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The enigmatic Placozoa part 2: Exploring evolutionary controversies and promising questions on earth and in space

Bernd Schierwater[1] | Hans-Jürgen Osigus[1] | Tjard Bergmann[1] | Neil W. Blackstone[2] | Heike Hadrys[1] | Jens Hauslage[3] | Patrick O. Humbert[4,5] | Kai Kamm[1] | Marc Kvansakul[4,5] | Kathrin Wysocki[1] | Rob DeSalle[6]

[1] Institute of Animal Ecology, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

[2] Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois, USA

[3] Gravitational Biology, Institute of Aerospace Medicine, German Aerospace Center (DLR), Cologne, Germany

[4] Department of Biochemistry & Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia

[5] Research Centre for Molecular Cancer Prevention, La Trobe University, Melbourne, Victoria, Australia

[6] American Museum of Natural History, New York, New York, USA

Correspondence
Bernd Schierwater, Institute of Animal Ecology, University of Veterinary Medicine Hannover, Foundation, Bünteweg 17d, 30559 Hannover, Germany.
Email: bernd.schierwater@ecolevol.de

Abstract

The placozoan *Trichoplax adhaerens* has been bridging gaps between research disciplines like no other animal. As outlined in part 1, placozoans have been subject of hot evolutionary debates and placozoans have challenged some fundamental evolutionary concepts. Here in part 2 we discuss the exceptional genetics of the phylum Placozoa and point out some challenging model system applications for the best known species, *Trichoplax adhaerens*.

KEYWORDS
genomics, gravitaxis, mitochondrial genome evolution, model system, Placozoa, Trichoplax, urmetazoon

INTRODUCTION

The first placozoan species, *Trichoplax adhaerens*, was (i) discovered in 1883,[1] (ii) ignored for the wrong reason for the first half of the 20th century,(see [2]) (iii) rehabilitated by Willi and Gertrud Kuhl in the 1960s,[3,4] (iv) little noticed for two decades, (v) re-discovered by the first author (BS) in the 1990s (see[2,5]) and recently prepared for space mission. *Trichoplax* has been entering a steadily increasing number of research laboratories world-wide, taking advantage of the outstanding primitivity, simplicity and practicality of *Trichoplax*, both at the organismal and genetic level.

UNIQUE EVOLUTION OF PLACOZOAN MITOCHONDRIAL GENOMES

Comparative mitogenomics, particularly aiming towards understanding the early evolution of animal mitochondrial (mt) genomes, have been challenged by the mitogenome diversity of placozoans, which harbor some of the largest (non-fragmented) mt genomes of all metazoan animals (see e.g.,[6] for overview) (Figure 1). The first studies on placozoan mitogenomes identified the presence of unusual animal mtDNA features like introns and open-reading frames of unknown origin in different placozoans.[7,8] These features together with long

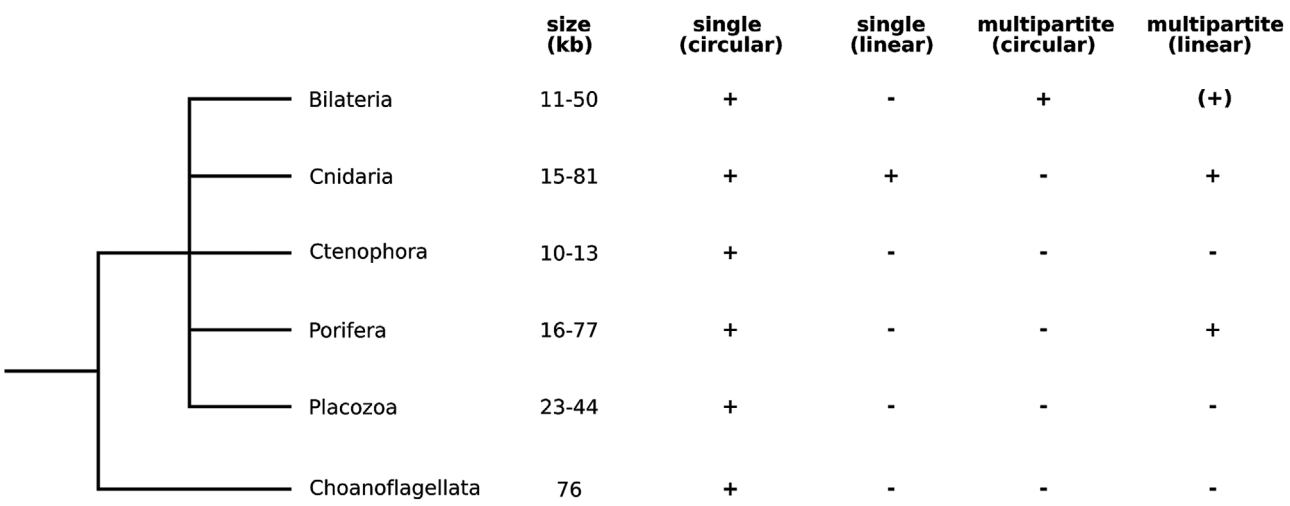

FIGURE 1 Mitochondrial genome structures are highly diverse between different metazoan groups. While placozoans were originally believed to harbor the largest circular mitochondrial genomes, new data from more placozoan species have created a size-overlap with other groups. A circular genome arrangement certainly represents the ancestral stage, while there are several independent cases of mt genome fragmentations. Data taken from[6–9,11–20]

non-coding intergenic spacers and comparatively large coding sequences contribute to the large mitogenome sizes in placozoans, which reach up to 44 kb.[9] Surprisingly the two standard mt genes, *atp8* and *atp9*, are missing in the otherwise complete mt genomes. The characterization of the small (23.4 kb) mitogenome of a recently discovered aberrant placozoan species, *Polyplacotoma mediterranea*,[10] surprisingly revealed that placozoan mitogenomes can also be compact. This smallest placozoan mt genome leads to a size overlap between circular placozoan and poriferan mitochondrial genomes. The unchallenged diversity of placozoan mt genomes possibly relates to a mito-nuclear incompatibility between different placozoan lineages. The accumulation of derived animal mitochondrial characteristics (e.g., hairpins) in *T. adhaerens*[9]—and also other members of its group (*clade I)*—indicates that the best known and most studied placozoan species, *T. adhaerens*, is a member of a rather derived group within extant Placozoa.

We have 14 fully sequenced and annotated mt genomes from all placozoan clades available (see[9] and references therein), but there are still some crucial questions open. Such questions include the (i) nature of control regions, (ii) mechanisms of transcriptional regulation, (iii) replication start, (iv) overall range of mt genome structure diversities, and (v) ancestral placozoan mt genome structure and size. The number of fully sequenced placozoan mt genomes is steadily increasing and will help to answer some of these questions.

PLACOZOAN GENOMICS—INSIGHTS INTO EARLY METAZOAN GENOME EVOLUTION

After sequencing the *T. adhaerens* genome,[21] two more high quality draft placozoan genomes have been analyzed and published.[22,23] Three incomplete genome drafts from another three haplotypes have mainly been used for phylogenetic analyses.[24] Given the morphological uniformity and the comparatively small number of species in the phylum, one might be tempted to accept the available data as representative for the genome organization in Placozoa. But we still know very little about genome evolution and the genomic mechanisms underlying speciation in the phylum. The almost invariable morphology and our lack of knowledge on the placozoan life-cycle force us to use genetic evidence from partial or whole genomes for the erection of taxonomic units (species, genera, and higher taxonomic orders).

The three high-quality and near complete placozoan nuclear genomes could be assembled up to the scaffold level in the megabase range[21–23] and represent two closely related taxa, *T. adhaerens* (H1) and *Trichoplax* sp. H2, and one distantly related species, *Hoilungia hongkongensis*, in the phylum (Figure 2). We can deduce that placozoan genomes range in size from 87–95 megabases, contain around 12 000 protein coding genes,[21–23] and are presumably the smallest not secondarily reduced metazoan genomes.[21] This conclusion is based on the finding that the *Trichoplax* genome—in contrast to other small invertebrate genomes—shows a significant amount of conserved synteny to eumetazoans like anthozoans or vertebrates.[21] Within the phylum, gene, exon, and intron sizes are similar[21–23] and genomic rearrangements vary from none[22] to amounts that are observed between different mammalian orders.[23] At the sequence divergence level, two closely related placozoan haplotypes show sequence variation comparable to human vs. chimpanzee, while less related haplotypes (i.e., presumably belonging to different genera or even families) show substantial variation.[22,23] However, if phased allelic information is analyzed between closely related haplotypes, intraspecific allelic variation can be higher than interspecific allelic variation, suggesting that deviating mitochondrial haplotypes exist in the same species. This allelic variation can be substantial and even affect highly conserved genes like transcription factors.[22] Interestingly and importantly this obscures

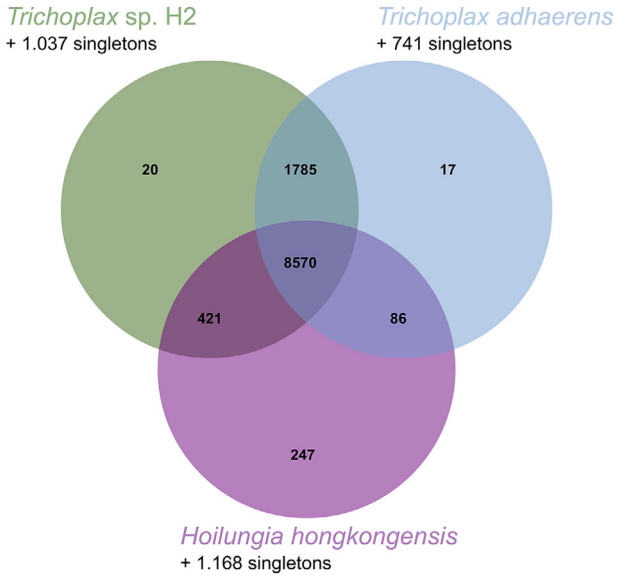

FIGURE 2 Whole genome comparisons between three placozoans. Shown are the orthologous gene clusters[25] of the predicted proteins (data from[22,23]): The Venn-Diagram shows that all three placozoans share 8.570 clusters. The clustering also clearly shows that *Trichoplax adhaerens* & *Trichoplax* sp. H2 are close relatives since they share 10.355 gene clusters, compared to only 8.991 and 8.656 clusters, respectively, that the two *Trichoplax* specimen share with *Hoilungia hongkongensis*.

species boundaries in a fascinating way, because it suggests that evidently "separated" placozoan haplotypes (or "species", respectively) have been able to mate as long as the chromosomal architecture allows homologous chromosomes to pair properly during meiosis.

The complexity of the placozoan gene repertoire clearly mirrors a pre-cnidarian stage. Most gene families of cnidarians or bilaterians are present in a placozoan but the complexity within gene families is much simpler, and certain pathways are either absent or incomplete. For example, Placozoa harbor genes for Wnt- and TGF-β signaling while a complete Hedgehog pathway is absent.[21] Up to 38[21,22,26] homeobox genes (Figure 3) cover all major classes present in higher animals,[22,26] but a further diversification first happened in Cnidaria,[27–29] leading to the situation seen in Bilateria. Despite the absence of a nervous system, Placozoa use partial genetic toolkits for neuroendocrine signaling.[21,30,31] Likewise, placozoans possess only partial pathways for innate immunity.[32] On the other hand, placozoans exhibit several examples of phylum specific gene family expansions, and certain duplications likely coincide with speciation processes.[23] These include genes related to innate immunity and cell death[32] or the large group of G protein-coupled receptors. This group of cell membrane receptors is by far the largest gene group in Placozoa and may comprise more than 800 different genes in a single species.[22] A number that is comparable to humans[33] despite a much lower gene content in placozoans.

We are confident that not too many homologs of bilaterian genes have escaped our placozoan genomes analyses, for at least two reasons. First, the modern gene prediction tools incorporate multiple evidence like homology-based searches and thousands of transcriptome contigs. Second, three independent genome assemblies from different species should compensate for potentially missing genome portions. For example, the non-overlapping portion of the *Trichoplax* sp. H2 genome yielded the *Pasha* homolog of the Microprocessor complex and other genes[22] that were missing in the *T. adhaerens* reference genome. There are also some examples of more challenging homology assignments, where the domain architecture of a placozoan gene is difficult to predict because of substantial deviation from the bilaterian consensus. One example is the homology of the cellular stress response gene NF-κB in Placozoa[32] which could only be verified via secondary structure prediction.[34]

TRICHOPLAX: A PROMISING MODEL FOR BIOMEDICAL RESEARCH

There has been much effort worldwide in trying to understand the fundamental biological principles that regulate the development and homeostasis of multicellular organisms and, in particular, how they underpin regeneration and disease processes such as cancer. These research efforts, however, have been impeded by the genetic redundancies and physiological complexities associated with 650 million years of evolution of multicellular organisms to today's humans. With its simple body plan, low complexity in cell types and the majority of signaling pathways that regulate stem cells and organ growth in humans, *Trichoplax* provides the simplest multicellular animal model to identify the original rules and mechanisms that underpin how a multicellular tissue is built and repaired. This in turn provides opportunities to use *Trichoplax* as a model organism to understand human diseases.

Compared to other non-bilaterian model systems, like the cnidarian *Hydra vulgaris* (e.g.,[35]), *Trichoplax* is still in its infancy with respect to established in vivo molecular manipulation protocols. While RNAi approaches have already been used successfully in *Trichoplax*[36] (Figure 4), we are still lacking proper genetic tools for long term functional manipulation. However, recent progress in transgenesis in Porifera[37] will clearly stimulate new approaches and methodical progress also in placozoans. In addition, the *Trichoplax* genome, proteome and single cell transcriptomes have now been described[21,38,39] which provide important information like placozoan promoter sequences, although physiological information, for example on rates of protein turnover, are still missing. Functional characterization of placozoan genes which are expressed in human cell lines already provide important insights into functional conservation of genes in Metazoa (e.g.,[40]). Finally, its simple pseudo-two-dimensional body plan makes *Trichoplax* extremely amenable to live in vivo imaging approaches.[41]

Trichoplax provides a number of additional interesting properties which makes it attractive for the study of regeneration. First, it can heal wounds within minutes,[42] and also displays one of the fastest epithelial contractility observed to date, at least an order of magnitude faster than currently known examples.[41] This extends to an ability to incorporate transplanted tissue across individuals.[43,44] Remarkably,

	Trichoplax	*Nematostella*	*Amphimedon*
ANTP	14	78	8
NKL	NK2, NK5, NK6, Hex, Dlx, Dbx/Hlx+2 NK-related genes	all (bilaterian) families except Tlx	NK2(2/3), NK6, Msx, BarH-related, Hex-related, Tlx-related
Ext. Hox	Not, Mnx	all families except Eng and possibly Vax	-
Hox/ParaHox	Gsx+1 gene with some affinity to ext. Hox and Hox/ParaHox	Gsx, anterior Hox, posterior Hox/Cdx-like+several genes with unclear relation to bilaterian Hox/ParaHox genes	-
PRD	9 (Arx, Ebx/Arx-like, Pax3-like, PaxB, Prd/Pax-like, Pitx, Gsc, Otp)	33 (PaxA, B, C, Pax6-like, Arx, Rx, Pitx, Otx, Otp, Gsc & several unassigned)	9 (Arx, PaxB, Rx, OG12)
POU	2 (Pou3, 4,)	5 (POU1, 3, 4, 6)	4 (POU 1, 6, 2–5)
LIM-HD	5 (LIM1, 1/5, 3/4, 2/9, Isl)	6 (Lim1, 2, 3/4 Awh, Isl)	3 (LIM3, Lin-11, Isl)
SIX	2 (Six1/2, Six3/6)	5 (Six2, 3, 4, 4/5)	1 (Six1/2)
TALE	4 (PBX/PBC, Pknox, Irx, Meis)	7 (PBC, TGIF, Meis, Irx)	6 (Meis, PBC, Irx)
HNF	1	1	-
Total	37	134	31

FIGURE 3 *Trichoplax* has become one of the three prominent non-bilaterian model systems, together with the anthozoan *Nematostella vectensis* and the sponge *Amphimedon queenslandica*. The *Trichoplax* homeobox gene complement shows a slightly higher diversity compared to the sponge *Amphimedon*, particularly in the ANTP class, but clearly represents a pre-cnidarian stage. Note: In the genome of the closely related *Trichoplax* sp. H2,[22] an additional homeobox gene of the PRD class has been identified, the ortholog of which is present in the *Trichoplax adhaerens* genome but has not been recognized as a homeobox gene because it is disrupted, probably due to sequencing or assembly errors. This makes a total of 38 homeobox genes in the genus *Trichoplax*. Image taken from[26]

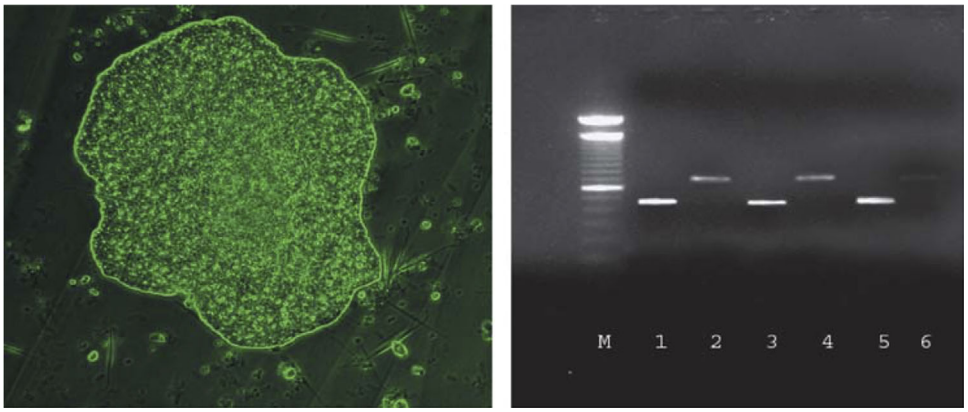

FIGURE 4 Functional gene studies in *Trichoplax adhaerens* have been successful by means of a modified RNAi protocol[36]. *Left*: Image of an animal after transfection with double-stranded *Trox-2* RNA. *Right*: Reduced expression of *Trox-2* after RNAi (lane 6) compared to the controls (lane 2 and 4). Lane 1, 3, 5: actin control. Images taken from[36]

the animals can be re-aggregated from disaggregated tissue[45] opening opportunities for tissue mosaic studies. Finally, due to its mode of vegetative fission, similarly to *Hydra* it is essentially immortal. Despite the availability of single cell sequencing data,[39] there is however a current lack of understanding of stem and progenitor cells in *Trichoplax*, and the lineage relationships that generate the varied repertoire of differentiated cells within *Trichoplax*. This information will be critical to maximize the benefit of *Trichoplax* for the study of regeneration.

What can we learn from *Trichoplax* for cancer biology?

The advent of multicellularity required new molecular mechanisms that allowed cellular cooperation and suppressed any cellular conflicts that enhance individual cell fitness to the detriment of the organism.[46] From this point of view, cancer would represent a breakdown of this multicellular cooperation with over-competitive cells effectively "cheating", leading to overall loss of fitness of the organism.[46,47] Protective mechanisms for emerging metazoans would include newly acquired controls of cell proliferation, cell death, differentiation and tissue architecture, all of these required to coordinate the establishment and maintenance of multicellularity. Supporting a breakdown of these mechanisms in cancer, examination of the evolutionary origin of cancer related protein domains suggests two peaks, one at the time of the origin of the first cell and the other around the time of the evolution of the first multicellular organisms.[48] Importantly, this second peak dubbed "gatekeeper" genes consist of oncogenes and tumor suppressors whose mutations promote tumor progression through altering cell proliferation, inhibiting

differentiation or inhibiting cell death. Therefore, examining the mechanisms that evolved with multicellularity and how the breakdown of multicellular cooperation occurs in basal metazoans such as *Trichoplax* is likely to lead to fundamental insights into the origins of cancer.

Of these various protective mechanisms, most progress has been made on the control of programmed cell death. *Trichoplax* harbors numerous genes involved in the control of programmed cell death. Genome analysis has revealed that whilst key necroptosis regulatory proteins such as RIK1/2 are absent in *Trichoplax*, key proteins involved in apoptosis regulation are present.[32,49] An initial survey identified 12 homologs of the intrinsic apoptosis mediator APAF-1,[32] a crucial caspase activating adaptor protein, as well as multiple caspase-like genes,[32,49] however a clear *Trichoplax* sp. H2 homolog of caspase-9 appeared to be absent. Bcl-2 like proteins are also found in *Trichoplax*, and their presence together with APAF1 and caspase-like proteins suggests the existence of a fully functional intrinsic apoptosis pathway.[32,49] Molecular characterization of four identified Bcl-2 proteins in *Trichoplax* has revealed the presence of two anti-apoptotic and two pro-apoptotic members of the Bcl-2 family that cooperate to control apoptosis via mechanisms reminiscent of intrinsic apoptosis in humans, both in terms of detailed interactions between members and structure of Bcl-2 proteins.[40] Intriguingly, no BH3-only like initiators of apoptosis were identified in *Trichoplax*, however the Bak-like *Trichoplax* homolog trBak was shown to act more like a BH3-only protein sensitizing cells to cell death rather than as an executor Bcl-2 protein similar to its human Bak counterpart. These findings have raised the possibility that modern BH3-only proteins may in part have originated from a Bak-like ancestor as found in *Trichoplax*. Whether or not this apoptosis pathway is primarily utilized in the context of tissue architecture maintenance or immune defense remains to be clarified, however its presence supports the notion that *Trichoplax* may also be subject to conditions associated with apoptosis dysregulation such as cancer. Of note, *Trichoplax* also possesses a functional homolog of the human tumor suppressor p53, *tap53*.[21] Experimental studies in heterologous systems have shown that tap53 can also initiate apoptosis and is regulated by the ubiquitin ligase taMdm2.[50,51] Functional studies of these putative *Trichoplax* oncogenes and tumor suppressor genes are now needed in *Trichoplax* itself to confirm their mode of action and are likely to provide important insights into basic mechanisms of cancer initiation.

Trichoplax thus constitutes an important new model to understand the fundamental mechanisms that evolved in multicellular organisms such cell death, developmentally coordinated proliferation and differentiation, and tissue architecture and its repair. In addition, *Trichoplax* may provide a new model to examine the emergence of developmental mechanisms regulating tissue architecture and homeostasis such as cell competition. Cell competition is a surveillance mechanism that measures relative cell fitness in a tissue, resulting in the recognition and elimination of mutant less-fit ("loser") cells, whilst the wild-type more-fit ("winner") cells undergo compensatory proliferation.[52–54] The ability to generate tissue mosaic animals in *Trichoplax* thus makes it an attractive system to examine how emerging cancer mechanisms such as cell competition may have evolved at the dawn of Metazoa.

FIGURE 5 Start of a sounding rocket during the MAPHEUS 5 campaign. Image by DLR

TRICHOPLAX FLIES INTO SPACE

In addition, and within the model system framework, *Trichoplax* is a highly suitable organism for understanding the evolution of gravity sensing in animals. Gravity is the only constant force that has been acting on organisms during all of evolution and it has a fundamental impact on life,[55] while all other parameters in the environment changed substantially over billions of years. Graviperception is found in single cells, fungi, plants, and animals.[56] The auxiliary tools to sense the gravity vector can be anything from heavy particles, the statoliths, to light oil drops, sedimenting or floating in specialized cells or organs. Little is known about the graviperception in *Trichoplax*. Preliminary experiments and microscopical studies suggest that *Trichoplax* uses gravity for spatial orientation (gravitaxis) and uses aragonite crystals in the crystal cells to function as statoliths.[57] This is a completely unique scenario for a metazoan animal since *Trichoplax* is lacking a nervous system and the question how signal transduction may lead to spatial orientation in a *Trichoplax* is quite challenging.[58] Probably *Trichoplax* uses mechanisms known from gravity sensitive protists or fungi or plants, which use calcium signals, cytoskeleton changes, membrane interactions, and ion channel activities.[56] In gravitactic ciliates the mechanosensitive ion channels, which are involved in gravity signaling,

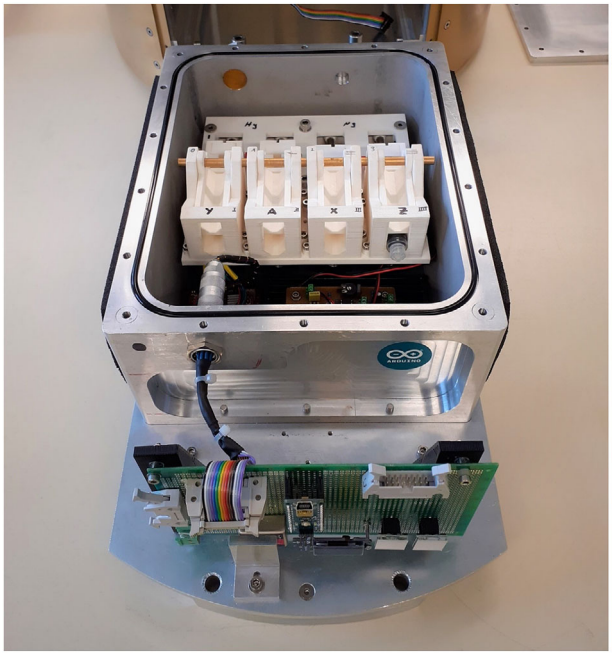

FIGURE 6 The Graviplax space chamber. The chamber allows the fixation of four different *Trichoplax* cultures at different time points during a sounding rocket flight. The late access unit shows the opened pressure vessel with the four syringe mechanics and the green interface board in the front. Image by DLR

are polarily organized.[59,60] We may expect that the distinctive cell and body polarities in *Trichoplax* are somehow interacting with gravity determination and gravitactic behavior. We are studying the effects of gravity in *Trichoplax* in a reverse way, that is, we take gravity *cum grano salis* away and look for resulting deficiencies in behavior, morphology and physiology. In so-called ground-based facilities we can simulate microgravity conditions. For example, two-dimensional fast clinorotation, in which a sample is rotated at 60–90 rpm in a very small diameter (see below), has been validated as low shear stress environment and promising simulation approach.[61] For exposing biological samples to real microgravity conditions different platforms are available, which provide different times of exposure and quality of microgravity. Microgravity can be achieved with drop towers (4.7 – 9 s), parabolic plane flights (22 s), sounding rockets (500 s), satellites and space stations (weeks—years). The German Aerospace Center performs sounding rocket flights (Figure 5) for *Trichoplax* within the MAPHEUS program to investigate microgravity effects in materials, testing of newly developed hard- and software and answering questions in gravitational biology.[62–64] The sounding rocket flight provides a high quality of microgravity (10^{-4} *g*) and a duration of approx. 6 min. The missions are implemented on Esrange, a rocket range and research center located about 40 km east of Kiruna in northern Sweden. The Graviplax experiments investigate the transcriptional changes and the moving behavior of *Trichoplax* in microgravity by means of a special fixation- and microscope-unit, including a temperature control unit with heater system (Figure 6 and Figure 7). The unit allows remote controlled feeding and fixation of the animals at different time points. An OLED display and touch buttons allow the use of the fixation unit also on the bench to perform ground control experiments in the laboratory. For the first time the movement of *Trichoplax* in space will be recorded using a microscope built on basis of the Raspberry Pi Cam V2 with 8 mega pixels connected to a Raspberry Pi Zero for video storage.[63] 2021 is the launch year for the *Trichoplax* model system in space. In order to further complement the results gained from sounding rocket microgravity approaches, additional experiments under simulated microgravity conditions will be conducted in the laboratory by using two

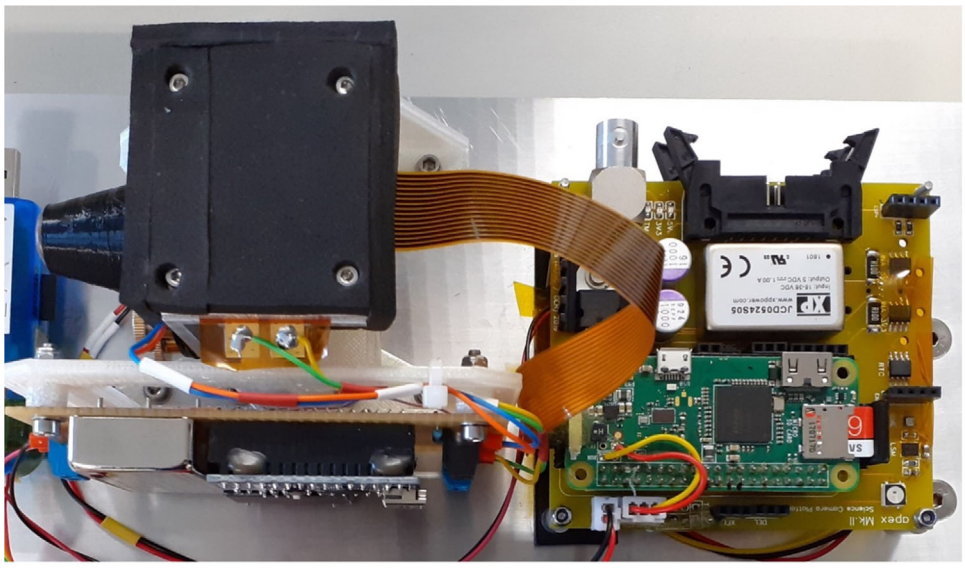

FIGURE 7 The *Trichoplax* Space Microscope allows recordings of the *Trichoplax* behavior during a sounding rocket flight. On the left side (under the black insulation) the microscope with heating system coupled to a vacuum tight cuvette is located. Animals are placed in the stainless-steel cuvette. At the bottom the Arduino and the DC/DC converter power supply is visible. The microscope and the Raspberry Pi Cam V2 are connected to a Raspberry Pi Zero piggyback on the apex MK.II board on the right side. (see[63])

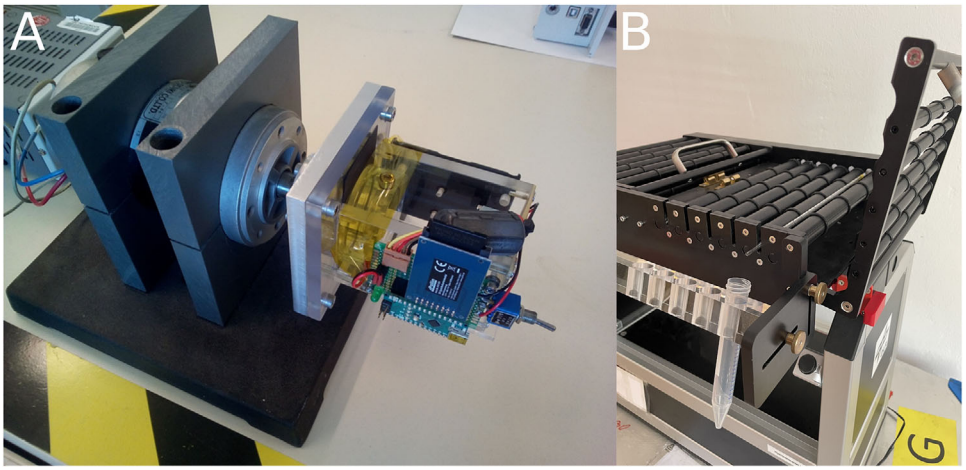

FIGURE 8 (A) Lab-bench format two dimensional clinostat with video camera set up adapted for use with *Trichoplax adhaerens* (Design: DLR; Picture: Pia Reimann). (B) Two dimensional pipette clinostat with fixation unit for processing of *Trichoplax* samples immediately after microgravity experiments. (Design: DLR; Picture: Pia Reimann)

dimensional (pipette) clinostats (Figure 8). These clinostat experiments will allow us to study the long-term effects of microgravity (i.e., for several hours or even days) on placozoan behavior and gene expression profiles.

CONCLUSIONS

Despite numerous open questions regarding the biology, placozoans are nevertheless a versatile and promising model system to address fundamental questions in basic and applied research. From functional genetics to developmental biology, and from gravitational biology to biomedical research, placozoans represent a critical jigsaw piece in comparative analyses.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors have provided input from their personal experience working with *Trichoplax* and written sections for the manuscript.

ORCID

Bernd Schierwater https://orcid.org/0000-0002-3410-3660
Neil W. Blackstone https://orcid.org/0000-0001-7195-3237
Rob DeSalle https://orcid.org/0000-0002-6490-7109

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