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Review

Placozoa and the evolution of Metazoa and intrasomatic cell differentiation

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ABSTRACT

The multicellular Metazoa evolved from single-celled organisms (Protozoa) and usually – but not necessarily – consist of more cells than Protozoa. In all cases, and thus by definition, Metazoa possess more than one somatic cell type, i.e. they show in sharp contrast to protists—intrasomatic differentiation. Placozoa have the lowest degree of intrasomatic variation; the number of somatic cell types according to text books is four (but see also Jakob W, Sagasser S, Dellaporta S, Holland P, Kuhn K, and Schierwater B. The *Trox-2* Hox/ParaHox gene of Trichoplax (Placozoa) marks an epithelial boundary. *Dev Genes Evol* 2004;214:170–5). For this and several other reasons Placozoa have been regarded by many as the most basal metazoan phylum. Thus, the morphologically most simply organized metazoan animal, the placozoan *Trichoplax adhaerens*, resembles a unique model system for cell differentiation studies and also an intriguing model for a prominent “urmetazoon” hypotheses—the placula hypothesis. A basal position of Placozoa would provide answers to several key issues of metazoan-specific inventions (including for example different lines of somatic cell differentiation leading to organ development and axis formation) and would determine a root for unraveling their evolution. However, the phylogenetic relationships at the base of Metazoa are controversial and a basal position of Placozoa is not generally accepted (e.g. Schierwater B, DeSalle R. Can we ever identify the Urmetazoon? *Integr Comp Biol* 2007;47:670–76; DeSalle R, Schierwater B. An even “newer” animal phylogeny. *Bioessays* 2008;30:1043–47). Here we review and discuss (i) long-standing morphological evidence for the simple placozoan bauplan resembling an ancestral metazoan stage, (ii) some rapidly changing alternative hypotheses derived from molecular analyses, (iii) the surprising idea that triploblasts (Bilateria) and diploblasts may be sister groups, and (iv) the presence of genes involved in cell differentiation and signaling pathways in the placozoan genome.

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1. Traditional knowledge and views

Attempts to explain the origin of metazoan life seek to unravel the transition from single-celled to multicellular organisms and

from a diploblastic (two embryonic epithelia) to a triploblastic (three embryonic epithelia) body plan. The most favored scenarios are based on four well known hypotheses on the “urmetazoon” bauplan: Haeckel’s gastraea, Jägersten’s bilaterogastraea, Metschnikoff’s phagocytella, and Bütschli’s placula (for overviews and references see Boero et al., 2007; Syed and Schierwater, 2002a,b). All these scenarios, as well as many (but not all) other scenarios accept a monophyletic origin of Metazoa. The ancestral protist form however, remains unknown and differs between

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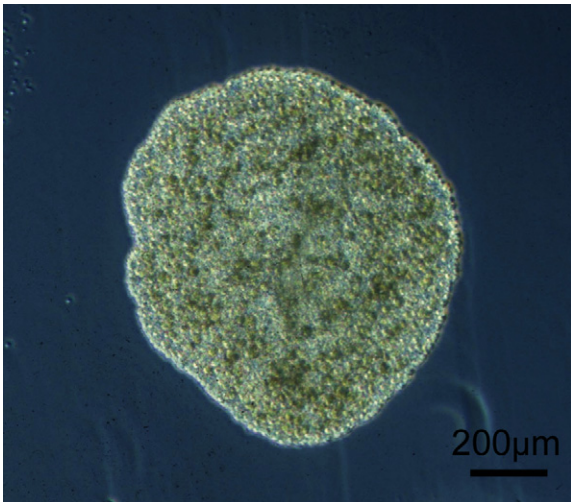


Fig. 1. Adult animal of the so-called “Grell lineage” of the placozoan, *Trichoplax adhaerens*. Shown is the parent of the clone used in the *Trichoplax* Genome Project. This single animal started clonal propagation in 1993 in the Schierwater Lab in Frankfurt. Some 20,000 offspring of this animal were used for harvesting the DNA needed for genome sequencing.

scenarios. Almost all recent molecular analyses use single-celled choanoflagellates (usually *Monosiga*) as an outgroup. This view is mainly based on a few character observations, the remarkable phenotypic similarity between choanoflagellate protists and the choanocyte cells of sponges (Porifera) (Gruner, 1993). In the absence of any convincing phylogenetic or cladistic analysis this view remains speculative, no matter how prominent it is (cf. Schierwater and DeSalle, 2007). Phenotypic, physiological, and genetic similarity between choanoflagellate individuals and choanocyte cells may also be explained by an endosymbiosis event instead of ancestry. The loose epithelial construction and the dynamics of cell migration and differentiation of sponges provide perfect prerequisites for the integration of symbiotic cells (cf. Maldonado et al., 2005; Regoli et al., 2004; Sakai et al., 2008). Neither of the two alternatives however, ancestry or endosymbiosis, can claim any convincing support over the other and thus both ideas have the status of speculations or early hypotheses. Hardly any other protist outgroup other than the choanoflagellates has been considered lately. The aforementioned urmetazoan hypotheses however, are more easily derived from colonial, i.e. multicellular protists, such as colonial flagellates like Volvocidae or even ciliates like Suctoria (Gruner, 1993). None of the latter have been used in molecular analyses which usually have fungi and/or choanoflagellates as the outgroup to Metazoa. This could be a bad mistake if choanoflagellates entered sponges as endosymbionts and if horizontal gene transfer took place.

2. The placula hypothesis

Since this review is on the possible importance of Placozoa (Schierwater, 2005) for metazoan evolution, we here consider only one out of several urmetazoan hypotheses, the placula hypothesis. The related planula hypothesis (Salvini-Plawen and Splechna, 1978) is discussed elsewhere (Schierwater et al., in revision), and for an overview and references on other urmetazoan hypotheses see Syed and Schierwater (2002a,b). Bütschli’s placula hypothesis has often been cited in connection with the placozoan *Trichoplax adhaerens* (Bütschli, 1884), which morphologically appears as a flattened disk attached to the substrate (Fig. 1). As a simplified *Trichoplax*, a flat, cilia-driven, benthic organism is described as a

hypothetical metazoan ancestor. In this model, the two-layered placula arises from a multicellular protist (e.g. flagellate) that adapts a benthic life stage (Fig. 2). Some authors have considered this a phylogenetic dead-end with no selective advantage for this process (see Syed and Schierwater, 2002a for review and references). On the other hand, escaping intraspecific competition by conquering a new ecological niche, leaving the pelagial and settling into the benthos, should provide compelling advantages if competition (e.g. for space and food) is reduced and the attachment to a substrate is not lethal to feeding, growth and reproduction.

In general, all metazoan animals possess an extracellular matrix (ECM), consisting of a basal lamina and a collagenous material. Only Placozoa completely lack an ECM, and thus any kind of basal membrane. Sponges lack a basal lamina but harbor other ECM material (Ax, 1995). From the view of anagenetic evolution one may assume that the hypothetical “urmetazoan” neither had a basal lamina nor an ECM. Both elements stiffen animal constructions and the lack of both elements allow quick shape changes, which are typical for the normal movement of *Trichoplax* (cf. Schwartz, 1984) and which are advantageous to another typical movement of *Trichoplax*, the extreme bulging which is reminiscent of gastrulation stages. A flattened, benthic-vagile placula would have had the privilege to feed directly from the ground and predigest underneath the lower epithelium in a temporary extracorporeal cavity. The lower epithelium then becomes a ‘nutritive epithelium’. The two epithelia of the hypothetical Placula show a remarkable resemblance to the lower (or feeding) and upper (or protective) epithelium of recent *Trichoplax* (see Fig. 3). Therefore from both an ecological and morphological perspective, the further development of the Placula to a placozoan bauplan would require only minor differentiation processes (see Fig. 2).

Bütschli argued that the three-layered *Trichoplax* is a comparatively derived organism, still mirroring the two-layered Placula as mode of life. It is important to note that both Schulze and Bütschli agreed in interpreting the upper epithelium of *Trichoplax* as an ectoderm and the lower epithelium as an entoderm homolog. Both authors hesitated to interpret the fiber cells as a mesoderm homolog because this would have implied a close affinity of *Trichoplax* to the triploblastic phyla. Schulze (1883) pointed out that observations on the ontogeny of *Trichoplax* would be required to solve this question (Schulze, 1883). Both researchers were aware of the principal counter-hypothesis that *Trichoplax* might be a secondarily simplified organism, as this was already proposed for some parasitic mesozoans. The latter alternative was clearly rejected by mitochondrial genome data (Ender and Schierwater, 2003) and recently also by whole genome data (Srivastava et al., 2008). After the first morphological descriptions and resulting phylogenetic interpretations, it was expected that elucidating the ontogeny and the life cycle of *T. adhaerens* would be the next crucial step in resolving the phylogenetic position of the Placozoa. One of the traditional and also recent problems with Placozoa is that the life cycle remains unknown. Recent studies show that the induction of sexual reproduction and development is at least possible in the lab (Fig. 4), giving us hope that this is an issue we will eventually be able to resolve (Eitel et al., in preparation-b). Of course we will never be able to see the hypothetical “urmetazoan” and all hypotheses on this subject will always remain nothing more than hypotheses. Nonetheless, hypotheses are made to be tested, and the placula hypothesis is the only one that can claim even a tiny grain of empirical support. Recently, Schierwater et al. (2008) reexamined the placula hypothesis in the context of a simple genetic model that could explain the transition from a Placula bauplan into a cnidarian bauplan in a most parsimonious way, i.e. by duplication of a single regulatory Proto-Hox/Para-Hox like gene, fuelling the manifestation of an oral–aboral body axis (see Fig. 2).

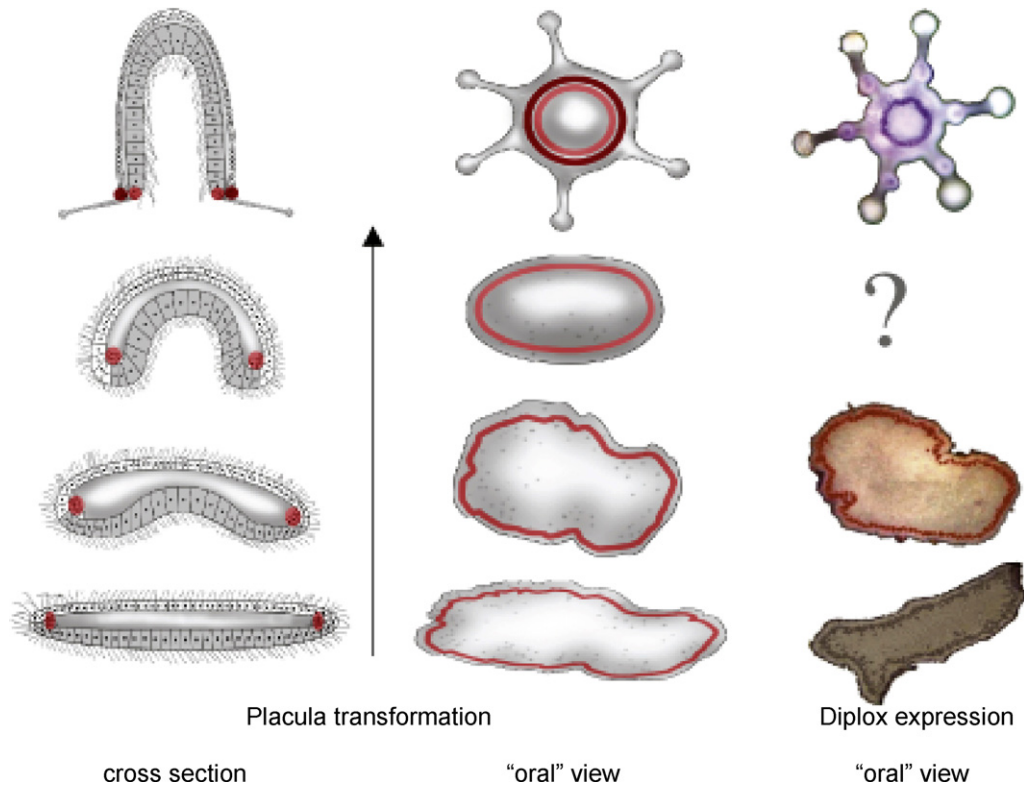


Fig. 2. Modern interpretation and modification of the placula hypothesis of metazoan origins. A non-symmetric and axis-lacking Placula bauplan transforms into a typical symmetric metazoan bauplan with a defined oral–aboral body axis. The Placula transforms from a primitive disk consisting of an upper and a lower epithelium (lower row) to a form with an external feeding cavity between its lower epithelium and the substrate (2nd row from bottom). For this the Placula lifts up the center of its body, as naturally seen in feeding *Trichoplax*. If this process is continued, the external feeding cavity increases (cross section, 3rd row) while at the same time the outer body shape changes from irregular to more circular (see oral views). Eventually the process results in a bauplan where the formerly upper epithelium of the Placula remains “outside” (and forms the ectoderm) and the formerly lower epithelium becomes “inside” (and forms the entoderm, upper row). This stage represents the basic bauplan of Cnidaria and Porifera. Three of the four transformation stages have living counterparts in form of resting *Trichoplax*, feeding *Trichoplax*, and cnidarian polyps and medusae (right column). From a developmental genetics point of view a single regulatory gene would be required to control separation between the lower and upper epithelium (three lower rows). We find this realized in *Trichoplax* in the form of the putative Proto-Hox/Para-Hox gene, *Trox-2*. More than one regulatory gene would be required to organize new head structures originating from the ectoderm–entoderm boundary of the oral pole (upper row) in Cnidaria. Indeed (and quite intriguing), two cnidarian orthologs of the *Trox-2* gene, *Cnox-1* and *Cnox-3*, show exactly these hypothesized expression patterns (Diplox expression upper row; for simplicity only the ring for *Cnox-1* expression is shown) (for details see Schierwater et al., in revision).

3. Intracellular differentiation in Placozoa

The main difference between the Placula and *Trichoplax* is the presence of (a) a third somatic cell type in the feeding epithelium, the gland cells, and (b) a fourth somatic cell type sandwiched in between the two epithelia, the so-called fiber cells. The latter are loosely connected to each other, tetraploid, contractile and capable of stimulus conduction (Fig. 3). Thus these cells seem to be proto-muscle as well as proto-nerve cells at the same time. Expression of a presumptive Proto-Pax gene indeed supports this idea (Hadrys et al., 2005).

While text books leave it with four somatic cell types, a study of the *Trox-2* gene, a Hox-like or Proto-Hox gene, has shown that there is at least one more cell type. The *Trox-2* gene is expressed at the margin of the upper epithelium meeting the lower epithelium cells (Jakob et al., 2004) (Fig. 5). In this region some cells are remarkably smaller than the epithelial, gland or fiber cells and likely represent pluripotent or even omnipotent cells which differentiate into the other cell types.

Current text book knowledge is that the fiber cells do not directly connect to the outer environment but stay loosely connected to epithelia cells and other fiber cells. This is however not true. A recent study has shown that fiber cells send openings directly to the outer surface by opening spaces between the upper epithelium cells (Eitel et al., in preparation-a) (Fig. 6). This recent work

will change our current view on both placozoan morphology and systematics, since these substantial differences in cell morphology relate to certain placozoan species lineages only (Eitel et al., in preparation-a).

Nonetheless, since only four or five (see above) somatic cell types are found in *Trichoplax*, and since non-cellular structures such as a basal lamina or other extracellular matrix (=ECM) material are lacking, *Trichoplax* remains the most simply organized metazoan animal known. People have argued that Placozoa do possess some kind of ECM, since several ECM related genes (both interstitial and basal membrane related) are found in the genome. It has been argued that a putative ECM in *Trichoplax* is just different enough from other ECMs that it escapes traditional staining techniques (Srivastava et al., 2008). It seems that it is yet an unsolved task to define an ECM. Neither a morphological, genetic or biochemical definition alone seems to be sufficient to decide on an ECM or related structure in Placozoa free of doubt. Current and only evidence for a putative ECM in Placozoa is the presence of several genes associated with ECM structures in other Metazoa. We do not know the function of these genes and thus we are left with three main alternatives. (i) Some kind of ECM related structure is present in Placozoa that has yet escaped all detection methods; (ii) some kind of ECM structure is present in a certain developmental stage of Placozoa, which has not yet been detected or investigated; (iii) the putative ECM related genes serve different functions and produce a

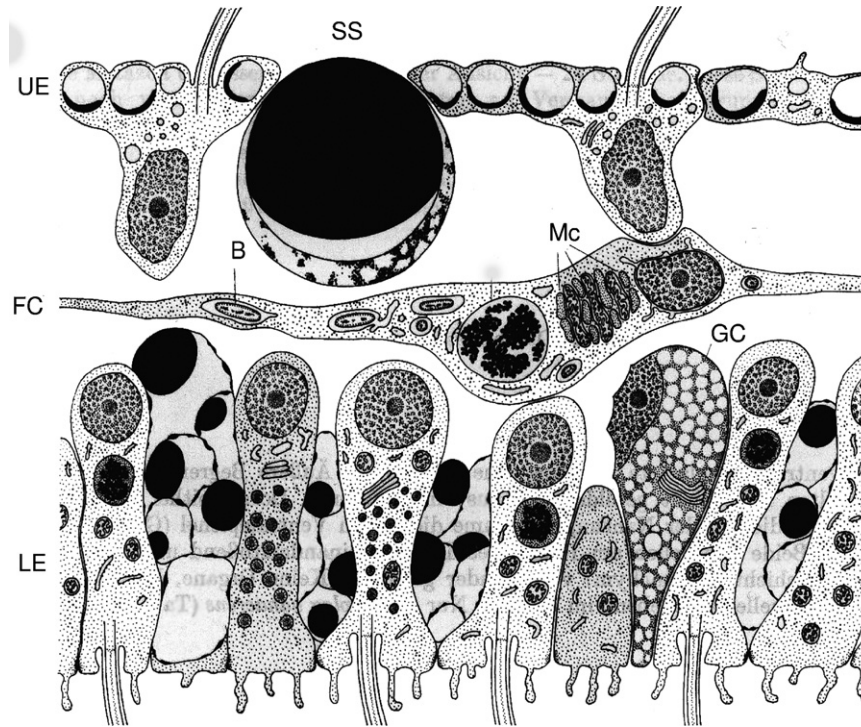


Fig. 3. Diagrammatic cross section of *Trichoplax adhaerens* (modified after Grell and Ruthmann, 1991). UE=upper epithelium, LE=lower epithelium, FC=contractile fiber cell, GC=gland cell, SS=shiny sphere, Mc=mitochondrial complex, B=(possibly endosymbiotic) bacterium in endoplasmic reticulum. Note that the interspace between fiber cells and epithelia is free from ECM, and that a basal lamina is lacking (from Syed and Schierwater, 2002b).

different overall product. Given the current evidence we favor the view that some genetic components for facilitating the evolution of an ECM are already present in Placozoa.

4. Short-lived molecular hypotheses and current views

Field et al. (1988) accomplished the first comprehensive examination of molecular hypotheses concerning the metazoan part of the tree of life (Field et al., 1988). Like many of the molecular studies generated at the time, this study was based on a small sampling of 18s rRNA sequences. This landmark paper, however, foretold many of the problems systematists interested in questions about the metazoan tree of life would face two decades later, including issues of robustness of inference from data, incongruence of molecular and morphological data and choice of phylogenetic method (at that time parsimony versus distance). This paper also prophesized some of the major controversial hypotheses regarding basal metazoan organisms that would be generated in light of potential conflicting anatomical and molecular evidence. However, the paper also predated high throughput sequencing techniques, sophisticated likelihood analyses (and hence a strong understanding of long branch attraction), data combination techniques and several other important conceptual advances in phylogenetics that have become relevant to deciphering the relationships of animals specifically, and the tree of life in general (Field et al., 1988).

Two specifically relevant reviews appeared a few years after the first comprehensive molecular analysis of metazoan phylogeny (Davidson, 1991; Valentine, 1994). Both of these reviews were of interest because they attempted to synthesize more information than the previous anatomical approaches to understand metazoan phylogeny. In addition, while a few studies utilized DNA based characters to examine phylogenetic questions in this part of the tree of life, the Field et al. (1988) paper set the pace for phylogenetic hypotheses about the metazoan tree of life for nearly a decade. The

turn of the 21st century ushered in a barrage of papers examining basal animal phylogeny that quickly out-distanced the Field et al. paper and generated even more novel hypotheses about relationships of organisms at the base of the metazoan tree. A relatively thorough review of the literature in the past decade for papers on this subject reveals at least 50 research articles published on the relationships of the major metazoan groups (see DeSalle and

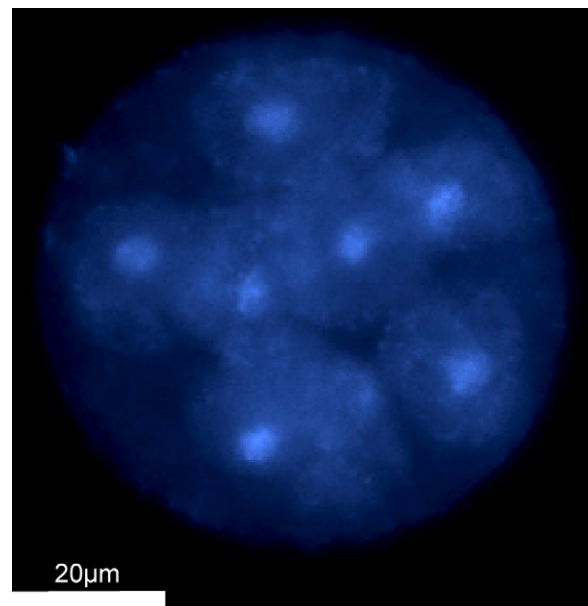


Fig. 4. Some placozoan species regularly reproduce sexually under some laboratory conditions. Shown is an 8-cell embryo of the genetic lineage H2. The embryo was stained with DAPI. Blue signals clearly mark the nucleus in each blastomere. The scale bar shows 20 μ m (Eitel et al., in preparation-b).

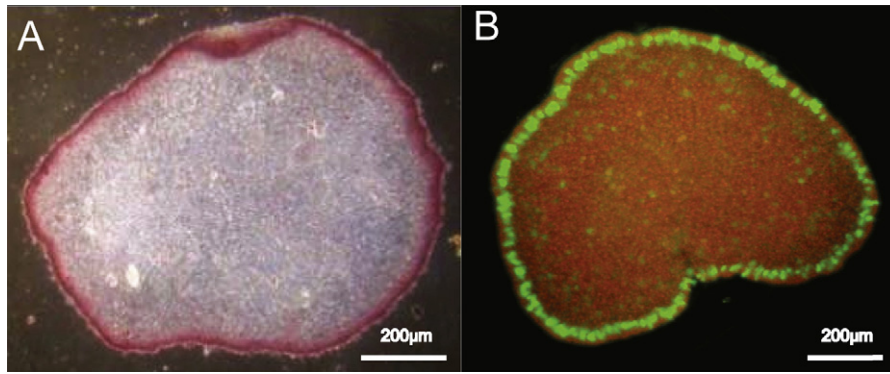


Fig. 5. Both RNA *in situ* (A) and immunolocalisation (B) of expression of the putative Proto/Para-Hox-like gene, *Trox-2*, suggest a role in defining epithelia separation, specifically lower and upper epithelia (Sagasser et al., in preparation). The observed pattern fits the modern placula hypothesis shown as Fig. 2.

Schierwater, 2008; Schierwater and DeSalle, 2007 for references). The number and diversity of hypotheses from these studies fulfills the prophecies of Field et al. (1988).

If we consider the number of major lineages at the base of the metazoan tree of life as consisting of Bilateria, Placozoa, Cnidaria, Porifera and Ctenophora, there are 105 possible ways to arrange these taxa in bifurcating trees with an outgroup (usually the out-

groups used are Choanoflagellates or Fungi, but see above). Yet scientists who have studied this problem have posited at least 8 different hypotheses concerning the relationships of these five major groups of animals at the base of the animal tree of life (Fig. 7). This means that, of the 100 or so possible hypotheses concerning these five taxa, nearly 10% of them have not been ruled out as possible explanations for how early Metazoa evolved.

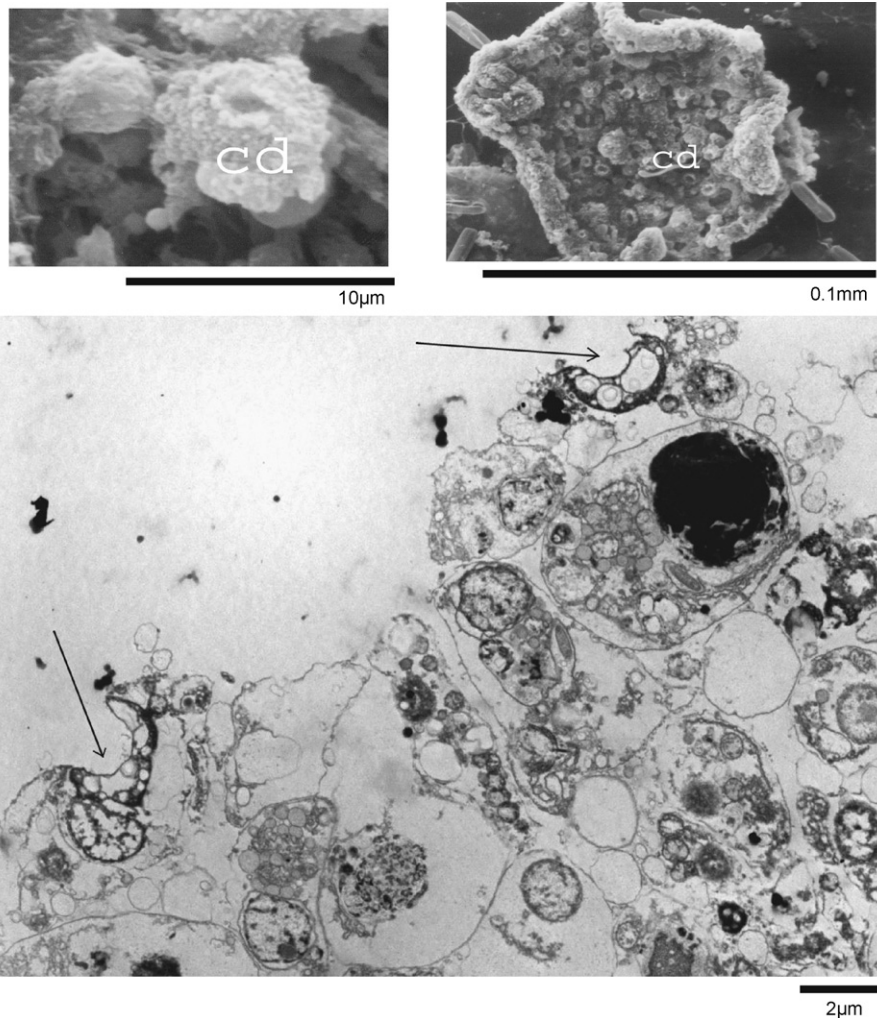


Fig. 6. Unique morphological features of a new placozoan genus or family. Only one genetic clade (*Placozoa* sp. H2) shows the 'concave discs' (cd, arrows) in the upper epithelium (modified from Eitel et al., in preparation-a).

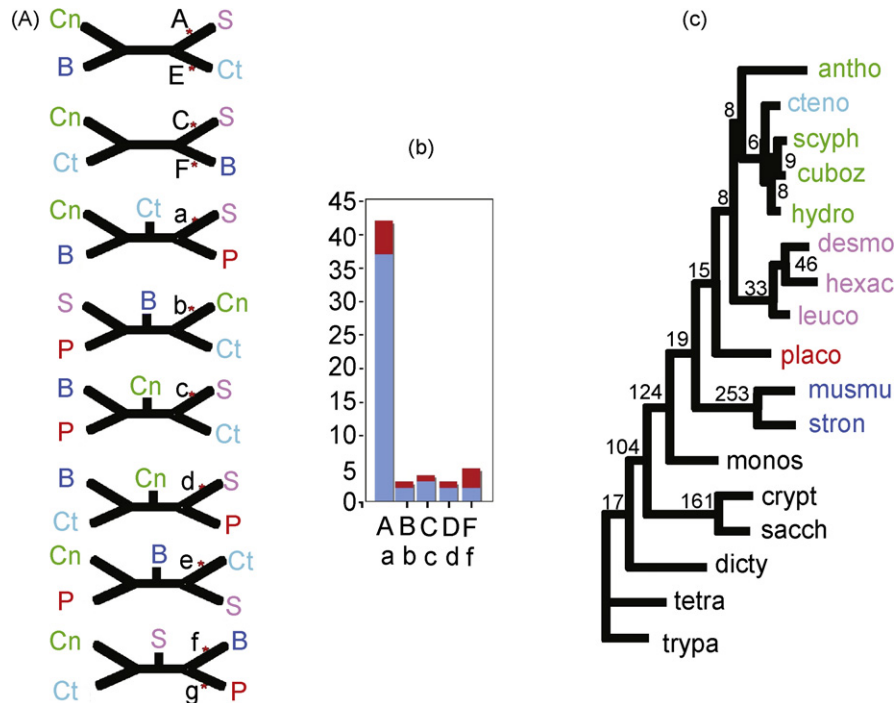


Fig. 7. (A) Potential arrangements of five critical taxa (Placozoa = P [red], Porifera = S [purple], Cnidaria = Cn [green], Ctenophora = Ct [cyan] and Bilateria = B [blue]) at the base of the metazoan tree on the right, and some hypotheses in the literature with only four taxa (Placozoa omitted). Red asterisks indicate root of the networks. The letters at the asterisks refer to roots obtained from recent publications. The upper case letters refer to publications that support the indicated root for trees without Placozoa. The lower case letters refer to publications that support the root for trees with all five taxa. (B) Histogram showing the distribution of hypotheses from papers published between 2000 and the present with respect to the major lineages of Metazoa. The letters refer to hypotheses indicated in Fig. 7A. Blue bars refer to studies that do not include Placozoa. Red bars refer to studies that do include Placozoa. (C) Concatenated evidence tree of metazoan relationships showing Bremer support values for an analysis where all characters have equal weight. This tree is also in complete agreement with the Bayesian analysis using Parsmodel. This hypothesis suggests monophyly of several well known groups such as the Deuterostomes, the Bilateria [blue], the Fungi, Porifera [purple], Cnidaria [green] with inclusion of Ctenophora [cyan] and the placement of Monosiga as ancestral to all animals. Fungi are hypothesized as sister to animals to the exclusion of the ciliate (Tetrahymena). Placozoa [red] is basal to all non non-bilaterian animals and Bilateria are sister to all non-bilaterian animals as opposed to some other hypotheses where Bilateria are embedded and highly derived in the animal clade. Our results suggest that Placozoa are not derived Cnidaria and in fact are the sister group to a monophyletic Sponge–Cnidaria clade. The inclusion of Ctenophora with the Cnidaria agrees well with other molecular studies (Kim et al., 1999).

The reasons for why such a large number of hypotheses are “still in the running” could be one or a combination of the following. (1) Some of the hypotheses, while good explanations for the data sources used, are not robust in and of themselves in terms of character support; (2) some of the hypotheses while validly emergent from the analyses performed in the various published papers, do not include broad enough taxonomic sampling to adequately address the radiation; (3) some of the studies are simply not robust with respect to genomic information and have limited taxonomic breadth.

The distribution of hypotheses with respect to the root of the metazoan tree is shown in Fig. 7A. The grand majority of hypotheses include only four of the five taxa, and do not include Placozoa. Most of these hypotheses place the root of the metazoan tree at Porifera. A “side effect” of this rooting pushes the Bilateria to a very derived position in the tree, because Bilateria are rarely inferred to be closely related to Porifera. However, when Placozoa are added to new analyses, the number of hypotheses supporting Porifera as the most basal metazoan group, drops relative to other hypotheses (Fig. 7A). In fact, hypotheses supporting Bilateria as the sister group to all other animals are obtained in nearly half of the studies listed in the survey. A side effect of adding Placozoa and pushing the root of the tree to Bilateria, is the basal positioning of Placozoa relative to the rest of the major lower animal groups—Ctenophora, Cnidaria and Porifera.

All of these hypotheses have been posited based on molecular evidence in the last year alone. For instance, Srivastava et al.

(2008) suggest Placozoa as the sister group to both Cnidaria and Bilateria with Porifera branching off first. Another recent study, suggests a basal position for Ctenophora and Anthozoa, but this study unfortunately, does not adequately address the issue of basal metazoan relationships, since it does not include Placozoa in the analysis (Dunn et al., 2008). A study that does include Placozoa (Ruiz-Trillo et al., 2008), suggests that Bilateria and Placozoa are derived metazoans. A final set of striking examples of the diversity of hypotheses generated on these taxa, are recent analyses of mitochondrial genome sequence data (Dellaporta et al., 2006; Lavrov et al., 2005; Signorovitch et al., 2007) that place Bilateria as sister to all diploblasts, with Placozoa as the most basal diploblast. The upshot of these previous studies have suggested that compression of cladogenetic events at the base of the metazoan tree of life causes the robust resolution of these nodes to be difficult, if not impossible (Baurain et al., 2006; Philippe and Telford, 2006; Rodriguez-Ezpeleta et al., 2007; Rokas, 2006; Rokas and Carroll, 2006). The implication of these studies is that even large amounts of data might not resolve the relationships of the major lineages at the base of the metazoan tree of life.

However, upon close examination of the topologies discussed in the literature, it becomes evident that there are actually only two unrooted topologies that have been preferred when Placozoa are left out (Fig. 7A). The most frequently observed topology after rooting is one where the Porifera are anchored to the root and Cnidaria and Bilateria are sister taxa, with Ctenophora sister to them. This way of looking at the literature is actually promising, because it

means that the internal relationships of the ingroup lineages are actually stable and suggest that the real problem is where to place the root of the tree. This observation leads us to suggest that problems of long branch attraction, and more precisely random rooting (Gatesy et al., 2007; Wheeler, 1990), have and will continue to plague this phylogenetic problem. The solution to the problem is to include as many taxa as possible in order to break up long branches, and the addition of Placozoa it appears, accomplishes this to a certain degree (besides, we suggest that no phylogenetic analyses at the base of Metazoa can leave out one of the two top candidates for a basal position).

Another solution to the problem is to include as many sources of data as possible when doing the phylogenetic analysis. We have recently undertaken a multi-partition phylogenetic analysis of taxa relevant to the metazoan tree of life (Schierwater et al., in revision). This analysis includes Placozoa and utilizes over 35 gene and morphology partitions. As well, as many taxa as possible within the major lineages are included in the analysis to break up the potential long branches that most certainly exist in these lineages. This new analysis includes representatives of all four classes of Cnidaria, all three classes of sponges and several multiple representatives of other major ingroup and outgroup lineages.

The results of this concatenated analysis are congruent with the view that Placozoa are the most basal diploblast group (Fig. 7C). As a side note, despite the phylogenetic position of the Placozoa in these analyses, we have no idea what the ancestor of Placozoa/diploblasts may have looked like; while it is the best “living fossil” we seem to have, we do not know how well the bauplan of *Trichoplax* reflects the bauplan of the hypothetical ancestor. Groups that are well accepted as monophyletic are observed as such in the analysis (Fungi, Bilateria and Porifera). An interesting result of the analysis is that Ctenophora, along with the four cnidarian classes (Anthozoa, Hydrozoa, Scyphozoa, and Cubozoa) form a strong monophyletic group, the Coelenterata. Both *Monosiga* and Placozoa are robustly excluded from a Porifera–Coelenterata monophyletic group. In addition, several different weighting schemes using parsimony as well as Bayesian analysis, resulted in the tree shown in Fig. 7C. The full analysis apparently misplaces the Bilateria clade as the sister to all diploblasts. The classical position of the Bilateria is in a highly derived position from within the diploblasts. This seemingly “weird” prediction of a basal Bilateria from the present analysis has been observed before in other studies (Lavrov et al., 2005; Signorovitch et al., 2007).

5. Insights from the placozoan genome

The recent sequencing of the placozoan genome has given us many clues into the genome of the hypothetical urmetazoan (Srivastava et al., 2008). The *Trichoplax* genome is, as expected, relatively small when compared to other metazoans (98 Mb, containing over 11,000 protein coding genes). Remarkably, this is not due to secondary reduction, as is the case with other similarly sized genomes such as that of *Caenorhabditis elegans* and *Drosophila*. The lack of secondary reduction is indicated by features such as the retention of ancient introns and conservation of linkage (i.e. synteny) in the *Trichoplax* genome when compared to the genomes of ‘higher’ animals. In addition, while the genome may be small, the gene content of *Trichoplax* is relatively complex, in sharp contrast to its low morphological complexity. While recognized as having only four (or five) cell types and no axis of symmetry, *Trichoplax* contains a wide array of transcription factors (e.g. Schierwater et al., 2008), both animal- and opisthokont-specific, including those involved in cell type specification and differentiation (e.g. LIM-homeobox genes, bHLH genes and GATA-family

zinc finger transcription factors) and embryonic (T-box family) and neuroendocrine development (POU homeodomain family). In addition it contains genes which specify structures in higher animals, which are simply not present in *Trichoplax*, for examples genes for extracellular matrix production, germline separation and neural signaling. This is similar to the results of the recent genome sequencing of the choanoflagellate *Monosiga brevicollis*, where many genes previously thought to be metazoan-specific were identified, and in general genetic complexity was higher than expected (King et al., 2008). Clearly, much research is needed in order to elucidate what roles these genes play in *Trichoplax* but it is clear it possesses many genes which are typical of ‘higher’ animals; *Trichoplax* gives us hints as to what may have been essential, at least genetically, for the evolution of the rest of the Metazoa. It may be that while there are only a few morphologically recognizable cell types the cell complement may indeed be more complex, as defined by specific gene expression patterns and/or functions. Whichever the case, the placozoan genome will undoubtedly aid our understanding of the genome of the last common cnidarian/bilaterian ancestor.

6. Cell differentiation and signaling genes in the placozoan genome

The transition from a unicellular protozoan to a multicellular metazoan was a key step in evolution, and relied upon the ability of neighboring groups of cells to acquire unique fates, ultimately in both a spatial and temporal manner. This ability was most likely permitted by the invention of metazoan-specific secreted signaling molecules, which, aside from single components, were not likely present in the last common ancestor of choanoflagellates and metazoans (King and Carroll, 2001; King et al., 2008; Snell et al., 2006). Typically, only a handful of signaling pathways are involved in cell differentiation and/or patterning of embryonic structures in metazoans, including the Hedgehog (Hh), bone morphogenic protein/transforming growth factor- β (BMP/TGF- β), Notch and Wntless (Wnt) pathways. In the recently published genome of *T. adhaerens* (Srivastava et al., 2008), while most major components of the Wnt and BMP/TGF- β pathways were found, the absence of critical components suggest there is no functioning Hedgehog or Notch signaling pathway. There is no recognizable Hedgehog ligand, receptors (Patched and Smoothed), or downstream GLI-like transcription factor, and although certain Notch pathway components are present, there appears to be no Notch domain-containing gene. Clearly, these data can be interpreted in three ways; it may merely indicate that genes may not have been identified due to incomplete data (unlikely as coverage is estimated to be 8-fold), it may indicate that the Notch or Hedgehog pathways were not present in the eumetazoan/placozoan ancestor, or it may indicate that placozoan specific gene loss has occurred—the most likely hypothesis as several of these component are present in both the sponge *Amphimedon queenslandica* (Adamska et al., 2007b; Nichols et al., 2006) and choanoflagellates (King et al., 2008; Snell et al., 2006). Interestingly sponges, choanoflagellates and the cnidarian *Nematostella vectensis* share unique Hedgehog-like genes with similar domain structures atypical of other higher metazoan Hedgehog proteins (Adamska et al., 2007a,b; King et al., 2008; Matus et al., 2008; Snell et al., 2006).

7. BMP/TGF- β and Wnt signaling

The *Trichoplax* genome contains all major components of two highly important pathways, the BMP/TGF- β and Wnt signaling pathway. The Wnt pathway is known to be involved in axial patterning

Table 1
Comparison of selected signaling pathway components within the Metazoa.

Pathway/ Component	Bilateria			Cnidaria	Porifera	Placozoa
	Vertebrates	<i>Drosophila</i>	<i>Caenorhabditis</i>	<i>Nematostella/Acropora/ Hydra</i>	<i>Amphimedon/Ephydatia/ Oscarella/Suberites</i>	<i>Trichoplax</i>
Hedgehog						
Hh	●	●	○	● ○	○	○
Patched	●	●	●	●	○	○
Smoothened	●	●	○	●	○*	○
Ci/GLI-1/2/3	●	●	●	●	●*	○
Fused	●	●	○	●	○*	●
SuFu	●	●	○	●	●*	○
BMP/TGF-β						
Signalling	●	●	●	●	●	●
Ligand(s)						
Receptor						
(Type I)	●	●	●	●	●	●
Receptor						
(Type II)	●	●	●	●	●	●
SMAD						
(Co-, I- and/or R- SMAD)	●	●	●	●	●	●
Sog/Chordin	●	●	●	●	○*	●
Wnt						
Wnt	●	●	●	●	●	●
Frizzled	●	●	●	●	●	●
β-catenin	●	●	●	●	●	●
GSK-3β	●	●	●	●	●	●
LRP	●	●	●	●	●	●
Axin	●	●	●	●	○*	●
Tcf/LEF	●	●	●	●	●	●
Notch						
Notch	●	●	●	●	●	○
Delta	●	●	●	●	●	●
Hes	●	●	●	●	●	●

A filled circle indicates presence of a homolog, an empty circle indicates absence, a shaded circle indicates irregular form/domain structure. Hh=Hedgehog; Ci/GLI-1/2/3=Cubitus interruptus/Glioma-1/2/3; SuFu=suppressor of fused; Wnt=*Drosophila* Wg, vertebrate Int; LRP=Lipoprotein Receptor-related Protein; Tcf/LEF=T-cell factor/lymphocyte enhancer binding factor; sog=short gastrulation; dpp/BMP=decapentaplegic/bone morphogenic protein; TGF-(=transforming growth factor-; GSK-3(=Glycogen Synthase Kinase; Hes=Hairy/enhancer of split. (Taken from Adamska et al., 2007a,b; Burglin, 2008; de Jong et al., in preparation; Holley and Ferguson, 1997; Kasbauer et al., 2007; Matus et al., 2008; Matus et al., 2006; Nichols et al., 2006; Srivastava et al., 2008; Suga et al., 1999; Technau et al., 2005.)

*Indicates no published literature or Genbank entry could be found, and therefore represents the authors' own preliminary search, which should not be taken as exhaustive.

in basal animals, in a system that has been compared to the Hox axial patterning system of higher animals. Cnidarians possess 11 of the 12 Wnt gene families of higher metazoans, which are serially expressed along the oral/aboral axis, and manipulation of which result in structural anomalies (Guder et al., 2006a,b; Hobmayer et al., 2000; Kusserow et al., 2005; Lee et al., 2007, 2006; Matus et al., 2006; Momose and Houliston, 2007; Muller et al., 2007; Sullivan et al., 2007). In addition, the Wnt pathway is also most likely involved

in axial patterning in demosponge larvae (Adamska et al., 2007a). A search of the *Trichoplax* genome reveals three *wnt* genes (belonging to different families), receptors (*frizzled* and *disheveled*), and all major downstream intracellular components (see Table 1).

In bilaterians, BMP/TGF-(signaling plays a role in the establishment of the embryonic dorsal/ventral axis (see (Holley and Ferguson, 1997), and is also thought to play roles in axial patterning in cnidarians (Finnerty et al., 2004; Hayward et al., 2002;

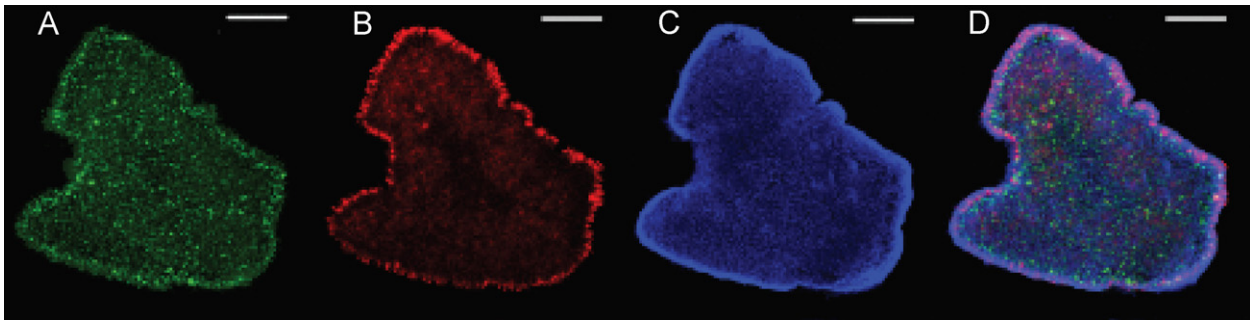


Fig. 8. Several families of regulatory genes have been known to play important roles in development in the Bilateria. Interestingly, almost all major gene families have counterparts in the morphologically simple Placozoa. Unraveling the function of these regulatory genes in Placozoa has attracted much attention in the evo-devo community. Shown here is an adult *Trichoplax adhaerens* showing autofluorescence (A), whole mount immunofluorescence staining with an antibody against phosphorylated Histone 3 (B), DAPI (C) and an overlay of all three (D). The scale bar shows 100 μ M. As a mitosis marker, the localization of phosphorylated Histone 3 provides insight into active areas of cell division in adult *Trichoplax adhaerens* (von der Chevallerie et al., unpublished data).

Matus et al., 2006; Rentzsch et al., 2006; Samuel et al., 2001) and demosponge larvae (Adamska et al., 2007a), despite no discernible homology between the bilaterian, poriferan and cnidarian axes (Adamska et al., 2007a; Ball et al., 2007; de Jong et al., 2006; Rentzsch et al., 2006). In the *Trichoplax* genome, there are representatives of all major genes which constitute the BMP/TGF- β pathway, including ligand, receptors and intracellular components (see Table 1). Taken together, a functioning Wnt and BMP/TGF- β pathway alone would give *Trichoplax* the ability to transduce many complex regulatory and developmental signals, and while not discussed here, the *Trichoplax* genome also contains many transcription factors, commonly associated in the gastrulation and development of higher animals (Srivastava et al., 2008). However, given the simple cellular morphology, the lack of any type of recognizable symmetry analogous to either the oral/aboral or anterior/posterior-dorsal/ventral axes of cnidarians/bilaterians, and as of yet, incompletely observed embryonic development, the finding *Trichoplax* contains a repertoire as complex as it does in its genome is intriguing. In addition, current work on the Wnt and BMP/TGF- β pathways show that at least some components are being actively transcribed in the cultured “adult” animal (de Jong et al., in preparation). It will undoubtedly be of high interest to fully elucidate both the spatial and temporal expression patterns of genes involved in these pathways, and determine why such a simple animal would need such an apparently complex signaling repertoire. We speculate that perhaps there is more to *Trichoplax* than meets the eye, and that such complex pathways may also be involved in as of yet unknown processes, such as sexual reproduction and embryonic development. This however does not detract from the theory that the original function of signaling pathways was for ‘simple’ cellular differentiation, and has since been co-opted in axial patterning in other ‘higher’ metazoan lineages, making *Trichoplax* a virtual ‘living fossil’. This supports the basis of “evo-devo”; evolution on the molecular level starts simple, and by the way of gene duplication and diversification, further players are co-opted to different and more specific roles and become involved in different contexts.

In any case, it is clear that the placozoan/eumetazoan ancestor already possessed a complex repertoire of signaling pathways which were most likely essential for the transition from Protozoa to Metazoa. Ultimately, the elucidation of the complete life cycle of *Trichoplax* will enable future studies to be carried out on the role these pathways play in development, although it is clear that the cultured “adult” form of *Trichoplax* actively expresses components of at least some of these pathways. We believe that *Trichoplax* provides an excellent model for the study of the evolution of signaling pathways and molecular interactions, due to its morphological simplicity, ease of culture and continued successful development of new tech-

niques. We postulate that genes expressed in each *Trichoplax* cell type will eventually be catalogued (aided by the recent advance of immunological techniques; see Fig. 8), allowing for molecular interactions between these different cells to be elucidated, leading to a better understanding of signaling pathways and their roles in cell differentiation and communication. This is of great interest not only to the “evo-devo” field, but also to other researchers who strive to better understand how signaling pathways function in more complex animals, and how these molecular interactions lead to the specification of diverse cell types.

8. Current efforts

Research on placozoans seems to be inverted. We have sequenced the genome before we have any good understanding of the life cycle and general biology of placozoans. Fortunately, a large number of labs have now started to work on placozoans, since this is clearly an ideal outgroup or reference group for all higher animals. Currently, most of the research efforts are molecular and aim to characterize genes known to play important roles in Bilateria. Our lab in Hannover for example, is actively involved in several international cooperations, studying cell proliferation, apoptosis, and developmental genes. Students in Hannover also perform graduate and postdoctoral research on the biodiversity, biogeography and systematics, development and life cycle, phototaxis and finally field ecology of placozoans. We hereby welcome any and all newcomers to the evo-devo and placozoan field. There is much to do and much to share with respect to this critical organism.

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