ON DEVELOPMENT AND EVOLUTION OF *TRICHOPLAX ADHAERENS* (PLACOZOA)

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Zusammenfassung

Die Evolution der Tiere hat bis heute zu einer ungeheuren Diversität von Formen bereits ausgestorbener und rezenten Tierarten geführt. Das Hauptaugenmerk der Evolutionsbiologie liegt auf dem Verständnis der grundsätzlichen Prozesse, welche die Spezialisierung und Komplexität der Arten bedingen. Insbesondere die Evolution entwicklungsbiologischer Mechanismen innerhalb der Tierstämme scheinen direkt die evolutionäre Geschichte der Arten widerzuspiegeln. Deshalb könnten Einblicke in die regulatorischen Grundlagen zur Musterbildung der ältesten Tierstämme, den Diploblasten, zu einem besseren Verständnis grundlegender Bauplanveränderungen führen.

Gene besonderer Klassen, die eine Homeobox beinhalten, stellen die Haupkontrollelemente für musterbildende Mechanismen dar. Da diese Gene hochkonserviert in allen Tierstämmen vorliegen, wird ihre Evolution direkt mit der Evolution des tierischen Bauplans in Verbindung gebracht. Während wir in abgeleiteten „modernen" Bilateria komplexe, hochspezialisierte Funktionen der Homeobox Gene untersuchen, wissen wir noch sehr wenig über die ursprünglichen Funktionen von homologen Homeobox Genen in basalen Diploblasten.

In der vorliegenden Arbeit wurden drei hauptregulatorische Gene in der am einfachsten organisierten rezenten Art, *Trichoplax adhaerens* (Placozoa), charakterisiert, um ein besseres Verständnis ihrer Funktion in einem der ersten vielzelligen Tierstämme zu erlangen. Die Arbeit umfasst: (i) Analysen der entwicklungsbiologischen Funktionen des ProtoHox/ParaHox Gens *Trox-2*, (ii) Expressionsanalysen und phylogenetische Einordnung des Paired-Box Gens *TriPaxB* und (iii) Expressionsanalysen des Paired-Box Gens *TriPaxC,* sowie funktionelle Untersuchungen von Effekten bei RNAi Knockdown. Zusätzlich wurde das komplette mitochondriale Genom von *Trichoplax adhaerens* charakterisiert, um die ursprünglich erscheinenden Musterbildungsprozesse von *Trichoplax* in einen stammesgeschichtlichen Kontext einordnen zu können. Die erzielten Daten geben einen detaillierten Einblick in zahlreiche neue Besonderheiten von Zellaufbau und anatomischer Struktur von *Trichoplax adhaerens.* Zusammengefasst liefern die Ergebnisse aus dieser Arbeit die Erkenntnis, dass es sich bei Placozoa tatsächlich um einen Tierstamm handeln könnte, der den gemeinsamen Vorfahren aller Metazoa widerspiegelt.

Schlüsselwörter: Evolution, ProtoHox/ParaHox, Pax, Mt genome, Placozoa

Summary

Animal evolution led to a tremendous diversity in form and number of extinct and extant animal species. Understanding the principle processes leading to specialization and complexity has been a key issue in evolutionary biology. The evolution of animal developmental mechanisms seems to directly mirror the evolutionary history of extant animal phyla.

Therefore, insights into the principle developmental regulatory mechanisms of earliest branching diploblastic phyla might lead to an understanding how key transition and new body plans have evolved. Homeobox containing genes of different distinct classes represent the master control elements for developmental processes. They are highly conserved among all animal species. Thus indicating that the evolution of animal bauplans is directly linked to the evolution of these master control genes. While most of their functions are characterized in derived bilaterian animals we know only little about their roles in basal diploblast animals.

In this thesis, three key regulatory genes were characterized in the most simple organized extant species, *Trichoplax ahaerens* (Placozoa).

To gain deeper knowledge on the developmental functions in *Trichoplax*, the studies comprised: (I) analyses on the developmental function and expression of the ProtoHox/ParaHox gene, *Trox-2*, (II) expression analyses and phylogenetic implications of the Paired box gene *TriPaxB*, (III) expression analyses and effects of a RNAi knock down of the Paired box gene *TriPaxC*. In addition, in an attempt to place the unique developmental mechanisms of *Trichoplax adhaeraens* into a phylogenetic background, the complete mitochondrial genome of *Trichoplax* was analyzed.

The data collected in this study provide detailed insights into the patterning processes during vegetative development of *Trichoplax adhaerens*. Several new features of cell composition and anatomical organization of *Trichoplax* were identified.

In summary, the collected data provide insights that Placozoans indeed represent a basal phylum, close to the hypothetical common ancestor of all metazoans.

Key words:

Evolution, Development, ProtoHox/ParaHox, Pax, Mt genome, *Trichoplax adhaerens*

1. Introduction

1.1 Brief History of Placozoa

A little over a century ago, in 1883, a new marine invertebrate species was discovered and introduced to the scientific community as *Trichoplax adhaerens* (Greek: *trich* = hair, *plax*= plate, Latin: *adhaerere* = to stick). It was recognized on the glass wall of a seawater aquarium by the German biologist F.E. Schulze in Graz, Austria. Schulze described the histological organization of the enigmatic organism in great detail [1]. Because of its extreme morphological simplicity compared to all other metazoans, Schulze claimed the most basal phylogenetic position for *Trichoplax adhaerens* [1, 2]. In the following years scientist doubted Schulzes hypothesis and claimed *Trichoplax adhaerens* to represent an aberrant planula larvae of a derived cnidarian. Soon after Krumbach published his interpretation considering *Trichoplax* as an "abnormal" cnidarian *Trichoplax* was pushed out of scientific research [3-6]. German zoologists rediscovered *Trichoplax adhaerens* in the 1960´s and continued research on histology and cytology of *Trichoplax* at the ultastructural level. In 1971 K.G. Grell proposed a completely new phylum for *Trichoplax adhaerens*, called Placozoa (Greek: *plakos* = flat, + *zōia* = animals) [7, 8]. Today the phylum Placozoa is established in all recent biological textbooks and *Trichoplax adhaerens* is, so far the only described species [9-11].

Placozoan Biology

Placozoa are found in the littoral of tropical and subtropical seas all over the world [12-16]. Because of its microscopic size and its ability to accumulate fluorochromes of the substrate it is feeding on, it is nearly impossible to observe Placozoa in the field [15, 17]. Knowledge about placozoan biology is primary gained by observations of laboratory-cultured animals. Under laboratory culture conditions the individuals move over the substrate and feed on biofilms of algae and bacteria [2, 18, 19].

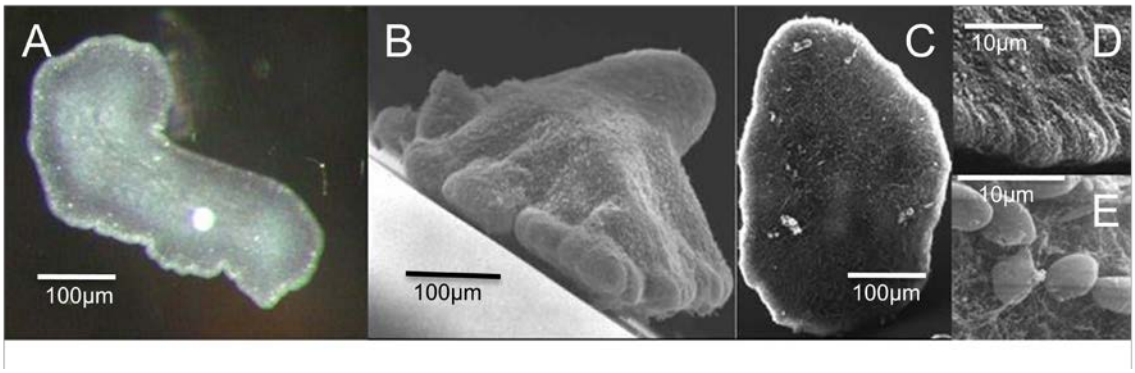

Figure 1. Light and s canning electron microscopic (SEM) micrographs of *Trichoplax adhaerens* habitus.
A) Light microscopic image
B) SEM image of the habitus
C) View lower epithelia with high cilia density
D) Detail of the marginal edge of *Trichoplax*
E) Detail of lower epithelia with attached partially digested algea

If the animals are moving, their bodies are changing shape randomly. Placozoan movement was previously interpreted as random or undirected [18, 20-23], but can be influenced by chemotaxis, phototaxis and mechanical stress (see also 1.3, 3.3). *Trichoplax* has neither organs nor any body axis [1, 2, 24-27]. The adult organism is described to consist of four to five differentiated somatic cell types building up three tissue layers [1, 2, 28-30]. Each layer lacks an extra cellular matrix and the cells of the tissues are connected by septated desmosomes [31-33]. The epidermis is built up by an upper and a morphological different lower tissue. A single ciliated, flattened cell type forms the upper functional epithelium. Under optimized culture conditions the upper epithelium shows inclusions of unknown origin, called shiny spheres [9, 28]. Under mechanical stress the animal is able to release the shiny spheres abruptly (Ana Signorovich, personal communication). Moreover, shiny spheres were recently associated with a possible defense mechanism of placozoans [34].

The lower functional epithelium is composed by at least two different, club shaped cell types – cylinder cells and gland cells. Ciliated cylinder cells are responsible for placozoan locomotion by cilia walking as well as for pinocytosis. Gland cells lack cilia and produce enzymes for exosomatic digestion of the substrate [9, 35].

The interspace (cavern) between the both epithelia is interspersed by fiber cells and filled with a fluid of unknown consistence. The fiber cells form a three dimensional, syncitium like network, with cell extensions connecting to the upper and l ower epithelia [31, 35, 36]. Active contraction of the fiber cells changes the animals shape [36-38]. Ultrastructural studies revealed that fiber cells contain bacteria of unknown

origin, presumed to represent endosymbionts. In addition fiber cells show sub cellular regions were mitochondria are located, so called mitochondrial complexes [2, 32, 39].

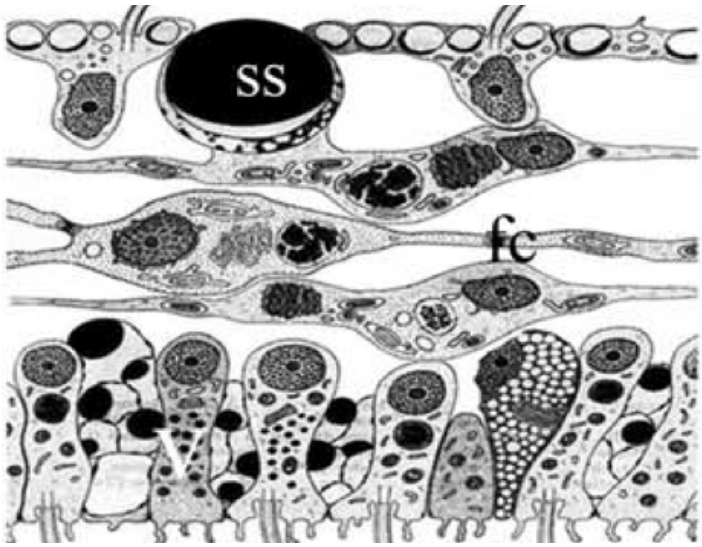

Figure 2. Simplified schematic cross section of the histological organization of *Trichoplax adhaerens*. Abbreviations: SS: Shiny spheres; FC: Fiber cells; V: Lower epithelia. Modified after Guidi et al. 2011.

Vegetative reproduction

Placozoa can reproduce by a n umber of vegetative mechanisms resulting in an increasing number of individuals of a single clonal lineage [40-42]. The most common form of vegetative reproduction is binary fission of the organism. Another mechanism of vegetative reproduction is the generation of spherical swarmers. These swarmers can appear internal or external of an adult organism. If released, the swarmers are floating until they settle and change their shape into a plate [42, 43].

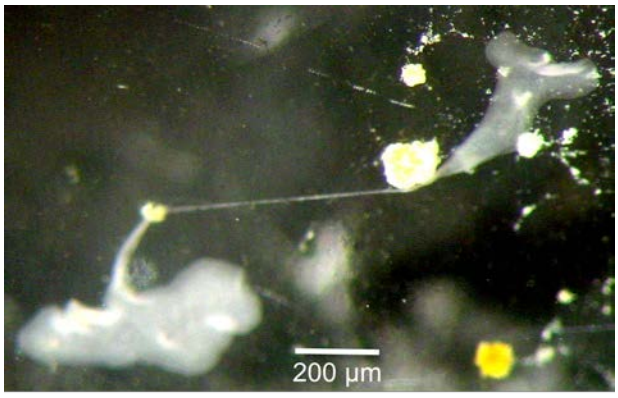

Figure 3. *Trichoplax adhaerens* during the last phase of binary fission process

Sexual Reproduction

At this point of knowledge, the mechanisms of placozoan sexual reproduction are uncertain [44, 45]. Putative egg like cells appear to grow in the interspaces of the animals epithelia. There is no evidence yet, if the putative egg cells derive directly of a meiotic cell or if they are a result of a conversion of already differentiated mitotic cells [43]. Putative egg cells, which undergo cleavage, show a kind of fertilization membrane. However, engaged cell cleavage results in an enigmatic developmental arrest in the 128-cell stage followed by a sudden death of the embryo. Neither sperm cells nor further development of gametes could be observed under laboratory conditions so far[39, 45, 46]. Investigations of molecular patterns in genomic DNA sequences from Caribbean Placozoa populations indicate that different clades within the phyla Placozoa carry molecular signatures of sexual reproduction. Identification of nucleotide polymorphisms, intergenic as well as interchromosomal recombination and shared alleles between heterozygotes and homozygotes gave first insights that sexual reproduction of placozoans might occur in the field [47].

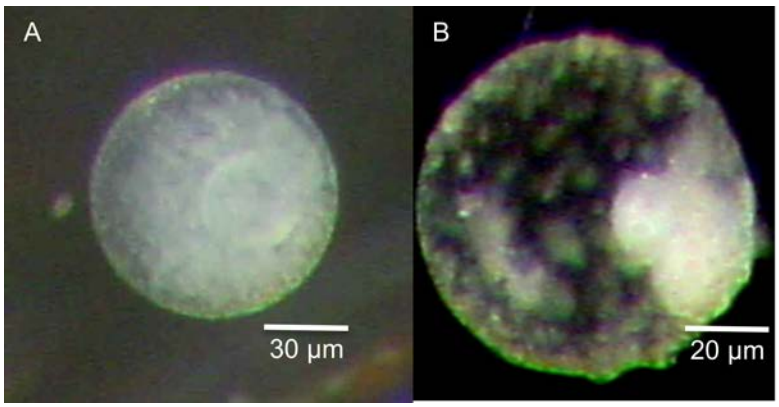

Figure 4. Egg Cells of *Trichoplax adhaerens*
A) Dissected early egg cell of *Trichoplax adhaerens.*
B) Egg with remains of a deceased “embryo”.

The Trichoplax Genome

The nuclear genome of *Trichoplax* was recently sequenced [48]. The genome size of *Trichoplax adhaerens* is about 90 Mbp, organized in 12 Chromosomes. Compared to bacteria, *Trichoplax adhaerens* has only about ten times the genome size of *E.coli* and represents the smallest animal genome known so far [11, 48-51].

Systematic classification

New metazoan systematic textbooks list Placozoa first, followed by the three other diploblast phyla, i.e. Porifera, Ctenophora and Cnidaria [9-11]. The most simple of all metazoan bauplan organizations is the common reason to claim *Trichoplax* to be a direct descendant of a pre-metazoan ancestor. The other diploblast phyla show (i) more complex, even symmetric organized bodyplans, (ii) a higher number of differentiated somatic cell types and/or (iii) an oral-aboral axis formation [6, 9, 27, 52, 53].

However, recent phylogenetic analysis of nuclear genome sequences brought up discussions about the phylogenetic relationships of diploblast phyla. The various studies led to all possible sister group relationships among the four diploblastic phyla and Bilateria (see Fig.5) [54-65].

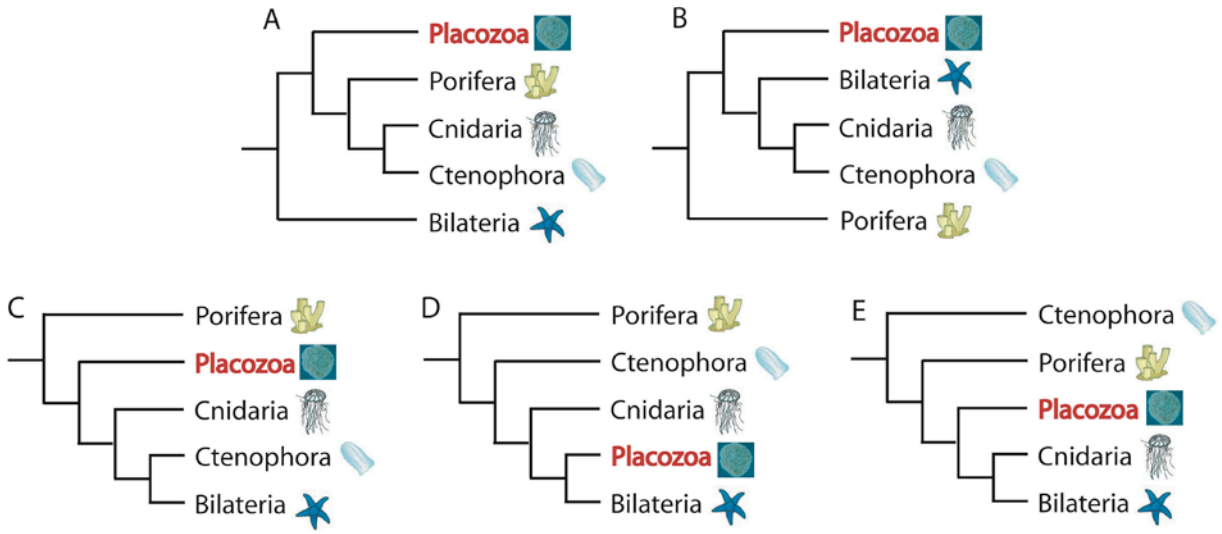

Figure 5. Postulated relationships among the five main metazoan clades. (A) Hypothesis by Schierwater et al. [62], with Bilateria as sister group to a clade that contains placozoans as sister to sponges, cnidarians and ctenophores. (B) Hypothesis with monophyletic Coelenterata by Philippe et al. [66]. (C) Traditional hypothesis based on morphology, with sponges as sister group to other metazoans, e.g. Nielsen [67]. (D) Hypothesis with monophyletic Eumetazoa but with Ctenophora as sister to all other eumetazoans by Pick et al. [55]. (E) Hypothesis based on phylogenomic analyses, with ctenophores as sister group to all other metazoans by Dunn et al. and Hejnol et al. [68, 69]. Modified after Edgecombe et al. [65]. Pictures are modified after Schierwater et al. [62].

The major problem of the conflicting analyses is the limited numbers of characters and/or the limited taxon sampling used in the phylogenetic data sets [59, 65]. Thus,

the phylogenetic position of the enigmatic phylum Placozao remains a matter of debate.

1.2 ParaHox and Hox genes

Highly conserved patterning processes control the development of metazoans. ParaHox genes and closely related Hox genes are crucial key regulators for establishment of axis and organ formation [70-72]. The common characteristic feature of ParaHox and Hox genes is the presence of the highly conserved 180 bp homeobox motive. The homeobox encodes a DNA binding domain responsible for transcriptional regulation of target genes [73]. ParaHox and Hox genes belong to the Antennapedia homeobox superclasss and are highly conserved in structure and genomic organization among all metazoan animals [71]. Their expression patterns during embryonic development are strictly spatial and temporal separated, resulting in axis establishment and organ formation in the embryo[70]. Moreover, their spatial and temporal expression patterns both mirror the order of the genes within the cluster [74]. Fundamental questions about the evolutionary origin of the ParaHox and Hox genes are explained by the evolutionary “invention” of an ancestral “ProtoHox” gene already present in a Diploblast/Bilateria ancestor, the Urmetazoa [59, 72, 75-81]. Several proposed scenarios about the evolution of ProtoHox/ParaHox/Hox genes are based on genomic sequence analyses and the resulting phylogenetic relationships to each other. Today the consensus view is that after establishment of a single ProtoHox gene at least two evolutionary duplication events must have occurred during the evolution of ParaHox/Hox genes (see Fig.6) [72, 74, 82, 83].

The single ProtoHox gene in an Urmetazoa builds up a ProtoHox cluster by gene duplication in early metazoan evolution. The established ProtoHox clusters underwent a complete duplication itself and led to a second cluster, resulting in the ParaHox cluster and closely related Hox gene cluster present in extant bilaterian metazoans (Holland 2002). Several phylogenetic attempts have been made to reconstruct the possible ancient nature of the putative ProtoHox gene. In all published datasets the outcome of the phylogenetic reconstructions suggested a close relationship of the ProtoHox gene to the extant *Gsx* class ParaHox gene [78, 81, 84-87].

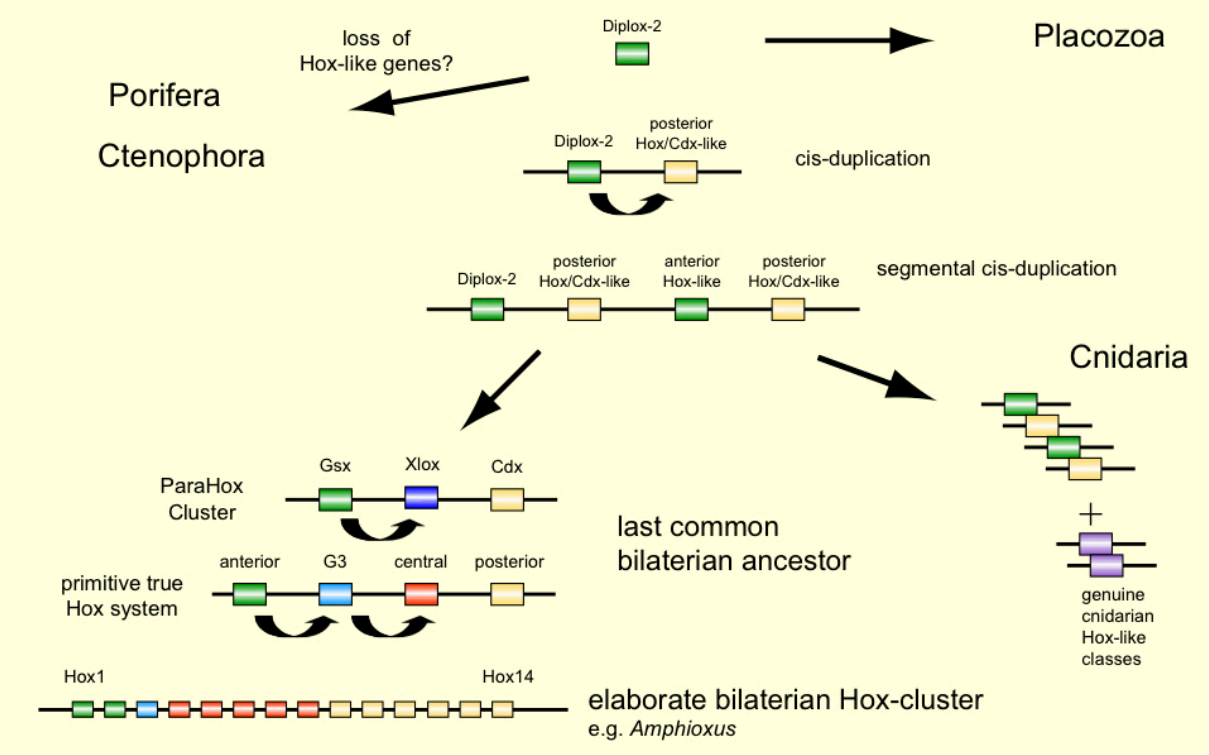

Figure 6. Simplified theoretical evolutionary model of cluster duplication during early metazoan evolution.
Starting from a single ProtoHox gene (Diplox-2 equals ProtoHox) the establishment of ProtoHox clusters and following ParaHox and Hox clusters can be explained by several cis- and/or segmental duplication events. Modified after Kamm [88].

However, the existing duplication models for ParaHox/Hox gene evolution can not be easily adapted to the ParaHox and Hox gene inventory of lower diploblastic metazoans phyla (i.e. Placozoa, Porifera, Ctenophores and Cnidarians). For example, one single ParaHox homologue, the *Gsx* like gene *Trox-2*, is present in Placozoa [6, 48, 83] and only two representatives ParaHox genes, i.e. *Gsx* and *Cdx*, are found in cnidarians [89-92].

Neither there is a genomic cluster organization of these genes in cnidarians (which would be expected by the common evolutionary models), nor details are known about their developmental function in early metazoans [79, 93, 94]. Moreover, representatives of Porifera and Ctenophora, seem to lack ParaHox genes at all [95, 96]. This fact resulted in a recently introduced terminological split of basal metazoans into non-Parahoxozoa (Porifera and Ctenophora) and ParaHoxozoa (Placozoa, Cnidaria and Bilateria) [96]. Since Porifera and Ctenophora lack the canonical ParaHox system, it has been suggested that non-ParaHoxozoa represent the earliest branching metazoan phyla, before the establishment of ProtoHox/ParaHox genes [97-99]. Nevertheless, others claim that the absence of ParaHox genes is the result

of a secondary loss in Porifera and Ctenophora. Since Porifera and Ctenophora show a high diversity of Antennapedia class Hox genes it is very likely that Porifera might have lost at least on ProtoHox/ParaHox/Hox gene during evolution [6, 75, 81, 87, 100]. This scenario could explain why Porifera do not rely neither on distinct patterning processes nor any specific tissue type for their bauplan organisation in adult individuals. Thus there would be no evolutionary pressure for the maintenance of ParaHox genes [6, 101-103].

Even more intriguing is the picture in Ctenophora. Ctenophores, which evolved a highly complex bodyplan, rely on a completely different developmental system compared to other metazoan Phyla [104-106]. While all other animal species can recover from a loss of embryonic stem cells during early embryonic development, by rescue compensation of neighboring stem cells (for review see [107]), ctenophores follow a different developmental strategy. If one of the specific "module like" progenitor cells is missing, the adult individual is lacking organ structures or whole quadrants along the oral-aboral axis. Based on these results, early embryonic progenitors of ctenophores display a strict stereotypic cleavage pattern with minimal transformational plasticity. Thus, ctenophores rely on a unique developmental "building block"- like cleavage program [104, 106, 108, 109]. Therefore the conservation or maintenance of a ProtoHox/ParaHox like system would be unnecessary. Consequently, it seems plausible that Porifera and Ctenophores might have lost a ParaHox inventory secondarily due to different developmental bauplan strategies [6, 75, 83, 87].

1.3 *Pax Genes*

Meatazoan PairedBox (Pax) genes represent key regulatory transcription factors, which are crucial during animal embryonic and sensory organ development [110-112]. Pax genes are identified in all metazoan phyla but their evolution and ancestral functions remain widely unresolved. [113-116].

The three characteristic domain features of this gene family are (i) the highly conserved Paired box encoding a 128 amino acid Paired domain, which is crucial for target DNA interaction, (ii) the presence or absence of a homeobox domain as second target DNA binding motif and (iii) the presence or absence of the Pax specific octapeptide [111, 117, 118].

Nine different Pax genes have been already identified in metazoans. According to domain architecture and sequence homology the genes are split into four distinct groups (see Figure 7) [115, 119, 120].

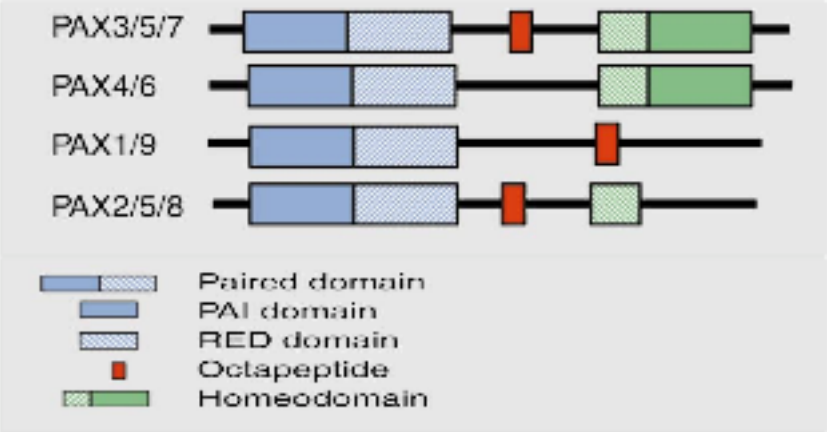

Figure 7. Overview on the four bilaterian Pax gene groups and their domain architecture. Modified after Hobert et al. 1999 [120].

The recently discussed evolutionary relationships of the family members implicate that a gene fusion between a Paired gene and a Homeobox gene occurred before Parazoa and Eumetazoa diverged [121]. The ancestral metazoan *Pax* gene is proposed to contain all three conserved domains found in “modern” Pax genes (the paired domain, the octapeptide, and the homeodomain). From here the different classes of Pax genes evolved by gene duplication [117, 122, 123]. Two alternative evolutionary scenarios were proposed for Pax gene evolution.

Catmull et al. claim that the split into the four groups of Pax genes has occurred at the root of an ancestral *Pax6* like gene [124]. In contrast, Sun et al. proposed that the divergence of the four Pax gene emerged from an ancestral *PaxB-2*/*5*/*8*/*4*/*6* like gene [119]. However, developmental analyses indicate that Pax genes are involved in the development of the animal nervous system and sensory organs. The most investigated Pax gene, *Pax6*, is crucial for the development of animal eyes and/or photoreceptor cells [125-127]. Intriguingly, ectopic expression experiments of squid and mouse *Pax6* genes in *Drosophila, Pax6* (*Ey*) knock out mutants induce the formation of compound eyes in the flies [128, 129]. In a reciprocal experiment *Drosophila Ey* is inducing ectopic expression of vertebrate eye structures in *Xenopus*. The overall ability of *Pax6* homologues genes to regulate eye development in even distantly related taxa supports the monophyletic evolutionary origin of all animal eyes

[126, 130]. Among Diploblast phyla, Cnidaria represent the most basal organism possessing true eyes [131]. Within Cnidaria, Cubozao show highly sophisticated lens eyes with complex structures such as iris, glass body, retina layer, and a pigmented cell layer [132, 133]. The Pax genes identified in the four classes of cnidaria result in a conflicting picture compared to bilatarian Pax genes. Although Cubozoa develop the most complex bauplan of cnidarian eyes, until now only two Pax genes (Pax-B and Pax-C) could be identified [134, 135]. Other cnidarian classes like Anthozoa (no eyes) and Hydrozoa (lens eyes) harbor at least four different Pax genes (*Pax-A, Pax-B, Pax-C, Pax-D*). Ectopic expression experiments revealed that at least three cnidarian Pax genes, i.e. *Pax-A* and *Pax-C* from Anthozoa and *Pax-B* from Cubozoa are sufficient to induce ectopic eyes in *Drosophila* [115, 117, 134, 135]. Since several cnidarian Pax genes seem to be capable to induce ectopic eye development, specific Pax gene functions cannot be assigned in cnidarians. In summary, no *bona fide Pax6* gene has been identified in cnidarians to date [131]. Nevertheless, the observation that cnidarians and bilaterians rely on genes on Pax family genes for eye development strongly support hypothesis of a monophyletic origin of all animal eyes [130, 131].

The two other diploblast phyla, i.e. Placozoa and Porifera were recently reported to harbor single representatives of the Pax-2/5/8 class. Intriguingly, there is growing evidence that Placozoa and Porifera both exhibit sensory- and effector systems that respond to external stimuli such as light and mechanical stress. While both phyla lack any organs or true nervous cell systems the presence of Pax genes was interpreted to predate the origin of proto neuronal and sensory cell types within Porifera and Placozoa [121, 122, 136].

1.4 Mitochondria

All metazoans share a characteristic sub cellular organelle with a semi autonomic genome, the mitochondria [137, 138]. Molecular phylogenetic studies on animal mitochondria support a common monophyletic ancestry of animal mitochondria [137, 139]. The origin of animal mitochondria is explained by the generally accepted endosymbiont hypothesis and is strongly supported by means of comparative mitochondrial genome analysis of eukaryotic mitochondria and extant α-Proteobacteria [138, 140].

Plants contain the greatest mitochondrial genomes (approx. 550Kb) known of multicellular organisms, followed by single cellular metazoans (i.e. Choanozoa and Ichtyosporea (40Kb-80Kb)). Fungi (18Kb-78Kb) and animal mitochondria (13Kb-43Kb) have followed a different evolutionary strategy by reducing the mitochondrial genome size and the number of encoded genes (see Fig.8) [138, 141].

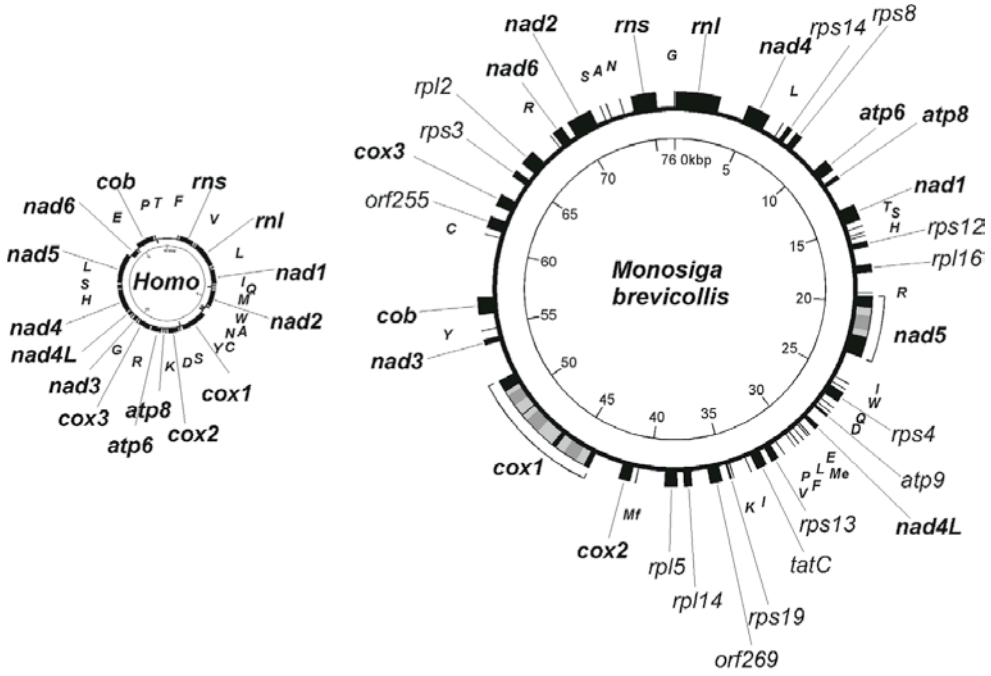

Figure 8. Schematic example of the differenzes in size and genome content of two metazoan species. Left: *Homo sapiens* (Vertebrata), 16.571 Kbp; right: *Monosiga brevicolis* (Choanozoa) 76.588Kbp. Modified after Lavrov [139]

Intriguingly diploblast mitochondrial genomes display a great variety in size, gene content and gene organization compared to bilateria mitochondria [139]. Thus mitochondrial phylogenetic studies are a powerful tool to mirror the evolutionary history of basal metazoan evolution. The possibility to trace the phylogenetic relationships of animal mitochondria back to single cellular metazoan organisms was recently fuelled by Lavrov and co-workers [142]. They were able to identify highly conserved Metazoa specific insertions and deletions in four different mitochondrial protein coding genes. These synapomorphies were found to be highly conserved among Metazoa, partially present in Amoebidia as a single cellular protistian metazoan, but completely absent in *Monosiga brevicollis* (Choanozoa). These findings have broad implications for our understanding of major evolutionary transitions from single cellular organisms to animals. At the same time the recent

findings of Lavrov and coworkers indicate that mitochondrial genomes harbour even more phylogenetic informative characters that have have not been included in the existing studies [139, 142, 143].

2. Aims of the thesis

The aim of this thesis is to provide crucial information about developmental mechanisms of the placozoan *Trichoplax adhaerens*. Therefore, analyses were performed regarding the functional role of the master control genes *Trox-2*, a member of the Proto/ParaHox gene family *Gsx* and two representative metazoan Pax genes, i.e. *TriPaxB* and *TriPaxC*. These genes were chosen, since earlier reports demonstrated that they evolved in the very beginning of animal evolution. Determining their functional role by means of expression analyses, characterization of morphological stuctures related to their expression, and implications for sensory based behavior in placozans could provide substantially knowledge on the developmental system in Placozoa.

In addition, the results on placozoan development are placed into a phylogenetic content. Genomic inventory, gene arrangement and phylogenetic analyses of the mitochondrial genome of *Trichoplax adhaerens* were utilized to gain a phylogenetic perspective of placozoan/metazoan relationships. All together, the obtained results generate a robust data set, which can be used for comparative analysis among basal metazoans and their evolutionary relationships.

3. Summary of Results and Discussion

3.1 The ProtoHox/ParaHox gene Trox-2 in Placozoa

(Sagasser et al. 2011 and references therein)

To address the possible evolutionary origin of metazoan ParaHox genes, i.e. *Gsx*, *Xlox* and *Cdx*, the role of the only *Gsx* like ProtoHox/ParaHox gene, *Trox-2,* present in *Trichoplax adhaerens* was investigated.

In all other metazoans, *Gsx*-like genes are involved in axis specific differentiation of neuronal progenitors. Intriguingly, Placozoans as the most basal organized non-symmetrical animal, harbor only a single *Gsx* like gene, *Trox-2*, but lack true neuronal cell types. This circumstance led consequently to the question if Placozoa might represent an extant ProtoHox dependent animal. To understand the developmental

function of *Trox-2*, the non-axial expression patterns, alternative transcript activation, and *Trox-2* dependent outcome of morphological structures were investigated in *Trichoplax adherens*.

By means of 3´ and 5´ rapid amplification of cDNA ends two independent alternative transcripts of *Trox-2* were identified. Further investigation by Northernblot and Westernblot demonstrated that both *Trox-2* mRNAs are expressed and translated on protein level. Histological *in situ* analyses demonstrated that alternative expression patterns of the *Trox-2* isoforms were strictly spatially separated in the developing animal. It was further shown that *Trox-2* expression was associated with the differentiation of enigmatic fiber cells during developmental processes. Consequently, a putative proto axis formation in *Trichoplax* was identified for the first time. Thus *Trox-2* represents a candidate ProtoHox gene that prepares the ground towards animal axis evolution and neuronal differentiation.

3.2 The Trichoplax *PaxB* Gene

(Hadrys et al. 2005 and references therein)

The placozoan PaxB like gene, *TriPaxB*, of Trichoplax adhaerens was isolated and characterized to gain a deeper knowledge in the evolution of Pax gene families among diploblast phyla. Phylogenetic analyses have shown that *TriPaxB* always comes out basal to PaxA, PaxB, and PaxC genes independent of the applied algorithms and also independent of whether paired domain sequences from bilaterian animals were included or not. Thus the *TriPaxB* gene meets expectations for a Proto-Pax gene or the early descendant of a Proto-Pax gene in metazoan animals. The data suggest that a PaxB similar gene was the ancestral gene involved in sensory organ development and evolution. We conclude therefore, that a functional split into bilaterian Pax2/5/8 and *Pax6* genes most likely occurred in the last common ancestor of diploblasts and triploblasts.

In addition, *in situ* analyses revealed expression of *TriPaxB* in cells of unknown function bordering a distinct area toward the center of the animal. Analyses of tissue sections revealed that *TriPaxB* expressing cells are not epithelial cells but lay upon the cells of the lower epithelia, inside the animal. The identified expression patterns of *TriPaxB* were found to correlate with the expression patterns of *Trox-2* and demark

a particular zone of cell proliferation and differentiation. While the Placozoa *TriPaxB* gene most likely predates the origin of nerve and sensory cells, its ancestral developmental function needs to be investigated in more detail.

3.3 The *TriPax-C* gene in *Trichoplax adhaerens*

(Sagasser et al. 2011 and references therein)

The previously reported isolation and characterization of *TriPaxB* (see 3.2) and subsequent phylogenetic analyses implicated that *TriPaxB* most likely represents the ancestral animal prototype like Pax gene. Here a second Pax gene in *Trichoplax adhearens*, *TriPaxC*, was characterized. Sequence analyses have shown that *TriPaxC* can be directly linked to Cnidarian Pax-C class and bilaterian *Pax6* class genes. Questions about its developmental role and its implications for placozoan sensory biology were addressed.

By means of *in situ* hybridization experiments the developmental functions of *TriPaxC* were characterized. *In situ* hybridization revealed the expression of *TriPaxC* transcripts in distinct, previously unknown cell types. Their distribution in an irregular pattern over central body parts of *Trichoplax adhaerens* does not fit any expression pattern of previously described developmental genes in *Trichoplax*. This study provides first experimental evidence that the *TriPaxC* gene might be involved in the differentiation of a formerly unknown cell type of Trichoplax adhaerens.

3.4 The mitochondrial genome of *Trichoplax adhaerens*

(Dellaporta et al. 2006 and references therein)

To gain a better understanding of the evolutionary relationship of the Placozoa *Trichoplax adhaerens* amongst other metazoan phyla, the molecular characterization of the complete Placozoa mitochondrial genome can provide an essential dataset to address the phylogenetic position of placozoans. The characterization and annotation of the complete mitochondrial genome of *Trichoplax* turned out to represent the largest animal mitochondrial genome known to date. Besides it unique size of more the 43Kb, it harbors unusual features like (i) intron interrupted genes, (ii)

numerous intergenic spacing regions, (iii) open reading frames coding for possible expressed proteins of unknown function and (iv) largely expanded gene sizes of common mitochondrial protein coding genes. Most intriguingly the placozoan mitochondrial genome displays common features known from other animal mitochondria but also shares characteristics with mitochondrial Genomes of single cell metazoans, i.e. Choanozoa and Ichtyosporea. The latter characteristics are similar open reading frames as well as numerous repetitive coding and non-coding sequences and large intergenic spacer regions. Based on the analyses of placozoan mitochondrial genome structure and gene content, it seems to be l ikely that mitochondrial genomes of early metazoan animals were of non-compact organization. Phylogenetic analyses place the mitochondrial genome of *Trichoplax adhaerens* at the root of animals and represents the least derived mitochondrial genome among diploblastic and bilaterian metazoans.

4. Conclusions

The origin of multicellular life has been explained by several different evolutionary hypotheses over the last 150 years. The consensus view of the different hypotheses proposes that multicellular life evolved almost certainly from single cellular marine protists.

Today we trace evolutional mechanisms down to the molecular level. Using comparative genomics, scientists identify a hierarchical complexity of genome inventories linking even distantly related metazoan taxa like earliest branching diploblast phyla and Bilateria. In addition, phylogenetic analyses of organism specific mitochondrial genomes, strongly support a monophyletic origin for all animals.

However, at present the phylogenetic relationships of the four earliest branching metazoan phyla (Placozoa, Porifera, Ctenophora and Cnidaria) remain ambiguous. In general, diploblast phyla show a tremendous structural complexity on cellular and molecular levels, sophisticated sensory properties and complex behavioral responses. It has been suggested that all these features of animal complexity were already established at a most basic level in a common ancestor of animals. Therefore, to identify the “missing link” from the common ancestor of animals and extant animal species has broad implications to further understand the evolution of animal complexity.

The thesis contributes substantial insight into developmental and evolutionary processes in the presumably most basal animal *Trichoplax adhaerens* (Placozoa). The expression analyses and the functional role of three key regulatory genes, crucial for animal development were investigated in *Trichoplax adhaerens.* The outcome of these studies provides a deeper knowledge for understanding principle bauplan transitions during placozoan evolution. The detailed cytological and functional investigations of developmental processes imply that *Trichoplax adhaerens* possess the most basal morphological and physiological state of specialization among animals. In addition complete mitochondrial genome analyses strongly support these findings.

However, even if *Trichoplax* appears to represent the most basal extant organism, we should keep in mind that Placozoa underwent their own evolutionary course and represents no steady state of early evolution. To verify the conclusions based on this thesis, more extensive comparative studies are needed for poriferan, ctenophoran and cnidarian species in order to achieve a robust picture about the basic nature of *Trichoplax adhaerens* and the urmetazoon in general.

Nevertheless, the combined analyses of developmental mechanisms and phylogenetic analyses suggest that *Trichoplax* represents a best fit model organism to explain the hypothetical “primitive” nature of the ancient multicellular metazoa, the so-called urmetazoon.

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6. Publications and manuscripts upon which the thesis is based

6.1 Sagasser S, Dellaporta SL and B Schierwater B (2011). Unconventional, non axial expression patterns of the ProtoHox/ParaHox gene *Trox-2* in *Trichoplax adhaerens* (Placozoa), submitted to *BMC Evolutionary Biology*

6.2 Hadrys T, DeSalle R, Sagasser S, Fischer N and B Schierwater B (2005). The Trichoplax *PaxB* Gene: A Putative Proto-*PaxA/B/C* Gene Predating the Origin of Nerve and Sensory Cells, *Molecular Biology and Evolution 22(7):1569–1578*

6.3 Sagasser S, Cramm M., Wagenseil N., v.d. Chevallerie K., Trabanco-Martin N., De Jong D. and B Schierwater B (2011). The *Trichoplax TriPaxC* gene, Spotlight on a prototypical photosensory system, prepared for submission to *Developmental Genes and Evolution*

6.4 Dellaporta S, Xu A, Sagasser SL, Jakob W, Moreno MA, Buss LW and B Schierwater B (2006). Mitochondrial genome of *Trichoplax adhaerens* supports Placozoa as the basal lower metazoan phylum, *Proc Natl Acad Sci U S A. 2006 Jun 6;103(23):8751-6*

Unconventional, non axial expression patterns of the ProtoHox/ ParaHox gene *Trox-2* in *Trichoplax adhaerens* (Placozoa)

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This is the author's version of a work submitted to *BMC Evolutionary Biology*.

Molecular work: Sven Sagasser

Manuscript: Sven Sagasser, Stephen L. Dellaporta, Bernd Schierwater

Abstract

Background

The only ProtoHox/ParaHox like gene *Trox-2,* present in the basal placozoon *Trichoplax adhaerens,* is known to play an indispensable role in developmental processes. While the bilaterian and cnidarian *Gsx* and *Cnox-2* homologues are involved in neurogenesis and axial patterning, the function of *Trox-2* in Placozoa is different.

Results

We show here that the developmental function of the ProtoHox/ParaHox gene *Trox-2* is much more limited compared to other Metazoa, while at the same time, its expression patterns are surprisingly complex during different developmental stages. We are the first to report the observation of differential transcript expression of a *Gsx*/ParaHox like gene during development in a basal, non-symmetrical metazoan animal. Two alternative transcripts of *Trox-2,* which differ in length at the 5´ coding region, are characterized. The full-length transcript is expressed in distinct regions towards the center of the animal. The shorter transcript lacks the *Gsx* typical octapeptide and is exclusively expressed in the outer margin of the animal.

Conclusions

The complex expression patterns of *Trox-2* are discussed in the context of an ancestral proto axis pattern formation. Moreover, a new formerly unknown level of organismal organization of *Trichoplax* was identified.

The results indicate that the *Gsx*-like *Trox-2* gene might be regarded as a extant surrogate of a ProtoHox gene in *Trichoplax adhaerens*.

Background

ParaHox genes resemble homeobox transcription factors of three gene families, *Gsx*, *Xlox* and *Cdx*, which are crucial for the development in metazoan animals [1]. ParaHox genes show a striking conservation of structural and functional properties implicating that these genes originated from ancestral ProtoHox genes by duplication events in early metazoan evolution [2]. The typical clustered organization of ParaHox genes in bilaterian taxa mirrors spatial and temporal separation of gene expression patterns [3, 4].

ParaHox like genes in diploblastic organisms, here known from Placozoa and Cnidaria, show differences to their bilaterian counterparts in terms of number of genes, organization and expression patterns [5-10]. Moreover, it seems that two diploblast phyla like Porifera and Ctenophora do not rely on ParaHox like genes at all. All animals having ParaHox like genes, i.e. Placozoans, Cnidarians and Bilataria were recently termed “ParaHoxozoa” [11]. Whether the lack of ParaHox like genes in Porifera and Ctenophora is a secondarily derived or an ancestral feature remains a subject of debate [12-15]. Ascertaining the function of ParaHox like genes in basal diploblast taxa may therefore hold a key for understanding the early evolution of Proto/ParaHox dependent bauplan setups [4, 16-21].

Recent phylogenetic studies imply that all four diploblast phyla split off very early in the metazoan tree of life and dependent on the analysis of the data sets, almost all possible sister group relationships to bilaterians have been suggested [10, 22-33]. Nevertheless the presence of homologous ParaHox genes in Placozoa, Cnidaria and bilaterian taxa implicates the origin of a Proto/ParaHox gene in the common ancestor of Bilateria and diploblasts [1, 7, 12, 13, 18]. In the past decade data emerged to show a conserved role for ParaHox genes among all “ParaHoxozoa” in order to establish an oral- aboral axis in diploblasts or an A-P axis in Bilateria, respectively [2, 6, 9, 17, 34, 35].

Within cnidarians the extensively investigated *Gsx*/ParaHox like gene *Cnox-2* has been shown to be involved in neuron and nematocyst differentiation along the oral-aboral axis [16, 36-43]. *Gsx* gene expression studies in bilaterian taxa show a striking conservation of a similar role for the developing central nervous system in both lineages, protostomes and deuterostomes [44-48].

Apparently this function of *Gsx* is conserved since the radiation of Placozoans Cnidarians and Bilaterians and appears to precede its basic function [19-21, 42, 43, 49-51].

Placozoans, until now only represented by *Trichoplax adhaerens*, display by far the most basic animal bauplan. *Trichoplax* neither possesses real organs or a nervous system, nor displays any body axis and is thought to have evolved along with the earliest Phyla during animal evolution. They are organized into two cell layers separating an inter epithelia cavity which is dispersed by a syncytial wickerwork of enigmatic fiber cells [52-54]. Evidence based analysis of mitochondrial and nuclear genome organization suggest that the evolutionary position of Placozoa might be basal to both - cnidarians and Bilateria [10, 24-26, 55]. Yet we know very little about the life cycle of placozoans. Vegetative reproduction appears to play the prominent role in placozoan development under laboratory conditions [56]. Differentiation of putative gametes can be monitored frequently in placozoan cultures but engaged proliferation of the cells results in disintegration of the so far called “embryo” [57-59]. However, we focused on the functional role *Trox-2* in the generative processes of *Trichoplax adhaerens*. In this case *Trox-2* expression specifies the temporal and regional identity of fiber progenitor cells in a nonsymmetrical organism. Thus knowledge of the basic functions of the *Trox-2* gene is essential for understanding the transformation processes in early placozoan evolution.

Results

Different transcripts of Trox-2

Figure 1 A&B

To analyze possible *Trox-2* transcripts we synthesized RNA-anchor ligated, full length cDNA as template for 5´RACE RT PCR.

We identified two structurally independent cDNA populations referred to as *Trox-2 a* and *Trox-2 b*. The two transcripts differ in length of the 5´ coding region. The shorter cDNA species, *Trox-2 b*, has a truncated 5` coding region, lacking the *Gsx* specific octapeptide. Each of the two cDNA´s contain the common *Gsx* /Diplox-2 characteristic homeobox. Expression of the different transcripts was verified by Northern Blot analysis, using a radioactive labelled probe, specific for a common

region 5` of the *Trox-2* homeobox. Two hybridization signals were observed by Northern Blot analysis indicating that the two isolated cDNA transcripts result of single mRNA species of *Trox-2*.

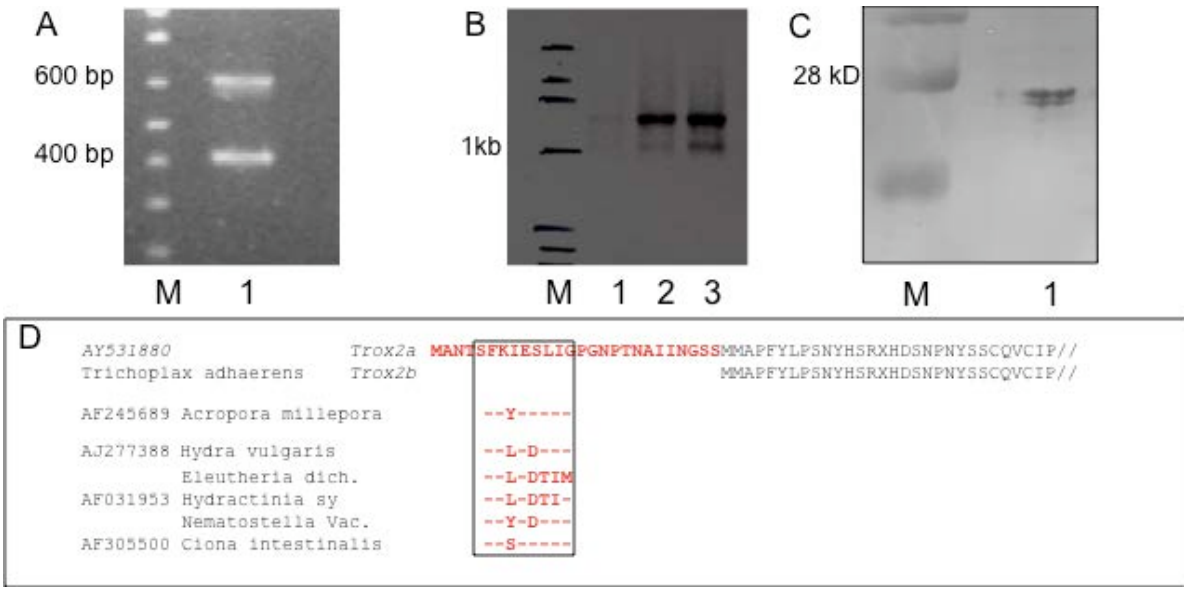


Figure1. Expression of *Trox-2* isoforms in *Trichoplax adhaerens*
A) 5´RACE analyses of the full length 5´region of *Trox-2* revealed two different RT PCR products. M: 1 kb size standard (Invitrogen); lane1: 5´Race *Trox-2* RT PCR products
B) Autoradiogram of total RNA northern blot hybridization of *T. adhaerens*. Two alternative mRNA populations are detected using a universal antisense probe against *Trox-2*. M: RNA II size standard (Roche); lane1: total RNA of 40 animals; lane 2: total RNA of 100 animals; lane 3: total RNA of 250 animals
C) Western Blot analysis of *T. adhaerens* total protein extract revealing two independent *Trox-2* isoforms by a narrow doublet of bands.
D) Alignment of the *Gsx* specific octapeptide of the conserved N-terminal domain of *Trox-2a* with Metazoan specific *Diplox-2/Gsx* orthologs. Homologue amino acids of the octapeptide are indicated by dashes. Note: Domain is missing in the alternative transcript Trox2-b.

Different Trox-2 transcripts encode different Proteins

Figure 1C

Using an affinity purified rabbit polyclonal antiserum we examined *Trox-2* protein expression by Western Blot analysis.

Trichoplax total protein extract samples were subjected to SDS PAGE and transferred to a nitrobond membran. The applied antibodies bind to a narrow doublet of bands of approximately 25 kD. This is consistent with the predicted translated *Trox-2 protein* isoforms with calculated molecular weights of 24.7kD and 22.1kD respectively. In accordance to the RNA transcript analysis, the Western Blot analysis confirms that the two different *Trox-2* messages are translated into different proteins.

Whole mount in situ hybridization of Trox-2

Figure 2

In order to identify differential activation of the two identified transcripts we took advantage to detect *Trox-2 a* separately from the *Trox-2 b* message using a specific probe for the oktapeptide coding 5´m-RNA region.

We find the *Trox-2 a* transcript to be temporarily co-expressed with *Trox-2 b* expression during a s pecific developmental stage only (Figure 2). *Trox-2 b* expression can be monitored in two different developmental stages. Stage one can be characterized by no *Trox-2 a* expression. In stage two, co expression of *Trox-2 a* and *Trox-2 b* is present.

Concerning the spatial distribution of the *Trox-2 a* the hybridization signals exclusively characterize early fiber cells from the periphery towards central areas of the animal body. Spatial distribution of *Trox-2 b* is restricted to marginal cells. Expression of *Trox-2 b* results in a uniform ring of fiber cells at the very outer edge of the animal (Figure 2B).

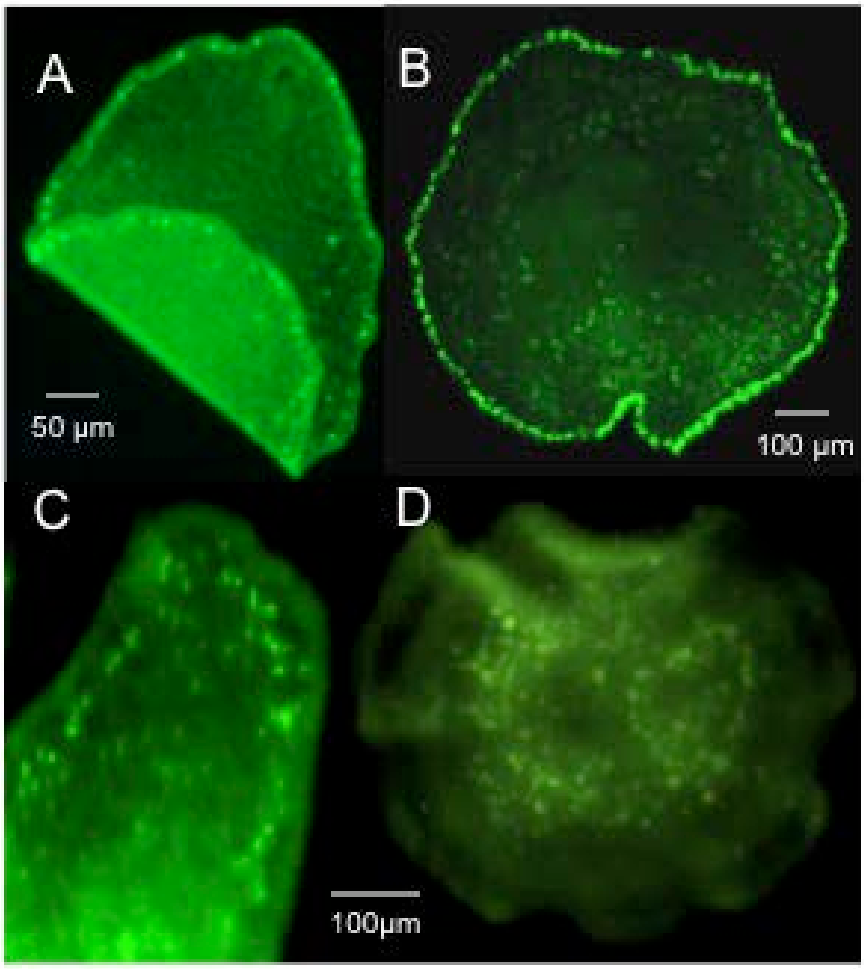


Figure 2. In situ hybridization analysis and spatial distribution of *Trox-2* isoforms, *Trox-2a* & *Trox-2b*. *Trox-2* universal probe hybridizes to both isoforms of the Trox-2 message (A & B), *Trox-2a* probe hybridizes specific to the unique 5´ region of the message (C &D).
A) A universal Trox-2b probe indicates Trox-2 expression in the outer edge of an animal which underwent binary fission.
B) An larger individual still growing. Enriched Trox-2 expression at the edge and central regions of the animal
C) Individual hybridized to the unique 5´ region of the Trox-2a message. Expression is apparent in the periphery towards the centre of the animal. Note: Lack of a hybridization signal in the edge of the animal.
D) Expression of Trox-2a message patterns the central regions of the animal. No detectable hybridization signal of Trox-2a at the edge or periphery

Whole mount immunolocalization and differential protein expression of Trox-2

Figure 3b

In early daughters of *Trichoplax, Trox-2* protein expression starts synchronized around the outer edge of the organism and is restricted to a single ring. This

expression pattern is in accordance to *Trox-2 b* hybridization analysis. This expression pattern is found in animals, which grow slowly, independent of size stages (Figure 3B). The second expression pattern of *Trox-2* marks peripheral and central cells in animals. A region with no *Trox-2* expression strictly separates simultaneous marginal and central expression patterns. Central cell expression starts with a distinct ring, undergoing in a diffuse, almost disappearing pattern, indicating a later on dynamic distribution of the cells (Figure2b).

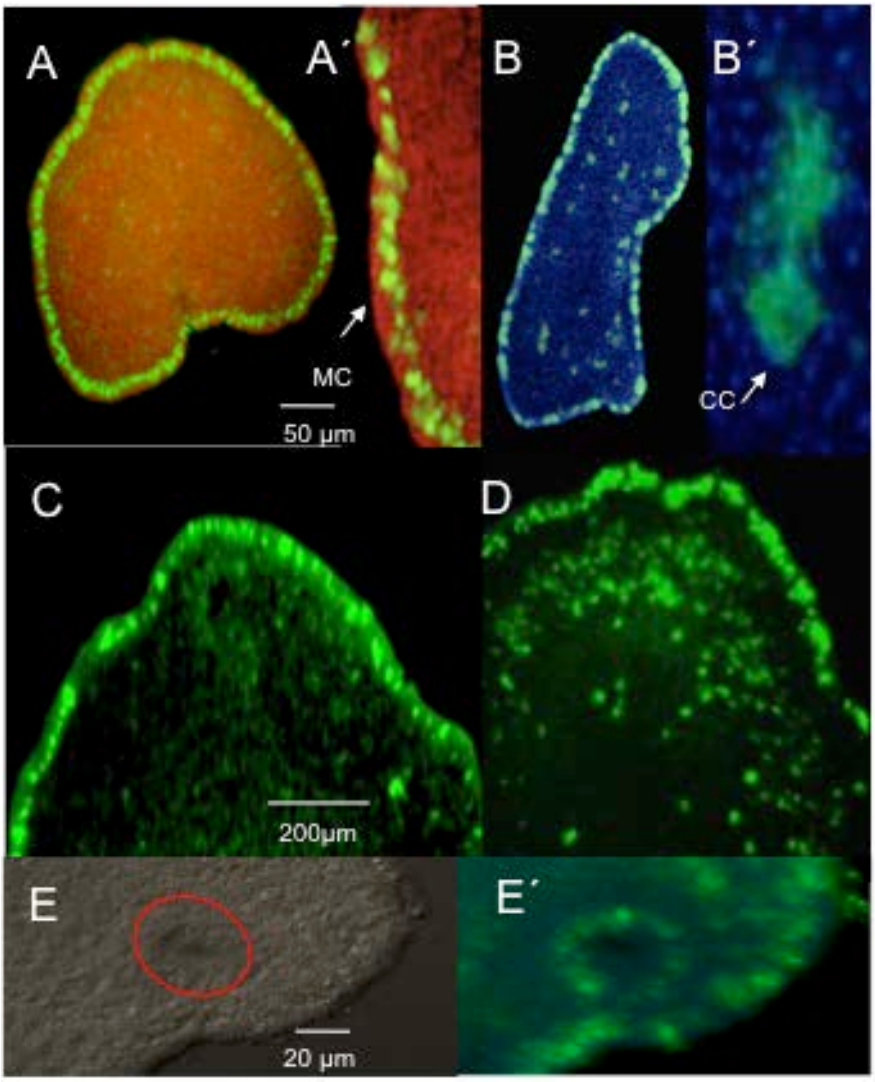


Figure 3. Immunohistological expression analysis of *Trox-2* in *Trichoplax adhaerens*
A) Individual after binary fission showing exclusive expression of Trox-2 along the outer edge.
A´) Detail of A showing prominent stained marginal cells
B) *T. adhaerens* with expression of Trox-2 protein in marginal cells and central cells (cc).
B´ Detail of B. Trox-2 exoression in central fibre cells.
C & D) Close up of large individuals with different Trox-2 expression patterns independent of animals size
E) Close up of an established oocyte.
E´) Expression of Trox-2 in nursery cells bordering the egg cell

Discussion

Our study revealed the first observation of the differential expression of a *Gsx*/ParaHox like gene. The complex expression patterns relate to different developmental stages in Placozoa.

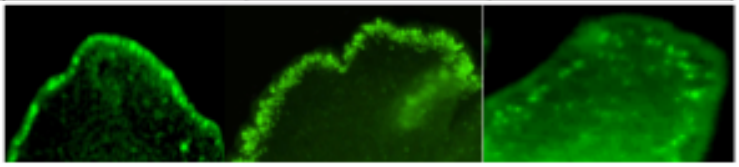
Different analyses of phylogenetic relationships and developmental functions implicate that the *Trox-2* gene might have directly evolved from an ancestral 'ProtoHox' gene [10, 13, 60-62]. While all other animal *Gsx*/ParaHox genes are involved in axis dependent development of neuronal structures, *Trox-2* expression patterns in Placozoa show a completely new picture. Understanding the developmental role of *Trox-2* in *Trichoplax adhaerens* is clearly crucial for the reconstruction of major key transitions that relate to the evolution of *Gsx*/ProtoHox/ParaHox genes. In other words resembles the best living surrogate of an ancestral ProtoHox gene [2, 10, 40, 50, 63].

Trox-2 is expressed in A&B

We have characterized two alternative transcripts of the *Trox-2* gene, *Trox-2 a* and *Trox-2 b*. The transcripts show substantial differences in the N-terminal domain architecture and are differentially activated. Each transcript is active along separated boundaries, patterning the organism. Evidently we identified for the first time different developmental functions for each transcript and can define different fiber cells in separated body regions of *Trichoplax adhaerens*.

A

Trox-2 **Expression**	Edge	Periphery	Central
RNA a	-	-	**a**
RNA b	**b**	**b**	-



B

Trox-2 **in development**	Oocyte development	Growth	After fission
RNA a	not detected	**a**	-
RNA b	not detected	**b**	**b**

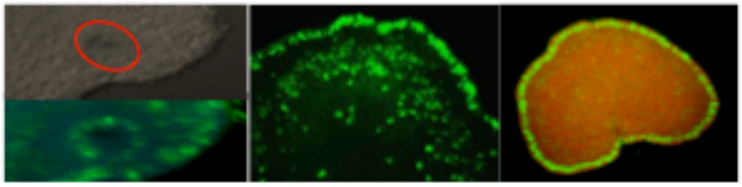


Table 1. Overview of spatial and temporal *Trox-2* expression.
A) Spatial distribution of identified *Trox-2* transcript isoforms reveals a strict separation of isoform *Trox-2a* in the center, *Trox-2b* at the edge of the animal.
B) Temporal activation of *Trox-2* during different developmental stages.

No axis - but the first step

So far all *Gsx*/ParaHox related genes have been found to play essential roles in axis formation [60, 64, 65]. We suggest *Trox-2* expression in Placozoa resembles an *a priory´* step towards axis formation.

The two expression patterns of *Trox-2* are consistent with the view of a polarity setup as a possible precursor for axis formation. Expression of the *Trox-2 b* transcript exclusively in the outer margin resembles the separation from the lower to the upper epithelium. In addition the segregated expression of *Trox-2 a* and *Trox-2 b* displays a clear separation of the margin and the center. This separation creates a polarity, which can be seen as the sum of a cum grano salis infinite number of short axis. From here the “new placula hypothesis” provides a simple and straight forward

scenario for the evolution of an oral-aboral/anterior-posterior axis from a non symmetrical polarity bauplan [66].

Moreover, the origin of *Trox-2* expressing fiber cells support previous findings, where cleavage of progenitor cells was observed in a distinct region close to the edge of the organism. This region was previously termed as the area of regeneration [67-70]. In addition to this our results clearly demonstrate that the different *Trox-2* transcripts border this regenerative boundary. The presence of two alternative transcripts not only enables us to distinguish between different fiber cell populations but also provides insights into the high plasticity of placozoan developmental processes. By altering the spatial-temporal activation pattern of *Trox-2*, *Trichoplax adhaerens* is capable to switch between different modes of fiber cell development.

Regulation

Since the alternative transcripts are strictly tempo -spatially separated it appears that the *Trox-2* locus must be under strict genetic control. The inductive signals, responsible for differential transcript activation need to be elaborated. Recent studies on bilateria *Gsx* genes identified *Nk2* genes, *Pax6* and *Dbx* (developmental brain homeobox) genes to be i nvolved in the regulation of the *Gsx* during neuronal progenitor maturation [44-47, 71]. Intriguingly conserved homologues of these genes were recently identified in *Trichoplax adhaerens* [72, 73]. These findings provide the base for comparative studies to elucidate if the common transcriptional activation mechanisms of *Gsx* are conserved in Placozoa.

Trox-2 and Neurons

In all known cases *Gsx* are also expressed in neurogenesis. According to the traditional view there is no neuron in placozoans. Since we have only little information about the nature of *Trox-2* expressing fiber cells it would be premature to compare them with *Gsx* expressing neurons of other animals. However, as Schierwater et al. [26] and others have shown, placozoans harbor up to 80% of molecules and related genes as key features for a possible pre-nervous integrative system in *Trichoplax* [74, 75]. Moreover early studies on fiber cells identified microtubules, bundles of actin filaments, terminal membrane vesicles and unique synaptic like structures. These features already encouraged biologist to speculate if fiber cells eventually might indeed define a possible “pre-neuronal” system [54, 58,

76-82]. Future attempts on characterization of “neuronal genes” already identified in the complete genome of *Trichoplax adhaerens* might unravel the enigmatic nature of the fiber cells [73]. However, data presented here give strong support for the existence of an evolutionary line indicating the ProtoHox/ParaHox gene *Gsx* to be involved in the establishment of integrative, network like structures in metazoans. Ranging from the non-axial establishment of fiber cells in placozoans to axial related neuronal progenitor differentiation in all other ParaHoxozoa.

Conclusions

In sum, expression characteristics of *Trox-2* are congruent with the idea that *Trichoplax* might be basal to bilaterians and cnidarians.

While the single ProtoHox/ParaHox gene *Trox-2* in *Trichoplax* is not involved in creating any body axis, the organism achieves a high degree of pattern specification by recruiting alternative *Trox-2* transcripts. This appears as a parsimonious alternative to the establishment or maintenance of a basal Proto/ParaHox cluster. In view of this concept the origin of ProtoHox/ParaHox genes seem to enable Proto/ParaHoxozoa to achieve a higher transdetermination state of plasticity and/or complexity for development. Thus, if *Trox-2* acts as a real ProtoHox gene in placozoans it would prepare the grounds toward the evolution of axial bauplans in metazoans.

Methods

Animal material

Experiments were performed on the clonal *Trichoplax* lineage “Grell”.

For culture conditions we refer to Schierwater et al. [6]. Prior to any experimental approach individually collected animals were rinsed three times with sterile filtered artificial seawater and subsequently used for the experiments.

Northern Blot analysis

Different amounts of *Trichoplax* total RNA (1 µg, 5 µg, 10 µg) were separated on a denaturing (Glyoxal/ DMSO/ Formaldehyde) 1,8 % agarose gel and blotted on to

Hybind Nylon membrane (Roche) following manufacturers protocol. The transferred membrane was baked at 80°C for 1 h.

A [^{32}P]dCTP-labeled *Trox-2* single stranded antisense RNA probe was denatured at 65°C for 5 min and added directly into hybridization solution. Hybridization was performed at 65°C for 16 h. After hybridization, the membrane was rinsed with a series of washing solutions that descend from 2× SSC, 0.5% SDS to 0.5× SSC, 0.1% SDS, each at 65°C for 20 min. The autoradiogram hybridization signal of *Trox-2* mRNA species was visualized by exposure of a Kodak X-ray film at -80°C for 8 h.

Amplification of 5' end cDNA with 'new RACE'

Total RNA was extracted from *Trichoplax adhaerens* employing the RNeasy Mini Kit (Qiagen). An additional digestion of residual genomic DNA was performed using the RNase-free DNase I (Roche) according to the manufacturer's instructions. 5′ RACE was performed by RNA ligase-mediated rapid amplification method using the GeneRacer™ Kit (Invitrogen). First-strand cDNA syntheses and subsequent PCR were carried out using the Transcriptor kit (Roche) and FastStart Polymerase (Roche), according to the manufacturer's instructions. For the amplification of partial *Trox-2* sequences, intron spanning oligonucleotide primers were designed according to 5'-ATGGACTTGCCATTATGGT-3´, to amplify an expected fragment of ~650 bp. The full-length *Trox-2 a* sequence and its isoform were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and cloned into the pGem-T Easy/DH5a system (Promega). Independent clones per PCR product species were sequenced on an ABI 310 capillary sequencer according to the manufacturer's instructions.

Production of Rabbit polyclonal Trox-2 antiserum

A synthetic peptide according to *Trox-2* C-terminal AA residues 201-215 (ACCS# AAS54997) was used to produce a polyclonal antiserum in rabbits (Bioscience, Göttingen). Rabbit immunization was performed by injection of 100µg purified fusion protein in complete Freund's adjuvant, followed by five booster injections in incomplete adjuvant at one-week intervals. Serum was collected from the rabbits one week after the last boost.

Raw serum was purified using a Protein-A column (Amersham) and specifity of the IgG fraction was subsequently tested by western blot analysis.

Detection of Trox-2 protein isoforms by Western blotting

Individuals of *Trichoplax adhaerens* were homogenized for 10 min in SDS lysis puffer (2% SDS, 100 mM NaCl, 150 mM Tris buffer, pH 8.0, 0.2% ß-Mercaptoethanol). Total protein extract was treated with Benzoinase (Roche) and subsequently denatured at 99°C for 5 min. After centrifugation at 13000×*g* for 10 min, supernatant was collected. The homogenate was subjected to 12% SDS-PAGE and Western blotting. Rabbit anti-*Trox-2* antibody was used in a dilution of 1:250. Subsequently, AP-conjugated goat anti-rabbit IgG (Invitrogen) was used and detection was performed with a s ubstrate solution (NBT/BCIP, Roche) following manufacturers instructions.

Detection of Trox-2 protein isoforms by immunohistochemistry (IHC)

For immunohistology the animals were fixed in Lavdowsky's fixative for 10 min [60]. Animals were washed in TBS adjusted to pH 7.5 containing 0.5% Triton X-100, 0.5% Tween 20 (TBSTT) for permeabilization of cell membranes. Permeabelized animals were blocked in TBSTT containing 5% FCS for 1 h. Primary polyclonal *Trox-2* antibody was applied in a dilution of 1:1000 in TBSTT/FCS and incubated for at least 2 h. After three wash steps in TBSTT for 15 min the animals were incubated for one hour with Alexa 488-coupled goat anti-rabit secondary antibody (Invitrogen), 1: 5000 in TBS containing 5% Tween (TBST), followed by three wash steps in TBST and one final wash in TBS containing 0.5% DAPI or propidium jodide respectively. Preparations were mounted in Vectashield medium (Vector Laboratories Inc.) and examined with a Zeiss Axiovert 700 Fluorescent microscope.

IHC Controls were performed with rabbit pre immunization serum and epitope blocked anti *Trox-2* serum.

Whole-mount in situ hybridization

Whole-mount in situ hybridization experiments were performed using a modified protocol developed for Placozoa [60]. Animals were fixed in Lavdowsky's fixative as described above.

Trox2-, and *Actin*-RNA-Probes were synthesized from subcloned cDNA fragments (pGEM-T easy; Promega) using digoxigenin (DIG) and fluorescein isothiocyanate–

uridine triphosphate (FITC-UTP) labeling (Roche, Mannheim, Germany) according to the manufacturer's manual.

Authors' contributions

S.S performed the molecular work. S.S. and B.S. wrote the manuscript.

All authors proofed and read the final manuscript.

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The Trichoplax *PaxB* Gene:
A Putative Proto-*PaxA/B/C* Gene
Predating the Origin of Nerve and Sensory Cells

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Abstract

Pax genes play key regulatory roles in embryonic and sensory organ development in metazoans but their evolution and ancestral functions remain widely unresolved. We have isolated a *Pax* gene from Placozoa, beside Porifera the only metazoan phylum that completely lacks nerve and sensory cells or organs. These simplest known metazoans also lack any kind of symmetry, organs, extracellular matrix, basal lamina, muscle cells, and main body axis. The isolated *Pax* gene from *Trichoplax adhaerens* harbors a paired domain, an octapeptide, and a full-length homeodomain. It displays structural features not only of *PaxB* and *Pax2/5/8*-like genes but also of *PaxC* and *Pax6* genes. Conserved splice sites between Placozoa, Cnidaria, and triploblasts, mark the ancient origin of intron structures. Phylogenetic analyses demonstrate that the *Trichoplax Pax*B gene, *TriPaxB*, is basal not only to all other known *PaxB* genes but also to *PaxA* and *Pax*C genes and their relatives in triploblasts (namely *Pax2/5/8*, *Pax4/6*, and *Poxneuro*). *TriPaxB* is expressed in distinct cell patches near the outer edge of the animal body, where undifferentiated and possibly multipotent cells are found. This expression pattern indicates a developmental role in cell-type specification and/or differentiation, probably in specifying-determining fiber cells, which are regarded as proto-neural/muscle cells in *Trichoplax*. While *PaxB*, *Pax2/5/8*, and *Pax6* genes have been linked to nerve cell and sensory system/organ development in virtually all animals investigated so far, our study suggests that *Pax* genes predate the origin of nerve and sensory cells.

Key words: *PaxB*, *Pax* gene evolution, Proto-*Pax*, *Trichoplax*, Placozoa.

Introduction

Transcription factors of the *Pax* gene family serve crucial functions in several developmental processes, particularly with respect to the development and differentiation of the central nervous system and sensory organs, both in vertebrates and invertebrates (Walther et al. 1991; Halder, Callaerts, and Gehring 1995; Rinkwitz-Brandt, Arnold, and Bober 1996; Torres, Gomez-Pardo, and Gruss 1996; Callaerts et al. 1999; Czerny et al. 1999; Holland et al. 1999; Kavaler et al. 1999; Kozmik et al. 1999; Groger et al. 2000; Kozmik et al. 2003). A structural characteristic of *Pax* genes is a paired-type DNA-binding domain, which was first identified in the

Drosophila pair-rule gene paired (Frigerio et al. 1986). In addition, most *Pax* genes contain a complete or partial homeodomain, and some *Pax* genes also possess an octapeptide close to the C-terminal of the paired domain. Regions between domains are less well conserved. Based on their structural properties *Pax* genes are grouped into five subfamilies in triploblasts: namely *Pax1-9/Poxmeso*, *Pax2-5-8/sparkling*, *Pax3-7/paired/ gooseberry*, *Pax6-4/eyeless*, and *Poxneuro* (Breitling and G erber 2000; Miller et al. 2000). In diploblasts, *Pax* genes from Cnidaria and Porifera belong to four classes, *PaxA–D* (Balczarek, Lai, and Kumar 1997; Sun et al. 1997; Catmull et al. 1998; Hoshiyama et al. 1998; Groger et al. 2000; Miller et al. 2000; Kozmik et al. 2003). Orthological relationships between diploblast and tripoblast *Pax* genes are not finally resolved yet, but it has been proposed that cnidarian *PaxB* genes are related to triploblast *Pax2/5/8* genes, *PaxC* genes to *Pax4-6*, and *PaxD* genes to *Pax1-9* and *Pax3-7* genes (Sun et al. 1997; Miller et al. 2000).

Pax2/5/8-related genes seem to be primarily associated with the development of mechanosensory systems in both invertebrates and vertebrates. In higher vertebrates *Pax2*, *Pax5*, and *Pax8* genes are present in multiple paralogs (likely as a result of chromosomal or whole-genome duplications) and are expressed in the developing inner ear and c entral nervous system in mammals (Rinkwitz-Brandt, Arnold, and Bober 1996; Torres, Gomez-Pardo, and Gruss 1996). In *Drosophila* only one *Pax2/5/8* gene (*D-Pax2*; *sparkling*) is present, which has a crucial function for the development of mechanosensory bristles (Fu et al. 1998; Kavaler et al. 1999), ommatidial cone, and pigment cells (Fu and Noll 1997). A *Pax2/5/8* gene identified in ascidians is expressed in the atrial primordium (Wada et al. 1998), a structure that comprises sensory cells similar to those of the vertebrate inner ear (Bone and Ryan 1978), and in gastropods, a *Pax2/5/8* gene is expressed in the statocyst (O'Brien and Degnan 2003). Interestingly the cnidarian *Pax2/5/8* counterpart, *PaxB*,is implicated in nerve cell differentiation in a hydrozoan (Groger et al. 2000) and in sensory organ (statocyst and eye) development in the cubozoan *Tripedalia cystophora* (Kozmik et al. 2003). The latter mirrors the combined expression (and function) of *Pax6* (eye) and *Pax2/5/8* (statocyst) genes in triploblastic animals. The *Tripedalia Pax* gene, *TcPaxB*, not only unites functional but also structural features of *Pax2/5/8* and *Pax6*-like genes. The paired domain is similar to *Pax2/5/8* genes, whereas the homeodomain displays features of *Pax6*-like genes. Kozmik et al. (2003) demonstrated that the *PaxB* protein is a functional hybrid of *Pax2/5/8* and *Pax6*.

Different hypotheses on the origin of metazoan *Pax* genes have been proposed. One hypothesis suggests that a *PaxA*-like paired domain was fused to a homeodomain and founded the *Pax* gene family (Galliot and Miller 2000; Miller et al. 2000). Breitling and Gerber (2000) postulated that *Pax*-like genes evolved by fusion of a DNA-binding domain of an ancestral transposase (Proto-*Pax* transposase) to a homeodomain shortly after the emergence of metazoan animals about 1 billion years ago. The authors further propose a single homeodomain fusion event followed by an early duplication of *Pax* genes before the divergence of Porifera. In order to unravel the early evolution of *Pax* genes we need data from all putative basal metazoan groups. While *Pax* genes have been isolated from sponges and cnidarians, no data have been available from the last and possibly most crucial diploblast phylum, the Placozoa.

Here we report the isolation and characterization of a single *Pax* gene from the morphologically most simple organized metazoan animal, the placozoan *Trichoplax adhaerens*, which lacks any kind of nervous system and/ or sensory organs. It is important to note that Placozoa are not secondarily reduced cnidarians (Ender and Schierwater 2003), and thus lack of nerve cells most likely is a plesiomorphy. The *Trichoplax Pax* gene, *TriPaxB*, is expressed in distinct cell patches in a ring-shaped pattern near the lower-upper epithelium boundary.

Our structural and phylogenetic analyses show that the *Trichoplax Pax* gene is basal to *PaxA*-, *B*- and *C*-type genes and harbors structural features of both *Pax2/5/8* and *Pax6* genes. These findings suggest that *TriPaxB* gave rise to at least four of the five *Pax* gene families in higher metazoan animals and provide support for Millers' hypothesis on the origin of *Pax* genes (Miller et al. 2000). The *TriPaxB* gene meets expectations for a Proto-*Pax* gene or the early descendant of a Proto-*Pax* gene in metazoan animals.

Materials and Methods

Polymerase Chain Reaction Amplification of Paired-Box Sequences

Trichoplax genomic DNA was isolated as described previously (Ender and Schierwater 2003). Messenger RNA (mRNA) from growing and reproducing *Trichoplax* individuals was isolated using the Invitrogen (San Diego, Calif.) ''Micro-Fast Track'' Kit according to the manufacturer's protocol. Different sets of degenerate primers were used to amplify a 344-bp fragment of the paired domain. Two sets of

degenerate primers are described in Hoshiyama et al. (1998). A third set was designed based upon conserved paired domain sequences from other diploblastic *Pax* genes by using the CODEHOP program (http://blocks.fhcrc.org/blocks/codehop.html). Complementary DNA (cDNA) preparations from growing and reproducing *Trichoplax* individuals served as template DNA. Double-stranded cDNA was synthesized from mRNA using the ‘‘Creator Smart’’ System (Clontech, Palo Alto, Calif.) according to the manufacturer’s protocol. Polymerase chain reaction (PCR) fragments were obtained only with the primer pairs S1-AS3 and S2-AS3; the sequences are as follows (see also Hoshiyama et al. 1998): forward: S1 5’-CAGGATCCCARYTIGGNGGNGTNTT-3’ (corresponding to the ‘‘QLGGVF’’ motif); reverse: AS3 5’-GTGAATTCATYTCCCANGCRAADAT-3’ (corresponding to IFAWEI). Other primers designed from highly conserved amino acid sequences within the paired domain did not result in the amplification of *Pax*-specific PCR products. These primers were forward: S2 5’-GAGGATCCTTYGTNAAYGGNMGNCC-3’ (corresponding to FVNGRP); reverse: AS1 5’-GTGAATTCYKRTCNKDATYTCCCA-3’ (corresponding to WEIRD[RK]); see Hoshiyama et al.(1998), and two primers designed using CODEHOP: forward: S3 5’-CAAGATCCTGTGCCGGTACTAYGARACNGG-3’ (corresponding to KILSRYYETG) and reverse: 5’-CTCCAGCAGGCAGTCCCKDATYTCCCA-3’ (corresponding to WEIRDCLLQ). PCR conditions were 30s 95°C, 30s 50°C, and 60s 68°C, and 40 cycles were performed. PCR fragments were subcloned in pGEM-T vector (Promega, Madison, Wisc.). Plasmid minipreparations were sequenced in both directions using ABI (Foster City, Calif.) BigDye terminator chemistry on an ABI-310 capillar sequencer.

Rapid Amplification of cDNA Ends and Genome Walk PCR

Starting from the paired-box cDNA fragment, the coding sequence of *Trichoplax PaxB* was amplified using the ‘‘SMART RACE’’ system (Clontech). The following primers were designed from the sequence of the isolated paired-box cDNA fragment (forward: 3’ Walk 1: ATCAACTACCGTTGGTGTTGCCACCT; 3’ Walk 2: CGATATGACGACGTATTGCTTCACGC; reverse: 5’ Walk 1: CTTGCTTCCTCCAATAATACCTGGGC; 5’ Walk 2: CTTCCATTTTCAAACACACCACCCAG). The 3’ and 5’ rapid amplification of cDNA ends (RACE)PCR reactions were performed according to the manufacturer’s manual

(Clontech). PCR conditions were 95°C 15s, 68°C 3 min, 35 cycles. The obtained RACE products were subcloned (pGEM-T) and sequenced.

To characterize the corresponding *Trichoplax PaxB* gene structure a ‘‘Genome Walk’’ (Clontech) was carried out. PCR reactions were performed using long-template *Taq*-polymerase as described in the manufacturer’s manual. PCR fragments were subcloned (pGEM-T) and characterized by sequencing.

Expression Analyses

Whole-mount in situ hybridization experiments were performed using a modified protocol developed for Cnidaria and Placozoa, respectively (Groger et al. 2000; Jakob et al. 2004). Animals were fixed in Lavdowsky’s fixative as described in Jakob et al. (2004). *TriPaxB*-, *Trox2*-, and *Actin*-RNA-Probes were synthesized from subcloned cDNA fragments (pGEM-T easy; Promega) using digoxigenin (DIG) and fluorescein isothiocyanate–uridine triphosphate (FITC-UTP) labeling (Roche, Mannheim, Germany) according to the manufacturer’s manual. Reverse transcriptase (RT)–PCR was done as described previously (Hadrys et al. 2004).

Phylogenetic Analyses

Distance and Maximum Parsimony analyses were carried out in order to infer phylogenetic relationships between *Pax* genes. All known paired domain sequences from diploblasts were included in the analysis. For rooted tree analyses a *Pseudomonas* transposase sequence served as an out-group (Breitling and Gerber 2000).

Bayes analysis was done with MrBayes (Huelsenbeck and Ronquist 2001). The parsmodel was applied and the following parameters were used: Markov chain Monte Carlo (MCMC) with 100,000 four chains and sampling frequency of 10. The trees generated from the MCMC simulation were imported into PAUP, and a Bayesian tree was visualized using the 50% majority rule option in PAUP (Swofford 2002).

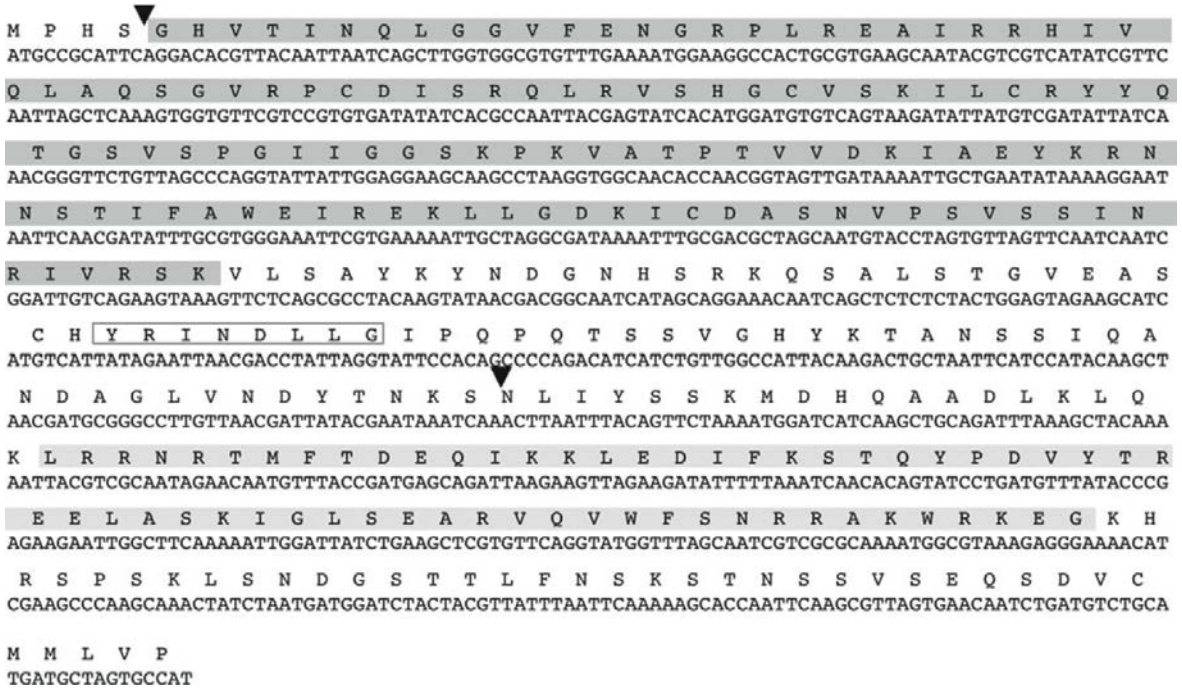

FIG. 1.—Nucleotide and amino acid sequence of *TriPaxB*. The isolated coding region comprises 955 nucleotides (318 amino acids). The *TriPaxB* protein includes a paired domain (underlayed in gray), an octapeptide (boxed) and a ‘‘full’’ homeodomain (underlayed in light gray). The positions of two introns are indicated by arrowheads (black). An intron located directly upstream the paired domain was found in all *Pax* genes investigated so far. An intron located between the octapeptide and the homeodomain was also found in other *Pax2/5/8* genes, for example, in *Drosophila D-Pax2* (*sparkling*; here the intron is much longer, however).

For likelihood ratio tests the method of Shimodaira and Hasegawa (1999; 2001) as implemented in the PROML program in PHYLIP ((Felsenstein 2004), http://evolution.genetics.washington.edu/phylip.html) was used to calculate likelihood ratios of the best neighbor-joining (NJ) tree and the parsimony tree relative to trees that were generated by RETREE (PHYLIP package) that removed and regrafted the *TriPaxB* gene at all nodes in the parsimony tree.

In this way the basal position of the *TriPaxB* gene could be tested in comparison to its position within the *PaxA–C* clade and within the *PaxB* clade (see fig. 3). The likelihoods of over 20 trees generated by RETREE were included in these tests using a likelihood that took into account site-specific rate differences using a gamma correction.

Accession numbers for sequences included in the analyses are *Acropora millepora PaxA* (*AmPaxA*): AF053458; *A. millepora PaxC* (*AmPaxC*): AF053459; *A. millepora PaxD* (*AmPaxD*): AF241311; *Chrysaora quinquecirrha PaxA1* (*CqPaxA1*):

U96195; *Cladonema californicum PaxB* (*CcPaxB*): AF260128; *Chrysaora quinquecirrha PaxB* (*CqPaxB*): U96197; *Drosophila melanogaster eyeless* (*ey*): X79493; *Drosophila melanogaster paired* (*prd*): M14548; *Ephydatia fluviatilis Pax2/5/8* (*EfPax258*): AB007462; *Halocynthia roretzi Pax-37* (*HrPax-37*): D84254; *Hydra littoralis PaxA* (*HlPaxA*): U96194; *Hydra littoralis PaxB* (*HlPaxB*): U96194; *Mus Musculus Pax2*: A60086; *Mus musculus Pax3*: NM_008781; *Mus Musculus Pax5*: M97013; *Mus musculus Pax6*: BC011272; *Paracentrotus lividus Pax258* (*suPax258*): AF016884; *Podocoryne carnea* (*PcPaxB*): AJ249563; *Pseudomonas syringae transposase*: AF169828; *T. cystophora PaxB* (*TcPaxB*): AY280703.

Results

Isolation and Structural Features of the T. adhaerens PaxB Gene

Using different sets of primers, we obtained PCR fragments of the expected size (344 bp) with the primer combination S1/AS3 only. Because primers were designed according to the most conserved regions of the paired-box motif, they are expected to amplify paired-box sequences of all *Pax* gene subfamilies. From a total of 20 clones sequenced all of which were 100% identical in sequence. By means of 5' and 3' RACE 955 n ucleotides of the coding sequence, including the paired and homeodomain (318 amino acids) were isolated (fig. 1). The 3'RACE reactions also revealed the presence of two weaker, slightly larger PCR products, indicating the presence at least two alternative transcripts (data not shown).

The *Trichoplax Pax* gene contains a paired domain, an oc tapeptide, and a ''full-length'' homeodomain (figs. 1 and 2). The paired domain displays structural features of *PaxB* and *Pax2/5/8* genes, and it harbors several amino acid positions that are regarded as diagnostic for this class of proteins (Kozmik et al. 2003) (fig. 1). The full-length homeodomain, however, is more similar to *PaxC/Pax6*-like genes (fig. 2*B*).

Two exon-intron junctions were mapped via genome walk PCR. The first intron is located directly upstream and adjacent to the paired box (fig. 2*A*). The location of this first intron is conserved in all *Pax* genes investigated so far. The second intron is located upstream of the homeodomain and comprises 350 bp. The accession number of the coding sequence is DQ22561.

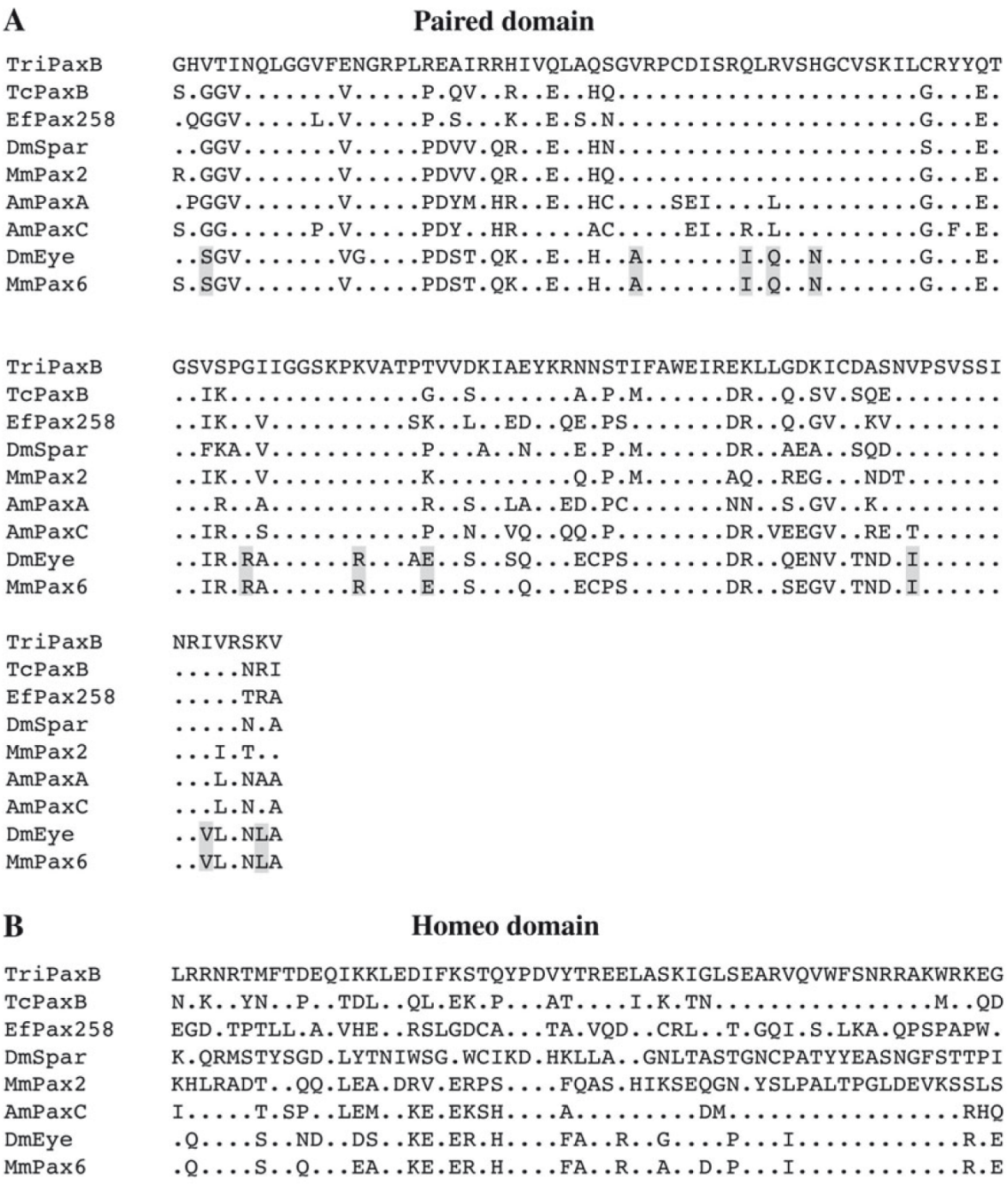

FIG. 2.—Alignment of paired domain (*A*) and homeodomain (*B*) sequences of several *PaxA*, *PaxB*, *PaxC*, *Pax2/5/8*, and *Pax6* genes. Amino acids underlayed in gray are *Pax6* specific. *Trichoplax PaxB* and *Tripedalia PaxB* harbor paired domains that are *Pax2/5/8* related and homeodomains that are *PaxC/Pax6* related. Am: *Acropora millepora*; Dm: *Drosophila melanogaster*; Ef: *Ephydatia fluviatilis*; eye: *eyeless*; Hl: *Hydra littoralis*; Mm: *Mus musculus*; Pc: *Podocoryne carnea*; spar: *sparkling*; Tc: *Tripedalia cystophora*; Tri: *Trichoplax adhaerens*.

Phylogenetic Analyses

In phylogenetic analyses the *TriPaxB* paired domain clusters basal to the *PaxB/Pax2/5/8* subfamily (fig. 3). Furthermore, *TriPaxB* appears to be basal also relative to all but one *Pax* family. *TriPaxB* always comes out basal to *PaxA*, *PaxB*, and *PaxC* genes, independent of the algorithm and also independent of whether paired domain sequences from triploblastic animals were included or not. The topology shown in figure 3*A* does not change when randomly chosen paired domain sequences from triploblasts are added to the analysis (fig. 3*C*).

To test the robustness of the basal position of the *TriPaxB* gene relative to *PaxA*, *PaxB*, and *PaxC* genes, we used the likelihood ratio test as developed by Shimodaira and Hasegawa (1999; 2001). The results of tests using the PROML program in PHYLIP (Felsenstein 2004) indicated that the tree with *TriPaxB* placed basal to all other *Pax* genes (except for *PaxD*) was the best tree according to likelihood scores (table 1). Furthermore, any tree tested where the *TriPaxB* gene was placed in the *PaxA* clade was highly statistically significantly indicated as worse than the *TriPaxB* basal tree. Placement of the *TriPaxB* gene into the clade in figure 3*A* that holds most of the other *PaxB* genes, however, indicates that while these trees have worse likelihood scores than the *TriPaxB* basal tree, the trees are not statistically significantly worse. In table 1, tree 1 is the ‘‘*TriPaxB* basal’’ tree. Trees 2–5 and 12 and 13 are trees where *TriPaxB* was grafted onto a *PaxA* or *PaxC* branch in the tree in figure 3*A*. All other trees except for tree 15 are cases where *TriPaxB* was grafted into places in the *PaxB* clade in figure 3*A*. Tree 15 retained *TriPaxB* as basal but as sister to the single *PaxD* gene.

A second approach we took was to examine the support for the NJ tree and the parsimony tree using Bayesian statistics. The Bayesian analysis suggests that the *Tri-PaxB* gene is not supported as a member of either the *PaxA–C* or *PaxB* clades and supports at 95% Bayesian proportion, the basal position of the *TriPaxB* gene. The Bayes proportions are shown on the branches of the tree in figure 3*B*.

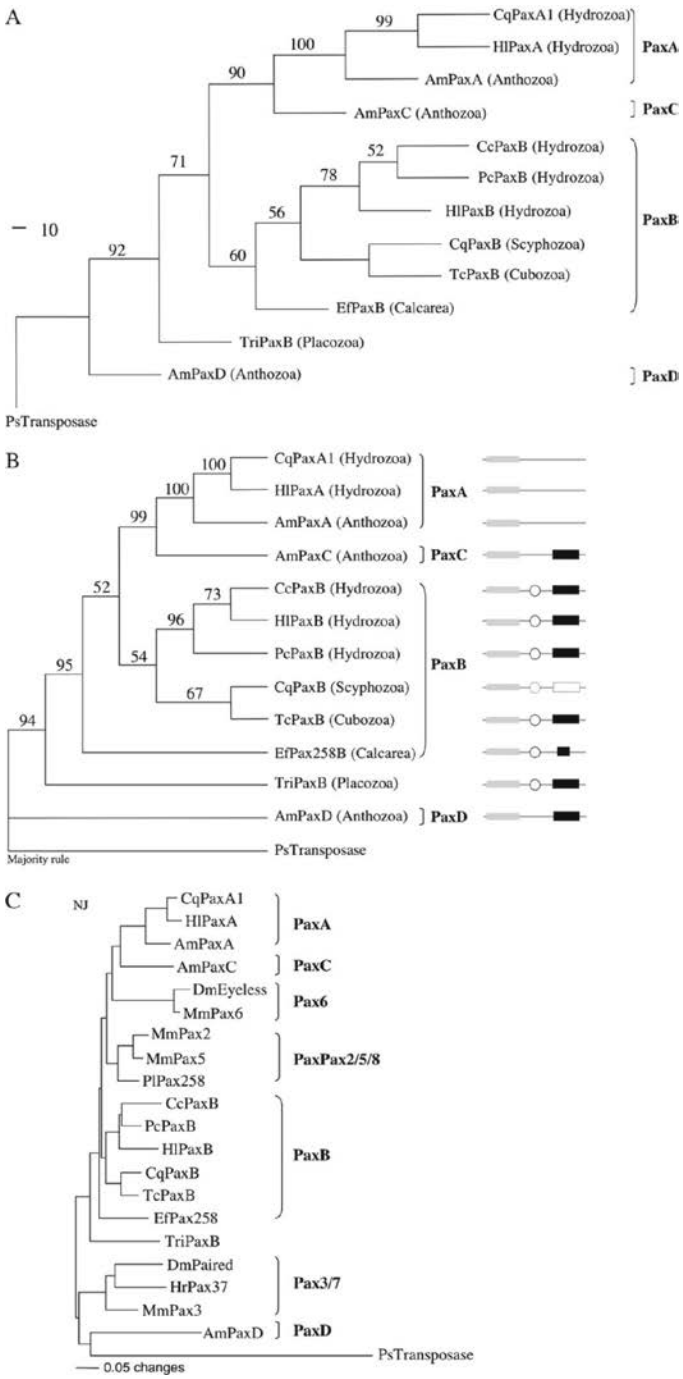

FIG. 3.—(*A*) Rooted neighbor-joining tree of paired domain sequences of all known diploblast *PaxA*, *PaxB*, *PaxC*, and *PaxD* genes. Bootstrap values result from 1,000 stepwise addition replicates. The shown topology is identical to the parsimony tree. A *pseudomonas* transposase sequence serves as out-group. (*B*) Bayesian analysis for all known diploblast *Pax* genes. The Bayes proportions are shown on the branches. At 95% Bayesian proportion, a basal position of the *TriPaxB* gene compared to *PaxA–C* and *PaxB* clades is supported. In this figure the structural features of the corresponding *Pax* genes are also illustrated. Paired domains = light gray boxes, homeodomains = black boxes, octapetides = white circles. For *CqPaxB* so far only sequences of the paired domain are available. *EfPax258* contains a partial homeodomain. (*C*) Neighbor-joining tree of all known diploblast together with several triploblast *Pax* gene paired domains. Note that the inclusion of triploblast sequences results in a loss of bootstrap support. Am, *Acropora millepora*; Cc, *Cladonema californicum*; Cq, *Chrysaora quinquecirrha*; Dm, *Drosophila melanogaster*; Ef, *Ephydatia fluviatilis*; Hl, *Hydra*

littoralis; Hr, *Halocynthia roretzi*; Mm, *Mus musculus*; Pc, *Podocoryne carnea*; Pl, *Paracentrotus lividus*; Tc, *Tripedalia cystophora*; Tri, *Trichoplax adhaerens*.

Table 1 Results of Likelihood Ratio Tests (as Developed by Shimodaira and Hasegawa, 1999, 2001) Using the PROML Program in PHYLIP (Felsenstein 2004).

Tree	Log L	Differentiated Log L	P Value	Significantly Worse
1	1,773.0) Best			
2	1,839.3	66.3	0.000	Yes
3	1,838.6	65.6	0.000	Yes
4	1,822.9	49.9	0.002	Yes
5	1,800.1	27.2	0.041	Yes
6	1,788.3	15.3	0.284	No
7	1,784.2	11.2	0.429	No
8	1,785.5	12.5	0.358	No
9	1,790.5	17.6	0.174	No
10	1,788.2	15.3	0.268	No
11	1,781.2	8.2	0.573	No
12	1,822.9	49.9	0.002	Yes
13	1,800.3	27.3	0.039	Yes
14	1,781.2	8.2	0.569	No
15	1,773.0	0.0	0.889	No
16	1,781.0	8.1	0.579	No
17	1,780.9	7.9	0.588	No
18	1,783.8	10.8	0.425	No
19	1,785.5	12.5	0.358	No
20	1,788.8	15.8	0.214	No

Expression of TriPaxB

Semiquantitative RT-PCR experiments revealed that *TriPaxB* is expressed in adult, i.e., growing, and vegetatively reproducing animals. Here, *TriPaxB* expression is significantly higher than that of the regulatory *Antp* superclass gene, *EMX* but lower than expression of the *HSP70* gene (fig. 5). Whole-mount in situ hybridization studies revealed expression in distinct cell patches along a ring region close to the outer edge of the animal body (fig. 6*A*, *E*, and *F*). Control hybridization with an *actin* antisense probe shows homogeneous expression throughout the entire body, as expected for a housekeeping gene (Fig. 6*B*). Control experiments with sense probes did not reveal any specific hybridization signals (data not shown, but see Jakob et al. 2004).

Interestingly, expression signals found in smaller animals were weaker than those found in larger animals (compare fig. 6*A* and *E*; data not shown). Analysis of tissue sections revealed that *TriPaxB*-expressing cells are not epithelial cells but cells inside the animal (fig. 6*C* and *D*). Possibly these cells are undifferentiated fiber cells.

Interestingly, the *Hox/ParaHox* gene, *Trox2*, shows a similar spatial expression pattern in this region of cell differentiation (on average the *Trox2* signal, however, is stronger and more evenly spaced; fig. 6*G* and Jakob et al. 2004).

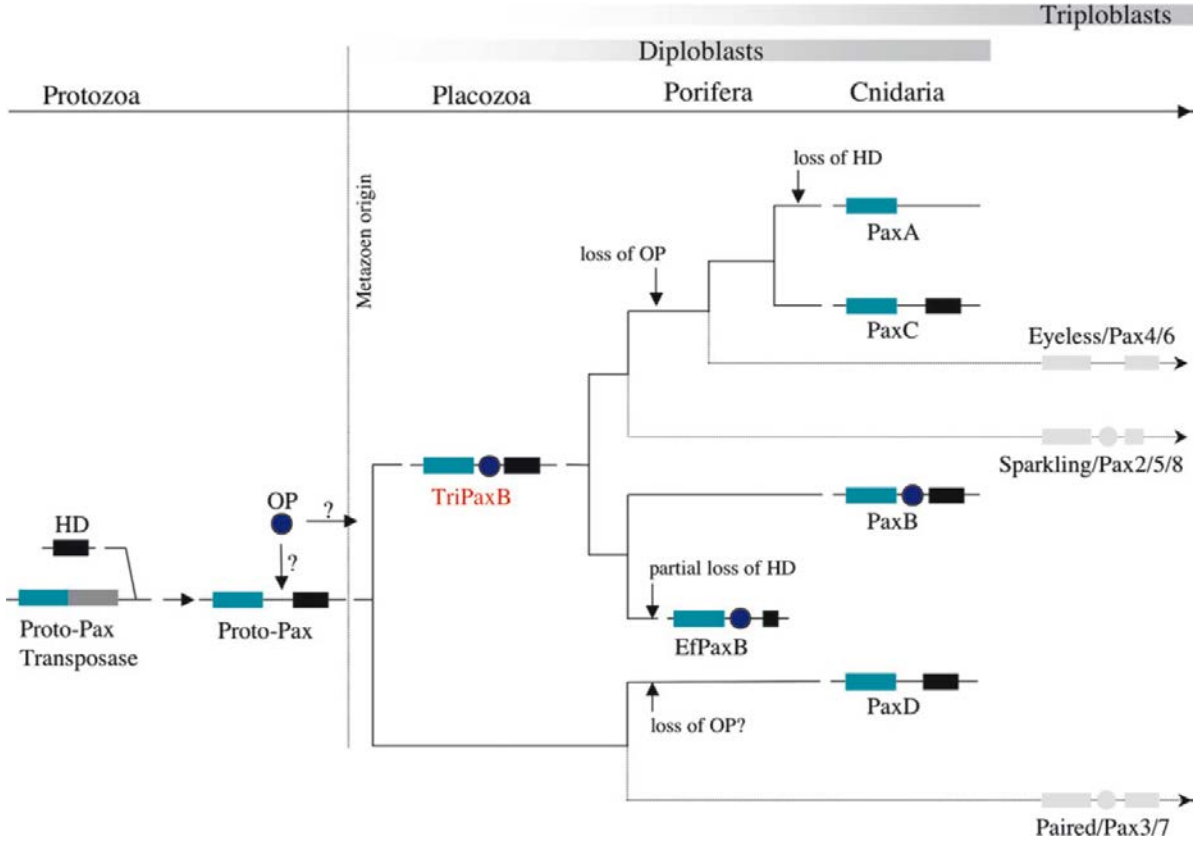

FIG. 4.—*Pax* gene evolution model. A Proto-*Pax* gene (B or D like) derived from a gene fusion event between a Proto-*Pax* transposase and a homeodomain (HD) in protozoans, as first proposed by Breitling and Gerber (2000). The octapeptide (OP) capturing occurred either after or before the first gene duplication event. In the latter case the octapeptide got lost in the D lineage. Two more rounds of gene duplication (B > C and C > A) followed by partial losses of homeodomain and/or octapeptide sequences did lead to the current *Pax* gene assembly in cnidarians. In sponges a partial loss of the homeodomain took place. Proposed relationships to triploblast *Pax258*, *Pax6*, and *Pax37* genes are indicated by dotted lines. For abbreviations see figure 3. Paired domains = green boxes, homeodomains = black boxes, octapetides = blue circles.

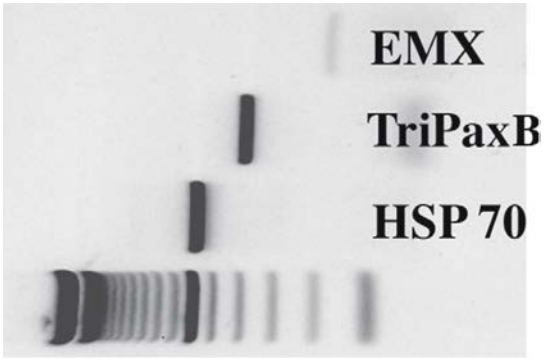

FIG. 5.—Semiquantitative RT-PCR analysis of *HSP70*, *TriPaxB*, and *EMX* using a cDNA preparation from reproducing stages of *Trichoplax adhaerens*. *TriPaxB* expression is significantly higher than expression of the regulatory *Antp* superclass gene, *EMX*, but lower than expression of the *HSP70* gene.

Discussion

The structure of the putative ancestral *Pax* gene has been controversially discussed (Sun et al. 1997; Catmull et al. 1998; Hoshiyama et al. 1998; Breitling and Gerber 2000). The data obtained from *T. adhaerens* strongly suggest that a putative ''Proto-*Pax*'' gene harbored at least two of the three *Pax* gene motifs, that is, a paired domain and a homeodomain, and possibly also the third motif, the octapeptide (fig. 4). The transition from an ''Ur-*Pax*'' gene (a homeodomain fused to a paired domain; (Breitling and Gerber 2000; Miller et al. 2000)) to the *Trichoplax PaxABC* gene, requires only a single step, the incorporation of the octapeptide. From this ''fully loaded'' ancestral *PaxABC* gene other *Pax* genes may be derived by subsequent deletion events, that is, loss of the (1) homeodomain, (2) partial homeodomain, and/or (3) octapeptide (fig. 4). The supported model is consistent with the paired domain gene trees in figure 3, current knowledge on the phylogenetic position of Placozoa, and the comparison of *Pax* gene functions.

The *Pax* evolution model in figure 4 incorporates the assumption that a Proto-*Pax* gene derived from a gene fusion event between a paired domain (e.g., from a Proto-*Pax* transposase) (Breitling and Gerber 2000) and a homeodomain in protozoans. In addition to the original arguments, this scenario seems plausible also because no *Pax* genes have been found in protists and the best-supported *Pax* paired domain phylogeny is obtained with transposase as out-group. The diploblast *Pax* gene tree (fig. 3*A* and *B*) is in accordance with a proposed basal position of Placozoa (for overview and references see Syed and Schierwater 2002). If Porifera

were basal, however, the *Pax* evolution model would require a slight modification. Here, the last common ancestor of Placozoa and Porifera would be assumed to have harbored a *Trichoplax*-type *PaxB* gene, whose structure remained unchanged in Placozoa but experienced a partial loss of the homeodomain in the lineage leading to Porifera. Because it is known that Placozoa are not secondarily reduced Cnidaria (Ender and Schierwater 2003) and because their bauplan cannot easily be derived from a sponge bauplan (Syed and Schierwater 2002), one has to argue that *Pax* genes of the A class, and—particularly interesting—also of the B and C class, predated the invention of nerve and sensory cells.

With respect to the evolution of *PaxD*-like genes additional research is needed to decide whether *PaxD* branched off even earlier (as shown in our evolution model) or if the *PaxD* paired domain originated in the next common ancestor of Cnidaria and triploblasts. In the first scenario a *PaxD*-type gene either got lost or escaped surveys in Placozoa and Porifera. In the second scenario insufficient taxon sampling or nonoptimal out-group choice may have hindered phylogenetic resolution in the analysis. Although it seems unlikely that a *PaxD*-type gene escaped our PCR screen, we cannot rule out, however, that Placozoans posses more than one *Pax* gene. To decide between the alternatives more data will be needed, which will likely come from ongoing whole-genome sequencing efforts in Placozoa, Porifera, and Cnidaria.

Functional information from triploblast *Pax* genes may also add to our understanding of early duplication events in diploblastic animals. It was previously assumed that *PaxB* is a precursor of *Pax2/5/8*, whereas *PaxC* could be a precursor of *Pax6* genes in triploblasts. Plaza et al. (2003) recently demonstrated that DNA-binding characteristics of cnidarian PaxB and PaxC proteins display no simple relationship to *Pax2/5/8* and *Pax6* genes. The authors showed that *A. millepora* PaxB and PaxC proteins can both bind to *eyeless* (*ey*) targets in vivo and in vitro, which casts doubt on the postulated direct relationship between cnidarian *Pax* genes and the bilaterian *Pax6* and *Pax2/5/8* classes. Given that our analysis suggests that Cnidarian *PaxB* and *PaxC* (and also *PaxA*) genes are derived from a gene with similar organization to the placozoan *PaxB*-like gene, one could speculate that the ancestral *PaxABC* gene unites functional features which were retained in cnidarian *PaxB* and *PaxC* as well as in triploblast *Pax2/5/8* and *Pax6* genes. This hypothesis is indeed supported by Kozmik et al. (2003) who showed that *T. cystophora PaxB* (the only *Pax* gene in cubomedusa found so far) contains a *Pax2/5/8*-type paired domain

and octapeptide but a *Pax6*-type homeodomain. The *Tripedalia PaxB* gene is expressed in larval stages, in the retina, lens, and statocyst. According to functional properties, that is, binding specificity, the ability to rescue *spa* (a *Drosphila Pax2* mutant) and to induce ectopic eyes in *Drosophila*, the authors suggest that the ancestor of the cubozoan *PaxB*-like protein was the primordial *Pax* protein in eye evolution and that *Pax6*-like genes evolved in triploblasts after separation from Cnidaria. *Trichoplax PaxB* meets these expectations for an ancestral Proto-*PaxABC* gene. Most interestingly, *Trichoplax* does not possess any kind of sensory organs or nerve cells. Expression of *TriPaxB* in small irregular cell patches along the outer edge of the animal possibly relates to undifferentiated cells and is spatially overlapping with the *Trox2* expression domain (the only *Hox/ParaHox* gene found in Placozoa; see fig. 6*G* and Jakob et al. 2004). Quite noteworthy, *TriPaxB* is not expressed in differentiated fiber cells, which represent putative proto-neural/muscular cells and are located between the upper and lower epithelium throughout the center region of the body (fig. 6*C* and *D*). *Tri-PaxB* could, however, function in cell determination of fiber cells from undifferentiated and multipotent precursor cells (cf. Jakob et al. 2004). We propose that *TriPaxB* and *Trox2* both demark a particular zone of cell proliferation and differentiation.

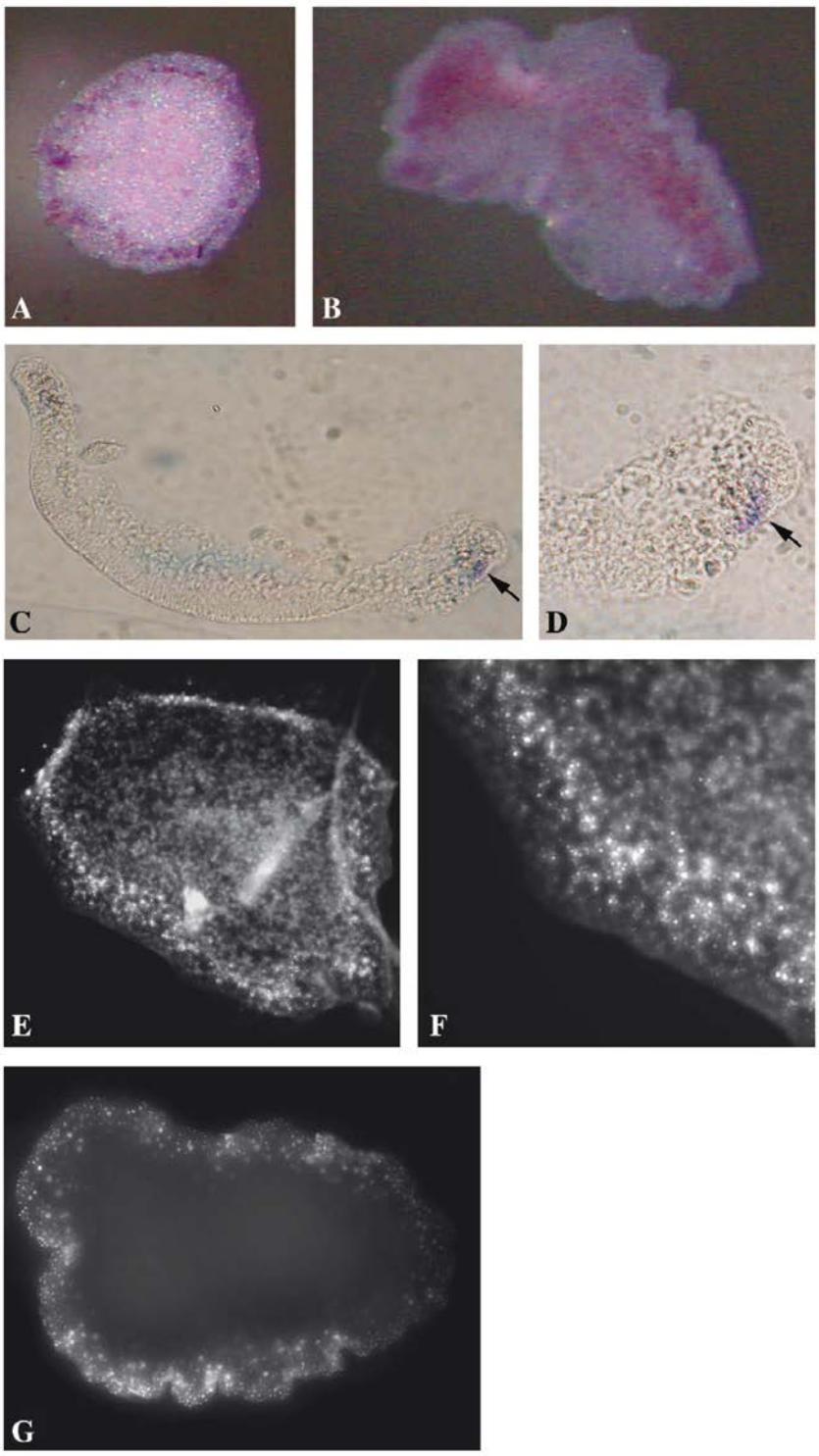

FIG. 6.—Whole-mount in situ expression analyses of *TriPaxB*, *Trox2*, and *actin*. The *TriPaxB* gene is expressed in growing and vegetatively reproducing stages of *Trichoplax adhaerens* (cf. fig. 5) in cell patches at some distance to the outer edge of the animal body (*A*, *C–F*). *Actin* (*B*) and *Trox2* (*G*) expression is shown for comparison. *A–D* represent DIG-labeled whole-mount in situ hybridizations, whereas *E–G* were labeled with FITC-UTP. Note that *TriPaxB* is not expressed in differentiated fiber cells that are located in between the upper and the lower epithelium in the center of the animal body (*C* and *D*). *Trox2* (*G*) is also expressed in the proposed zone of cell proliferation and differentiation close the outer edge of the animal body, but on average stronger and more homogeneously (Jakob et al. 2004).

We further propose that *PaxB* was co-opted in the last common ancestor of cnidarians and triploblasts for sensory organ and nerve cell development and that two rounds of gene duplication (B → C and C → A) followed by partial losses of homeodomain and/or octapeptide sequences led to the current *Pax* gene assembly in cnidarians (fig. 4). In the triploblast lineage additional duplication-deletion events have taken place and among others resulted in the functional split of protein function of *Pax2/5/8* and *Pax6* genes (fig. 4). Because *TriPaxB* is basal to all other known *PaxB* genes (and also to *PaxA* and *PaxC* genes), it is basal also to *Pax2/5/8* and *Pax6* genes (Sun et al. 1997) (fig. 4). Our data suggest that a *PaxB* similar gene (harboring functional features of both *Pax2/5/8* and *Pax6* genes) was the original gene involved in sensory organ development and evolution. A functional split into *Pax2/5/8* (mechanosensory) and *Pax6* (eye/light sense) likely occurred in the last common ancestor of diploblasts and triploblasts. While the placozoan *TriPaxB* gene most likely predates the origin of nerve and sensory cells, its ancestral developmental function needs to be investigated in more detail.

Acknowledgments

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The *Trichoplax TriPaxC* gene:
Spotlight on a prototypical Pax6 like gene

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Abstract

Pax genes comprise a diverse family of homeodomain transcription factors, crucial for developmental processes of neuronal and sensory structures in animals. Distinct Pax gene classes have been isolated in diploblast phyla, i.e. Porifera, Placozoa and Cnidaria and all major bilaterian groups. We recently demonstrated the presence of a *PaxB* like gene in *Trichoplax adhaerens* and its implications for early Pax gene evolution among basal metazoans. Here we report on a second Pax gene in *Trichoplax*, *TriPaxC*. We analyzed its phylogenetic characteristics, expression patterns and implications for light dependent behavioral tasks utilizing RNAi knockdowns. *Cum grano salis*, *TriPaxC* fits in to the evolutionary scenario claimed by Hadrys et al 2005 and sheds light on early evolution of prototype sensory abilities in placozoans.

Introduction

The Phylum Placozoa with *Trichoplax adhaerens* as it's only representative species, is traditionally viewed as the simplest living animal (Schulze 1883; Grell 1991; Grell 1981; Schierwater et al. 2010). Although the distinct placozoan phylogenetic position is controversially discussed, several arguments strongly support Placozoa to integrate among the lowest level of metazoan evolution. The most basal animal bauplan of *Trichoplax* lack any kind of axis, organs, true neurons and is composed of only four to five different cell types, for review see (Schierwater et al. 2010). Despite their simple organization, little is known about cellular physiological properties of *Trichoplax*. However, the identification of a limited inventory of key regulator genes for metazoan development, i.e. the limited repertoire of Antennapedia class genes (Schierwater et al. 2008; Monteiro et al. 2006b), the single ProtoHox/ParaHox (Jakob et al. 2004a) and the presence of Pax genes (Srivastava et al. 2008; Hadrys et al. 2005), indicate that *Trichoplax* already possess a higher physiological state of specialization then generally believed. As shown by Hadrys et al. (Hadrys et al. 2005), *Trichoplax* expresses the *TriPaxB* gene in cells of unknown morphological characteristics. Thus led to the speculations about an additional cell type in *Trichoplax* (Hadrys et al. 2005). Expression of highly conserved Pax genes seems to be common among basal metazons since derived PaxB genes were isolated from Porifera (Hoshiyama et al. 1998; Hill et al. 2010) and at least four different Pax genes

were isolated from cnidarians (Miller et al. 2000; Catmull et al. 1998; Sun et al. 1997; Plaza et al. 2003; Matus et al. 2007). However, recent attempts to identify distinct functional homologues of cnidarian Pax genes and their Bilateria counterparts failed. Attempts have been made to identify a cnidarian counterpart to the bilaterian *Pax6* gene – a key regulator for eye development (Onuma et al. 2002; Stierwald et al. 2004; Suga et al. 2010; Gehring and Ikeo 1999). By ectopic expression experiments three different cnidarian Pax genes were found capable to induce ectopic eye development in *Drosophila* (Kozmik et al. 2003; Plaza et al. 2003; Suga et al. 2010). These results indicate overlapping functional properties among the Pax genes (Kozmik et al. 2003; Kozmik 2008; Miller et al. 2000). As a consequence, there is no bona fide Pax-6 gene known from diploblasts yet (Kozmik 2008). Despite the crucial roles of Pax genes for sensory organ development among bilaterians, we know very little about their developmental function and physiological outcome during development in diploblast phyla (Breitling and Gerber 2000). To gain insight into the developmental role of early metazoan Pax genes in *Trichoplax*, we characterized the identified Pax gene *TriPaxC*. By means of *in situ* Hybridization expression analyses of *TriPaxC*, we were able to identify a distinct cell type of unusual morphology, not recognized in *Trichoplax* so far.

Material and Methods

Animal cultures

All experiments were carried out on the clonal placozoan lineage “Grell”. For precise culture conditions see Schierwater and Kuhn (Schierwater and Kuhn 1998). Prior to any experimental approach individually collected animals were rinsed three times with sterile artificial seawater and subsequently used for the experiments.

RNA isolation and cDNA synthesis

Total RNA was extracted from *Trichoplax adhaerens* employing the RNeasy Mini Kit (Qiagen). An additional digestion step was performed using the RNase-free DNase I (Roche), in order to remove residual traces of genomic DNA according to the manufacturer's instructions.

Total RNA was used to synthesize cDNA using the Transcriptor cDNA Kit (Roche) following manufacturers protocol.

Rapid amplification of cDNA ends (RACE), molecular cloning and sequencing

Primer sets according to the Pax-like annotated gene were designed to genomic sequence information (accession number: XM 002115668.1) (5'-GTGTATTCATAAACGGCCGTCC-3', 3'-CCTCGCCGTAATAGAACTACATTC-5'). The 3' rapid amplification of cDNA ends (RACE)PCR Race were performed using FastStart (2x) DNA polymerase (Roche) following standard protocol recommended by the manufacturer. PCR was performed as follows: (30´´@98°C (1x) ((30´´@95°c,15´´@60°C, 1´@72°c (38x))).

The obtained PCR product was cloned into *E.coli* "one shot" (Invitrogen), utilizing pGem-t-easy TA ligation kit (Promega). Bacteria were screened by single colony T7/Sp6 standard PCR and positive clones were subsequently sequenced using an ABI 310 automated sequencer.

In situ hybridization

Whole-mount *in situ* hybridization experiments were performed using a m odified protocol developed for placozoans (Jakob et al. 2004b). Hybridization was done for at least 10 hours. Hybridization and s ubsequent wash steps were performed at 65°C. Sense and antisense probes were synthesized via SP6 and T7 RNA polymerases respectively using FITC labeling kit (Roche). Digital microphotographs were obtained using a Zeiss Axoivert fluorescent Microscope

Phylogenetic analyses

Distance and Maximum Likelihood analyses were carried out in order to infer phylogenetic relationships between Pax genes. Paired domain sequences from diploblasts were included in the analysis (for GenBank accession numbers see Hadrys et al. (Hadrys et al. 2005)). Both analyses were conducted using Mega5 (Tamura et al. 2011). For distance method the JTT+G model was applied. For maximum likelihood analyses the more complex WAG+G+I+F was applied (model not available for distance methods in Mega5). Support for both analyses was assessed with 1000 Bootstrap replicates.

As previously analyses have shown that the *PaxD* gene from *Acropora millepora* is basal to all other diploblast Pax genes (Hadrys et al. 2005) it was used as outgroup in the phylogenetic analyses.

Results

Sequence and phylogenetic analysis of TriPaxC cDNA transcripts

The sequence analyses of cloned RT-PCR products are in accordance with the predictions of the *Trichoplax* genome database. We could not find any differences to the computational in silico intron/exon prediction of the genomic *TriPaxC* loci. The transcript is lacking an octapeptid, present in several Pax classes of diploblast metazoans. Moreover, the two identified domains, the Paired box and homeobox (Figure 1), show highest homology to the cnidarians Pax genes of *Acropora millepora* PaxC*Am* followed by slightly weaker affinity to *Acropora millepora* PaxA*Am* (Miller et al. 2000) (Figure 2).

Figure 1. Obtained cDNA sequences of *TriPaxC*.
Dark grey resembles the Paired box motif. Light grey indicates the Homeobox motif. Red marks the intron 1 postion so far only known for Placozoa. Blue marks a common conserved intron position amongst diploblasts

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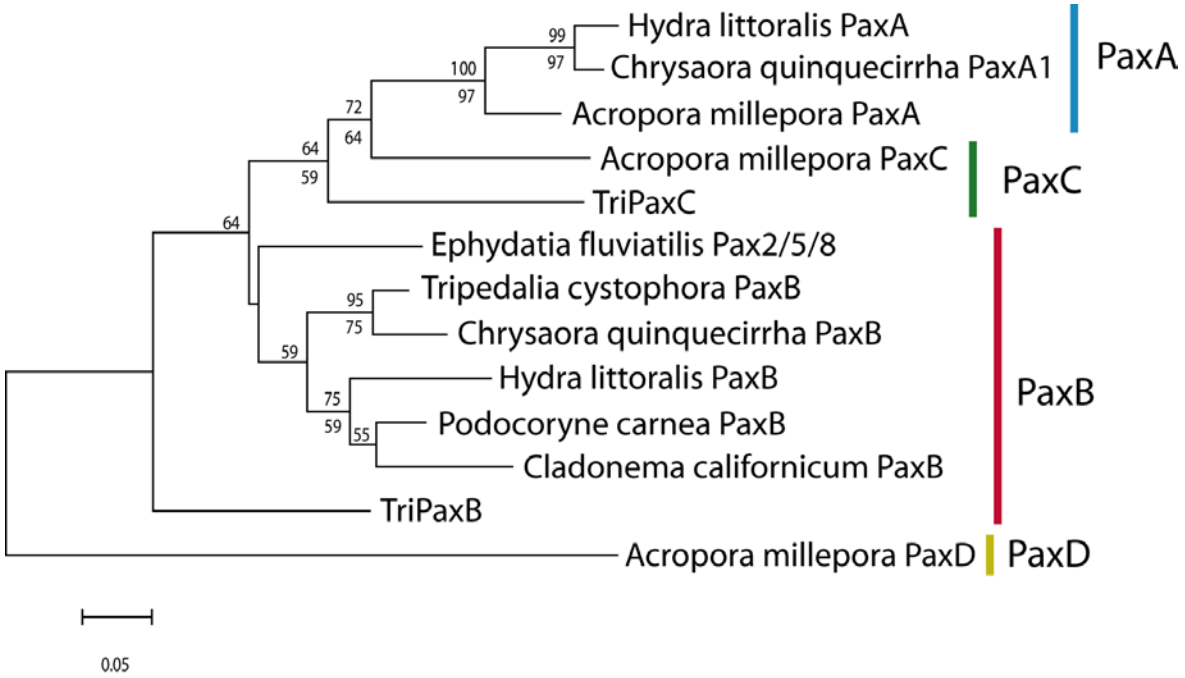

Figure 2. Rooted neighbor-joining tree of paired domain sequences of known diploblast *PaxA*, *PaxB*, *PaxC*, and *PaxD* genes. Bootstrap values result from 1,000 stepwise addition replicates (distance: above nodes/likelihood: below nodes). The shown topology is identical to the maximum likelihood tree. *Acopora millepora PaxD* sequence serves as outgroup.

In situ Hybridization

Expression of *TriPaxC* in *Trichoplax adhaerens* appears in a single distinct cell type (Figure 4). *TriPaxC* expressing cells show a characteristic morphology which cannot be accounted to any previously described cell type. Expression pattern appear in a non-structured, scattered pattern throughout the center of the animal. No expression of *TriPaxC* could be observed in the marginal regions of the animal.

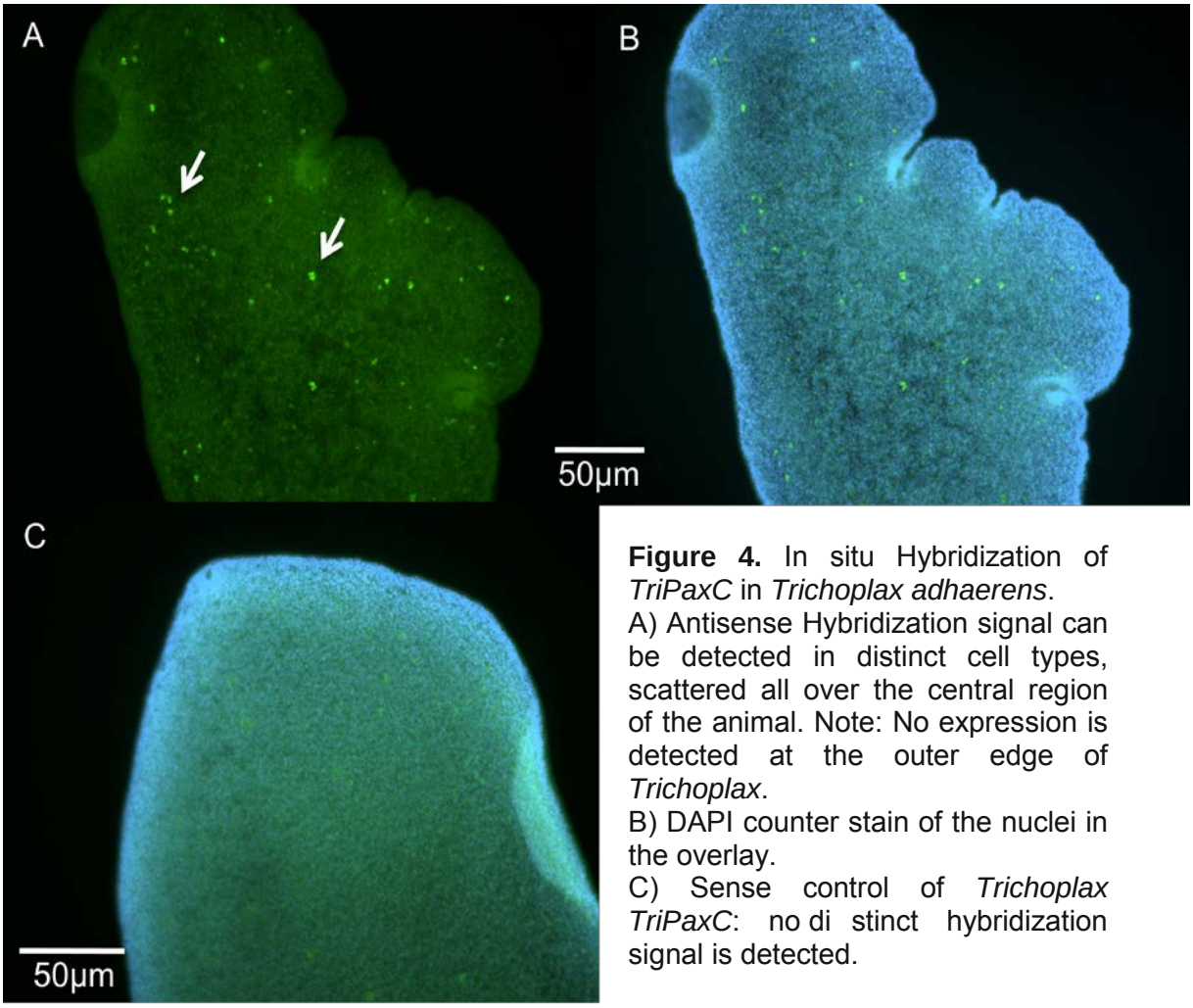

Figure 4. In situ Hybridization of *TriPaxC* in *Trichoplax adhaerens*.
A) Antisense Hybridization signal can be detected in distinct cell types, scattered all over the central region of the animal. Note: No expression is detected at the outer edge of *Trichoplax*.
B) DAPI counter stain of the nuclei in the overlay.
C) Sense control of *Trichoplax TriPaxC*: no di stinct hybridization signal is detected.

Discussion

Pax-like genes were isolated from every major animal group, ranging from early branching Diploblast to bilaterian phyla. Recent studies interpreted the course of Pax genes evolution using phylogenetic analysis and concluded a major functional split of Pax genes likely occurred in the last common ancestor of diploblasts and triploblasts (Miller et al. 2000; Kozmik et al. 2003; Catmull et al. 1998; Hadrys et al. 2005).

In this study we characterized the *TriPaxC* in *Trichoplax* adhaerens in terms of gene structure, expression patterns and its implication for placozoan sensory and behavioral capabilities.

The gene structure of *TriPaxC* harbors two of the three Pax gene motifs which are

the paired domain and a homeodomain while a octapeptid domain is missing. Both domains revealed highest sequence homology to the cnidarian Pax-C class and the bilaterian *Pax6* class.

Phylogenetic analyses of *TriPaxC* compared to representatives of diploblast Pax classes reveal that it descends from ancestral PaxB like genes and groups basal to cnidarian PaxC and PaxA genes. These results show that *TriPaxC*, cum granum salis, fulfills evolutionary considerations, previously hypothesized for the evolutionary course of animal Pax genes (Hadrys et al. 2005; Miller et al. 2000). Starting from an ancestral PaxB gene PaxC and PaxA diverged. While the *Trichoplax TriPaxC* gene most likely provides the ground for the evolution of PaxA and PaxC classes in the anthozoan, the most ancient cnidarian class (Bridge et al. 1992; Ender and Schierwater 2003).

Since diploblast Pax genes are expected to be directly linked to the origin of nerve and sensory cells, their ancestral developmental functions are not elucidated yet (Sun et al. 1997; Catmull et al. 1998; Hoshiyama et al. 1998).

We performed *in situ* hybridization experiments to identify the expression pattern of *TriPaxC*. Based on the expression analysis we can identify a specific cell type in *Trichoplax*. Intriguingly its morphology shows no clear homology to the previously described cell type. Its scattered distribution over the central regions of the *Trichoplax* body cannot be compared with any known developmental pattern process in *Trichoplax* so far. All other developmental genes investigated so far, like the Proto/ParaHox gene *Trox-2* (Sagasser and Schierwater 2002; Jakob et al. 2004b), the ProtoPax like gene *TriPaxB* (Hadrys et al. 2005) or the homeobox genes *Dll*, *Mnx* (Monteiro et al. 2006a), show expression patterns with distinct regional identities. Thus we conclude that *TriPaxC* occupies a central role for the development of a formerly unknown cell type in Placozoa. Considering the phylogenetic position of *TriPaxC* (basal to cnidarian PaxC and PaxA), it might be possible that *TriPaxC* prepared the grounds for an early key regulator of light sensory system in animals. No attempts will be made here to assign a distinct developmental function for *TriPaxC*. Whether *TriPaxC* is (i) directly involved into the development of photosensitive receptor cells, (ii) necessary for differentiation of stimulus conducting cells or (iii) responsible for the supply of effector cells mediating behavioral responses, has to be elucidated in ongoing experiments.

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Mitochondrial genome of *Trichoplax adhaerens* supports Placozoa as the basal lower metazoan phylum

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Abstract

Mitochondrial genomes of multicellular animals are typically 15-to 24-kb circular molecules that encode a nearly identical set of 12–14 proteins for oxidative phosphorylation and 24–25 structural RNAs (16S rRNA, 12S rRNA, and tRNAs). These genomes lack significant intragenic spacers and are generally without introns. Here, we report the complete mitochondrial genome sequence of the placozoan *Trichoplax adhaerens*, a metazoan with the simplest known body plan of any animal, possessing no organs, no basal membrane, and only four different somatic cell types. Our analysis shows that the *Trichoplax* mitochondrion contains the largest known metazoan mtDNA genome at 43,079 bp, more than twice the size of the typical metazoan mtDNA. The mitochondrion's size is due to numerous intragenic spacers, several introns and ORFs of unknown function, and protein-coding regions that are generally larger than those found in other animals. Not only does the *Trichoplax* mtDNA have characteristics of the mitochondrial genomes of known metazoan outgroups, such as chytrid fungi and c hoanoflagellates, but, more importantly, it shares derived features unique to the Metazoa. Phylogenetic analyses of mitochondrial proteins provide strong support for the placement of the phylum Placozoa at the root of the Metazoa.

Key words

animal evolution . phylogenetics

Introduction

Trichoplax adhaerens [Shulze 1883] is a marine invertebrate distributed in tropical waters worldwide (1-3). It is the simplest known free-living animal, displaying no axis of symmetry, lacking a basal membrane, possessing only four somatic cell types (4-6), and having one of the smallest known animal genomes (7-9). Until recently, *T. adhaerens* was the sole representative of the phylum Placozoa, but recent field studies and molecular analyses indicate genetic diversity underlying apparent morphological uniformity within the Placozoa (3, 10). In the laboratory, placozoans reproduce asexually by either binary fission or budding dispersive propagules called swarmers. Eggs have been observed, and recent DNA polymorphism analysis has provided evidence for sexual reproduction within the Placozoa (10).

The phylogenetic placement of Placozoa among the metazoans, i.e., the animals, remains unresolved. In particular, its placement among lower metazoans, that is, the phyla Cnidaria, Ctenophora, and Porifera, has been controversial. Most studies place Porifera at the base of the metazoan tree of life (11-15), but others support placozoans as one of the earliest branching lineages of Metazoa (16-20). Conflicting data, including 18S, 28S, and 16S analysis, have suggested that Placozoa form a sister clade to all bilaterians or a sister clade to both cnidarians and bilaterians (14, 21-27).

Comparative mitochondrial genomics is becoming an effective tool to resolve phylogenetic placements because of several unique properties of mitochondrial genomes, including uniparental inheritance, orthologous genes, and lack of substantial intermolecular recombination (reviewed in refs. 28, 29, 30). Although some have questioned the utility of comparative mitochondrial genomics based on problems of convergence (31), in many cases, mitochondrial data have provided robust phylogenetic trees capable of resolving evolutionary relationships among fungi (32), protists (33), diploblasts (34), and bilaterians (35-42).

The closest living relatives of animals, the choanoflagellates and fungi, possess large mitochondrial genomes with extensive intragenic spacers, introns, and several ORFs of unknown function. The unicellular choanoflagellate, *Monosiga brevicollis*, has mtDNA that is nearly four times larger (76,568 bp) than the typical animal mtDNA genome and encodes 55 different genes, often separated by large intragenic spacer regions, including two genes interrupted by introns (43). Metazoans, on the other hand, have compacted 15-to 20-kb circular mitochondrial genomes that encode a nearly identical set of 12–14 proteins for oxidative phosphorylation and 24–25 structural RNAs (16S rRNA, 12S rRNA, and tRNAs) without significant intragenic spacers and, generally, without introns. Mitochondrial DNA variants exist in metazoans, such as the presence of type I introns and linear mtDNA molecules found in cnidarians (34, 44, 45), the presence of the atp9 gene in sponges (15, 46), and the secondary expansion of mtDNA found in some mollusks (47, 48) and insects (49).

Our analysis shows that the *Trichoplax* mitochondrion possesses the largest known metazoan mtDNA genome, at 43,079 bp, more than twice the size of the typical metazoan mtDNA. Its large size is due not to secondary expansion but to features shared with metazoan outgroups, such as intragenic spacers, several

introns, ORFs of unknown function, and protein-coding regions that are generally larger than that found in animals. The large *Trichoplax* mtDNA is the least derived mitochondrial genome of any animal. Moreover, the *Trichoplax* mitochondrion shares unique derived features with other lower metazoans, notably the loss of all ribosomal protein genes. These structural features of the *Trichoplax* mitochondrial genome, along with Bayesian and maximum-likelihood (ML) analyses of mitochondrial proteins from metazoans and outgroups, provide robust support for the phylogenetic placement of the phylum Placozoa at the root of the Metazoa.

Results and Discussion

We cloned the full-length mitochondrial genome from the placozoan *T. adhaerens*, determined its complete sequence and organization (Fig. 1), and compared it with the mtDNA genomes of other diploblasts (15, 44, 45) and the choanoflagellate *Monosiga brevicollis* (43). The 43,079-bp circular mtDNA genome of *Trichoplax* is more than twice the size of the mtDNA found in most metazoans (17–25 kb), including those of poriferans (15, 46) and cnidarians (44, 45) (Table 1), making it the largest known animal mtDNA genome. The size and composition of the *Trichoplax* mtDNA genome resembles that of the choanoflagellates and is in striking contrast to the streamlined genomes found in virtually all metazoans. In rare cases of relatively large mtDNAs (up to 42 kb) found in animals, these instances are known to be a result of secondary duplications, repeat expansions, or extensive A + T-rich regions (47-49).

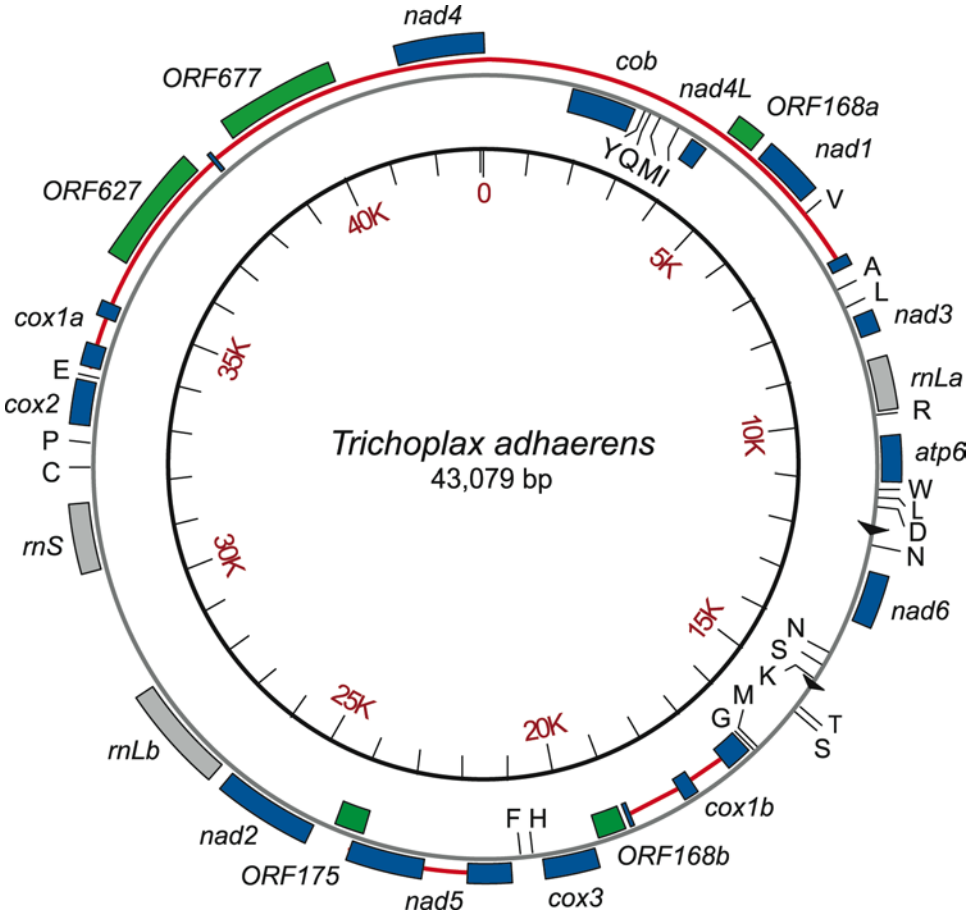

Fig. 1. Scale drawing of the mitochondrial genome of *T. adhaerens*. The complete sequence of the 43,079-bp mitochondrial genome from a Red Sea isolate of *T. adhaerens* (50) was determined and annotated by identifying ORFs with the National Center for Biotechnology Information's ORF FINDER using genetic code 4. Known mitochondrial proteins (blue rectangles) were identified by BLAST and by alignment to corresponding proteins found in poriferans (NC_006894, NC_006990, and NC_006991), cnidarians (NC_000933 and NC_003522), and the choanoflagellate *Monosiga* (NC_004309) to infer the start of translation. Genes transcribed in the clockwise direction are shown on the outer circumference; genes transcribed in the counterclockwise direction are shown on the inner circumference. Large (*rnLa* and *rnLb*) and small (*rnS*) ribosomal genes are represented as gray rectangles. The tRNAs (black lines) were identified by using TRNASCAN-SE (51) and are annotated by their International Union of Pure and Applied Chemistry (IUPAC) amino acid codes. ORFs encoding unknown proteins >100 aa in length are identified by their amino acid coding capacity (green rectangles). Introns in the *cox1* and *nad5* genes are shown as red lines connecting exons (blue rectangles). A 103-bp imperfect direct repeat is shown as black triangles. Note that the carboxy-terminal region of *cox1* (exons 5–7) is inverted with respect to *cox1* exons 1–4 because of the presence of a large 16-kb inversion encompassing the region from *trnP* to *trnV*. This inversion has been confirmed experimentally in the Red Sea isolate but does not exist in another placozoan taxon (A. Signorovitch, L. Buss, and S.L.D., unpublished data).

The *Trichoplax* mtDNA genome encodes a typical complement of animal mtDNA genes, including ATP synthase subunits (*atp6*), cytochrome oxidase subunits (*cox1*, *cox2*, and *cox3*), apocytochrome b (*cob*), reduced nicotinamide adenine

dinucleotide ubiquinone oxireductase subunits (*nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, and *nad6*), a full complement of tRNAs (24 in all), and small and large ribosomal RNAs (*rrnS* and *rrnL*) (Fig. 1 and Table 1). Additional features of the *Trichoplax* mtDNA include extra introns in the *cox1* gene and the physical separation of the *rrnL* and *cox1* genes into two discrete domains (Fig. 1). Several unknown ORFs are also found in the *Trichoplax* mitochondrial genome, including one that encodes a 627-aa protein containing reverse transcriptase (pfam00078) and Type II intron maturase (pfam01348) domains, located just downstream of the second exon of *cox1*. We have not identified the *atp8* gene in *Trichoplax* mtDNA.

Notably, *Trichoplax* mtDNA shares characteristics found in the mitochondrial genomes of known metazoan outgroups. The large size of the *Trichoplax* genome is due to the presence of large intragenic spacers, several ORFs of unknown function, and additional *cox1* introns, two of which share identical positions within the choanoflagellate Monosiga and the fungus Monoblepharella *cox1* genes (32, 43). Furthermore, most mtDNA genes in *Trichoplax* encode larger proteins than those found in other animals. On average, protein-coding regions are 10% larger in *Trichoplax* than in other diploblasts, which is comparable to the difference between choanoflagellates and diploblasts (43). Similar to *Monosiga* (43), only about half of the *Trichoplax* mtDNA contain coding regions, whereas other diploblast mtDNA genomes range from 76% to 98% coding capacity in the 25.6-and 18-kb mtDNAs of the poriferans *Axinella* and *Geodia* (15, 46), respectively (Table 1).

Table 1. Comparison of representative mitochondrial genomes

Organism	Size, bp	Coding, %	tRNAs	rRNA	ORFs	Introns	RPs	RC subunits
Choanoagellata								
Monosiga	76,568	47	25	rrnL, rrnS	6	4	rps 3, 4, 8, 12–14, 19	atp 6, 8, 9, cob, cox 1–3, nad1–4, 4L, 5, 6
Placozoa								
Trichoplax	43,079	50	24	rrnLa, rrnLb, rrnS	5	3	0	atp 6, cob, cox 1–3, nad1–4, 4L, 5, 6
Porifera								
Axinella	25,610	76	25	rrnL, rrnS	0	0	0	atp 6, 8, 9, cob, cox 1–3, nad1–4, 4L, 5, 6
Geodia	18,020	98	24	rrnL, rrnS	0	0	0	atp 6, 8, 9, cob, cox 1–3, nad1–4, 4L, 5, 6
Tethya	19,565	92	25					atp 6, 8, 9, cob, cox 1–3, nad1–4, 4L, 5, 6
Cnidaria								
Metridium	17,443	86	2	rrnL, rrnS	1	2	0	atp 6, 8, cob, cox 1–3, nad1–4, 4L, 5, 6
Acropora	18,338	85	2	rrnL, rrnS	0	1	0	atp 6, 8, cob, cox 1–3, nad1–4, 4L, 5, 6

Data compiled from mitochondrial genomes taken from GenBank: *Monosiga brevicollis* (NC_004309), *T. adhaerens* (this study) the poriferans *Axinella corrugata* (NC_006894), *Geodia neptuni* (NC_006990), and *Tethya actini* (NC_006991), and the onidarians *Metridium senile* (NC_000933) and *Acropora tenuis* (NC_003522). Coding percentage calculated from the proportion of sequence having protein coding (known mitochondrial proteins or ORF>100aa) tRNA genes, and rRNA coding sequences; ORFs, number of reading frames

encoding unknown proteins >100 aa; RPs, ribosomal protein genes; RC subunits, respiratory chain subunit genes.

The *Trichoplax* mitochondrial genome shares metazoan features lacking in choanoflagellates and fungi. Notably, we find no evidence of the presence of ribosomal protein genes in Trichoplax, a property shared with other metazoan mtDNAs, suggesting that loss of ribosomal protein genes may be a synapomorphy for the animal kingdom. Mitochondrial DNA features that had heretofore been thought to be restricted to either sponges or cnidarians are all found in *Trichoplax*. Specifically, the mitochondrial genomes of both cnidarians and *Trichoplax* mtDNA have conserved introns in the *nad5* and *cox1* genes as well as unknown ORFs.

To further examine the phylogenetic position of *Trichoplax* among the lower metazoans, we performed Bayesian and ML analyses on 2,730 amino acid positions derived from 12 well conserved protein sequences (*cox1–3*, *cob*, *atp6*, *nad1–4*, *4L*, and *5–6*) common to the mitochondrial genomes of *T. adhaerens*; the cnidarians *Metridium senile* (NC_000933), *Acropora tenuis* (NC_003522), *Anacropora matthai* (NC_006898), and *Montipora cactus* (NC_006902); the poriferans *Geodia neptuni* (NC_006990), *Axinella corrugata* (NC_006894), and *Tethya actinia* (NC_006991); and the choanoflagellate *Monosiga brevicollis* (NC_004624). *Monoblepharella* sp. JEL15 (NC_004624) was included as an outgroup taxon for this analysis because chytrids are regarded as the basal fungal taxon (32). The predicted amino acid sequences for each of the 12 genes were aligned by using CLUSTALW (52) and edited, manually and computationally, by using GBLOCKS (53), to remove ambiguous sites. These alignments were concatenated to produce a final data set of 2,730 aa (see Data Set 1, which is published as supporting information on the PNAS web site).

Partitioned Bayesian analysis, implemented in MRBAYES 3.1.1 (54), was performed by using the mtREV amino acid substitution model, with substitution-rate variation among sites modeled by a discrete approximation of the γ-distribution with a proportion of invariable sites (I + Γ). This analysis produced the phylogeny depicted in Fig. 2A. The posterior probabilities exceeded 99% for each node, with overwhelming support for *Trichoplax* being basal to both poriferans and cnidarians. ML analysis, implemented in PAML 3.14 (55), using star decomposition tree search and the mtREV amino acid substitution model, produced an identical tree topology with the bootstrap values shown in Fig. 2. Using site-wise log-likelihoods generated by PAML, statistical tests, implemented in CONSEL 0.1i (56), were conducted to test

all possible placements of *Trichoplax* among lower metazoans (Table 2). The *P* values of the Approximately Unbiased (57), the weighted and unweighted Kishino–Hasegawa (58), and the weighted and unweighted Shimodaira–Hasegawa (57) tests all exceeded 0.999 for the tree shown in Fig. 2A, with no other topology supported by *P* values >0.002.

Inclusion of bilaterian mtDNA data from the deuterostomes *Strongylocentrotus purpuratus* (NC_001453) and *Saccoglossus kowalevskii* (NC_007438) and the protostomes *Artemia franciscana* (NC_001620) and *Katharina tunicata* (NC_001636) in the phylogenetic analyses yielded a bifurcation at the base of metazoans between two clades (Fig. 2B), one comprising all bilaterians and the other comprising all diploblasts. Inclusion of additional bilaterian taxa in the analysis did not change this topology (data not shown). This result is consistent with that reported by Lavrov et al. (15) and may be due to long branch attraction that is known to affect analyses of fast evolving metazoan sequences (59). A relative-rates test comparing bilaterians to diploblasts using *Monosiga* and *Monoblepharella* as outgroups was performed by using RRTREE (60). The *P* value for bilaterians evolving at the same rate as diploblasts was 10^{-7}, indicating that the conditions for long branch attraction are present. Most importantly, regardless of whether bilaterian sequences are included or not, the basal phylogenetic position of Placozoa within the lower metazoans is robust, with *P* values between 0.924 and 1.000 for the various statistical tests (see Table 3, which is published as supporting information on the PNAS web site). Finally, the ''placozoan-basal'' topology was robust to the choice of outgroups, including the addition or substitution of chytrid fungi (*Allomyces macrogynus* NC_001715 and *Rhizophydium* sp. 136 NC_003053) as outgroups (see Fig. 3, which is published as supporting information on the PNAS web site).

Our results demonstrate that the placozoan *Trichoplax* possesses an unusual and unique mitochondrial genome, with structural and compositional features characteristic of both choanoflagellate mtDNAs, the closest relatives to animals, and typical lower metazoan mtDNAs. Like choanoflagellates, the *Trichoplax* mtDNA is much larger than the typical metazoan mtDNA, with substantial noncoding regions, genes generally larger than those found in other metazoans, several unknown ORFs, and conserved introns in both *nad5* and *cox1* genes. The large mtDNA genome found in *Trichoplax*, although consistent with the idea that marked gene loss and mtDNA compaction occurred during the emergence of multicellular animals,

nonetheless indicates that pronounced compaction was not coincident with the origin of the Metazoa.

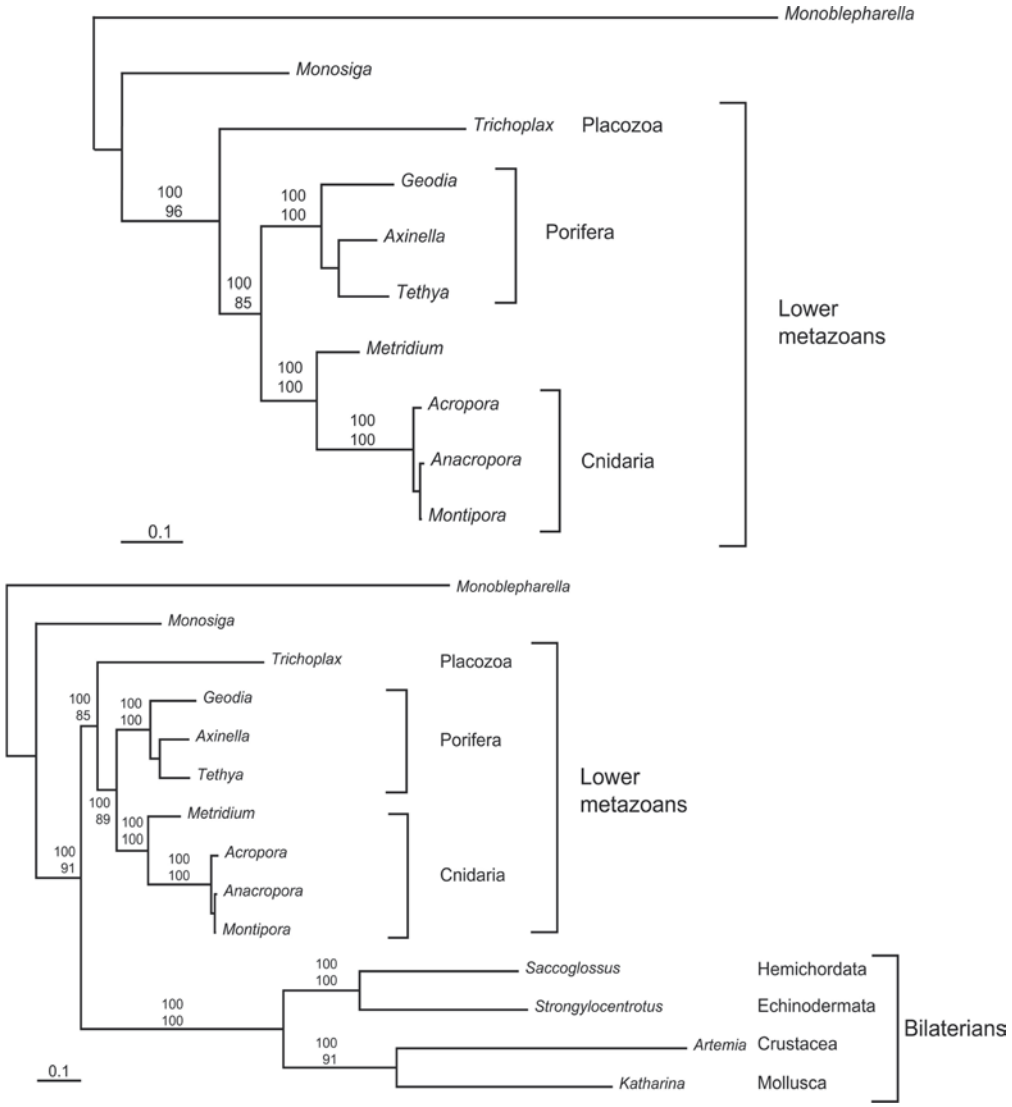

Fig. 2. Phylogenetic analysis of concatenated mitochondrial proteins. The data set consisted of a total of 2,730 amino acid positions concatenated from 12 mitochondrial protein sequences (*atp6*, *cob*,*cox1*–3, *nad1–4*, and *4L*, *5*, and *6*) .Partitioned Bayesian analysis was performed with MRBAYES for 500,000 generations by using four chains and the mtREV amino acid substitution model. Substitution rate variation among sites was modeled by a discrete approximation of the γ-distribution with a proportion of invariable sites (I + Γ). ML analysis performed with PAML using the mtREV amino acid substitution model and star decomposition tree search gave an identical tree topology. Posterior probability (*Upper*) and bootstrap (*Lower*) values are shown for each node. In these analyses, the output trees were rooted by using the chytrid fungus *Monoblepharella*.

Texts of invertebrate zoology have long been unanimous in placing the Porifera as the basal metazoan phylum, based largely on the striking resemblance of the collared cells of choanoflagellates to the choanocytes of sponges. Choanocytes, however, are adaptations for filter-feeding in these two taxa, and their absence is expected in

placozoans, which have a different mode of feeding. The data and analyses presented here provide strong support for the phylogenetic placement of Placozoa as the basal extant lower metazoan phylum.

Table 2. CONSEL statistical tests of the tree topology obtained by Bayesian and ML analysis

	P					
Hypothesis	au	kh	wkh	sh	wsh	pp
Trichoplax basal to Porifera Cnidaria	1.000	0.999	0.999	1.000	1.000	1.000
Trichoplax within Porifera	10^{-44}–10^{-4}	0–0.001	0–0.001	0–0.113	0–0.002	10^{-111}–10^{-29}
Porifera basal to Trichoplax Cnidaria	10^{-104}–10^{-4}	0–10^{-4}	0–10^{-4}	0–0.073	0–10^{-4}	10^{-291}–10^{-33}

Summary of statistical tests on all possible phylogenetic positions of *Trichoplax* within lower metazoans by using the Approximately Unbiased (56), the weighted and unweighted Kishino–Hasegawa (57); and the weighted and unweighted Shimodaira–Hasegawa (56) tests, as implemented in CONSEL. In addition, posterior probabilities pp are also displayed.

Materials and Methods

Cloning and Sequencing of *T. adhaerens* Mitochondrial DNA. Total genomic DNA was isolated from a cultured Red Sea isolate (50). Approximately 20 μg of genomic DNA was resuspended in 0.5 ml of Tris-EDTA buffer, sheared by two quick passages through a 20-gauge needle attached to a 1-ml syringe, end-repaired by using the DNA Terminator Kit (Lucigen, Middleton, WI) and size fractionated by pulse-field electrophoresis. The 30-to 40-kb DNA fraction was gel purified, ligated into the pCC1FOS vector, packaged in vitro, and plated on EPI300 *Escherichia coli* cells according to manufacturer's instructions (EPICENTRE Biotechnologies, Madison, WI). Several independent, overlapping fosmid clones containing near-full-length (36- to 40-kb) mitochondrial DNA inserts were identified by colony hybridization using a 16S rRNA probe (27). Purified fosmid DNA was isolated and sheared by sonication and end-repaired and fractionated by gel electrophoresis. The 2-to 4-kb fraction was gel purified and ligated to pSMART LC-Kan vector and transformed into *E. coli* 10G-competent cells according to manufacturer's instructions (Lucigen). Approximately 384 random subclones were chosen for sequencing. Template DNA was prepared by using TempliPhi amplification (GE Healthcare) and sequenced by BigDye Terminator version 3.1 cycle sequencing on ABI PRISM 3700 DNA analyzers (Applied Biosystems) with both forward and reverse vector primers (Lucigen). Selected regions of poor quality or low coverage were resequenced by using fosmid DNA

template and custom DNA primers designed by the Autofinish feature of CONSED (61).

Sequence Assembly and Annotation. DNA sequence chromatograms generated from random subclones and custom primer sequencing were processed and assembled by using the PHRED– PHRAP–CONSED software suite release 13.0 (www.phrap.org). The assembled mitochondrial genome sequence was analyzed with the National Center for Biotechnology Information's ORF FINDER using genetic code 4. Predicted ORFs were subjected to a similarity search using BLASTP. A custom-made Perl script, available upon r equest, automated this process. Each identified mitochondrial protein sequence was aligned to the corresponding sequences from related taxa, including poriferans (NC_006894, NC_006990, and NC_006991), cnidarians (NC_000933, NC_003522, NC_006898, and NC_006902), and the choanoflagellate *Monosiga* (NC_004309) to infer translational startsites, intron–exon boundaries, and estimated boundaries of ribosomal RNA genes. The transfer RNAs were identified with TRNASCAN-SE 1.21 (www.genetics.wustl.edu/eddy/tRNAscan-SE). Twelve conserved mitochondrial proteins (*atp6*, *cox1–3*, *cob*, *nad1–4*, and *4L*, *5*, and *6*) from *Trichoplax*, and other species were individually aligned by using CLUSTALW (52), edited manually and computationally by using GBLOCKS (53) to remove ambiguous sites, and concatenated to give a final data set of 2,730 aa for phylogenetic analysis (Data Set 1).

Phylogenetic Analysis. Partitioned Bayesian analysis, as implemented in MRBAYES 3.1.1 (54), was performed for 500,000 generations by using four independent chains and the mtREV amino acid substitution model. Substitution-rate variation among sites was modeled by a discrete approximation of the γ-distribution with a proportion of invariable sites (I + Γ). The first 1,250 samples (25%) were discarded as burn-in. ML analysis, implemented in PAML 3.14 (55), was performed by using the mtREV amino acid substitution model and star decomposition tree search. For bootstrap analysis, 100 r esampling replicates were generated by using SEQBOOT (62) and analyzed by ML analysis using PAML. The topology given by MRBAYES and PAML was statistically tested for robustness against other possible

tree topologies with CONSEL 0.1i (56) using site-wise log-likelihood outputs from PAML.

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Supplementary Material

Table 3. Statistical test of all possible positions of *Trichoplax* within lower metazoans and bilaterians using the Approximately Unbiased (au), the weighted (wkh) and unw eighted (kh) Kishino–Hasegawa, and the weighted (wsh) and unweighted (sh) Shimodaira–Hasegawa tests

Hypothesis	Probabilities					
	au	kh	wkh	sh	wsh	pp
Trichoplax basal to Porifera + Cnidaria; Bilateria as sister group (Figure 2*B*)	0.945	0.924	0.924	1.000	1.000	1.000
Trichoplax basal to Bilateria; Cnidaria + Porifera as sister group	0.074	0.076	0.076	0.835	0.397	$10^{-}10$
Trichoplax basal to Bilateria + Porifera + Cnidaria	0.008	0.009	0.009	0.719	0.048	$10^{-}15$
Trichoplax basal to Porifera; Cnidaria as sister group	$10^{-}4$	0.001	0.001	0.295	0.003	$10^{-}32$
Porifera basal to *Trichoplax* basal to Cnidaria	$10^{-}4$	$10^{-}4$	$10^{-}4$	0.234	$10^{-}4$	$10^{-}37$

Trichoplax within Porifera	$10^{-}80$–0.001	0	0	$10^{-}4$–0.006	0	$10^{-}116$–$10^{-}90$
Trichoplax within Cnidaria	$10^{-}52$–$10^{-}4$	0	0	0-0.001	0	$10^{-}299$–$10^{-}110$
Trichoplax within Bilateria	$10^{-}53$–$10^{-}4$	0	0	0	0	0–$10^{-}253$

Twelve conserved mitochondrial proteins (atp6, cox1-3, cob, nad1-4, and 4L, 5, and 6) from *Trichoplax* and comparative species were individually aligned by using CLUSTALW (1), edited manually and computationally by using GBLOCKS (2) to remove ambiguous sites, and concatenated to give a final data set of 2,730 aa (Data set 1). Partitioned Bayesian analysis, as implemented in MRBAYES 3.1.1 (3), was performed for 500,000 generations using four independent chains, the mtREV amino acid substitution model, and the assumption of discrete □-distributed sites with some invariable sites (I + □) using the JTT substition model . Maximum likelihood (ML) analysis, implemented in PAML 3.14 (4), was performed using the mtREV amino acid substitution model and star decomposition tree search. The topology given by MRBAYES and PAML was statistically tested for robustness against other possible tree topologies with CONSEL 0.1i (*5*) using site-wise log-likelihood outputs from PAML.

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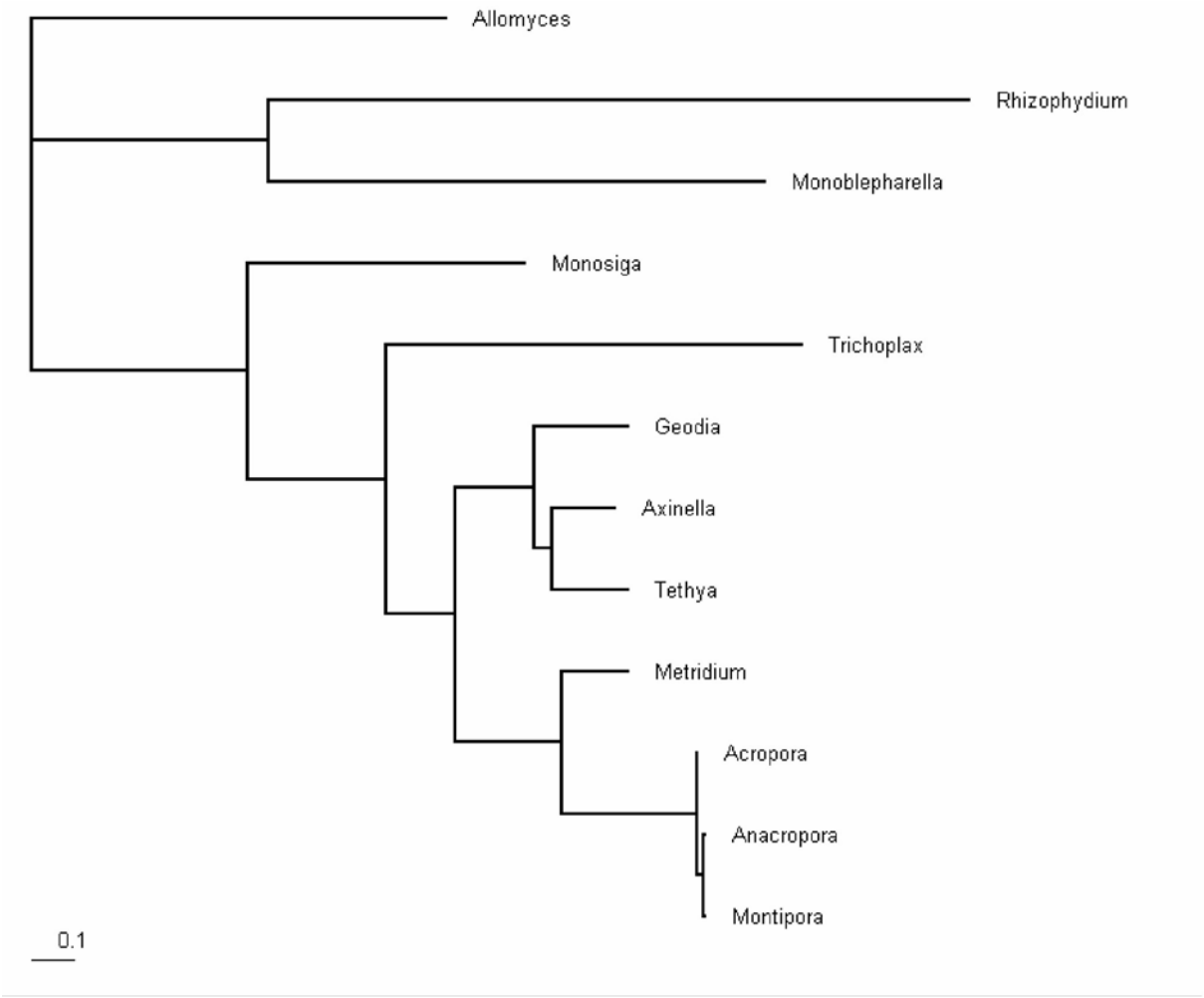

Fig. 3. The data set (available upon r equest) consisted of a total of 3,001 aa pos itions concatenated from 12 mitochondrial protein sequences (atp6, cob, cox1-3, nad1-4, and 4L, 5, and 6) from *Trichoplax adhaerens* (this study); the chytrid fungi *Allomyces macrogynus* (NC_001715), *Rhizophydium* sp. 136 (NC_003053), and *Monoblepharella sp. JEL15* (NC_004624); the cnidarians *Metridium senile* (NC_000933), *Acropora tenuis* (NC_003522), *Anacropora matthai* (NC_006898), and *Montipora cactus* (NC_006902); the poriferans *Geodia neptuni* (NC_006990), *Axinella corrugata* (NC_006894), and *Tethya actinia* (NC_006991); and the choanoflagellate *Monosiga brevicollis* (NC_004624). Partitioned Bayesian analysis was performed with MRBAYES for 250,000 generations using four chains and the mtREV amino acid substitution model. Substitution-rate variation among sites was modeled by a discrete approximation of the g-distribution with a proportion of invariable sites (I + G). All nodes show a posterior probability value >0.999.

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9. List of Publications

Publications

Sagasser S, Dellaporta SL and B Schierwater B.
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prepared for submission to *Developmental Genes and Evolution*

Sagasser S., Knorr K., Schierwater B.
Magnetic field effects in Cnidarians and the early evolution of animal magnetic senses
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Asymmetrical expression of retinal cryptochrome in a migratory bird: a possible transducer for the avian magnetic compass
Möller, A, Sagasser, S, Schierwater, B, Wiltschko W.
IV. Symposium des German-Brain-Endocrine-Immune-Network, Bayreuth, Oktober 2006

Melanopsin in the retina of passerine birds: Restricted expression to retinal ganglion cells
Sagasser, S, Heyers, D, Möller, A, Mouritsen, H.
IV. Symposium des German-Brain-Endocrine-Immune-Network, Bayreuth, Oktober 2006

Cryptochromes as a possible transducer molecule for magnetoreception in birds
Sagasser, S, Möller, A.
Gordon Research Conference on Photosensory Receptors & Signal Transduction, Il Ciocco, Italien, Mai 2006

Tracking an ancient function of the *Antennapedia* class gene *Trox-2* in *Trichoplax adhaerens* (Placozoa)
Sagasser, S, Schierwater, B.
96. Meeting of the German Zoological Society, HU Berlin, Juni 2003

Expression analysis of the Antennapedia superclass Gene Trox-2 in the placozoon *Trichoplax adhaerens*
Sagasser,S, Dellaporta, S, & Schierwater, B.
95. Meeting of the German Zoological Society, Halle, Mai 2002

10. Erklärung

Hiermit erkläre ich, dass ich meine Dissertation mit dem Titel:

On Development and Evolution of *Trichoplax adhaerens* (Placozoa)

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe. Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Fürth im Odw., 29.09.2011