



HAL
open science

Globins in the marine Annelid *Platynereis dumerilii* shed new light on hemoglobin evolution in Bilaterians

Solène Song, Viktor Starunov, Xavier Bailly, Christine Ruta, Pierre Kerner, Annemiek J M Cornelissen, Guillaume Balavoine

► **To cite this version:**

Solène Song, Viktor Starunov, Xavier Bailly, Christine Ruta, Pierre Kerner, et al.. Globins in the marine Annelid *Platynereis dumerilii* shed new light on hemoglobin evolution in Bilaterians. 2020. hal-03456385v2

HAL Id: hal-03456385

<https://hal.science/hal-03456385v2>

Preprint submitted on 8 Dec 2020 (v2), last revised 30 Nov 2021 (v3)

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27

**Globins in the marine annelid *Platynereis dumerilii* shed new light on hemoglobin
evolution in bilaterians.**

28
29
30
31
32

Solène Song^{1,2}, Viktor Starunov³, Xavier Bailly⁴, Christine Ruta⁵, Pierre Kerner¹, Annemiek
J.M. Cornelissen² and Guillaume Balavoine^{1*}

- 33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
1. Université de Paris, CNRS, Institut Jacques Monod, Paris, France
 2. Université de Paris, CNRS, Laboratoire Matière et Systèmes Complexes, Paris, France
 3. Laboratory of Evolutionary Morphology, Zoological Institute, Russian Academy of Sciences, Universitetskaja nab. 1, 199034 Saint Petersburg, Russia
 4. CNRS, Sorbonne Université, Centre de Ressources Biologiques Marines, Station Biologique de Roscoff, Roscoff, France
 5. Laboratory of Integrative Biology of Marine Organisms, Institute of Biology, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

* Corresponding author
guillaume.balavoine@ijm.fr

Abstract

Background

How vascular systems and their respiratory pigments evolved is still debated. While many animals present a vascular system, hemoglobin exists as a blood pigment only in a few groups (vertebrates, annelids, a few arthropod and mollusk species). Hemoglobins are formed of globin sub-units, belonging to multigene families, in various multimeric assemblages. It was so far unclear whether hemoglobin families from different bilaterian groups had a common origin.

Results

To unravel globin evolution in bilaterians, we studied the marine annelid *Platynereis dumerilii*, a species with a slow evolving genome. *Platynereis* exhibits a closed vascular system filled with extracellular hemoglobin. *Platynereis* genome and transcriptomes reveal a family of 19 globins, nine of which are predicted to be extracellular. Extracellular globins are produced by specialized cells lining the vessels of the segmental appendages of the worm, serving as gills, and thus likely participate in the assembly of a previously characterized

1
2
3
4
5
6
7
8
9
10
11
12
13
14
annelid-specific giant hemoglobin. Extracellular globin mRNAs are absent in smaller juveniles, accumulate considerably in growing and more active worms and peak in swarming adults, as the need for O₂ culminates. Next, we conducted a metazoan-wide phylogenetic analysis of globins using data from complete genomes. We establish that five globin genes (stem globins) were present in the last common ancestor of bilaterians. Based on these results, we propose a new nomenclature of globins, with five clades. All five ancestral stem-globin clades are retained in some spiralian, while some clades disappeared early in deuterostome and ecdysozoan evolution. All known bilaterian blood globin families are grouped in a single clade (clade I) together with intracellular globins of bilaterians devoid of red blood.

15 *Conclusions*

16
17
18
19
20
21
22
23
24
25
26
27
28
29
We uncover a complex “pre-blood” evolution of globins, with an early gene radiation in ancestral bilaterians. Circulating hemoglobins in various bilaterian groups evolved convergently, presumably in correlation with animal size and activity. However, all hemoglobins derive from a clade I globin, or cytoglobin, probably involved in intracellular O₂ transit and regulation. The annelid *Platynereis* is remarkable in having a large family of extracellular blood globins, while retaining all clades of ancestral bilaterian globins.

30 **Keywords**

31
32
33
34
globin, metazoan, bilaterian, annelid, convergent evolution, blood

35 **Background**

36
37
38
39
40
41
42
43
44
The exchanges of gas, nutrient and waste relying on diffusion are impaired when body size and tissue thickness increase. Most animals develop at least one type of circulatory system to circumvent this limitation. Vascular systems are diverse, representing different solutions to the same purpose in animals with varied body plans.

45
46
47
48
49
50
51
52
53
54
One major function of the blood vascular system is performing gas exchanges, bringing dioxygen to the tissues and taking back waste products (eg. CO₂). To perform this respiratory function efficiently, many species use respiratory pigments that bind dissolved gases in a cooperative way and considerably increase their solubility in the blood or hemolymph. A general picture of the nature of circulating respiratory pigments used in bilaterians (figure 1) suggests a complex evolutionary history.

55
56
57
58
59
60
61
62
63
64
65
Among the circulating respiratory pigments, hemoglobins in particular display a complex situation and the evolution of globins is still not understood in depth. Globins are ancient proteins present in all groups of living organisms (1). They are characterized by a

1 unique molecular structure, the “globin fold” made of eight alpha helices which shelters a heme
2 prosthetic group, itself responsible for binding diatomic gases such as O₂ or NO. The globin
3 motif domain is sometimes found in composite proteins that have evolved by fusion of pre-
4 existing protein domains but all respiratory “hemoglobins” derive from proteins with a stand-
5 alone globin motif. The oxygen transport function is well described in specific groups
6 (vertebrates, annelids) but the globin superfamily is prevalent across the animal kingdom and
7 the functions of globins are still poorly known (2). Many phylogenetic studies have focused on
8 the hemoglobins of mammals and vertebrates in general, starting with the seminal work of
9 Zuckerkandl and Pauling (3). At a much larger phyletic scale, there has been studies on the
10 evolution of globins at the level of the tree of life (1,4), and at the level of the eukaryotic tree
11 (5). These studies emphasize the universality of the globin fold motif present since the last
12 common ancestor of all living cells (LUCA, last universal common ancestor) and the likely
13 functions of these proteins as enzymes or sensors. Within metazoans, many works have
14 analysed globin evolution in specific metazoan groups such as annelids (6,7), deuterostomes
15 (8), echinoderms (9), pancrustaceans (10), insects (11), cephalochordates (12), chordates (13)
16 or agnathans (14,15).

17 However, a smaller number of studies have focused on the evolution of globins at the
18 metazoan scale (16–19). These studies have shown that vertebrate hemoglobins and the
19 related myoglobins are only a small branch in a vast tree of animal globins, some of which are
20 used for respiratory functions but many others have still unknown functions.

21 One of these sub-families, the neuroglobins (Ngb) are hexacoordinate intracellular globins
22 first discovered in the mammalian nervous system and whose function(s) remain poorly
23 understood (20–22). Neuroglobin orthologues have been discovered in a number of animal
24 groups other than vertebrates as well as in choanoflagellates. Expressions of neuroglobins in
25 mammals, cnidarians and the acoel *Symsagittifera* (18) all occur in neural or sensory cells.
26 Neuroglobins are proposed to be the most ancient clade of globins occurring in animals and
27 hemoglobins have been suggested to be derived from these ancestral neuroglobins by
28 independent, convergent functional divergence (18).

29 Another study (23) has identified globin X. Globin X proteins are widespread among
30 metazoans, present in teleost fishes and amphibians but not in amniotes. Another
31 panmetazoan clade of globins but missing in all vertebrates, called X-like, is also identified
32 (16). Globin X are expressed in neural cells in vertebrates (24). Globins X and X-like often
33 present one or two acylation sites at the N-terminus, either myristoylation or palmitoylation,
34 suggesting that these proteins are linked to cell membranes. The functions of globin-X proteins
35 are not known, although a role in protecting the cell from reactive oxygen species (ROS) is
36 suggested. Blank and Burmester (16) suggest that the blood globins of vertebrates may derive

1 from ancient membrane-bound globins, via the loss of acylation sites, opening, among other
2 things, the possibility to form multimers.

3 Last, a broad study on a large number of marine species transcriptomes including many
4 annelids focused on the question of extracellular globins (19). Extracellular globins form
5 soluble hemoglobin multimers. Extracellular globins had been known in a small number of
6 bilaterian groups and were thought to have evolved independently in these lineages. Authors
7 claim that extracellular globins similar and orthologous to the ones already well known in many
8 annelids can be identified in Echinodermata, Hemichordata, Brachiopoda, Mollusca,
9 Nemertea, Bryozoa, Phoronida, Platyhelminthes, and Priapulida. They come to the
10 conclusions that extracellular hemoglobin may exist in many more bilaterian groups than
11 previously thought and that the last common ancestor of bilaterians may have possessed this
12 hemoglobin.
13
14
15
16
17
18
19

20 Despite bringing forward interesting conceptions, these previous works on metazoan
21 globins all suffer from biased sampling. Gene sequences are identified from animals whose
22 complete genome was not available at the time of the study and therefore represent only a
23 subset of the diversity that must exist in metazoans. The sampling of globins of Blank and
24 Burmester (16) was made by searching NCBI Genbank with terms related to globins, while
25 the same database was screened with a Ngb probe in Lechauve et al (18). Belato et al's study
26 (19) focuses only on extracellular globins and uses only transcriptomes. A larger sampling of
27 complete metazoan genome sequences is now available and allows an update.
28
29
30
31
32

33 In this work, we explored the globin content of the genome of the marine annelid
34 *Platynereis dumerilii*. Many annelids, including *Platynereis*, are remarkable for the similarities
35 of their blood vascular system (BVS) with the vertebrate BVS. Annelid BVS are usually closed.
36 Annelid blood vessels, devoid of endothelium, are located in between spacious pairs of
37 coelomic cavities in each segment and have a metameric organisation along the trunk of the
38 animal. The blood of many annelids is red, containing a high concentration of respiratory
39 hemoglobin. In contrast to vertebrates, this circulating hemoglobin is often extracellular
40 instead of enclosed in red blood cells although a few annelid species have circulating red
41 blood cells with intracellular hemoglobin (25). The extracellular globin structure is very different
42 from the hemoglobin of vertebrates. Instead of a heterotetramer of globin sub-units, many
43 annelids possess a giant hexagonal bilayer hemoglobin molecule (HBL-Hb, originally referred
44 to as "erythrocrurin"), organised in an assemblage of no less than 144 individual globin
45 peptides with the help of linker proteins (26–28). *Platynereis* possesses red blood indicating
46 that it is composed of dissolved hemoglobin. Despite its status of emergent model animal for
47 evolution and development studies (29–31), the composition of the blood of *Platynereis* has
48 not been yet characterized, although it can be inferred that they possess the annelid
49 extracellular giant hemoglobin. We identified the multigenic family encoding for the globins in
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 *Platynereis* publicly available sequenced genomes and transcriptomes. We combined *in situ*
2 hybridizations, electron microscopy and 3D reconstructions to identify and characterize the
3 cells that produce these extracellular globins.
4

5 We analyse the evolutionary relationships of *Platynereis* globins at the metazoan-wide
6 phylogenetic scale, using a sampling of metazoans for which completely sequenced genomes
7 are available. We wanted in particular to put to the test the idea that animal hemoglobins are
8 derived convergently from "neuroglobin-like" proteins, involved in a neuronal function (18).
9

10 **Results**

11 ***Identification of globin genes in the *Platynereis* genome and cluster mapping***

12 We identified in the transcriptomes and genome of *Platynereis* 19 different genes coding
13 for proteins with a single globin motif (figure 2A). Nine of these genes code for a peptide with
14 a secretion signal at their N-terminus, (*Pdu Egb-A1a*, *-A1b*, *-A1c*, *-A1d- α* , *-A1d- β* , *-A1d- γ* , *-A2*,
15 *-B1*, *-B2*), making them likely to represent extracellular globins (additional file 1). Six of these
16 genes, distinct from the predicted extracellular globins, code for peptides that are likely to be
17 membrane-bound, thanks to N-terminal acylation sites. All 19 sequences were identified both
18 in the genome assemblies and in the transcriptomes, indicating that they are not cross species
19 contaminations. In addition, all sequences were mapped to different loci in the latest assembly
20 of the *Platynereis* genome (figure 2B and additional file 2). They therefore represent genuine
21 paralogues instead of divergent alleles. Six of the nine potential extracellular globins (*Pdu*
22 *Egb-A1d- α* , *-A1d- β* , *-A1d- γ* , *-A2*, *-B1*, *-B2*) are comprised in a chromosomal cluster (figure 2B)
23 and two other closely related genes (*Pdu Egb-A1a*, *-A1c*) form a tandem pair in a distinct
24 contig. All 10 other genes, coding for putative intracellular globins, as well as the extracellular
25 *Pdu Egb-A1b* are found as isolated genes on rather large contigs.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43

44 ***Phylogenetic analysis of the *Platynereis* globins at the metazoan scale***

45 To understand better the origins of the multiple globin genes present in *Platynereis*
46 genome, we carried out a phylogenetic analysis of metazoan globins. Except for extracellular
47 globins in a couple of annelid species, we used exclusively species for which a complete
48 genome sequence is available. We chose species representative of all major metazoan clades
49 (phyla). We designed a screening technique with a concatenated globin probe and a low E-
50 value cut-off in order to detect even the most divergent globins. We decided however not to
51 treat in this study the globin domains that are part of multidomain proteins, such as the
52 androglobin of vertebrates. We also did not take into account the globins with multiple serially
53 arranged globin folds as occurring for example in echinoderms (9,32). We included the
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

pancrustacean *Daphnia pulex*. In *Daphnia*, 11 proteins have a double globin domain organization. As it suggested multiple tandem duplications of an ancestral didomain globin, we chose to separate the N- and C- terminal globin domains in each of these peptides and to treat them as individual OTUs in our trees. Our complete genome surveys uncovered a highly variable number of globin sequences in the different metazoan species, ranging from none in the ctenophore *Pleurobrachia bachei* and the rotifer *Adineta vaga*, to 32 in the annelid *Capitella teleta* (additional file 3). We also searched all metazoan genomes for the specific linker proteins that are associated with the assembly of the HBL-Hb. These proteins are only present in the four annelid species that have also extracellular globins (additional file 3).

We conducted a maximum-likelihood phylogenetic analysis of this sampling of 272 protein sequences (sequence alignment in additional file 4; complete tree in additional file 5; simplified version in figure 3). A major issue in phylogenetic analyses is to determine the root of the tree, as this directly impacts all the evolutionary interpretations that can be deduced from the tree. In this particular analysis, there are no obvious molecules that could serve as outgroups for globins inside the metazoans. We also decided not to use globins from other eukaryotic groups, as we were concerned that using very divergent outgroups with a small number of alignable positions could result in an artifactual rooting of the tree. Instead of determining where the root of the tree could possibly be, we decided to deduce where it could not be. To this goal, we took into account the solid groupings found in the unrooted tree, as indicated by their aLRT scores, and their composition in species to determine which groupings are natural clades, which we describe in details below. We also excluded a root position within well supported mono-species clades. The root of the tree that is represented in figure 3 (and additional file 5) is thus chosen arbitrarily among the positions that we consider possible based on that criterium. We looked for groupings that would indicate pan-metazoan or pan-bilaterian genes, because they contain globins that are derived from a large sampling of metazoans species and do not contain sub-trees that are themselves composed of a large panel of metazoan species. These solid clades are likely to derive from a single ancestral gene. We found four well supported clades (aLRT > 0.95), that we named clade I to IV, that contain a broad sampling of bilaterian species, but no non-bilaterian species. These are likely descendants of single pan-bilaterian globin genes. In addition, one species rich clade containing both bilaterian and non-bilaterian genes (Porifera, Placozoa, Cnidaria) was found but with only a marginal aLRT support. We named this clade number V as we consider that it likely contains descendants of a single pan-metazoan gene. Last, distinct of these five pan-bilaterian or pan-metazoan clades, we found a well-supported clade containing only spiralian genes (Mollusca, Annelida), which we termed Sp-globins (Sp-gb). In addition to these multi-species clades, two other smaller clades are visible. One of them contains mostly globins of

1 the cnidarian *Nematostella vectensis* as well as a very derived globin of *Drosophila*
2 *melanogaster* that might be there because of long branch attraction. The other clade contains
3 only globins from the nematode *Caenorhabditis elegans*. These mono-species clades
4 presumably represent relatively recent gene radiations of a single divergent globin and remain
5 unclassified. These eight well supported clades are connected by nodes with low support.
6 Note that our arbitrary root chosen for representation in figure 3 (and additional file 5) is outside
7 of these eight clades. We conducted bayesian phylogenetic analyses of the same sampling
8 of genes and found good support for most clades (types I, II, III, IV, Sp-gb), but not for clade
9 V (additional file 5).

10
11
12
13
14
15
16 We mapped on our ML tree the globins that are thought to be membrane bound because
17 they harbor predicted dual N-terminal acylation signals (additional file 6; additional file 5,
18 magenta rectangles) (16,33). None of the clade I globins is predicted to be membrane-bound.
19 This is in contrast with the clade II, III, IV and Sp-gb, that all contain numerous predicted
20 acylated globins. We retained as likely membrane-associated only those peptides that show
21 strong predictions of both N-terminal myristoylation and palmytoylation. It is likely however
22 that more globins of these clades are actually membrane-bound, for example by combining
23 myristoylation with a basic motif. Based on the large proportion of predicted acylated peptides,
24 there is a strong probability that the ancestral globins of clades II and IV, at least, were
25 themselves linked to the membranes.

26
27
28
29
30
31
32
33 This analysis thus suggests that all bilaterian globins derive from five ancestral genes that
34 were present in *Urbilateria*, thus defining five clades of bilaterian globins. Interestingly,
35 *Platynereis* globins are distributed in all five clades plus the spiralian-specific clade. In the
36 genomes of non-bilaterian metazoans, a very variable number of globin genes are found. To
37 analyse further the pre-bilaterian evolution of globins, we performed a second ML analysis
38 with an extended sampling of non bilaterian metazoans (sequences: additional file 7; tree:
39 additional file 8), two choanoflagellates and one acoel. This extended analysis supports the
40 presence of a single Clade V globin in the last common ancestor of metazoans (additional file
41 6 and 8). The situation remains unclear in cnidarians as hydrozoans and one ctenophore
42 appear to have clade III-related sequences. Additional complete genomes of non bilaterians
43 and resolving the controversy of their phylogenetic relationships will be needed to understand
44 fully the complement of globins in the last common ancestor of metazoans.

45
46
47
48
49
50
51
52
53 All blood respiratory globins, or suspected to have a respiratory function, either
54 intracellular (human) or extracellular (annelids, *Daphnia*) are found in clade I in the ML tree
55 (figure 3). They also form species-specific clades inside clade I. All human red cell hemoglobin
56 genes are found in a strongly supported clade with the human myoglobin and the human
57 cytoglobin. All the *Daphnia* globin domains of their large family of didomain proteins are found

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

in two separate clades, the N-domains and the C-domains, again suggesting a radiation from a single ancestral didomain-coding gene. All extracellular annelid globins are found in one moderately supported clade, containing two globin "A" and "B" clades. These extracellular globins are present in four of the six annelid species selected (*P. dumerilii*, *Arenicola marina*, *Lumbricus terrestris* and *Alvinella pompejana*), representing groups far apart in the annelid tree. This grouping reinforces the interpretation that *P. dumerilii* putative extracellular globins have indeed a respiratory function, as this function is demonstrated by a number of works in the three other annelid species (27,28,34). It also shows that the acquisition of a secretion signal and the gene A/B duplication that gave birth to these respiratory proteins must have taken place early in annelid evolution, possibly before the last common ancestor of all living annelids. To strengthen this interpretation, we also searched two other annelid genomes that do not present any putative extracellular globins. One is the leech *Helobdella robusta* in which no clade I globins are detected. The other is *Capitella teleta* that presents a large family of strongly related clade I intracellular globins, that are suspected to have a respiratory function (35). All the families of circulating respiratory globins present in distant groups in our analyses (vertebrate red cell globins, *Daphnia* extracellular globins, annelid extracellular globins, *Capitella* red cell globins) thus correspond to independent events of gene recruitment and duplications, seemingly from a single ancestral clade I globin.

Expression profiling of *Platynereis* extracellular globins

While our phylogenetic analysis suggests a circulating respiratory function for *Platynereis* extracellular globins, we wanted to obtain strong evidence of the linkage between extracellular globins and the development of the vascular system of *Platynereis*. We particularly wanted to establish which cells are responsible for the production of *Platynereis* putative hemoglobin. To this end, we compared and combined two types of analyses: an expression analysis of globin genes by *in situ* hybridization *in toto* (WMISH) on a series of *Platynereis* developmental stages and a transmission electron microscopy study of juvenile stages to explore the vascular system development and characterize the globin-producing cells. *Platynereis* shows a succession of larval and juvenile stages, as well as a spectacular sexual metamorphosis, called epitoky (additional file 9). As the larvae, juvenile and mature worms have transparent tissues, the accumulation of hemoglobin in the blood is readily visible at all life cycle stages. Larvae and small juveniles, that are colorless, show no sign of expressing hemoglobin. The blood becomes visibly red only when the worm reaches a certain size, around 10-15 segments. Then, the quantity of blood increases progressively as the worm grows longer and thicker, peaking in mature swarming worms.

Specific RNA probes were produced for each globin gene. It must be noted however that the *Pdu-Egb-A1d α* , *-A1d β* and *-A1d γ* genes have very similar nucleotide sequences, as well

1 as being close chromosomal neighbors. The signal obtained with the *Pdu-Egb-A1d α* probe
2 could be a mix of the expressions of all three genes. None of the globin probes gave a
3 significant signal on early larval stages (24 hpf trochophore, 48 hpf metatrochophore, 72 hpf
4 nectochaete). The 10 intracellular globins gave no signal or non-specific signal in silk glands
5 after long staining periods (not shown). Only six of the seven extracellular globin probes
6 showed specific patterns. They all display expressions in the same cells, but not necessarily
7 at the same time. Typical expression patterns for Egb-A2 are shown in Figure 4. In particular,
8 we saw no expression of Egb-A1c in juvenile worms. The expression for this gene starts only
9 in worms that are approaching sexual maturity and are about to enter epitoke metamorphosis.
10 For all the most precocious extracellular globins, the expression starts when the worm is about
11 5 segments (not shown) in the posterior-most segment. In older juveniles, the expression is
12 located along presumptive lateral vessels in the trunk (figure 4A', red arrowheads). The
13 expression shows a graded pattern along the anterior-posterior axis: stronger in the mid-body
14 segments and absent in the rostral most and caudal most segments. This graded expression
15 continues as the worms continue to develop. In bigger worms, the expression starts in the
16 appendages of the mid-body along putative vessels that irrigate the part of the appendage
17 that serves as a respiratory gill. The central most segments display expression in the
18 parapodia when more rostral and caudal segments still display expression around lateral
19 vessels in the trunk (figure 4C', red arrowheads). There is a more intense expression in the
20 mid-body segments and seemingly a progressive spatial shift of the hemoglobin-producing
21 activity from the trunk to the parapodia (figure 4C; additional file 10). In worms engaged in
22 sexual metamorphosis, we find a decreasing intensity of expression of all extracellular globins
23 in parapodia (figure 4E-F). In fully mature swarming worms, hemoglobin content is peaking
24 but there is no mRNA of any blood globin left (figure 4G).

25 In addition to the spatial pattern evolution, our WMISH analyses suggest that different
26 extracellular globin isotypes might be expressed at different times. To establish further
27 evidence for globin isotype switching during the life cycle, we turned to quantitative PCR
28 analysis. The stages for qPCR were divided as follows: 48hpf larvae, 6 weeks larvae, 50
29 segments larvae, sexual metamorphosis stage I, sexual metamorphosis stage II, mature
30 swimming worms. We designed specific primer pairs for each Egb gene, but again it is
31 expected that primers for *Pdu-Egb-A1d α* are going to capture expressions of the two closely
32 related genes *-A1d β* and *-A1d γ* as well. The relative expression levels of the extracellular
33 genes with respect to reference genes show important variations from one biological triplicate
34 to the other (additional file 12). Different individuals of the same apparent stage can display
35 substantially different levels of globin mRNA. We have no clear explanation for this important
36 variability but globin expression at the mRNA level is known to be highly responsive to
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 physiological conditions, in particular the amount of O₂ available in the environment (36).
2 Notwithstanding, the general trend is that blood production picks up at 6 weeks, culminates
3 between 50 segments and the beginning of the sexual metamorphosis (75-80 segments) and
4 collapses rapidly after, ending by the time the worm starts swimming, which will be followed
5 by reproduction and death (figure 4I). Individual gene expressions, despite variability, show
6 trends that confirm our WMISH observations for some genes. The globins A2, B1 and B2
7 (figure 4I) are expressed at levels that follow the general tendency described above and at
8 higher levels than the four A1 globins. Four paralogous A1 globins (figure 4I), meanwhile, are
9 expressed at different levels. While A1a and "A1d- α " follow the general tendency, A1c is
10 expressed significantly only in sexually maturing animals maybe representing an adult-specific
11 HBL-Hb sub-unit. In contradiction with at least some of our WMISH results, A1b was detected
12 only at very low levels in all biological replicates.
13
14
15
16
17
18
19
20
21

22 ***Characterization of *Platynereis* hemoglobin-producing cells***

23 Next, to understand how the *Platynereis* BVS develops and to localize the extracellular
24 globin-producing cells and analyse their cytological properties, we observed stained semi-thin
25 sections of worms at various developmental stages (example in figure 5K-L) and made 3D
26 reconstructions of their blood vessels (figure 5A-J). We also observed ultra-thin sections of
27 worms by electron transmission microscopy (figure 5M-R). The *Platynereis* BVS is built
28 progressively during development. The first signs of a BVS appear when a feeding juvenile
29 with 3-4 segments has settled on the substrate around 15 days after fertilization, in the form
30 of a pulsatile dorsal vessel and a non-contractile ventral vessel (figure 5A-B). Then the juvenile
31 starts to add segments at the posterior tip of the body and this sequential addition last during
32 most of the benthic life of the animal (figure 5C-F). New metameric BVS unit are put in place
33 in each new segment added. Each metameric unit communicate with the contiguous
34 segments by the pulsatile dorsal vessel, the quiescent ventral vessels and derivations of the
35 lateral vessels. New vessels appear in the growing trunk, especially at the level of the
36 segmental appendages, which serve both as legs and branchiae (gills).
37
38
39
40
41
42
43
44
45
46

47 The numbers of putative hemoglobin-producing cells (HPC) increase progressively as the
48 worm ages, in correlation with the overall quantity of blood observed. One stage corresponds
49 to juvenile worms with around 25-30 segments (figure 5G-H). In these worms, the BVS
50 metameric unit is still fairly simple with dorsal and ventral vessels and a couple of lateral
51 vessels (figure 5G-H, red tracks). The lateral appendage vasculature is still little developed.
52 At this stage, sheaths of characteristic putative HPC (hc, in blue) are already present around
53 a pair of transverse blood vessels (tbv, Figure 5G-H). The other stage corresponds to pre-
54 mature worms with around fifty segments. These worms have much more developed lateral
55 appendage network with lateral vessels colonizing the gill-like parapodia (figure 5I-J, red;
56
57
58
59
60
61
62
63
64
65

1 movie in additional file 11). In the mid body segments of these worms, the putative HPC
2 sheaths are still visible around the transverse vessels (tbv) but new HPC appear around
3 parapodial vessels, more densely on the dorsal side.
4

5 Putative HPC semi-thin and TEM images are illustrated in figure 5K-R. On semi-thin
6 sections, they appear as thick sheaths of cells surrounding the lateral vessel lumen (figure 5K-
7 L). The HPC are organized in a simple epithelium with basal sides facing the vessel lumen
8 and apical sides facing the coelomic cavity (figure 5M). A clear basal lamina delineates the
9 vessel lumen on the basal side (figure 5O). HPC are therefore meso-epithelial cells in clear
10 continuity with the meso-epithelium that surrounds the coelomic pockets. They show all the
11 characteristics of an intense protein synthesis and secretion activity (figure 5N-Q). The
12 cytoplasm is mainly filled with a dense rough endoplasmic reticulum (figure 5N-O). Electron-
13 dense bodies are visible in the cytoplasm of HPC and are interpreted as hematin vesicles
14 (figure 5P). Last, secretion vesicles are clearly identified on the basal side (figure 5Q),
15 indicating intense exocytosis process. The vesicles contain a uniformly granulated matrix
16 identical to the aspect of the blood. These small granules are thought to represent the giant
17 particles of HBL-Hb, characteristic in many annelid species. These HPC are remarkably
18 similar to the previous descriptions of hemoglobin secreting cells in the heart-body (an organ
19 inside the dorsal vessel) of several sedentarian annelids (37). Small juvenile worms with 4-6
20 segments already have HPC, visible in the posterior most segments as small groups of cells
21 (blue) (figure 5A-F).
22

23 In juveniles of various stages, we also identify secretory cells with clear signs of apoptotic
24 degenerescence (figure 5R). We believe they represent dying HPC. Their location is
25 correlated with the globin-production shift we detected in the WMISH analysis. We find
26 degenerating cells around the transverse vessels in the mid-body segments at the time when
27 globin production shifts from the trunk to the appendages. We find these degenerative cells
28 as early as in small juvenile with 6 segments (figure 5E-F, in the forlast posterior segment,
29 green).
30

31 Discussion

32 We report the first exhaustive screening for globin genes in an annelid and more
33 generally in a protostome animal. We performed a broad phylogenetic study of the metazoan
34 globins based on complete genome sequences. This study helps in understanding the
35 evolution of the globin superfamily in metazoans. Combined with the existing knowledge, it
36 gives some hints in the evolution of the red blood itself. Bilaterian hemoglobins evolved several
37 times independently, but from a single intracellular clade I stem globin or cytoglobin (38) as it
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 is termed in mammals. This stem cytoglobin appears to be still present in most bilaterians.
2 Our study strongly contradicts earlier suggestions that annelid-like extracellular globins
3 existed in bilaterian ancestors (19) or that bilaterian hemoglobins evolved by functional shift
4 of neuroglobin-like proteins (18) .
5
6
7

8 ***A phylogenetic nomenclature of metazoan globins*** 9

10 In this work, we have screened only whole genome sequences of a representative
11 sampling of metazoans, using a concatemer sequence probe representing the diversity of
12 human and *Platynereis* sequences. We are thus confident that our sampling of globins
13 represents the most complete description to date of globin diversity in animals. In trees based
14 on maximum-likelihood and bayesian algorithms, a number of robust nodes emerged that
15 correspond to globins present in a large sampling of bilaterian species, thus likely to represent
16 ancestral bilaterian genes.
17
18
19
20

21 The existing classification of globins is mostly mammalian and function oriented, which is
22 of course relevant in certain research contexts. In this article, we want to give a different
23 viewpoint by providing a classification that is based on gene phylogeny at the metazoan level,
24 exactly as it exists for other conserved gene families. Previous publications have tended to
25 develop additional nomenclature by keeping strong mammal- and hemoglobin-centered
26 (hence an evolutionary derived function) points of view. Globins evolved in functional contexts
27 that were entirely different from the eventual evolution of a blood function in a few animal
28 groups and this is what our phylogenetic nomenclature reflects.
29
30
31
32
33
34

35 The five ancestral groups of globin molecules we identified (figure 6A) are the following:

36 - Clade I: this includes the totality of circulating blood globins, either extracellular or
37 carried by red cells, found in metazoans. In addition, this group also includes many non-
38 circulating globins. These additional non-blood clade I globins are present in animals with
39 hemoglobin blood, with a non-hemoglobin blood or with no blood at all. The large majority of
40 bilaterian taxa are represented in this group but no globin of non-bilaterian taxa (cnidarians or
41 sponges) is present, indicating that this clade originated in an ancestor of bilaterians. Globin
42 proteins can display two different chemistries, depending on whether the heme is attached to
43 the globin by one or two histidine side chains, respectively referred to as “pentacoordinate” or
44 “hexacoordinate” (39). The vertebrate hemoglobins and myoglobins, responsible for
45 conveying and storing oxygen are pentacoordinate. Cytoglobins and *Drosophila Glob1*
46 however are hexacoordinate suggesting that the vertebrate blood globins could descend from
47 a hexacoordinate globin.
48
49
50
51
52
53
54
55
56

57 - Clade II: this clade contains multiple globins from all bilaterian superphyla
58 (deuterostomes, ecdysozoans, spiraliens). It has been called formerly « globin X-like » (16).
59 Clade II globins are not identified in non-bilaterian taxa, indicating that this clade originated in
60
61
62
63
64
65

1 an ancestor of bilaterians. No clade II globin is present in vertebrates (although it is present in
2 cephalochordates), indicating that a clade II globin was present in the common ancestor of
3 chordates and lost secondarily in vertebrates.

4
5 - Clade III: this clade contains globins from all bilaterian superphyla. It corresponds to
6 « globin X » (16). Clade III globins are not identified in non-bilaterian taxa in our main
7 sampling, but the presence of Clade III-like genes in hydrozoans and a ctenophore indicates
8 that this clade may have originated in an ancestor of eumetazoans (figure 6B). Our sampling
9 contains only one vertebrate species, human, in which clade III globins are not present. It is
10 known from other studies that clade III globins are present in non-amniote vertebrates, but
11 absent from amniotes, indicating secondary loss.

12
13 - Clade IV: this clade contains globins from all bilaterian superphyla, although it is absent
14 from chordates and also from all ecdysozoans except Priapulida. This clade has gone
15 undetected in previous studies. Clade IV globins are not identified in non-bilaterian taxa,
16 indicating that this clade originated in an ancestor of bilaterians.

17
18 - Clade V: this clade is the least statistically supported of all. It contains however globins
19 from a broad variety of metazoans, including sponges and cnidarians, as well as
20 choanoflagellates, one of the closest eukaryotic sister group of metazoans (additional files 7
21 and 8). This is the only globin type whose existence is strongly suggested in the last common
22 ancestor of metazoans. Paradoxically, it is also the most "dispensable" globin. It has
23 disappeared from the genomes of the majority of the species in our sampling (especially all
24 ecdysozoans). Clade V is represented in human by the neuroglobin protein and corresponds
25 to some of the « neuroglobins » identified in previous studies. This is however a much
26 narrower group than the « neuroglobin-like » proteins previously described (18). These
27 authors included many globins in a broad « neuroglobin-like » class, that does not appear to
28 be a clade in our study because they are split between clade II, III and V.

29
30 - In addition, a strongly supported clade contains only globins from spiralian species
31 (Sp-gb). It may have appeared by a gene duplication of any of the other globin clades.

32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 ***The animal globin superfamily has an ancient history independent from the history of blood***

50 The existence of these clades clearly indicates that at least five globin genes existed in
51 *Urbilateria*. We call these five hypothetical molecules the "bilaterian stem-globins". Few globin
52 sequences fall outside of these five well-defined clades (figure 6B). These additional globins
53 fall into two cases. In one case, these sequences may derive from the stem globins but could
54 have evolved rapidly so as to blur sequence similarities. This is what we would suppose for
55 the large clade of *C. elegans* globins found alongside a single clade I globin. The second case
56 concerns the clade of sea anemone globins, which includes almost all the sea anemone
57
58
59
60
61
62
63
64
65

1 globins in our dataset (except from a single clade V globin). Most of the members of this group
2 are membrane-bound, suggesting that it could be related to any of the bilaterian stem globins
3 that were potentially membrane-bound (clade II, III and IV). Alternatively, the sea anemone
4 membrane-bound globins may derive from an ancestral Eumetazoan membrane-bound globin
5 that would also have given birth to bilaterian clade II, III and IV. Reconstituting metazoan globin
6 history prior to bilaterian diversification is thus at this stage still tentative because of the small
7 sampling of species.
8
9

10
11 The deep relationship between bilaterian stem globins is not solved by our trees (that are
12 arbitrarily rooted in any case). The idea that bilaterian blood globins (and all other clade I
13 globins as defined by our trees) may derive from molecules that were initially membrane-
14 bound (16) is thus not corroborated by our study. It is in our view equally possible that clade I
15 derives from an ancestral duplication of the cytoplasmic clade V, while clade II, III and IV would
16 have acquired membrane tethering independently.
17
18
19
20

21 What were the functions of these numerous non-blood globins derived from the
22 Urbilaterian stem-globins? Here again, very few functional studies have been performed and
23 we can only speculate. Burmester and Hankeln (21) have reviewed the possibilities for the
24 special case of neuroglobins (clade V), but the same possibilities seem to exist for the other
25 clade of non-blood globins (clade I to IV) (40). These intracellular globins, cytoplasmic or
26 membrane-bound, or in some cases located in the nucleus (41), can display a “classic”
27 function, similar to the vertebrate myoglobin (Mb) in storing O₂ in hypoxic conditions or
28 facilitating the delivery of O₂ to the mitochondria. New functions have also emerged in the
29 literature, such as the regulation of reactive oxygen species (ROS) (42) or reactive nitrogen
30 species (RNS) that can be deleterious to the cell. It has been also been speculated that they
31 could function as O₂ or redox sensors by interacting with other proteins such as G proteins
32 (43) or cytochromes to transmit a signal.
33
34
35
36
37
38
39
40
41

42 The five Urbilaterian stem globins have undergone remarkably dissimilar fates in different
43 bilaterian groups (figure 6B). They have all been conserved in the annelid *Platynereis dumerilii*
44 and the mollusks *Pinctada* (bivalve) and *Lottia* (snail). In other bilaterian groups, one or more
45 of the ancestral clades have been lost. Amniotes have kept only clade I and clade V globins.
46 The urochordate *Ciona* has only clade I globins left but six clade I paralogues are present,
47 maybe compensating for the loss of the other types. The most extreme case was however the
48 rotifer *Adineta*, in which we could not identify any globin-related gene. This species lineage
49 has thus lost all five ancestral globins.
50
51
52
53
54
55
56

57 ***A novel scenario for the evolution of blood globins***

58 Oxyphoric circulatory globins have evolved several times during the course of
59 metazoan evolution. All circulatory multigenic families in our dataset derive from a likely unique
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

clade I globin, possibly after recruitment for the circulatory function and several rounds of gene duplications. These multigenic families encompass for example the human hemoglobin family as well as the extracellular globin families of annelids and *Daphnia*.

In the case of the hemoglobins of vertebrate erythrocytes (RBC), heterotetramers comprising alternate globin isotypes circulate depending on the development stage. Adult human hemoglobins form heterotetramers comprising sub-units of two isotypes, α and β , coded by paralogous genes (44). During the course of human embryogenesis and foetal development, different isotypes of hemoglobins are produced (δ , γ , ϵ , ζ), coded by other paralogous genes located in two clusters of α and β -related paralogues. The crucial emerging property of the hemoglobin tetramer is cooperativity: the binding of an O_2 molecule to one of the sub-unit induces a conformational change in the tetramer that makes the binding of O_2 easier to the three remaining sub-units.

Elsewhere among metazoans, other gene radiations correlated with hemoglobin-based blood evolution have occurred. Three of the concerned groups are sampled in our tree: Four species representing a wide diversity of annelids, the special case of the annelid *Capitella teleta*, the pancrustacean *Daphnia*.

In annelids, our phylogenetic analysis is in accordance with the scenario that early gene duplications, probably in the last common ancestor of modern annelids, produced the initial two families (A, B) encompassing the four extracellular globin sub-families A1, A2, B1, B2 that form the HBL-Hb. A recent work based on a massive sampling of annelid species transcriptomes recovered a variable number of extracellular globins, ranging from 1 to 12 depending on the species (7,19). Gene trees made in this later work suggest that, while the early gene duplication A/B indeed occurred prior to annelid diversification, the sub-families A1, A2, B1 and B2 are not recognizable at the scale of the annelid tree. This is in accordance with what we obtain at the much smaller scale of our annelid sampling. This fact might be interpreted as evidence that the annelid last common ancestor has only two clade I paralogous globin genes, A and B, and that additional gene duplications occurred independently in many annelid lineages. Alternatively, four paralogous genes (A1, A2, B1, B2) may have existed early on in annelid history, accounting for the HBL-Hb found in divergent annelid groups, but in this case the gene sequences are not informative enough to reconstitute early duplication steps. Our work differs however in a striking way from previous results (19). These authors identify a number of extracellular globins related to the annelid A and B extracellular globins from a large and diversified subset of bilaterian species including echinoderms, hemichordates, brachiopods, nemertines, mollusks and priapulids. We find no evidence for the existence of globins related to the annelid extracellular globins in bilaterian complete genomes, other than annelids. We argue for a thorough reconsideration of transcriptome screens (19), as many

1 protein globin sequences these authors found in non-annelid bilaterians are strikingly similar
2 if not identical to individual annelid sequences (as illustrated in their Figure 4). This is
3 suggesting multiple tissue or DNA contaminations between species transcriptomes. Despite
4 *Platynereis* blood hemoglobin having never been directly characterized by spectrometric tools,
5 our EM and sequences similarities show that *Platynereis* must exhibit HBL-Hb. In *Platynereis*,
6 additional duplications have affected the A1 gene, making several paralogue sub-units. These
7 sub-units, according to our *in situ* hybridization and qPCR data, may be used in producing
8 alternative composition of circulating hemoglobin that would be used for physiological
9 adaptations as the worm develops, grows and metamorphoses.

10 The clade I stem-globin evolved into a family of intracellular globins in some annelid
11 species. In the case of the sedentarian worm *Capitella*, which does not have blood vessels,
12 the hemocoel contains many red cells with intracellular globins with oxygen binding properties
13 (25). We have identified 17 intracellular globin paralogues, all closely related, in the genome
14 of *Capitella* Spl and no extracellular globins. It appears as a case where the ancestral annelid
15 extracellular globins were lost secondarily and replaced by RBC globins.

16 Although hemocyanin is the main respiratory pigment in pancrustaceans (21),
17 branchiopods such as *Daphnia*, a few malacostracans and a few Insects (such as *Chironomus*
18 larvae) rely on hemoglobin for respiration. *Daphnia* hemoglobins have been thoroughly
19 studied (45), as a model of adaptation to the environment by the extensive use of alternative
20 globin isotypes. *Daphnia* possesses a large family of extracellular two-domain globin-coding
21 genes. It is proposed that the hemolymph of *Daphnia* contains a crustacean hemoglobin made
22 of 16 identical globin peptides with two hemes each (46). For our phylogenetic analysis, we
23 treated the two globin fold sequences independently, resulting in trees indicating that the
24 multigenic family of *Daphnia* globins has emerged from the tandem duplications of an
25 ancestral didomain globin gene.

26 However, globin gene radiations are not necessarily linked to recruitment for a function in
27 the blood. This is exemplified by Urochordates and Cephalochordates. The ascidian *Ciona*
28 *intestinalis* possesses 6 clade I globins, all closely related. The cephalochordate
29 *Branchiostoma* has eight clade I globins found in two clusters in this analysis. Both species
30 have nevertheless a colorless blood and to date, no functional study has shown either oxygen
31 binding properties or their presence in the circulatory system.

32 All clade I globins likely derive from a single ancestral clade I sequence. This ancestral
33 clade I globin was duplicated several times in different clades independently to give small
34 families of blood respiratory proteins. This raises two questions: What was the ancestral
35 function(s) of this clade I globin that made it so well pre-adapted for a role in blood function
36 and is there a gene, in current bilaterian species, that still carries this ancestral function(s)?

1 We can only speculate at this point because little is known on the function of the non-
2 circulating clade I globins in the vast majority of bilaterian species. But the existing knowledge
3 can at least help defining the direction of future research. Part of the answer may come from
4 vertebrates that possess both circulatory and tissue-expressed clade I globins. The “tissue”
5 clade I globins in vertebrates are myoglobins and cytoglobins. Myoglobins are monomeric
6 clade I globins present in all vertebrates. Their function is well known and very specific: they
7 are expressed in muscles and are capable of storing large quantities of O₂ necessary for
8 sustained muscular effort. They also facilitate intracellular transport of O₂ (47). Cytoglobins
9 were first identified as distinct intracellular globins in amniotes (38,48). They harbour a
10 structural difference with hemoglobins (and myoglobin) in having a hexacoordinated heme
11 iron, rather than pentacoordinated. They form homodimers, also by contrast to the former.
12 Phylogenetically, they are firmly identified as clade I globins in our study. Contrary to
13 myoglobins, they are expressed in a large range of tissues and organs, with maybe higher
14 expressions in cells producing large amount of cell matrix such as fibroblasts and
15 chondroblasts (49). Information on the function (or functions) of cytoglobins remains scarce to
16 this date. It is known that they bind dioxygen, carbon monoxide but also nitric oxide with high
17 affinity (50). It has thus been proposed that cytoglobins could play multiple roles in intracellular
18 homeostasis. The first suggestion is of course related to dioxygen binding including roles in
19 oxygen buffering, sensing, transport and storage. The activity of cytoglobin as nitric oxide
20 deoxygenase, a very ancient function of globin proteins in eukaryotes, is also proposed to play
21 an important role in the biology of the cell. Last, oxidative stress is another circumstance in
22 which cytoglobins may play a role (50).

23 Remarkably, molecular phylogenies have demonstrated that large families of oxyphoric
24 circulatory hemoglobins have evolved by gene duplications two times in the vertebrates: once
25 in the gnathostomes and once in agnathans (14,51). Interestingly, in these molecular trees,
26 the cytoglobins of gnathostomes and agnathans are found as sister groups to both their large
27 radiations of circulatory globins, suggesting that cytoglobin existed prior to the origin of red
28 cell globins in vertebrates. Cytoglobins evolve conservatively (13), suggesting that they keep
29 important functions. There are good reasons to propose that cytoglobins (rather than the more
30 specialized myoglobin) may be close to a clade I globin with ancestral functions, as was
31 suggested before (16).

32 The fruitfly *Drosophila*, like other insects, has a tracheal respiration and no circulatory
33 globins. Yet, genome screens have revealed three globin genes in the *Drosophila* genome
34 (52). Two of these globins possess derived sequences. But the third one (Dme_524369;
35 “Glob1” in Burmester et al, 2006) is found solidly clustered with clade I globins in our tree
36 (figure 3, additional file 5). This globin is hexacoordinated and may represent a derived
37 cytoglobin-like molecule. It is found expressed in many tissues in the developing embryo and

1 larva (53) but prominently in the tracheal cells. Gene knock-downs (53,54) lead to reduced
2 survival of flies under hypoxic conditions. This suggests a role in intracellular O₂ homeostasis.

3 Both cytoglobins and *Drosophila Glob1* may thus be close to the functions of a clade I
4 stem globin. It has to be noted from the ML tree (figure 3) that several species (arthropods
5 and mollusks) have a single clade I globin that may represent the unduplicated descendants
6 of the stem globin. Few species have completely lost the clade I (only the annelid *Helobdella*
7 and the cephalopod *Octopus*). This may reflect the initial functional importance of clade I stem
8 globin.
9

10
11
12
13 Why have multiple gene duplications affected the clade I stem globin each time it has
14 been recruited for a blood function? As we have mentioned earlier, cooperativity is an
15 important functional mechanism for a multimeric globin whose role is to store large quantity of
16 O₂ in the blood, yet be able to deliver it on demand to all tissues in need. It may be best
17 developed in a heteromultimeric assemblage and thus requires gene duplication and rapid
18 divergence of the O₂ storing protein. This idea has received crucial support from recent
19 biochemical studies using synthetic ancestral proteins inferred from the diversity of extant
20 vertebrate globins (55). The second factor is the developmental switch of globins. This is well
21 illustrated by the lamprey and the gnathostome cases. The lamprey *Petromyzon* possesses
22 18 hemoglobin genes that are expressed differentially at three different developmental stages,
23 embryo, larva and adult (14). These developmental switches are similar to those found in
24 gnathostomes. Yet, all the agnathan circulatory globins are found in a single monophyletic
25 group completely distinct from the radiation of gnathostome hemoglobins (51). The lamprey
26 globin family was convergently recruited for doing the same kind of developmental switch as
27 seen in gnathostomes. We may see to a more limited extent the same developmental switch
28 in *Platynereis*, with different A1 isotypes specialized for the juvenile and the adult blood
29 functions.
30
31
32
33
34
35
36
37
38
39
40
41
42

43 ***The hemoglobin-producing cells: a specialized type of blood-making cells***

44
45 Little was known on the nature of the cells that produce and secrete respiratory pigments,
46 outside of vertebrates. We demonstrate in this work the existence of a specialized category of
47 vessel-lining cells in *Platynereis*, that produces the blood globins, in other words the
48 hemoglobin-producing cells (HPC). We analysed the development of these cells during the
49 life cycle of *Platynereis*. They first appear in the posterior most segments in a five-segment
50 worm. The youngest feeding juvenile worms with 3 or 4 segments probably do not have HPC
51 and therefore no hemoglobin in their blood. These are minute worms (less than 500 µm long)
52 that probably perform gas exchanges with the seawater by simple diffusion through their tissue.
53 In intermediate size juvenile worms (25-40 segments, less than one cm long), HPC form
54 sheaths around transverse trunk vessels, in most segments of the worm. As the worm grows,
55
56
57
58
59
60
61
62
63
64
65

1 the circulatory system structure becomes more complex and a rich network of vessels and
2 capillaries develop inside the parapodia, the worm segmental appendages. These parapodia
3 take on the role of gills. HPC develop around most vessels inside the parapodia. We currently
4 do not know whether these are the cells of the wall of existing vessels that are differentiating
5 *in situ* to become HPC, or whether HPC colonize the parapodia by emigrating from another
6 location. We also show that, at this stage (worms larger than 40 segments), HPC within the
7 trunk degenerate and are presumably digested by phagocytotic coelomocytes. Worms
8 accumulate more red blood as they get close to sexual metamorphosis. When they have a
9 maximal quantity of blood and start metamorphosing into swarming epitokes, the HPC stop
10 progressively their production. It is possible that these cells degenerate but we have not
11 demonstrated this phenomenon in metamorphosing worms.

12 To our knowledge, this is the first time the full cycle of these HPC is described in any
13 annelid species and this is also the first time they are found associated with the parapodia
14 serving as gills. HPC with very similar cytological properties have been described in other
15 annelid species. They have been called perivascular cells because of their position around the
16 lumen of vessels or extravascular cells, when they are located in the mesodermal peritoneum
17 covering the gut in earthworms (56), siboglinids (57) or *Arenicola* (58). In some sedentarian
18 annelids such as *Amphitrite* (59), a specialized organ, the heart-body, produces the
19 hemoglobins. The heart-body is a solid mass of tissue that grows inside the lumen of the
20 anterior dorsal vessel (60). There is thus a remarkable plasticity in the location of this
21 hemoglobin-producing tissue in annelids that will be worth studying further.

22 How do these HPC relate to the hematopoiesis processes? It should be noted that, in
23 *Platynereis* and in annelids in general, HPC are not circulating, contrary to vertebrates RBC.
24 They are part of a mesodermal epithelium that is in contact with the coelomic cavity on the
25 apical side and with the blood vessel lumen on the basal side. They are functionally polarized
26 cells that secrete hemoglobin only on the basal side toward the vessel lumen. Besides HPC,
27 *Platynereis* (and annelids in general) have several classes of coelomocytes/hemocytes that
28 are freely floating in the coelomic cavity and can cross the mesoepithelium to populate the
29 blood. Can HPC be considered as cells produced by an extended hematopoietic process?
30 This is not only a question of convention, when considering the diversity of HPC in metazoans.

31 Is there a phylogenetic connection between the different metazoan HPC or more
32 generally speaking between the different metazoan respiratory pigment-producing cells
33 (hemoglobin, hemocyanin, hemerythrin)? In the various bilaterian groups that possess either
34 a hemocoel or coelom/blood compartments, HPC can be either free floating or static. In
35 vertebrates, intracellular globins are massively produced and stored in red blood cells.
36 Accumulating respiratory pigments in circulating cells is also the solution retained in a number
37 of other groups. Within annelids, the bloodworm *Glycera* have red cells filled with monomeric

1 and polymeric hemoglobins, circulating in the coelom as the BVS is much reduced in these
2 worms (61). Capitellids have also a reduced BVS and red cells. Also in annelids, Sipunculidae
3 have pink cells filled with hemerythrin, illustrating the evolutionary pigment swapping which
4 has occurred in several groups. Data on the existence and location of pigment producing cells
5 in other protostome invertebrates are still patchy. In the marine chelicerate *Limulus*, whose
6 remarkable blue blood has been exploited for preparing bacterial endotoxin tests, the
7 hemocyanin is secreted by cells called cyanoblasts. Cyanoblasts are found floating in the
8 hemocoel and burst to liberate their hemocyanin content (62). The tissue of origin of these
9 cyanoblasts is unknown. In gastropod mollusks, cells responsible for hemocyanin production
10 have been identified as the pore cells or rhogocytes (63), abundant in the connective tissue.
11 In pulmonate snails that have hemoglobin instead of hemocyanin, it has been shown that
12 these rhogocytes have switched to hemoglobin production (64). One can hope that in the
13 future single-cell transcriptomics as well as more powerful cell lineage tracing methods will
14 help to solve the interesting question of the homology or convergence of respiratory pigment
15 producing cells.

26 Conclusion

27
28
29
30 The annelid *Platynereis* has retained the complete set (clade I to V) of ancestral
31 bilaterian globin genes plus a spiralian-specific globin. *Platynereis* may be an excellent model
32 in the future to determine the initial functions of these stem globins, for instance by selective
33 CRISPR-Cas9 inactivation. It possesses nine clade I globins which are all extracellular. This
34 is consistent with the known presence in several annelids of HBL-Hb with respiratory function.
35 Although the presence of HBL-Hb in *Platynereis* has not been formally proven by
36 crystallography, our results strongly suggest HBL-Hb existence through phylogenetic,
37 expression and morphological evidence. We identified the cells that secrete these extracellular
38 globins. Their location and activity depends on the worm size and life stages. These cells are
39 part of the meso-epithelial walls of some particular vessels: in the trunk in juveniles, and then
40 in the appendages in the pre-mature worms. The blood production level peaks at the onset of
41 the maturation process, likely in preparation for the adult locomotory activity peak. The HPC
42 morphology established with TEM are similar to pigment producing tissues described in other
43 annelids but their location in the appendages vessels serving as gills is original.

44
45
46
47
48
49
50
51
52
53
54 The finding of HPC within the gill organs in a marine animal is an important step forward.
55 *Platynereis* is easily tractable to molecular biology experiments and can be used for obtaining
56 transgenic strains. The molecular characterization of these cells will be very useful for
57 comparative studies and exploring the diversity of HPC in bilaterians. One possibility would
58
59
60
61
62
63
64
65

1 be to use single-cell transcriptomics, as HPC should be easily singled out in the data because
2 of their massive production of extracellular globins. However, scRNAseq remains relatively
3 expensive and shallow. Another possibility would be to develop a CRISPR-Cas9 protocol for
4 tagging an extracellular globin gene with a GFP coding sequence. This would allow sorting
5 HPC with flow cytometry and the bulk sequencing of their transcriptomes.
6
7

8 Another goal will be to understand the embryonic origin not only of the HPC but also
9 of the other mesoepithelial cells that are involved in forming the blood vessels. Is there a
10 developmental and lineage connection between these mesoepithelial cells of the vessels, the
11 HPC and also the coelomocytes of *Platynereis*? In other words, do we have the equivalent of
12 a hemangioblast lineage in the annelid? This question is all the more important if we consider
13 the hypothesis that the BVS evolved in the first place to distribute nutrients and was later on
14 recruited for a gas exchange function.
15
16
17
18
19
20
21
22

23 **Methods**

24 **Animal culture and collection**

25 *Platynereis* embryos, juveniles and adults were bred in the Institut Jacques Monod
26 according to the protocols compiled by A. Dorresteyjn (www.platynereis.de).
27
28
29
30
31
32

33 **Survey of *Platynereis dumerilii* globin genes**

34 *Platynereis dumerilii* globin genes were identified with consecutive steps of sequence
35 similarity searches. We first used a concatenated probe of all 12 distinct human globin
36 sequences (Ngb + Mb + Cygb + Hb- α 2, - θ , - μ , - ζ , - β , - δ , - γ 1, - γ 2, - ϵ) as query against expressed
37 sequence tags (ESTs) from *Platynereis* Resources (4dx.embl.de/platy/), and Jékely lab
38 transcriptome (<http://jekely-lab.tuebingen.mpg.de/blast/#>). Complete coding sequences were
39 assembled from EST fragments using CodonCode Aligner (CodonCode Corporation, USA)
40 and consensus sequences were determined due to high level of polymorphism in this species.
41 The sequences were reciprocally blasted against Genbank non redundant protein database.
42 This allowed constituting a first list of sequences that were recognized as *bona fide* globins.
43 In order to get an exhaustive repertoire of *Platynereis* globin genes, including highly divergent
44 sequences and sequences that might be missing from the transcriptomes, we performed a
45 tblastn search against the most recent *Platynereis* genome assembly using as query a
46 concatenation of all *Platynereis* transcriptome globin sequences. Exons recovered with this
47 method were systematically reblasted on the transcriptome sequences (additional file 2). No
48 additional gene sequence was detected at this stage. Double or multiple scaffold hits for some
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 gene sequences likely correspond to divergent haplotypes of the same genomic region, the
2 *Platynereis* strain used for genome sequencing showing some highly polymorphic
3 chromosomal regions. Chromosomal globin clusters were annotated using Artemis
4 (<http://sanger-pathogens.github.io/Artemis/Artemis/>) and for each *Platynereis* globin, intron
5 positions were mapped on genomic DNA using CodonCode Aligner.
6
7
8
9

10 **Survey of globin genes in animal genomes**

11 Gene searches were carried out using the tblastn or blastp algorithms implemented in
12 ngKlast (Korilog V 4.0, Questembert, France). We used a concatenated probe of all 17
13 *Platynereis* globins with distinct sequences as a query (*Pdu Egb-A1a, -A1b, -A1c, -A1d- α , -*
14 *A2, -B1, -B2 + Pdu-gb-IIA, -IIB, -IIC, -IID, -IIIA, -IIIB, -IVA, -IVB + Pdu-Sp-gb + Pdu-Ngb*). The
15 sequences were reciprocally blasted against Genbank non redundant protein database. This
16 concatenated query sequence covers as much as the molecular diversity of globins as
17 possible and even very derived sequences were recovered in the hit lists. To ensure that all
18 sequences with actual globin similarity are recovered, we reciprocally blasted hits with
19 decreasing E-value until at least ten consecutive non-globin hits turn up in the list. We used
20 publicly available files of peptide predictions from 22 genome datasets widely distributed
21 among the metazoan phyla: Porifera, Cnidaria, Placozoa, Ecdysozoa, Lophotrochozoa and
22 Deuterostomia (additional file 13). When available, the screen was complemented with
23 publicly available transcriptomes that allowed for the correction of a few annotation problems.
24 In addition, the globin genes search was performed on the transcriptomes of *Lumbricus*
25 *terrestris*, *Arenicola marina* and *Alvinella pompejana*.
26
27
28
29
30
31
32
33
34
35
36

37 The putative extracellular globin genes were identified using Phobius signal peptide predictor
38 (<http://phobius.sbc.su.se/>, Stockholm Bioinformatics Center) and SignalP-5.0 predictor
39 (<http://www.cbs.dtu.dk/services/SignalP/>) (additional file 6).
40
41

42 The putative N-terminal acylation predictions were carried out using GPS-Lipid predictor
43 (<http://lipid.biocuckoo.org/>, 65) and GPS-Palm predictor (<http://gpspalm.biocuckoo.cn/>, 66)
44 and compiled in additional file 6.
45
46

47 The giant hemoglobin linker proteins were screened in all genomes in the same fashion as
48 globins, using this time as a probe a concatenation of all linker proteins from the earthworm
49 *Glossoscolex* found in Genbank. Predicted sequences of Pdu-L1, -L2a and -L2b were
50 deposited in Genbank (MW075674-MW075676).
51
52
53
54

55 **Phylogenetic analyses**

56 The amino-acid sequences of the identified globin genes in 25 species were aligned with
57 MUSCLE 3.7 (67) as implemented on the LIRMM web
58 (http://phylogeny.lirmm.fr/phylo.cgi/one_task.cgi?task_type=muscle)
59 under default
60
61
62
63
64
65

1 parameters and adjusted manually in Bioedit. A selection of aligned positions was produced
2 to eliminate unaligned or ambiguously aligned regions (additional file 4). This resulted in a
3 alignment of 275 globin sequences displaying 231 phylogeny informative positions. The
4 phylogenetic trees were constructed using two different approaches: the maximum likelihood
5 (ML) and the Bayesian analyses. Maximum likelihood trees were generated using PhyML3.0
6 (http://phylogeny.lirmm.fr/phylo.cgi/one_task.cgi?task_type=phyml) (68), using the LG model
7 of amino-acid substitutions (69). This model has been shown to perform better than the more
8 widely used WAG model. The Bootstrap test for short sequences such as globins is
9 inappropriate. Therefore statistical support for nodes was assessed using SH-like test (aLRT
10 score) (70). Bayesian analysis was performed with MrBayes 3.2.6 (71) using either LG or
11 WAG fixed model, run for respectively 32 174 500 generations and 24 972 000 generations ,
12 using all compatible consensus and a burn'in value of 0.25. The calculation was performed
13 with 4 chains including one heated chain at temperature 0.5. The average standard deviations
14 of split frequencies were respectively 0.031 and 0.017.

15 Bayesian posterior probabilities were used for assessing the confidence value of each
16 node. Phylogenetic trees were visualized and rooted using FigTree V.1.5.0
17 (<http://tree.bio.ed.ac.uk/software/figtree/>).

28 **Cloning of extracellular globin genes and in situ probes design**

29 Large cDNA fragments, encompassing at least the whole coding sequences, were cloned
30 by nested PCR using sequence-specific primers on cDNA from mixed larval stages and
31 posterior regenerating segments. PCR products were cloned into the PCR2.1 vector following
32 the manufacturer's instructions (Invitrogen, France) and sequenced. The full list of primers
33 used is provided in additional file 14. Sequences were deposited in Genbank (MT701024-
34 MT701042). These plasmids were used as template to produce Dig RNA antisense probes
35 for whole-mount *in situ* hybridization (WMISH) using Roche reagents.

36 **Visualization of *Platynereis* extracellular globin genes expression patterns by whole 37 mount *in situ* hybridization**

38 The animals were fixed in 4% paraformaldehyde (PFA), 1 x PBS, 0.1% Tween20 and
39 stored at -20°C in methanol 100%.

40 NBT/BCIP whole-mount *in situ* hybridization was performed as previously described
41 ([https://www.ijm.fr/fileadmin/www.ijm.fr/MEDIA/equipes/Balavoine/IJM_HybridationInSituNB](https://www.ijm.fr/fileadmin/www.ijm.fr/MEDIA/equipes/Balavoine/IJM_HybridationInSituNB_T_BCIP.doc)
42 T_BCIP.doc) on larval stages (24h, 48h, 72hpf), juvenile larvae, different stage of maturing
43 worms and mature epitoke worms, as well as on regenerated posterior "tails", 9 days after
44 posterior amputation. Bright-field images were taken on a Leica DM5000B microscope
45 equipped with color camera.

Quantitative PCR

Total RNA was extracted using RNAeasy kit (Qiagen). The extraction was done from a batch of 48hpf larvae, a batch of 6 weeks worms, whole 50 segments juvenile worms, a stretch of 20 segments starting 24 segments after the head for maturing stages. The tissue was disrupted and homogenized in Qiagen RLT buffer using Eppendorf micropestles. First-strand cDNAs were synthesized using 100 ng of total RNA, random primers and the superscript II Reverse Transcriptase (Invitrogen, Life Technology). Specific primer pairs were designed with Applied Biosystems Primer Express software to amplify specific fragments between 50 and 60bp (additional file 15). The composition of the PCR mix was: 3 μ l of cDNA (representing cDNA synthesized from 15 ng initial total RNA) , 5 μ l of SYBR GREEN master mix, 1 μ l each of forward and reverse primers (500 nM final concentration), in a 10 μ l final volume. qPCR reactions were run in 96-well plates, in real-time Applied Biosystem StepOne thermocycler. The PCR FAST thermal cycling program begins with polymerase activation and DNA denaturation at 95 °C for 20 sec, followed by 40 cycles with denaturation for 3s at 95°C and annealing/extension for 30s at 60°C. After amplification, melting curve analyses were performed between 95°C and 60°C with increase steps of 0.3 °C to determine amplification product specificity. The slopes of the standard curves were calculated and the amplification efficiencies (E) were estimated as $E=10^{(-1/\text{slope})}$. qPCR were run with biological triplicates for juvenile worms of 50 segments and maturing worms, and each sample was run with technical duplicates and the ribosomal protein small subunit 9 (*rps9*) and *D-adenosylmethionine synthetase* (*sams*) housekeeping gene as references (72). Relative expression level of each target gene was obtained by $2^{(-\Delta\text{Ct sample})}$ where $\Delta\text{Ct sample} = \text{Ct sample} - \text{Ct gene of reference}$ (average of *rps9* and *sams*).

Transmission electron microscopy and 3D reconstruction

For electron microscopy animals were relaxed in 7,5% MgCl₂, fixed in 2,5% glutaraldehyde buffered in 0,1M phosphate buffer and 0,3M NaCl, rinsed 3 times in the same buffer, and postfixed in 1%OsO₄ in the same buffer for 1h. The specimens were dehydrated in ascending acetone series, transferred in propylene oxide and embedded in Epon-Araldite resin (EMS). Ultrathin sections (60-80nm) were cut with Reichert Ultracut E or Leica Ultracut UCT and counterstained with 2% uranyl acetate and Reynolds lead citrate. Images were acquired using Zeiss Libra 120, FEI Technai or Jeol JEM-1400 transmission Electron microscopes and processed with Fiji and Adobe Photoshop software. For 3D-reconstructions the series of semithin (700 nm) sections were cut with Diatome HistoJumbo diamond knife (Blumer et al., 2002), stained with methylene blue/basic fuchsine (D'amico, 2009), and

1 digitalized at 40x magnification using Leica DM2500 microscope with camera. The images
2 were aligned with IMOD and ImodAlign tool. The reconstructions were made with Fiji
3 TrackEM2 plugin.
4

5 6 **List of abbreviations**

7
8
9
10 BVS: blood vascular system

11 EST: expressed sequence tags

12 GFP: green fluorescent protein

13 HBL-Hb: giant hexagonal bilayer hemoglobin

14 HPC: hemoglobin producing cells

15 qPCR: quantitative polymerase chain reaction

16 RBC: red blood cells

17 TEM: transmission electron microscopy
18
19
20
21
22
23
24

25 **Declarations**

26 27 28 **Ethics approval and consent to participate**

29 "Not applicable" --- This research focuses on non cephalopod invertebrates. There are no
30 ethical considerations mentioned for these species according to EU Directive 86/609-STE123.
31
32

33 **Consent for publication**

34 "Not applicable"
35
36

37 **Availability of data and materials**

38 The data sets supporting the results of this article are available in the Figshare repository
39 (<https://figshare.com/account/home#/projects/72548>).
40
41

42 **Competing interests**

43 The authors declare no competing interests
44
45

46 **Funding**

47 S. Song obtained a PhD fellowship from the LABEX "WHO AM I?" (No.ANR-11-LABX-0071).
48 The Balavoine Lab was funded by the CNRS, the Université de Paris and two grants from the
49 ANR (METAMERE no. ANR-12- BSV2-0021 and TELOBLAST no. ANR-16- CE91-0007).
50
51

52 **Author's contributions**

53 SS performed all molecular biology experiments, analysed the data, composed the figures.
54 GB conceived the study, analysed the data, composed the figures and drafted the text. VVS
55 performed the semi-thin and TEM imagery, analysed the data and composed the figures. XB
56 participated in the phylogenetic analyses. PK participated in the genomic and transcriptomic
57
58
59
60
61
62
63
64
65

1 screenings. AJMC and CR participated in data analysis. All authors corrected the draft. All
2 authors read and approved the final manuscript.

3 **Acknowledgements**

4 We warmly thank Detlev Arendt's laboratory (EMBL Heidelberg, Germany) and Oleg
5 Simakov's laboratory (University of Vienna, Austria) for letting us use the data of the
6 *Platynereis* whole genome sequencing and assembly ahead of publication. We thank the
7 Balavoine Lab Members and Biofluidics Lab members for helpful discussions during the
8 course of this work. We acknowledge Nathalie Luciani for equipment and training for qPCR
9 experiments and analysis. We acknowledge the ImagoSeine facility (member of the France
10 Biolmaging infrastructure supported by the French National Research Agency, ANR-10-INSB-
11 04, 'Investments of the future') and its experienced staff for their assistance in photonic and
12 electron microscopy imaging and image analysis. We acknowledge the facilities «Chromas»
13 and «Center for Molecular and Cell Technologies» of St.Petersburg State University Research
14 park for the help with semithin sectioning and image analysis. We also would like to thank four
15 anonymous referees for valuable comments and advises.

26 **References**

- 27 1. Vinogradov SN, Hoogewijs D, Bailly X, Mizuguchi K, Dewilde S, Moens L, et al. A
28 model of globin evolution. *Gene*. 2007;398:132–142.
- 29 2. Vinogradov SN, Moens L. Diversity of globin function: Enzymatic, transport, storage,
30 and sensing [Internet]. Vol. 283. American Society for Biochemistry and Molecular Biology;
31 2008. 8773–7 p.
- 32 3. Zuckerkandl E, Pauling LB. Molecular disease, evolution, and genetic heterogeneity.
33 In: Kasha M, Pullman B, editors. *Horizons in Biochemistry*. New York: Academic Press; 1962.
34 p. 189–225.
- 35 4. Vinogradov SN, Hoogewijs D, Bailly X, Arredondo-Peter R, Gough J, Dewilde S, et al.
36 A phylogenomic profile of globins. *BMC Evol Biol*. 2006;6:1–17.
- 37 5. Vinogradov SN, Bailly X, Smith DR, Tinajero-trejo M, Poole RK, Hoogewijs D. *Microbial*
38 *Eukaryote Globins* [Internet]. 1st ed. Vol. 63. Copyright © 2013 Elsevier Ltd. All rights
39 reserved.; 2013. 391–446 p.
- 40 6. Bailly X, Chabasse C, Hourdez S, Dewilde S, Martial S, Moens L, et al. Globin gene
41 family evolution and functional diversification in annelids. *FEBS J*. 2007;274(10):2641–2652.
- 42 7. Belato FA, Coates CJ, Halanych KM, Weber RE, Costa-Paiva EM. Evolutionary History
43 of the Globin Gene Family in Annelids. Betran E, editor. *Genome Biol Evol*. 2020 Jun
44 29;evaa134.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
8. Hoffmann FG, Opazo JC, Hoogewijs D, Hankeln T, Ebner B, Vinogradov SN, et al. Evolution of the globin gene family in deuterostomes: Lineage-specific patterns of diversification and attrition. *Mol Biol Evol.* 2012;29(7):1735–1745.
9. Christensen AB, Herman JL, Elphick MR, Kober KM, Janies D, Linchangco G, et al. Phylogeny of echinoderm hemoglobins. *PLoS ONE.* 2015;10(8):e0129668.
10. Burmester T. Evolution of respiratory proteins across the pancrustacea. *Integr Comp Biol.* 2015;55(5):792–801.
11. Herhold HW, Davis SR, Grimaldi DA. Transcriptomes reveal expression of hemoglobins throughout insects and other Hexapoda. Robinson-Rechavi M, editor. *PLOS ONE.* 2020 Jun 5;15(6):e0234272.
12. Ebner B, Panopoulou G, Vinogradov SN, Kiger L, Marden MC, Burmester T, et al. The globin gene family of the cephalochordate amphioxus: Implications for chordate globin evolution. *BMC Evol Biol.* 2010;10(1):370.
13. Storz JF, Opazo JC, Hoffmann FG. Phylogenetic diversification of the globin gene superfamily in chordates. *IUBMB Life.* 2011;63(5):313–322.
14. Fago A, Rohlfing K, Petersen EE, Jendroszek A, Burmester T. Functional diversification of sea lamprey globins in evolution and development. *Biochim Biophys Acta - Proteins Proteomics.* 2018;1866(2):283–291.
15. Rohlfing K, Stuhlmann F, Docker MF, Burmester T. Convergent evolution of hemoglobin switching in jawed and jawless vertebrates. *BMC Evol Biol.* 2016 Dec;16(1):30.
16. Blank M, Burmester T. Widespread occurrence of N-terminal acylation in animal globins and possible origin of respiratory globins from a membrane-bound ancestor. *Mol Biol Evol.* 2012;29(11):3553–3561.
17. Dröge J, Pande A, Englander EW, Makalowski W. Comparative Genomics of Neuroglobin Reveals Its Early Origins. *PLoS ONE.* 2012;7(10).
18. Lechauve C, Jager M, Laguerre L, Kiger L, Correc G, Leroux C, et al. Neuroglobins, pivotal proteins associated with emerging neural systems and precursors of metazoan globin diversity. *J Biol Chem.* 2013;288(10):6957–6967.
19. Belato FA, Schrago CG, Coates CJ, Halanych KM, Costa-Paiva EM. Newly discovered occurrences and gene tree of the extracellular globins and linker chains from the giant hexagonal bilayer hemoglobin in metazoans. Eyre-Walker A, editor. *Genome Biol Evol* [Internet]. 2019 Jan 21 [cited 2019 Feb 11];
20. Burmester T, Weich B, Reinhardt S, Hankeln T. A vertebrate globin expressed in the brain. *Nature.* 2000 Sep;407(6803):520–3.
21. Burmester T, Hankeln T. What is the function of neuroglobin? *J Exp Biol.* 2009;212(10):1423–1428.
22. Ascenzi P, Gustincich S, Marino M. Mammalian nerve globins in search of functions.

IUBMB Life. 2014 Apr;66(4):268–76.

23. Roesner A, Fuchs C, Hankeln T, Burmester T. A Globin Gene of Ancient Evolutionary Origin in Lower Vertebrates: Evidence for Two Distinct Globin Families in Animals. *Mol Biol Evol.* 2005 Jan;22(1):12–20.
24. Blank M, Wollberg J, Gerlach F, Reimann K, Roesner A, Hankeln T, et al. A Membrane-Bound Vertebrate Globin. Uversky VN, editor. *PLoS ONE.* 2011 Sep 20;6(9):e25292.
25. Mangum CP, Colacino JM, Grassle JP. Red Blood Cell Oxygen Binding in Capitellid Polychaetes. *Biol Bull.* 1992 Feb;182(1):129–34.
26. Royer WE, Strand K, van Heel M, Hendrickson WA. Structural hierarchy in erythrocrucorin, the giant respiratory assemblage of annelids. *Proc Natl Acad Sci U S A.* 2000;97(13):7107–11.
27. Royer WE, Sharma H, Strand K, Knapp JE, Bhyravhatla B. Lumbricus Erythrocrucorin at 3.5 Å Resolution: Architecture of a Megadalton Respiratory Complex. *Structure.* 2006 Jul;14(7):1167–77.
28. Royer WE, Omartian MN, Knapp JE. Low Resolution Crystal Structure of Arenicola Erythrocrucorin: Influence of Coiled Coils on the Architecture of a Megadalton Respiratory Protein. *J Mol Biol.* 2007;365(1):226–236.
29. Fischer A, Dorresteyn A. The polychaete *Platynereis dumerilii* (Annelida): A laboratory animal with spiralian cleavage, lifelong segment proliferation and a mixed benthic/pelagic life cycle. *BioEssays.* 2004;26(3):314–325.
30. Fischer AHL, Henrich T, Arendt D. The normal development of *Platynereis dumerilii* (Nereididae, Annelida). *Front Zool.* 2010;7(1):31.
31. Zantke J, Bannister S, Veedin Rajan VB, Raible F, Tessmar-Raible K. Genetic and genomic tools for the marine annelid *Platynereis dumerilii*. *Genetics.* 2014;197(1):19–31.
32. Bailly X, Vinogradov SN. The Bilatarians Sea Urchin and the Radial Starlet Sea Anemone Globins Share Strong Homologies with Vertebrate Neuroglobins. *Dioxygen Bind Sens Proteins.* 2008;191–201.
33. Resh MD. Covalent lipid modifications of proteins. *Curr Biol.* 2013;23(10):R431–R435.
34. Jouan L, Marco S, Taveau J-C. Revisiting the structure of *Alvinella pompejana* hemoglobin at 20Å resolution by cryoelectron microscopy. *J Struct Biol.* 2003 Jul;143(1):33–44.
35. Wells RMG, Warren LM. The function of the cellular haemoglobins in *Capitella capitata* (Fabricius) and *Ivotomastus latericeus sars* (capitellidae: polychaeta). *Comp Biochem Physiol.* 1975;51A:737–40.
36. Gorr TA, Gassmann M, Wappner P. Sensing and responding to hypoxia via HIF in model invertebrates. *J Insect Physiol.* 2006;52(4):349–364.
37. Dales RP, Pell JS. Cytological aspects of haemoglobin and chlorocruorin synthesis in

polychaete annelids. Z Für Zellforsch Mikrosk Anat. 1970;109(1):20–32.

38. Burmester T, Ebner B, Weich B, Hankeln T. Cytoglobin: A Novel Globin Type Ubiquitously Expressed in Vertebrate Tissues. Mol Biol Evol. 2002 Apr 1;19(4):416–21.

39. Kakar S, Hoffman FG, Storz JF, Fabian M, Hargrove MS. Structure and reactivity of hexacoordinate hemoglobins. Biophys Chem. 2010 Nov;152(1–3):1–14.

40. Burmester T, Hankeln T. Function and evolution of vertebrate globins. Acta Physiologica. 2014.

41. Geuens E, Brouns I, Flamez D, Dewilde S, Timmermans JP, Moens L. A globin in the nucleus! J Biol Chem. 2003;278(33):30417–20.

42. Koch J, Burmester T. Membrane-bound globin X protects the cell from reactive oxygen species. Biochem Biophys Res Commun. 2016;469(2):275–280.

43. Wakasugi K, Nakano T, Morishima I. Oxidized human neuroglobin acts as a heterotrimeric G α protein guanine nucleotide dissociation inhibitor. J Biol Chem. 2003;278(38):36505–36512.

44. Hardison RC. Evolution of Hemoglobin and Its Genes. Cold Spring Harb Perspect Med. 2012;2(12):1–18.

45. Zeis B. Hemoglobin in Arthropods-Daphnia as a Model. Subcell Biochem. 2020;94:163–194.

46. Ilan E, Weisselberg E, Daniel E. Erythrocrucorin from the water-flea Daphnia magna. Quaternary structure and arrangement of subunits. Biochem J. 1982;207(2):297–303.

47. Postnikova GB, Shekhovtsova EA. Myoglobin: Oxygen Depot or Oxygen Transporter to Mitochondria? A Novel Mechanism of Myoglobin Deoxygenation in Cells (review). 2018.

48. Trent JT, Hargrove MS. A ubiquitously expressed human hexacoordinate hemoglobin. J Biol Chem. 2002;277(22):19538–19545.

49. Hankeln T, Wystub S, Laufs T, Schmidt M, Gerlach F, Saaler-Reinhardt S, et al. The cellular and subcellular localization of neuroglobin and cytoglobin - A clue to their function? IUBMB Life. 2004;56(11–12):671–679.

50. Oleksiewicz U, Liloglou T, Field JK, Xinarianos G. Cytoglobin: biochemical, functional and clinical perspective of the newest member of the globin family. Cell Mol Life Sci. 2011 Dec;68(23):3869–83.

51. Schwarze K, Campbell KL, Hankeln T, Storz JF, Hoffmann FG, Burmester T. The globin gene repertoire of lampreys: Convergent evolution of hemoglobin and myoglobin in jawed and jawless vertebrates. Mol Biol Evol. 2014;31(10):2708–2721.

52. Burmester T, Storf J, Hasenjäger A, Klawitter S, Hankeln T. The hemoglobin genes of Drosophila. FEBS J. 2006;273(3):468–480.

53. Yadav R, Kundu S, Sarkar S. *Drosophila glob1* expresses dynamically and is required for development and oxidative stress response: *Drosophila glob1* Expresses Dynamically.

genesis. 2015 Dec;53(12):719–37.

54. Gleixner E, Ripp F, Gorr TA, Schuh R, Wolf C, Burmester T, et al. Knockdown of *Drosophila* hemoglobin suggests a role in O₂ homeostasis. *Insect Biochem Mol Biol*. 2016;72:20–30.

55. Pillai AS, Chandler SA, Liu Y, Signore AV, Cortez-Romero CR, Benesch JLP, et al. Origin of complexity in haemoglobin evolution. *Nature*. 2020;581(July 2019):1–6.

56. Lindner E. Ferritin und Hemoglobin im Chloragog von Lumbriciden (Oligochaeta). *Z Zellforsch Mikrosk Anat*. 1965;66(6):891–913.

57. Nakahama S, Nakagawa T, Kanemori M, Fukumori Y, Sasayama Y. Direct Evidence That Extracellular Giant Hemoglobin is Produced in Chloragogen Tissues in a Beard Worm, *Oligobranchia mashikoi* (Frenulata, Siboglinidae, Annelida). *Zoolog Sci*. 2008;25(12):1247–52.

58. Breton-Gorius J. Etude au microscopie électronique des cellules chloragogènes d'*Arenicola marina* L: leur rôle dans la synthèse de l'hémoglobine [Internet]. Masson; 1963.

59. Friedman MM, Weiss L. An electron microscopic study of hemoglobin synthesis in the marine annelid, *Amphitrite ornata* (Polychaeta: Terebellidae). *J Morphol*. 1980 May;164(2):121–38.

60. Kennedy GY, Dales RP. The Function of the Heart-Body in Polychaetes. *J Mar Biol Assoc U K*. 1958 Feb;37(1):15–31.

61. Mangum CP, Colacino JM, Vandergon TL. Oxygen binding of single red blood cells of the annelid bloodworm *Glycera dibranchiata*. *J Exp Zool*. 1989 Feb;249(2):144–9.

62. Fahrenbach WH. The cyanoblast: Hemocyanin formation in *Limulus Polyphemus*. *J Cell Biol*. 1970;44(2):445–453.

63. Albrecht U, Keller H, Gebauer W, Markl J. Rhogocytes (pore cells) as the site of hemocyanin biosynthesis in the marine gastropod *Haliotis tuberculata*. *Cell Tissue Res*. 2001;304(3):455–462.

64. Kokkinopoulou M, Spiecker L, Messerschmidt C, Barbeck M, Ghanaati S, Landfester K, et al. On the Ultrastructure and Function of Rhogocytes from the Pond Snail *Lymnaea stagnalis*. Knight M, editor. *PLOS ONE*. 2015 Oct 21;10(10):e0141195.

65. Xie Y, Zheng Y, Li H, Luo X, He Z, Cao S, et al. GPS-Lipid: A robust tool for the prediction of multiple lipid modification sites. *Sci Rep*. 2016;6(June):1–9.

66. Ning W, Jiang P, Guo Y, Wang C, Tan X, Zhang W, et al. GPS-Palm: a deep learning-based graphic presentation system for the prediction of S-palmitoylation sites in proteins. *Brief Bioinform*. 2020;

67. Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 2004;32(5):1792–1797.

68. Guindon S, Gascuel O. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. Rannala B, editor. *Syst Biol*. 2003 Oct 1;52(5):696–704.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
69. Le SQ, Gascuel O. An improved general amino acid replacement matrix. *Mol Biol Evol.* 2008;25(7):1307–1320.
70. Anisimova M, Gascuel O. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Syst Biol.* 2006;55(4):539–552.
71. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics.* 2003;19(12):1572–1574.
72. Schenk S, Krauditsch C, Fruhauf P, Gerner C, Raible F. Discovery of methylfarnesoate as the annelid brain hormone reveals an ancient role of sesquiterpenoids in reproduction. *eLife.* 2016;5(NOVEMBER2016):1–23.
73. Mangum CP, Kondon M. The role of coelomic hemerythrin in the sipunculid worm *Phascolopsis gouldi*. *Comp Biochem Physiol A Physiol.* 1975 Apr;50(4):777–85.
74. Klippenstein GL. Structural Aspects of Hemerythrin and Myohemerythrin1. *Integr Comp Biol.* 2015 Aug 1;20(1):39–51.
75. Zhang JH, Kurtz DM. Two distinct subunits of hemerythrin from the brachiopod *Lingula reevii*: an apparent requirement for cooperativity in oxygen binding. *Biochemistry.* 1991 Sep;30(38):9121–5.
76. Martín-Durán JM, De Mendoza A, Sebé-Pedrós A, Ruiz-Trillo I, Hejnal A. A broad genomic survey reveals multiple origins and frequent losses in the evolution of respiratory hemerythrins and hemocyanins. *Genome Biol Evol.* 2013;5(7):1435–1442.
77. Schmidt-Rhaesa A. The Evolution of Organ Systems. *The Evolution of Organ Systems.* 2007.
78. Ota M, Isogai Y, Nishikawa K. Structural requirement of highly-conserved residues in globins. *FEBS Lett.* 1997 Sep 29;415(2):129–33.
79. Cannon JT, Vellutini BC, Smith J, Ronquist F, Jondelius U, Hejnal A. Xenacoelomorpha is the sister group to Nephrozoa. *Nature.* 2016;530(7588):89–93.
80. Philippe H, Poustka AJ, Chiodin M, Hoff KJ, Dessimoz C, Tomiczek B, et al. Mitigating Anticipated Effects of Systematic Errors Supports Sister-Group Relationship between Xenacoelomorpha and Ambulacraria. *Curr Biol.* 2019;1–9.

48 **Figure legends**

50
51
52 **Figure 1: Distribution of circulatory systems and respiratory pigments in the metazoan consensus tree.** Some species have no known respiratory pigments despite having a circulatory system or at least a fluid filled cavity (nematodes, most echinoderms, urochordates, cephalochordates). It is assumed that in these species, gases either diffuse freely through thin layers of tissues or are freely dissolved in the hemolymph. Some groups have circulating

60
61
62
63
64
65

1 hemoglobins (“red blood”). The status of these hemoglobins is very diverse. They can be
2 extracellular hemoglobins dissolved in the blood, as in many annelids. They can be contained
3 in red cells as in the vertebrates or in some annelids such as capitellids (25). Some groups
4 have circulating dissolved hemerythrins (“pink blood”), like priapulids, brachiopods and some
5 annelids (Sipunculidae, Magelonidae) (73–75). Mollusks on one side and many arthropods on
6 the other side have circulating dissolved hemocyanins (“blue blood”) of different molecular
7 origins (76). Circulating respiratory pigments of different types are generally not present
8 together in the blood, suggesting that the recruitment of each type of pigment for the
9 respiratory function occurred multiple times independently in the evolution of bilaterians (76).
10 HG: haemoglobin; HC: hemocyanin; mHC: molluscan “hemocyanin”; HE: hemerythrin; BVS:
11 blood vascular system; CCS: coelomic circulatory system. Synthetic information on respiratory
12 pigments can be found in (77).
13
14
15
16
17
18
19
20
21

22 **Figure 2: The globin genes of *Platynereis dumerilii*.** A - Schematic representation of
23 aligned primary structures of globin genes identified in *Platynereis* transcriptomes and
24 genome. Five invariant amino acid positions provide solid anchor sites for precise homology
25 alignments. Small Alignment gaps are not represented. The predominantly conserved introns
26 positions B12.2 and G7.0 are marked by green stapled lines. Additional introns positions are
27 shown with green arrowheads. Predicted N-terminal signal peptides and acylations are
28 represented as shown. B - Schematic representation of the two globin gene clusters found on
29 *Platynereis* scaffolds. Orange and red block arrows indicate the length and strand position of
30 the genes. Each gene is interrupted by 3 - 4 introns. Gene *Egb-B1* partially overlaps with gene
31 *Egb-B2*, by a small exon located inside one of the other gene introns.
32
33
34
35
36
37
38
39

40 **Figure 3: Maximum likelihood tree of metazoan globin sequences.** LG model of amino
41 acid substitution was used and a SH-like test of a dataset of 272 sequences from 24 metazoan
42 species. To improve readability and facilitate annotations, a simplified tree version is used
43 (extensive version of the tree in additional file 5). Gene names do not appear and branches
44 have been color-coded according to the clade of the metazoan tree in which they occur (colour
45 code in the box on the right). The tree is arbitrarily rooted outside of the natural clades. Green
46 and red diamonds indicate a number of nodes that are supported by aLRT values superior to
47 0.75 and 0.95, respectively. The tree shows 5 groups (clade I, clade II, clade III, clade IV,
48 clade V), 4 of the 5 with well-supported nodes (clade I, clade II, clade III, clade IV),
49 encompassing sequences of most bilaterian phyla. An additional well-supported node groups
50 together only spiralian species sequences. *Platynereis* sequences are indicated by their
51 names and thicker red branches.
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 4: Expression profiling of *Platynereis* extracellular globins. A – H: Expression patterns of *Pdu-Egb-A2*. The expression patterns of other extracellular *P. dumerilii* globins are spatially very similar and located in the same hemoglobin-producing cells of transverse trunk vessels and parapodial vessels (additional file 10). A - 18-segment juvenile show expression in transverse vessels in the trunk in a series of adjacent segments (red arrowheads). There is no expression in the rostral-most and caudal-most segments. A' - magnified view of three central segments. B and C - 22-segment juvenile and 35-segment juvenile show expression in the parapodia (segmental appendages) for central segments and expression in lateral vessels in a few segments more rostrally and caudally located. C' - magnified view of a few anterior segments of worm (C) showing some residual expression in transverse vessels (red arrowheads). D - drawing of a pre-mature worm explaining the dissection and mounting of parapodia. E-G - decreasing expression of *Egb-A2* in parapodia of worms undergoing sexual metamorphosis. Notice the delineation of vessels by HPC in the dorsal part of parapodium (E). I: Relative abundance of mRNA of *P. dumerilii* extracellular globin genes, detected by real-time qPCR, at different life cycle stages. The sub-adult is a 12-week old worm. Metamorphosis stage 1 is defined according to the following criteria: eyes starting to bulge, parapodia starting to transform. Metamorphosis stage 2: eyes grown to maximal volume, parapodia fully transformed, but the animal is not displaying active swimming behaviour yet. Reference genes are *rps9* and *sams*. For stage 50 segments, stage I pre-matures, stage II pre-matures and swarming worms, the data points correspond to the median value of biological triplicates.

Figure 5: Developing vasculature and hemoglobin producing cells in *Platynereis* as revealed by semi-thin sections and TEM. A-J: 3D reconstruction of the blood vascular systems of early *P. dumerilii* juveniles. All reconstructions are made from stacks of semi-thin sections using Fiji TrackEM2 plugin. 4- (A-B), 5- (C-D), and 6- (E-F) segments juveniles of *Platynereis dumerilii*. A, C, E: ventral views. B, D, F: Lateral left views. Arrowheads in E and F label putative degenerative HPC (green). G, H: single segment of a 25-segment juvenile worm, carrying a pair of appendages (parapodia) in anterior view and dorsal view, respectively. The outside body wall is lined in light blue. The intestine endoderm is in green. I, J: Vascular system in three contiguous segments of a 50-segment sub-adult worm. For the sake of clarity, the dense intestinal blood network (or gut blood sinus) is not shown. *dbv* – dorsal blood vessel, *hc* – hemoglobin-producing cells (blue), *i* – intestine, *ibv* – intestinal blood vessels, *npv* – nephridial vessels, *tbv* – transversal blood vessels, *vbv* – ventral blood vessel, *tbs* – transverse blood sinus (pygidium), all vessel lumens are in red. K-R: Ultrastructure of *P. dumerilii* HPC in a juvenile worm, 25-30 segments. K: transversal semithin section of a mid-body segment in a 25-segment juvenile worm, showing the position of HPC around the transversal blood vessel. L: magnified view of the transversal vessel surrounded by HPC (rectangle selection in K). M:

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Transmission electron micrograph of the overall hemoglobin-producing tissue organization around a transversal blood vessel. N-R: Transmission electron micrographs of different parts of the HPC. N: Apical part of a hc, containing nucleus (*nu*) and rough endoplasmic reticulum (*rer*). O: Basal part of the cell with mitochondria (*mt*), rough endoplasmic reticulum (*rer*), and ferritin granules (*fg*). P: closer view of a hematin vesicle (*hm*). Q: Invaginations of the basal membrane (arrowheads) indicating active exocytosis process. R: Transmission electron micrographs of the degenerating HPC in sub-adult *P. dumerilii* (about 50 segments); cross-section through the transversal blood vessel at the level of HPC. Notice the cytoplasm, filled by electron-lucent vesicles (*arrowheads*), the large electron-lucent vesicle (*asterisk*) and the granulocytes filled with electron-dense and electron-lucent granules. *bv* – blood vessel lumen, *dmc* – muscular cells of the dissepiment, *gc* – granulocyte, *nu* – cell nucleus, *coe* – coelomic cavity, *dbv* – dorsal blood vessel, *ecm* – extracellular matrix (vascular lamina), *hm* – hematin granules, *hc* – hemoglobin-producing cells, *mt* – mitochondria, *nu* – cell nucleus, *rer* – rough endoplasmic reticulum, *vnc* – ventral nerve cord. Scale bars : K-L: 50 μm , M: 10 μm , N: 4 μm , O-Q: 2 μm , R: 10 μm .

Figure 6: Phylogenetic classification of metazoan globins. A - Table of the clades suggested in this study. The current name column indicates the denominations used in previous literature. B - Table of the number of genes of each clade present in the species genome we sampled. For clade I, the number of extracellular globins is indicated between parentheses. An asterisk shows all the species in which HBL-Hb linker proteins have been detected. Interrogation marks indicate missing data because the whole genome is not available for the relevant species. On the right, the animal tree shows the inferences of gain and loss of globin gene clades in key metazoan ancestors. Colored disks indicate the gain of a new gene clade by duplication from existing ancestral genes while crossed disks illustrate the numerous secondary losses of globin clades. The green circles with a question mark illustrate the uncertainty on the appearance of a clade III gene in metazoan ancestors, as some cnidarians and a ctenophore possess genes with possible clade III affinities (additional file 8). In the deuterostome branch, the extinction of clade III (green circle with a cross) occurred independently in *Branchiostoma*, *Ciona* and *Homo* lineages because clade III genes (globin X) have been identified in several vertebrate species (40).

Additional information

Additional file 1: Amino acids alignment of the *Platynereis* extracellular globins with the respiratory globins of *Arenicola marina*, *Lumbricus terrestris* and human hemoglobins. The

1 highly conserved residues of globins in dark red (78). The conserved cysteines NA2 and H7
2 residues known to be involved in the formation of an intrachain disulfide chain in annelids are
3 shown in black (6).
4
5

6 **Additional file 2: Table of the *Platynereis* globin sequences extracted from**
7 **transcriptomes, blasted on the *Platynereis* genome assembly.** Sheet 1: the hits for each
8 mRNA sequence are listed in order of decreasing E-Value. Each hit partial sequence was
9 reciprocally blasted on the *Platynereis* mRNAs and on Genbank for unidentified sequences.
10 Some of the hits correspond to likely distant haplotypes. Others do not correspond to a globin
11 sequence. Sheet 2: the genome assembly was screened with a concatenated *Platynereis*
12 transcriptome globin probe to detect potential additional genes. The hits are listed by
13 decreasing E-value. Partial hits were reciprocally blasted on Genbank and *Platynereis*
14 transcriptome. Most high E-value hits correspond to the genes already identified in the
15 transcriptome. A series of low E-value, short sequence globin hits on Genbank were found to
16 code for *Platynereis* proteins that bear no similarity to globins, indicating that no additional
17 globin gene is present in the assembly.
18
19
20
21
22
23
24
25
26
27

28 **Additional file 3: List of all predicted metazoan globin protein sequences and of all**
29 **predicted hexagonal bilayer linker protein sequences used in this study.**
30
31
32

33 **Additional file 4: Nexus file of aligned globin sequences used for the phylogenetic**
34 **analysis.** The unaligned sequences at the N- and C- termini were cropped as well as some
35 insertions which likely correspond to undetected intronic sequences.
36
37
38
39

40 **Additional file 5: Maximum likelihood tree using LG model and a SH-like test of a dataset**
41 **of 272 sequences from 24 metazoan species, (simplified in Figure 3).** The letter and color
42 codes for species names is explained at the right of the figure. The tree is arbitrarily rooted
43 outside of the clear natural groups. The tree shows 5 groups (clade I, clade II, clade III, clade
44 IV, clade V), 4 of the 5 with well-supported nodes (clade I, clade II, clade III, clade IV),
45 encompassing sequences of most bilaterian phyla. An additional well-supported node groups
46 together only spiralian species sequences. Support values for maximum-likelihood with LG
47 model, bayesian posterior probability with LG model and bayesian posterior probability with
48 WAG model are indicated on the key nodes discussed. *Platynereis* sequences are indicated
49 in red labels. The globin peptides predicted to be extracellular or membrane-bound are
50 indicated with blue or green rectangles, respectively.
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
Additional file 6: Table of all globin sequences indicating their clade affinity in the ML tree, the signal peptide prediction and N-terminal acylation prediction. Signal peptide prediction is done with SignalP 5.0. Myristoylation is predicted with GPS-lipid algorithm and palmitoylation with GPS-Palm algorithm. The dual acylation prediction is based on two closely located myristoylation and palmytoylation sites only.

10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
Additional file 7: List of additional globin protein sequences, from non bilaterian metazoans (*Aurelia aurita*, *Clytia hemisphaerica*, *Hydra vulgaris*, *Amphimedon queenslandica*, *Mnemiopsis leydyi*), one acoel (*symsagittifera roscoffensis*), and two choanoflagellates (*Salpingoeca rosetta*, *Monosiga brevicollis*). The two globins from the acoel are extracted from a transcriptome (18). Acoels are part of Xenacoelomorpha, a group of worms that has been proposed to be the sister-group of all remaining bilaterians (79). This view remains however challenged as they are also proposed to belong with the deuterostomes (80). Choanoflagellates are a group of eukaryots closely related to metazoans, that had been reported to possess neuroglobin-like proteins (18).

27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
Additional file 8: Maximum likelihood tree using LG model and a SH-like test of a dataset of 293 sequences from 32 metazoan species. The tree is arbitrarily rooted outside of the natural clades. Green and red diamonds indicate a number of nodes that are supported by aLRT values superior to 0.75 and 0.95, respectively. The six clades of globins previously highlighted are again recovered. as Hydrozoans (in our analysis, *Hydra* and *Clytia*) possess genes related to the clade III. One ctenophore, *Mnemiopsis*, also display a clade III related gene. One occurrence of a clade I gene in the hydrozoan *Clytia* appears suspicious as the protein does not show any particular similarity with clade I globins in a blast search. Last, the acoel is shown to possess clade III and V globins. In the absence of a complete genome sequence and the phylogenetic position of acoels being ambiguous, it does not inform on the early evolution of globins.

47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
Additional file 9: The life cycle of *Platynereis dumerilii*. *Platynereis dumerilii* is a medium-sized annelid that can be easily cultured in the laboratory, giving a large number of offspring all year round. The life cycle is fairly typical of marine annelids. It includes a microscopic (160 μ m diameter) lecithotrophic trochophore larva that elongates after 2,5 days in a minute three-segment worm (400 μ m long). Once settled in the benthos in a silk tube, the larva will grow by posterior addition of segments and in cross-section to reach a considerably larger size (5-6 cm). The worm lives a relatively sedentary life, feeding on a variety of fresh or decaying food in the benthos for the longest part of its lifespan. Nearing the end of its life, the worm

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

undergoes a rather dramatic sexual metamorphosis: the coelom, from head to tail, entirely fills up with gametes; the worm segmental appendages, called parapodia, change shape and acquire a swimming locomotory function; the gut degenerates as adults do not eat. Both males and females exit their tubes to swarm at the surface of the sea. At this stage they acquire a very fast swimming behaviour and have only a few hours for mating before they die of exhaustion.

Additional file 10: Expression patterns of five extracellular globin genes in *Platynereis dumerilii*. WMISH on juvenile stages (30-35 segments). The expression patterns of all extracellular globins are located in the same HPC of transverse trunk vessels (red arrowheads) and parapodial vessels (blue arrowheads).

Additional file 11: Movie of a 3D reconstruction of the vasculature of a 50-segment sub-adult worm. 3 consecutive segments are shown.

Additional file 12: Table of relative normalized expression of *Platynereis* extracellular globins measured by qPCR.

Additional file 13: Table of genomic and transcriptomic resources used for 32 species.

Additional file 14: List of primers used for PCR amplification of *Platynereis* globin cDNAs.

Additional file 15: List of primers used for qPCR amplification of *Platynereis* extracellular globins.

The figure shows a phylogenetic tree on the left and a corresponding table on the right. The tree branches from a common ancestor at the bottom left, indicated by a dashed line, and splits into several major groups. The table lists 21 animal groups, their respiratory pigments, and their circulatory systems. The respiratory pigments are color-coded: red for Hemoglobin (HG), pink for Hemerythrin (HE), blue for Myoglobin (mHC), and cyan for Hemocyanin (HC). The circulatory systems are color-coded: purple for Blood Vessel System (BVS), orange for Coelom (CO), and brown for Coelomic Cavity System (CCS). Shaded triangles in the tree point to the groups Cephalopoda, other Mollusca, and Branchiopoda.

	Respiratory pigment	Circulatory system
Porifera	—	—
Ctenophora	—	—
Placozoa	—	—
Cnidaria	—	—
Vertebrata	HG	closed BVS + coelom
Urochordata	—	open BVS + hemocoel
Cephalochordata	—	closed BVS + coelom
Hemichordata	—	open BVS + hemocoel
Echinodermata	HG in few species	BVS + CCS
Platyhelminthes	—	—
Brachiopoda	HE	open BVS
Nemertea	—	CCS
Cephalopoda	mHC	closed BVS
other Mollusca	mHC sometimes HG	open BVS + hemocoel
Annelida	HG sometimes HE	closed BVS + coelom
Priapulida	HE	pseudocoelom
Kinorhyncha	—	pseudocoelom
Nematoda	—	pseudocoelom
Tardigrada	—	—
Onychophora	HC	open BVS + hemocoel
Chelicerata	HC	open BVS + hemocoel
Myriapoda	HC	open BVS + hemocoel
other Pancrustacea	HC or HG	open BVS + hemocoel
Branchiopoda	HG	open BVS + hemocoel
Insecta	HC	open BVS + hemocoel
Acoelomorpha	—	—

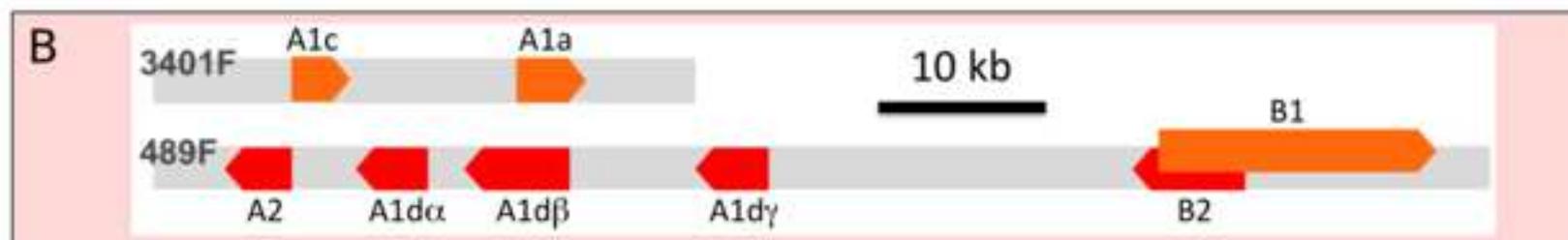
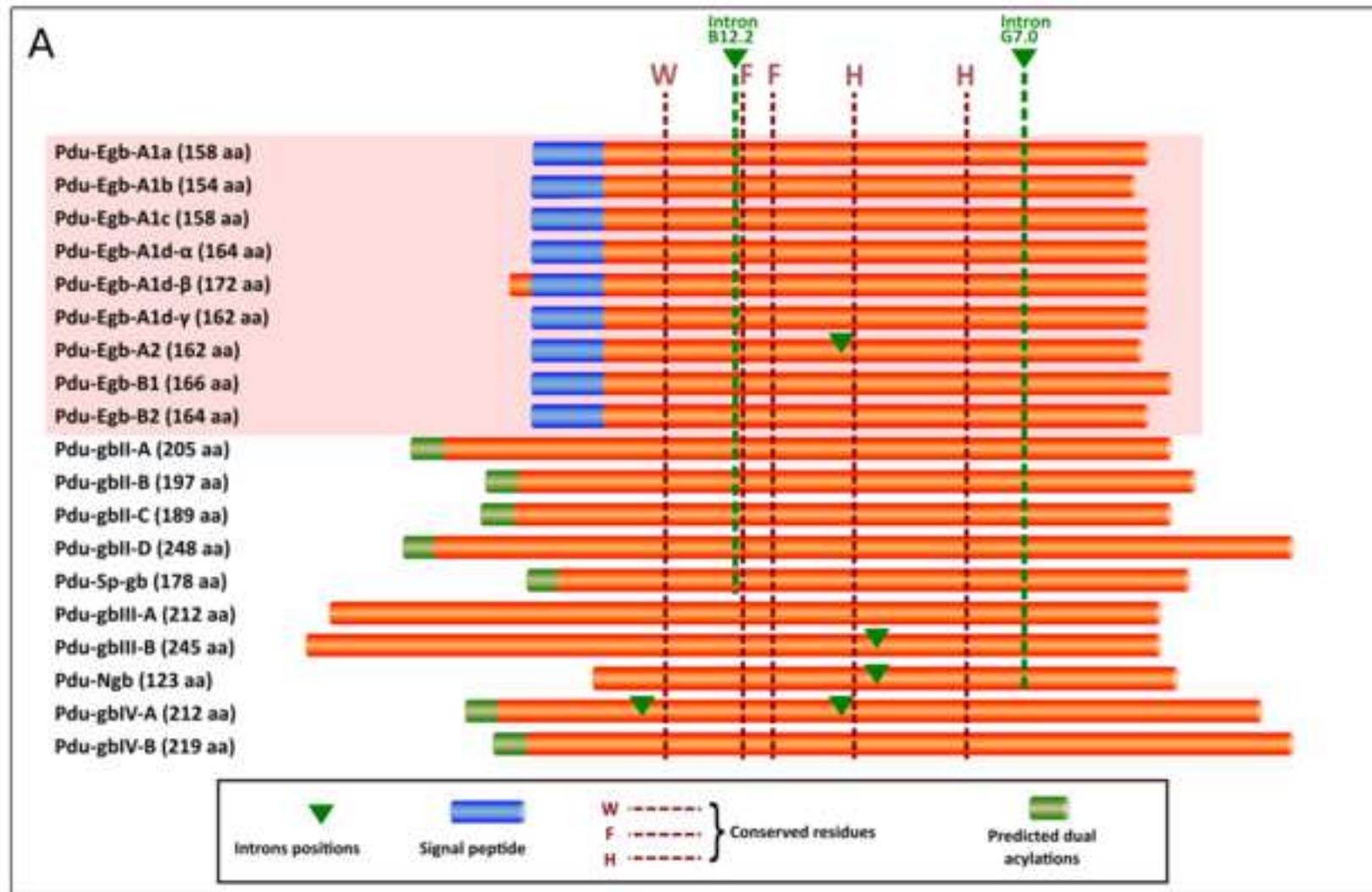
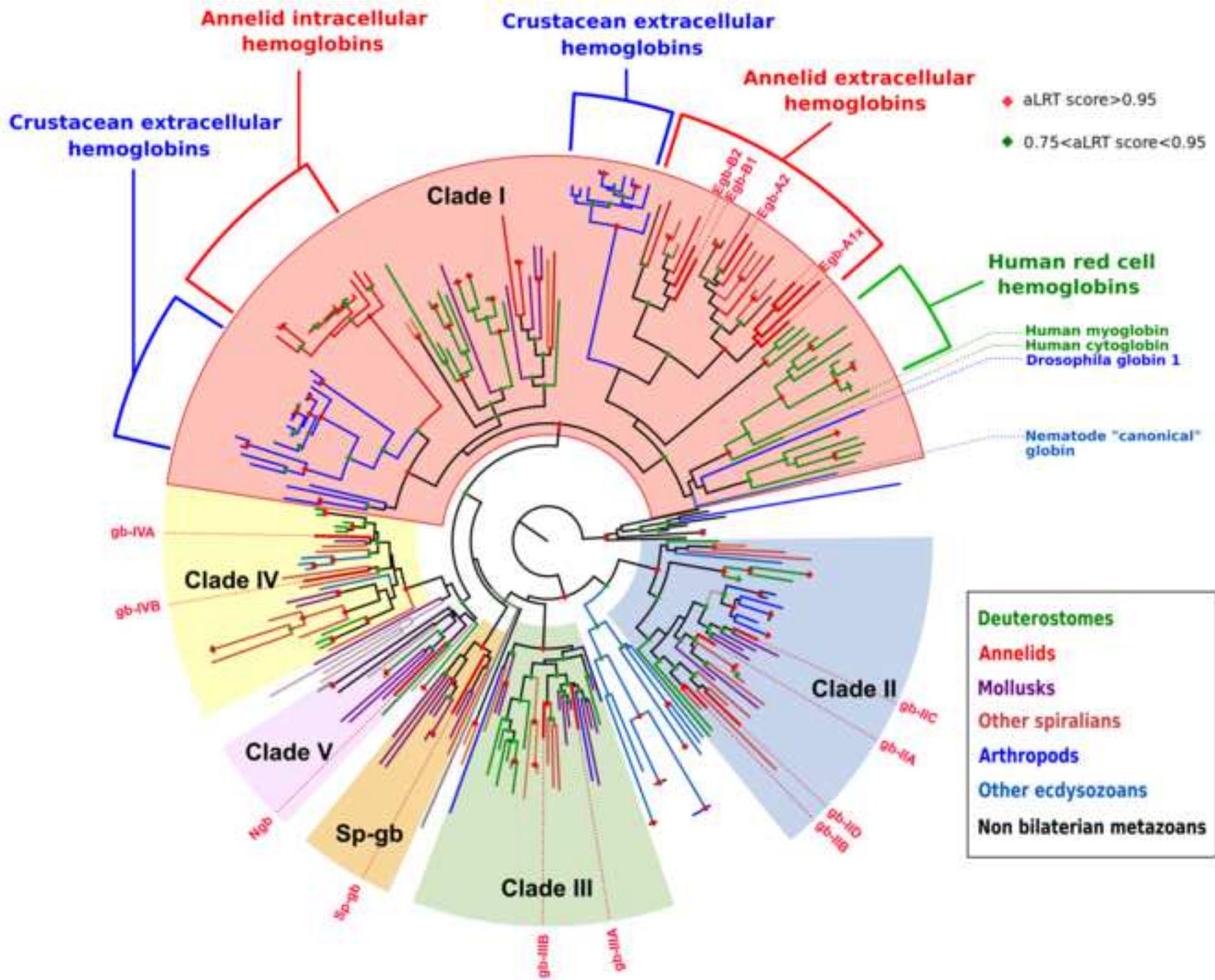
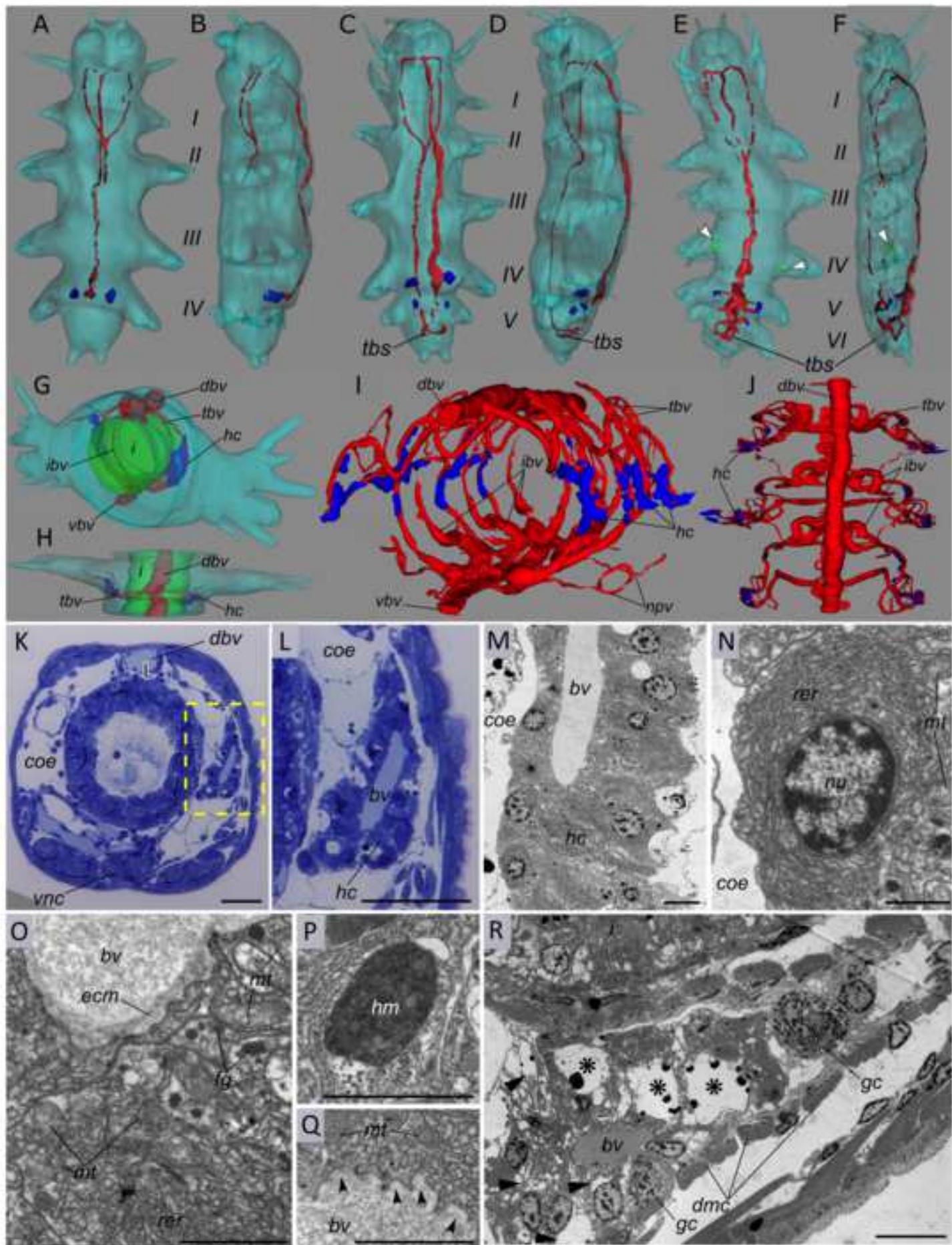


Figure 3





A

Phylogenetic classification	Current name	Cytological localization	Phylogenetic distribution	References	Remarks
Clade I	gnathostome red blood cell hemoglobins (Hb)	cytoplasmic	all gnathostomes except icefish	Jensen et al, 1998 Harrison, 2012 Gao, 2018	α and β hemoglobins forming heterotetramers is cooperative O ₂ binding
	agnathan red blood cell hemoglobins (Hb)	cytoplasmic	lampreys and hagfish	Hoffmann et al, 2010 Schwarze et al, 2014 Fago et al, 2018	evolved convergently respective to gnathostome Hb form dimers and tetramers cooperative O ₂ binding
	myoglobin (Mb)	cytoplasmic	most gnathostomes	Brunori, 2009 Stors et al, 2011	monomeric muscle globin paralogous with Hb and CgH through whole genome duplication
	globin E (GbE)	cytoplasmic	some sarcopterygians	Lidenharn et al, 2019	GbE is a myoglobin paralog with specialized functions in the eye or oocytes
	cytoglobin (CgH)	cytoplasmic nuclear	all vertebrates	Burmester et al, 2002 Olskiewicz et al, 2011	forms homodimers paralogous with Hb and Mb through whole genome duplication
	globin Y (GbY)	cytoplasmic	many gnathostomes	Fuchs et al, 2006	possible early Hb paralog
	"insectahemoglobins"	mostly cytoplasmic extracellular in Chironomus	most hexapods	Herfeld et al, 2020	mostly non circulating globins intracellular respiratory role orthologous to all vertebrate clade I globins
	crustacean extracellular blood globins	soluble in hemolymph	branchiopods	Burmester, 2015	bidomain or multidomain globins
	annelid extracellular blood globins "erythrocrurin", "chlorocrurin"	soluble in blood	most annelids	Bally et al, 2007 Belato et al, 2020	forms giant hexagonal bilayer particle orthologous to all vertebrate clade I globins
	annelid red blood cell hemoglobins	contained in circulating coelomic cells	some annelid families	Margum et al, 1992	evolved several times in correlation with the loss of a closed vascular system
Clade II	globin X-like	mostly membrane-bound	most bilaterians	Blank and Burmester, 2012	functions unknown
Clade III	globin X	mostly cytoplasmic some membrane-bound	many bilaterians absent in mammals and birds	Blank et al, 2011 Blank and Burmester, 2012 Koch and Burmester, 2016	one of globin X/Clade III globins proposed function is to protect cells from ROS
Clade IV	previously unnamed	mostly membrane-bound	many bilaterians	this work	functions unknown
Clade V	neuroglobins sensu stricto	cytoplasmic	non bilaterian metazoans some bilaterian groups	Burmester et al, 2000 Leclauve et al, 2013	the most ancient animal globin clade, present in choanoflagellates and in metazoans devoid of nerve cells (sponges) but presence is sporadic in bilaterians
Spiralian globins	previously unnamed	mostly cytoplasmic some membrane-bound	spiralian	this work	functions unknown

B

