae as an unpaired anterior coelomic vesicle connected by a ciliated canal with the dorsal epidermis (Figure 3E)[47]. *Eya*, *six1/2*, *sall* and *lhx1/5* are expressed in the larval protonephridium (Figure 3F), with *eya* and *six1/2* restricted to the terminal and canal portion of the organ, respectively (Figure 3F). Expression of *six1/2* and *eya* has been also reported in the presumptive larval nephridium of a sea urchin[48, 49], another non-vertebrate deuterostome, which belongs to the sister group of hemichordates, the echinoderms. Furthermore, the expression of *lhx1/5* has been reported in the larval nephridium of amphioxus [50], supporting conservation of this TF also in the nephridial development of non-vertebrate chordates. We did not detect transcripts of *pou3* and *osr* in the developing protonephridium of the investigated developmental stage of *S. californicum*. Instead, *pou3* shows a broad ectodermal expression (Figure 3F), while *osr* is expressed in the digestive tract in the pattern similar to what was observed in lophophorates and annelids (Figure S2D).

These results show that the molecular similarities of vertebrate kidney and protostome nephridial development are also observed in the UF-based excretory systems of non-vertebrate deuterostomes. These data support the idea that the conserved set of nephridial developmental TFs has been inherited from the last common ancestor of protostomes and deuterostomes

(Figure 4A). Although recently the basal position of Xenacoelomorpha in the bilaterian tree of life has been challenged again[12, 13] and monophyly of deuterostomes has been questioned [51], these ambiguities do not directly influence our reconstruction of the ancestral set of nephridia-related transcription factors. Regardless of the topological controversies, the demonstrated molecular similarities could be traced back to the last common ancestor of protostomes and Ambulacraria (Figure 4A), in which the ancestral excretory organs were already present.



Conservation of the ultrafiltration proteins

We showed molecular similarities in the expression of several TFs during the development of anatomically diverse UF-based excretory organs. Next, we wanted to test whether these diverse excretory organs share also the expression of genes encoding for structural proteins known for

the formation of the filtering site in the excretory organs of vertebrates and flatworms[4, 7, 15]. Such molecular conservation would further support the presence of a filtration apparatus necessary for UF and production of primary urine in the ancestral excretory organs.

We investigated the expression of three structural genes (*nephrin*, *kirrel* and *zo1*) in the phoronid, brachiopod, annelid, onychophoran and hemichordate. All three genes are expressed in the filtering portion of the excretory organs in phoronids, annelids and hemichordates (Figure 4B). In onychophorans, *nephrin* and *zo1* are specifically expressed in the filtering sacculus at the base of each walking leg (Figures 4B, S3D), however the *kirrel* homologue is only transiently expressed in the posterior portion of the embryo and seems to be missing from the sacculi (Figure S3D). This indicates that the UF site of Onychophora might be formed without this otherwise conserved molecule, paralleling the situation in birds, in which, despite the loss of *nephrin* gene, a typical UF site is present[52-54]. In brachiopods, we did not observe expression of the UF genes in the larval nephridial structures (Figure S1E), which are likely non-functional rudiments. However, in post-metamorphic juvenile brachiopods, the UF genes are broadly expressed (Figure S1F), including the periesophageal coelom, the presumptive site of ultrafiltration in adult brachiopods[55].

The observed conservation of the UF genes among distantly related animals with ciliated excretory organs suggest that the ancestral excretory organ already used a filter composed out of Nephrin, KIRREL and ZO1 proteins and deployed ultrafiltration as a mechanism for excretion. The orthologues of the UF-related genes are also present in the potential sister group of Nephrozoa, the xenacoelomorphs (which lack excretory organs), where the genes are broadly expressed in tissues not related with excretion, e.g. in gonads and nervous system[56]. This suggests that the recruitment of those three structural genes into the formation of an excretory filter was an important step in the evolution of specialized excretory organs in the lineage of Nephrozoa. However, subsequent losses of ultrafiltration function of those genes were followed

in some animals, e.g. Nephrin in birds[52-54] and some flatworms[57], and KIRREL in onychophorans (this study). Interestingly, in numerous Nephrozoa *nephrin* and *kirrel* are expressed not only in the excretory organs but also in the nervous system, where they contribute to the neuronal guidance [53, 58-61], indicating that the potentially ancestral neural expression, shared with xenacoelomorphs[56], was not lost despite the gain of a new role in the UF site formation. On the other hand, if xenacoelomorphs represent a sister group to Ambulacraria[12, 13], then the expression of *nephrin*, *kirrel* and *zo1* in the nervous system and excretory organs is equally old (dating back to the common bilaterian ancestor) and lack of excretory function of those genes in xenacoelomorphs[56] should be seen as a consequence of secondary loss of excretory organs in that clade.

Conclusions

The homology of UF-based organs has been proposed by some comparative morphologists based on the continuity of different types of excretory organs (e.g., ontogenetic transition from proto- to metanephridia[3, 14, 24, 25] or existence of morphologically intermediate forms[16, 17]). Here we demonstrated for the first time that different types of excretory organs are not only similar structurally, but also molecularly, as several transcription factors (*eya*, *six1/2*, *pou3*, *sall*, *lhx1/5*, *osr*) are expressed during their development. Therefore, we showed the presence of a deeply conserved molecular signature of the excretory organs (Figure 4A), which provides further support for the homology of all types of UF-based organs. Whether those TFs were conserved independently or as a part of the same gene regulatory network remains an opened question that was beyond the scope of this paper and would requires the functional analyses. Nevertheless, this conserved set of transcription factors can be used for identification of rudimentary excretory structures never described before or as a molecular signature of excretory cell clusters in large datasets that emerge from single-cell transcriptomics.

Importantly, this does not contradict the idea that protonephridia gave rise to metanephridia several times independently over the course of animal evolution[14]. It indicates, however, that metanephridia and protonephridia can be seen as modifications of the same ancestral excretory organ[14, 16, 17]. Therefore, metanephridia are homologous as excretory organs (since it is possible to trace them to a common ancestral excretory organ) but are convergent as "metanephridial type" of organs (since this ancestral organ was not a metanephridium). A simple parallel can be made to vertebrate limbs: the wings of bats and birds are homologous as forelimbs (and patterned by homologous gene regulatory networks[62]) but are convergent as wings.

We also provide evidence for the evolutionary conservation of the proteins involved in the formation of the site for UF, suggesting the presence of UF in the common ancestor of protostomes and deuterostomes (Figure 4A). The ancestral nature of UF is further supported by the distribution of UF-based organs on the phylogenetic tree (Figure 1A) and that the UF-sites share morphological similarities between distantly related clades[3, 4, 14-17]. Therefore, despite the lack of unambiguous fossil record of the nephrozoan ancestor, it is possible to reconstruct its excretory organs as ciliated and UF-based.

Although we showed a conservation of TFs and structural gene expression in nephridia across animal phylogeny, we also found some intriguing clade-specific differences from the putative bilaterian ancestral state. Most importantly, it seems that *osr* lost its nephridia-related expression in one of the spiralian subclades, while the protostome-specific *hb* became co-opted into nephridial patterning at the base of the protostome lineage (Figure 4A). Further clade-specific or organ type-specific differences are expected to be revealed with more detailed studies of particular nephrozoan species. Those differences might be related with such phenomena as re-wiring of preexisting gene regulatory networks, developmental system drifts,

gene cooption or changes in the temporal or spatial expression of genes during nephridiogenesis.

Although we now have a better understanding of the early evolution of UF-based excretory organs, there are still many aspects of the evolution of excretion that require further investigation. In particular, the origin of secretory excretory organs, present in the most studied invertebrate model systems – nematodes (the so-called H-system) and insects (Malpighian tubules) – remains obscure. In *D. melanogaster* and *C. elegans* those organs neither express the conserved set of nephridial genes nor develop from any identifiable UF-based rudiments[35, 36, 63] and thus most likely emerged *de novo* (Figure 4A). Besides Malpighian tubules, insects also possess specialized excretory cells – nephrocytes – that perform an ultrafiltration-like process with a filter composed of Nephrin, KIRREL and ZO1[64]. The presence of this conserved UF apparatus as well as the morphological data from crustaceans, in which nephrocytes develop from metanephridial podocytes[65] and forms intermediate between both cell types are known[66], indicate that the ancestral UF-based organs of insects might became reduced to single excretory cells[16], in tandem with the development of Malpighian tubules. Further investigation of the molecular basis of the development of Malpighian tubules and H-system in additional, non-model arthropod and nematode species, is needed in order to understand how the ancestral UF-based organs got replaced by entirely new excretory systems in those two evolutionarily successful groups.

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Author contributions

A.H. conceived the study. L.G., C.A., A.H. and R.J. designed experiments and analyses. L.G., C.A., P.B., C.J. L., R. J. and A.H. performed collections. L.G. and R. J. performed phylogenetic analyses and gene family evolution studies. L.G., C.A., and R.J. performed gene expression analyses. All authors contributed to interpretation of the results, and L.G. and A.H. drafted the manuscript. C.A., C.J. L. and G.B. edited the manuscript.

Declaration of Interests

The authors declare no competing interests.

Figure legends

Figure 1. Phylogenetic distribution of excretory organs and their molecular patterning.
A, Most of the bilaterian clades possess excretory organs based on the principle of ultrafiltration (UF) (protonephridia and metanephridia). Specialized secretory-excretory organs are found in nematodes and some arthropods. Clades investigated in this study are marked in bold. **B**, Homologous transcription factors involved in regeneration of planarian protonephridium and development of the vertebrate kidney, in both organs homologous structural genes form the UF site (terminal cells and podocytes, respectively). Abbreviations: B, Bilateria; D, Deuterostomia; E, Ecdysozoa; L, Lophophorata; P, Protostomia; S, Spiralia.

Figure 2. Expression of the nephridia-related transcription factors in Lophophorates.
A, A pair of protonephridia (arrowheads) develops in the posterior end of the phoronid larva, ciliated cells forming in each protonephridium are outlined with magenta dotted lines. **B,** Transcription factors expressed in the developing protonephridia of *Phoronopsis harmeri* (arrowheads), **C**, Co-expression of the investigated transcription factors in the protonephridia of preactinotrocha larva of *P. harmeri*. **D**, A pair of ciliated nephridial rudiments (arrowheads) is present in the middle portion of the larval *Terebratalia transversa.* **E**, Transcription factors are expressed in various structures, including the area where nephridia develop (arrowheads). Abbreviations: A, anterior; D, dorsal; dt, digestive tract; vv, ventral view; lat, lateral view; P, posterior; V, ventral. DAPI stained cell nuclei are in cyan and acetylated tubulin immunoreactivity is in yellow. Nephridia on the schematic drawings are marked in red. Scale bars, 20 μm.
See also Figures S1 and S2.

Figure 3. Expression of the nephridia-related transcription factors in annelid, ecdysozoans and hemichordate.
A, A pair of ciliated protonephridia (outlined with magenta dotted line) is positioned on the lateral edges of the larval *Owenia fusiformis*. **B**, Transcription factors expressed in the developing protonephridia (black arrowheads) of *O. fusiformis*. **C**, Position and structure of the protonephridia of the adult *Priapulus caudatus* and expression of the genes in the dissected organs. **D**, Embryo of *Euperipatoides kanangrensis* possesses single metanephridium rudiment associated with each walking appendage, *pou3* is expressed in the proximal sacculi (red arrowheads), while *eya* and *six1/2* in the more distal canals (green arrowheads) of the

developing organs. **E**, Early tornaria larva of *Schizocardium californicum* possesses ciliated, anterior coelomic vesicle, which serves as protonephridium (arrowheads). **F**, Expression of *eya*, *six1/2*, *sall* and *lhx1/5* is detected in the larval protonephridium (arrowheads), while pou3 is broadly expressed in the ectoderm and its expression is not detected in the excretory organ. Abbreviations: A, anterior; fa, frontal appendage; ao, apical organ; dt, digestive tract; vv, ventral view; fp, filtering portion; j, jaw; l1–l6, walking legs 1–6; lat, lateral view; nd, nephroduct; neph, magnified nephridium; oe, oesophagus; P, posterior; sd, stomodaeum; sp slime papilla; to, terminal organ; tu, tubule; wr, worm rudiment. DAPI stained cell nuclei are in cyan, Cybr-green stained cell nuclei in green and acetylated tubulin immunoreactivity is in yellow. Nephridia on the schematic drawings are marked in red. Scale bars, 20 μm.
See also Figures S2 and S3.

Figure 4. Evolution of the animal excretory organs and expression of the UF-related structural genes.

A, Proposed scenario for the evolution of excretory organs ad their genetic control. The last common ancestor of protostomes and deuterostomes had ciliated, UF-based organs (light green), which were developing with an expression of the set of transcription factors, conserved in the contemporary members of the clade. The UF-based excretory organs has been lost and replaced by the secretion-based systems (red) in nematodes and insects. **B**, Putative UF-related structural genes are expressed in the filtering cells (arrowheads) of the excretory organs in investigated species. Areas shown on micrographs are outlined in green. Abbreviations: vv, ventral view; l1–l5, walking legs 1–5; lat, lateral orientation. Nephridia on the schematic drawings are marked in red.
See also Figures S1 and S3.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact

- Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Andreas Hejnol (Andreas.Hejnol@uib.no).

Materials Availability

- This study did not generate new unique reagents.

Data and Code Availability

- All newly determined sequences have been deposited in GenBank under accession numbers MT900856–MT900925.
- Multiple protein alignments used for orthology assignments are available upon request from the corresponding author.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Studied animals

Adult gravid animals were collected from Bodega Bay, California, USA *Phoronopsis harmeri* Pixell, 1912, San Juan Island, Washington, USA *Terebratalia transversa* (Sowerby, 1846), Station Biologique de Roscoff, France, *Owenia fusiformis* Delle Chiaje, 1844, Kanangra Boyd National Park, NSW, Australia *Euperipatoides kanagrensis* (Reid, 1996) and Morro Bay State Park, California, USA, *Schizocardium. Californicum* Cameron & Perez, 2012. The animals were spawned and larvae or embryos were obtained as described elsewhere[31, 40, 47, 67, 68]. Adult *P. caudatus* worms were collected from Gullmarsfjorden, Sweden and dissected in laboratory to obtain their protonephridia.

METHOD DETAILS

Animal fixation

Before fixation, larvae were relaxed in 7.4% magnesium chloride. All samples were fixed in 4% paraformaldehyde for 1 h at room temperature. After fixation, samples were washed in 0.1% Tween 20 phosphate buffer saline, dehydrated through a graded series of methanol, and stored at −20 °C in pure methanol or ethanol.

Gene identification

Transcriptomes from mixed developmental stages and/or adults were used for gene identification. Gene orthologs were identified based on reciprocal tBLASTn search and confirmed with phylogenetic analysis. Protein sequences were aligned with reference sequences downloaded from GenBank using CLC Main Workbench 7 and non-conserved regions where removed with TrimAl[69] (using the gappyout option). Phylogenetic analyses were performed in FastTree v2.1[70] (using the LG amino acid substitution model) and in RaxML[71] (using the best fitted model, chosen separately for each protein with ProtTest 3.4.2[72]). All phylogenetic trees and lists of reference sequences are available from corresponding author upon request.

Immunohistochemistry and in situ hybridization

Morphology of studied stages was investigated with mouse primary monoclonal antibodies against acetylated-tubulin (Sigma, T6793) in 1:500 concentration, visualized with the secondary goat anti-mouse antibodies (Life Technologies) conjugated with fluorochrome (AlexaFluor647) in 1:50 concentration. Cell nuclei were stained with DAPI (*P. harmeri*, *T. transversa*, *O. fusiformis*, *S. californicum*) or Cybr-green (*E. kanagrensis*) and samples were mounted in 80% glycerol (*P. harmeri*, *S. californicum*) or Murray Clear (*T. transversa*, *O. fusiformis*).

Single whole-mount colorimetric and fluorescent in situ hybridization was performed following an established protocols ([40]for *E. kanagrensis* and [73] for the other species) with probe concentration of 1 ng/µl and hybridization temperature of 67°C. Proteinase K treatment time was adjusted for each species and ranged from 2 (*P. harmeri*, *O. fusiformis*, *P. caudatus*, *S. californicum*) to 10 min (*T. transversa*). Samples were mounted in Murray Clear (fluorescently stained *T. transversa* larvae and nephridia of *P. caudatus*) or 70% glycerol (all remaining samples). Double fluorescent whole-mount in situ hybridization was performed as described elsewhere[74] and samples were mounted in 80% glycerol.

Imaging and image processing

Fluorescently labeled samples (immunochistochemical staining and fluorescent in situ) were scanned in a Leica SP5 confocal laser-scanning microscope. Samples investigated with colorimetric in situ hybridization were imaged with Zeiss AxioCam HRc connected to a Zeiss Axioscope Ax10 compound scope with bright-field Nomarski optics or Zeiss AxioCam MRc connected to Zeiss Discovery V8 dissecting scope. The expression of the studied genes in the developing excretory organs was assessed by the optical sectioning of multiple specimens in both lateral and dorsoventral focal planes to ascertain that the signal is present in the cells of the organs and not in adjacent tissues. Images were analyzed and adjusted for brightness and

contrast with IMARIS 9.1.2 (confocal scans) and Photoshop CS6 (Adobe) (light micrographs) and figure plates were assembled with Illustrator CS6 (Adobe). Some of the used silhouettes of animals were downloaded from PhyloPic.com.

QUANTIFICATION AND STATISTICAL ANALYSIS

- The presented qualitative data did not require statistical analysis.

KEY RESOURCES TABLE

Supplementary Information

Document S1. Figures S1–S3.

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