Two intracellular and cell type-specific bacterial symbionts in the placozoan Trichoplax H2

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Placozoa is an enigmatic phylum of simple, microscopic, marine metazoans1–4. Although intracellular bacteria have been found in all members of this phylum, almost nothing is known about their identity, location and interactions with their host1–4. We used metagenomic and metatranscriptomic sequencing of single host individuals, plus metaproteomic and imaging analyses, to show that the placozoan Trichoplax sp. H2 lives in symbiosis with two intracellular bacteria. One symbiont forms an undescribed genus in the Midichloriaceae (Rickettsiales)1–3 and has a genomic repertoire similar to that of rickettsial parasites4,5, but does not seem to express key genes for energy parasitism. Correlative image analyses and three-dimensional electron tomography revealed that this symbiont resides in the rough endoplasmic reticulum of its host’s internal fibre cells. The second symbiont belongs to the Margulisbacteria, a phylum without cultured representatives and not known to form intracellular associations1–3,5,6. This symbiont lives in the ventral epithelial cells of Trichoplax, probably metabolizes algal lipids digested by its host and has the capacity to supplement the placozoan’s nutrition. Our study shows that one of the simplest animals has evolved highly specific and intimate associations with symbiotic, intracellular bacteria and highlights that symbioses can provide access to otherwise elusive microbial dark matter.

Placozoa are marine invertebrates at the base of the animal tree and are considered among the simplest animals known. These millimetre-sized benthic animals can be easily cultured and are considered key models for understanding metazoan evolution, developmental biology and tissue formation1–4. Electron microscopy studies as early as in the 1970s revealed the presence of intracellular bacteria in these animals1–3. Remarkably, nearly five decades later, only very little is known about the biology of these symbionts and their interactions with their hosts.

The phylum Placozoa encompasses 19 cryptic species, on the basis of mitochondrial haplotypes5–8. These benthic animals do not have a mouth or gut and feed on algae and bacterial biofilms by external digestion and subsequent uptake via their ventral epithelium5–8. All placozoans have three cell layers and six morphologically differentiated cell types5,8,9. The thick ventral epidermis consists of ciliated epithelial cells in which glandular and lipophilic cells are irregularly interspersed10–12. Ciliated epithelial cells make up the thin dorsal epidermis in which crystal cells occasionally occur. An internal meshwork of fibre cells, sandwiched between the two epidermal layers, connects the ventral and dorsal body walls13. Intracellular symbionts were first described in these fibre cells13–15,17. The bacteria were present in all seven haplotypes examined, independent of sampling site or time, and were hypothesized to reside in the lumen of the rough endoplasmic reticulum (rER)1–3,5,6. Persistent and stable residence of a bacterium in the rER of a host would be remarkable as the vast majority of intracellular symbions live in the cytoplasm or vacuoles, and the few known exceptions inhabit the nucleus or mitochondria17,18.

In this study, we focused on the Trichoplax sp. haplotype H2 (Trichoplax H2), previously reported to host two bacterial morphotypes1–3. Sequencing of placozoan genomes consistently yielded rickettsial and other bacterial sequences1–3,5,6. However, as thousands of host individuals were pooled for these analyses, it was neither clear whether these bacterial sequences originated from contaminants or symbions nor whether they were present in all host individuals. Our recent advances in high-throughput sequencing of single placozoan individuals, together with correlative imaging analyses and three-dimensional (3D) reconstruction, allowed us to explore the patterns, structure and function of the placozoan symbiosis at the individual and cellular level.

The Trichoplax H2 microbiome is dominated by two bacterial symbions. We isolated a placozoan H2 haplotype lineage from a seawater tank at the Kewalo Marine Laboratory, University of Hawai‘i (Supplementary Fig. 1). To characterize the microbiome of this Trichoplax H2, we combined highly sensitive DNA and RNA extraction and library preparation protocols to sequence the metagenomes and metatranscriptomes of microscopic single individuals that have an estimated biovolume of 0.02 µl and from which we could isolate 0.5 to 4 ng of nucleic acids (n=5). All five individuals had similar microbial communities based on 16S ribosomal RNA (rRNA) gene reads, but only two taxa were consistently dominant in all five host individuals (Supplementary Fig. 2 and Supplementary Table 1).

The first and most abundant 16S rRNA phylotype was an alphaproteobacterium from the family Midichloriaceae (Rickettsiales)1–3 (Fig. 1a). Midichloriaceae are obligate intracellular, often pathogenic, bacteria found in protists and animals, including humans3. In 16S rRNA analyses, the Trichoplax H2 midichloriacean phylotype formed an unnamed lineage that consisted of sequences recovered from diverse invertebrate hosts and sequences from subsurface sediment samples (98.4–99.4% pairwise identity; Fig. 1a). We recovered a high-quality 1.26 Mb metagenome-assembled genome that
Fig. 1 | Phylogenetic analyses of the Trichoplax H2 symbionts. ‘G. incantans’ represents an undescribed genus in the Midichloriaceae (Rickettsiales) and ‘R. eludens’ is a Marinamargulisbacterium (Margulisbacteria). Bootstrap support values below 0.5 are not shown. Scale bars indicate substitutions per site. Colours and shades of grey indicate taxonomic groups. For each sequence, the accession number, the percentage identity to ‘G. incantans’ and the published taxonomic names and hosts (where available) are indicated. **b, c** Phylogenomic analyses using 43 conserved marker genes based on metagenome-assembled genomes and reference genomes: ‘G. incantans’ and related Midichloriaceae. For each sequence, the accession number, the percentage identity to ‘G. incantans’ and the published taxonomic names and hosts (where available) are indicated. **b, c** Phylogenomic analyses using 43 conserved marker genes based on metagenome-assembled genomes and reference genomes: ‘G. incantans’ and related Midichloriaceae. For each sequence, the accession number, the percentage identity to ‘G. incantans’ and the published taxonomic names and hosts (where available) are indicated.
H2 individuals revealed that the two bacterial symbionts were always intracellular, spatially segregated and specific to one of the six host cell types (Fig. 2b and Supplementary Figs. 5–7). 'G. incantans' was observed only in fibre cells and was the only bacterium located in these cells (Fig. 2b and Supplementary Figs. 5 and 6). All 'G. incantans' cells were surrounded by a host membrane that was densely

**Fig. 2 | ‘R. eludens’ and ‘G. incantans’ are specific to two spatially segregated host cell types.** a, A false-coloured FISH image using probes specific for ‘G. incantans’ (GRIN-61-2, Atto-647) and ‘R. eludens’ (RUEL-846-22, Atto 594); host nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI). The results are representative of five independent experiments. b, A TEM image of a cross-section of *Trichoplax* H2 with ‘G. incantans’ and ‘R. eludens’ indicated in false colour (for raw image data see Supplementary Fig. 5). c–f, TEM images of fibre cells. ‘G. incantans’ is indicated with white arrows and the rER with white arrowheads. e, f, TEM images of ventral epithelial cells containing ‘R. eludens’. Outer membrane vesicles are indicated with black arrowheads, fimbriae-like structures with black arrows and internal structures by a white asterisk. Results in b–f are representative of three independent experiments.
covered with ribosomes (Fig. 2c,d and Supplementary Fig. 6; n = 49 symbiont cells in 9 specimens). Similar host structures surrounding the bacteria in other Trichoplax lineages were interpreted as indicating that the bacteria reside inside the host’s rER. An alternative interpretation for such host membrane structures was shown for the human intracellular pathogens Brucella and Legionella, as well as the amoebal midichloriacean parasite Candidatus ‘jidibacter’. These bacteria remodel the phagosome surfaces of their hosts so that they become covered by host ribosomes as an effective strategy for avoid digestion by their hosts.

To resolve the subcellular architecture of ‘G. incantans’ symbiosis, we used high-resolution 3D TEM tomography to determine whether the structures surrounding the symbiont cells were remodelled phagosomes or rER. Our 3D electron tomographic reconstructions revealed that the ribosome-covered membranes, in which ‘G. incantans’ occurred, formed networks that were connected to the nuclear envelope. This indicates that the structure in which ‘G. incantans’ is embedded is in fact rER. ‘G. incantans’ symbionts were only observed in the rER, some even within the same rER lumen, and never in other host structures (Fig. 3; Supplementary Fig. 8; Supplementary Video 1). These analyses suggest that ‘G. incantans’ persistently resides in the rER of its host. The second symbiont, ‘R. eludens’, colonized only the ventral epithelial cells. These symbionts were always found within cytoplasmic vacuoles of the host (Fig. 2e,f). The vacuoles contained numerous membrane-bound vesicles, presumably outer membrane vesicles produced by ‘R. eludens’ (Supplementary Fig. 7). Thin, tubular structures that resemble fimbrae appeared to connect the bacterial cells to the host vacuole membrane (Fig. 2f; Supplementary Fig. 7).

Bacteria that live inside animal cells are known from only 6 of the 114 recognized bacterial phyla. The number of bacterial phyla with representatives that can live as intracellular symbionts has not increased since the characterization of Mycoplasmatales in the early 1960s, despite huge advances in the sequencing of animals from a wide range of phyla and environments that have led to the discovery of numerous lineages of microbiota. Marinimargarilbacteria is one of the most phylogenetically remote clades of bacteria, discovered through high-throughput sequencing of environmental samples. The remote position of the placozoans in the animal tree of life has probably contributed to this late discovery of Marginibacteria as the seventh bacterial phylum with intracellular symbionts of animals. Our study of the Trichoplax microbiome highlights how bacteria captured by eukaryotes provide a route for studying bacteria that are otherwise known only from sequences found in water or sediment samples.

‘R. eludens’ gains nutrition from lipids degraded by its host. We sequenced the metatranscriptomes of the same single placozoan individuals that were used for metagenomic analyses (n = 3) and generated metaproteomes from pooled samples of 10 to 30 individuals (n = 3) to investigate the physiology of ‘R. eludens’. Physiological modelling of these expression data revealed that ‘R. eludens’ is an aerobic chemoorganoheterotroph, with a complete tricarboxylic acid (TCA) cycle that generates energy and biomass from glycerol and the β-oxidation of fatty acids (Fig. 4a; Supplementary Table 3). The source of the glyceral and fatty acids is probably lipids from the algal diet of the host. Our analyses of the host’s transcriptome revealed that Trichoplax H2 expressed several lipases, most probably for the digestion of the alga it feeds on (Supplementary Table 4). These host lipases hydrolyze lipids to glycerol and fatty acids. The genome of ‘R. eludens’ also encodes lipases that would allow ‘R. eludens’ to digest lipids independently of its host. Interestingly, we found neither transcripts nor peptides for these symbiont lipases, suggesting that ‘R. eludens’ relies on the lipases expressed by its host (Supplementary Table 3).

The transfer of glycerol and even-chain fatty acids from the host to ‘R. eludens’ probably occurs passively, as they can easily diffuse through cell membranes. We predict that the fatty acids are taken up and activated by ‘R. eludens’ on the basis of its high expression of a long-chain fatty acid coenzyme A (CoA) ligase (among the top 25% of expressed genes; Fig. 4a; Supplementary Table 3). The fatty acids are then probably catabolized to acetyl-CoA and respired, as indicated by the expression of all the genes needed for β-oxidation and the oxidative TCA cycle. However, the anabolic incorporation of fatty acids is unlikely, as we could not detect the genes for the glyoxylate shunt.

‘R. eludens’ encoded genes for synthesizing all nucleotides and amino acids, including the nine amino acids considered essential for animals. However, we found no genomic or transcriptomic indications that ‘R. eludens’ exports nutrients to its host, for example via amino acid exporters (see Fig. 4a and Supplementary Note 3). Moreover, in our TEM analyses, we found no evidence for the intracellular, lyosomal digestion of ‘R. eludens’, such as lamellar bodies or tertiary lysosomes commonly observed in other nutritional symbioses. Our ultrastructural analyses did, however, reveal large numbers of putative outer membrane vesicles in the host vacuole surrounding ‘R. eludens’ (Fig. 2e,f and Supplementary Fig. 7). It is tempting to speculate that the host takes up outer membrane vesicles produced by ‘R. eludens’ via phagocytosis and thus supplements its diet, as the host lacks synthesis pathways for essential amino acids. However, the beneficial effects of such putative amino acid provisioning by ‘R. eludens’ are not clear, given that the animal’s algal diet may contain sufficient amounts of essential amino acids.

‘G. incantans’ has the genes for energy parasitism but does not express them: it lives in the rER of fibre cells and seems to be a typical Rickettsiales based on genomic features alone, namely a heterotroph that relies on its host for biomass and energy generation (Fig. 4b). The ‘G. incantans’ genome encodes the hallmark feature for intracellular energy parasites that is present in all Rickettsiales genomes: a fully functional ADP/ATP-translocase for importing ATP from its host. In contrast to all other known energy parasites, we found no transcripts or respective peptides of the ADP/ATP-translocase in ‘G. incantans’ (Supplementary Table 5). Instead, ‘G. incantans’ generated ATP with an ATP synthase, and the subunits a and b were highly expressed in the bacterium’s proteome (Supplementary Table 6). Compared to the typical energy-parasitic lifestyle of cytosolic Rickettsiales that rely on ATP imported from their hosts, the ability of ‘G. incantans’ to synthesize ATP by itself likely lowers its detrimental impact on its host considerably.

High transcription of key genes of the oxidative TCA cycle and the presence of a complete electron transport chain in the genome, with some of the subunits of the electron transport chain among the most highly transcribed genes, suggests that the proton gradient for ATP synthesis is fuelled by oxidative phosphorylation (Fig. 4b and Supplementary Table 5). An incomplete glycolysis pathway and several importers for α-ketoacids and C4-dicarboxylates suggest that the metabolites respired in the TCA cycle are imported from the host (Fig. 4b).

The genome and transcriptome of ‘G. incantans’ revealed a strong host dependence on both amino acid and nucleotide supply (Fig. 4b; see Supplementary Note 4 for details). In contrast, the transcription profile of ‘G. incantans’ suggested that it could supply its host with riboflavin (vitamin B2), an essential vitamin that cannot be synthesized by most metazoans. Our analyses of the transcriptomic data of Trichoplax H2, as well as the genome and proteome of the closely related haplotype H1, revealed that both seem to lack the known genes for synthesizing riboflavin (Supplementary Fig. 9) and rely on an external source of riboflavin (Supplementary Table 4). This suggests that when riboflavin availability is limiting for the host, ‘G. incantans’ could supplement the nutrition of its host.

‘G. incantans’ does not seem to be detrimental to Trichoplax H2, despite the fact that it has to import most of the compounds it needs for generating energy and biomass from its host. Our metagenomic,
FISH and TEM data revealed 2–20 symbiont cells per fibre cell, so that the total number of 'G. incantans' cells per host individual is roughly the same as the number of host cells (Supplementary Note 5). This indicates closely regulated control of symbiont growth by the symbiont, the host or both partners. Pathogen abundances are typically orders of magnitude higher per host cell and often result in rapid exploitation and destruction of host cells and the impairment of host reproduction.37 The relatively low abundance of 'G. incantans' in Trichoplax H2 together with the rapid doubling rates of these hosts (2–3 d in our aquaria) are in contrast to virulent pathogenic infections. Unlike all other known energy parasites, 'G. incantans' seems to generate its own ATP and might even modulate its host immune response to prevent apoptosis (Supplementary Note 4).

Bacterial phylotypes highly similar or identical to 'G. incantans' occur worldwide in aquatic environments. To assess how widespread the two Trichoplax symbionts are in other environments and hosts, we surveyed the ~300,000 publicly available amplicon-based 16S rRNA sequence libraries using the IMNGS pipeline. We did not find any sequences related to 'R. eludens', using a cut-off of 99% identity. In contrast, sequences highly similar or identical to 'G. incantans' were present in aquatic environments, both marine and limnic, from across the globe (Supplementary Table 7). Of the 8,026 libraries from aquatic environments, we found sequences that were at least 99% identical to 'G. incantans' in almost 10% of these libraries (n=845). One third of the sequences were identical to 'G. incantans' and almost all were attributed to the genus Grellia on the basis of evolutionary placement analysis (Supplementary Fig. 10). The presence of Grellia phytoplotype in such a wide range of environments, including limnic ones, indicates that these bacteria have host ranges beyond placozoans. Indeed, our phylogenetic 16S rRNA analyses showed that sequences that group with the genus Grellia have been found in marine protists (Eutreptiella), sea cucumbers (Apostichopus) and oysters (Crassostrea), as well as in the limnic cnidarian Hydra oligactis (see Fig. 1a). The Hydra sequences came from specimens
Fig. 4 | ‘R. eludens’ has versatile biosynthesis pathways, whereas ‘G. incantans’ depends on the import of most nutrients from its host. Physiological reconstructions based on RAST annotations and Pathway Tools metabolic modelling. Functions that are discussed in the text and highly expressed are indicated in red. a, ‘R. eludens’. b, ‘G. incantans’. Bold font indicates primary function. ABC, ATP-binding cassette; AdoMet, S-adenosyl-L-methionine; MFS, major facilitator superfamily; nt, nucleotide; nt-ACTUI, the nucleotides a cell can import (all but guanine); P, phosphate; PEP, phosphoenolpyruvate; T4SS, type IV secretion system; TRAP, tripartite ATP-independent periplasmic.

collected freshly from their natural environments and animals reared in the laboratory for more than 30 yrs, indicating the stability of this association in these hosts38.

The recent realization that human pathogens such as Chlamydiae, Legionellales and Rickettsiales have close relatives that live in hosts ranging from protists to fish and from aquatic and soil habitats has led to a paradigm shift in our view of the ecology and evolution of intracellular bacteria27,39,40. ‘G. incantans’ extends our conceptual understanding of the pervasiveness of such bacteria and shows that a single environmental rickettsial genus occurs worldwide in marine and limnic habitats. This remarkable distribution raises the question of whether all Grellia are host-associated. If ‘G. incantans’ had a free-living stage, this would be in contrast to all other known Rickettsiales that infect animals27.
why the midichlorian symbionts of Trichoplax H1 and H2 do not belong to the same clade, although their hosts are very closely related and separated only a few decades ago14. This split could have been caused by their midichlorian symbionts, as Rickettsiales are well known to induce reproductive incompatibility in insects41. Future studies of the microbiomes of the large number of extant haplotypes are needed to fully understand the ecology and evolution of symbioses between placozoans and their bacterial symbionts.

**Methods**

**Isolation and cultivation.** The placozoans were isolated from a coral tank at the Kewalo Marine Laboratory, University of Hawai’i at Mānoa in October 2015 by placing glass slides, mounted in plastic slide boxes that had the top and bottom cut out, into the tank for 10 d (ref. 13). Placozoans were identified using a dissection microscope, transferred to 400 ml glass beakers with 34.5% artificial seawater and fed weekly with 2 × 10⁶ cells ml⁻¹ of Isochrysis galbana from a log-phase culture. Doubling times were 2–3 d at 25 °C in 34.5% artificial seawater and with a 16:8 light:dark regime.

**Nucleic acid extractions.** DNA was extracted from two single individuals from the Trichoplax H2 cultures using the DNeasy Blood & Tissue Kit (Qiagen) and DNA and RNA from three additional single individuals were extracted using the AllPrep DNA/RNA Micro Kit (Qiagen), according to the manufacturer's protocols for both kits except for the following modifications. Proteinase K digests were performed overnight. Elution volumes were halved and all samples were eluted twice, reusing the first elute. Elutions were carried out with a 10-min-long waiting step before centrifugation.

**DNA and RNA sequencing.** Illumina-library preparation and sequencing were performed by the Max Planck Genome Centre. In brief, DNA/RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent) and genomic DNA was fragmented to an average fragment size of 500 base pairs (bp). For the DNA samples, the concentration was increased (MinElute PCR Purification Kit; Qiagen) and an Illumina-compatible library was prepared using the Illumina Ultralow Library Systems Kit (NuGEN) according to the manufacturer’s protocol. For the RNA samples, the Ovation RNA-seq System V2 (NuGen) was used to synthesize complementary DNA and sequencing libraries were then generated with the DNA Library Prep Kit Illumina (BioLABS). All libraries were size selected by agarose gel electrophoresis and the recovered fragments quality- assessed and quantified by fluorometry. For each DNA library, 14–22 million 150 bp single-end reads were sequenced on an HiSeq 4000 (Illumina) and, for the RNA libraries, 150 bp single-end reads were sequenced to a depth of 42–44 million.

**Host mitochondrial 16S rRNA gene phylogenetic analyses.** The metagenomic assembly was screened for the contig containing the host mitochondrial 16S rRNA gene (mt16S) using BLAST v2.7.1 as implemented in Geneious R11. The gene was extracted from the contig and aligned together with a database of publicly available mt16S sequences using MAFFT v7.394 in G-Insi mode. All libraries were size selected by agarose gel electrophoresis and the recovered fragments quality-assessed and quantified by fluorometry. For each DNA library, 14–22 million 150 bp paired-end reads were sequenced on an HiSeq 4000 (Illumina) and, for the RNA libraries, 150 bp single-end reads were sequenced to a depth of 42–44 million.

**Bacterial diversity 16S rRNA gene phylogenetic analyses.** For the 16S rRNA gene database of all metatypes recovered, the phyloFlash v3.0 beta1 pipeline (https://github.com/HRGV/phyloFlash) assembled full-length SSU genes for all samples. The dataset was aligned and phylogenetic trees were calculated and visualized as for the host mt16S dataset above. The tree was rooted with the Eukarya and only the bacterial part of the tree is shown in this Letter.

**Genome analyses.** Full-length 16S rRNA gene sequences were reconstructed for each metagenomic and metatranscriptomic library using phyloFlash v3.0 beta1 (https://github.com/HRGV/phyloFlash) from raw reads.

For assembly, adapters and low-quality reads were removed with bbduk v37.9 (https://sourceforge.net/projects/bbmap/) with a minimum quality value of 2 and a minimum length of 36; single reads were excluded from the analysis. Each library was error corrected using BasesHammer v3.624. A combined assembly of all the libraries was performed using SPAdes 3.6.2 (ref. 45) with standard parameters and k-mers 21, 33, 55, 77 and 99.

The reads of each library were mapped back to the assembled scaffolds using bmap v37.9 (https://sourceforge.net/projects/bmap/) with the option fast=1. Scaffolds were binned on the basis of the mapped read data using MetaBAT v1.0. The binning was refined using Bandage v0.8.1 by collecting all contigs linked to the contig that contained the full-length 16S rRNA gene of the target organism. The bin quality metrics were computed with QUAST v5.0.2 and the completeness for all bins was estimated using checkM v1.0.7 (ref. 46).

Annotating the symbiont draft genomes was performed using RAST and verified with PSI-BLAST v2.7.1 for selected genes discussed. Average nucleotide and amino acid identities between genomes were calculated with the ANI/AAI matrix calculator (http://envе-omics.ce.gatech.edu/g-matrix/). Comparative analyses were conducted using the PATRIC database and services47. Pathway analyses with the BioCyc database48 were used to analyse the metabolic capacities of ‘G. incantans’ and ‘R. eludens’. The genomes were screened for secretion systems and effectors using EffectiveDB.

**Transcriptomic analyses.** Adapters and rRNA gene reads were removed from the RNA-seq reads using bbduk v37.9. The gene expression for each symbiont genome H1 and H2 of the host (both of the published predicted proteomes of T. adhaerens H1) was calculated from RNA-seq libraries using kallisto v0.45.0 with default settings49. Transcription levels were mapped onto metabolic pathways using Pathway Tools v2.20.

**Proteomic analyses.** Peptide samples for proteomics were prepared and quantified from two samples of 10 Trichoplax each and one sample of 30 Trichoplax specimens, as described by Kleiner et al. and according to the filter-aided sample preparation protocol described by Wisniewski et al. In addition to minor modifications described in Hamann et al., we did not clear the lysate by centrifugation before boiling the sample in lysis buffer. Instead, as the sample size was extremely limited (10 Trichoplax specimens × 0.2 µl), we loaded the whole lysate onto the filter units used for the filter-aided sample preparation procedure. Centrifugation times before column washes with 100 µl UA (0.1 M Tris/HCi pH 8.5) were halved as compared to Hamann et al.. Peptides were not desalted. Peptide concentrations were determined with the Pierce Micro BCA assay (Thermo Fisher Scientific) following the manufacturer’s instructions.

For each sample, all peptides were analysed by one-dimensional LC-MS/MS as described in Kleiner et al. with the modification that a 75 cm analytical column was used. Briefly, the sample containing 30 specimens was measured in technical replicate, for the others the whole sample was used in one analysis. The peptide (0.8–3 µg) was loaded with an UltiMate 3000 RSLC NanoLiquid Chromatograph (Thermo Fisher Scientific) in loading solvent A (2% acetonitrile, 0.05% trifluoroacetic acid) onto a 5 mm ×300 µm ID C18 Acclaim PepMap 100 pre-column (Thermo Fisher Scientific). Elution and separation of peptides on the analytical column (75 cm ×75 µm analytical EASY-Spray column packed with PepMap RSLC C18, 2 µm material, Thermo Fisher Scientific, heated to 60 °C) was performed at a flow rate of 2.25 ml min⁻¹ using a 460 min gradient going from 98% buffer A (0.1% formic acid in 98% acetonitrile) to 5% buffer B (0.1% formic acid in 98% acetonitrile) in 363 min, then to 50% B in 70 min, then to 99% B in 1 min and ending with 99% B. The analytical column was connected to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) via an Easy-Spray source. Eluting peptides were ionized via electrospray ionization. Carry-over was reduced by two wash runs (injection of 20 µl acetonitrile, 99% v/v ethanol) between samples. Data acquisition in the Q Exactive Plus was performed as in Petersen et al. A database containing protein sequences from the Trichoplax host as well as the two symbionts was used. Sequences of common laboratory contaminants were included by appending the cRAP protein sequence database (http://www.thegpm.org/cRAP) to the database. The final database contained 13,801 protein sequences. The Expect values of the MS/MS spectra against this database were performed with the Sequest HT node in Proteome Discoverer v2.2.0.388 (Thermo Fisher Scientific) as in Petersen et al.

For protein quantification, normalized spectral abundance factors were calculated per species and multiplied by 100, to give the relative protein abundance as a percentage.

**Phylogenetic and phylogenomic analyses.** A 16S rRNA gene database for ‘G. incantans’ was constructed using the assembled 16S rRNA gene sequence from each metagenomic library, the 20 best BLAST hit hits in the nr database and all other sequences of described Candidatus taxa in the Midichloriaceae. We added the five type strains with the best BLAST hit score (five species of Rickettsia) as an outgroup. We also screened the trace reads from the Trichoplax H1 genome project for reads containing Midichloriaceae 16S rRNA gene fragments using BLAST v2.7.14, assembled them in Geneious R9 (http://www.geneious.com) and added the resulting sequence to the database. A similar search for margulisbacterial 16S rRNA fragments yielded no hits.

The 16S rRNA gene database was aligned using MAFFT v7.394 and the phylogenetic tree was calculated using FastTree v2.1.10 with a GTR model for nucleotide substitution. The tree was drawn with Geneious R9.

For ‘G. incantans’, the database of genomes for phylogenetic analysis was compiled from all available genomes from the Midichloriaceae as well as representatives for all genera of the Anaplasmataceae and Rickettsiaceae. We also searched the assembly of the H1 genome project to find contigs that belong to Midichloriaceae contamination using BLAST v2.7.14 with the ‘G. incantans genome as implemented in Geneious R9 (http://www.geneious.com). The identified set of contigs corresponded to the set found by Driscoll et al. and was added to the database. We similarly searched for sequences related to ‘R. eludens’ in the same genome project, but no hits were detected.

For genome-based alignments of the amino acids of 43 conserved phylogenetic marker genes, the tree workflow as implemented in CheckM v1.0.11 was used. For Rutfiannamia, the genome bin data were integrated into a taxonomically
selected part of the alignment from Hug et al. that covered all Melainabacteria and Cyanobacteria, WOR-1 and RBX-1 (Margulisbacteria), as well as five short branches forming Firmicutes as an outlier. The phylogenetic reconstructions of the concatenated alignments were calculated using FastTree v2.1.10 with the WAG model for amino acid substitutions and visualized and analysed using iTOL.

**Tag-sequence data analysis.** The 16S rRNA gene sequences from ‘G. incantans’, as well as representative sequences from all characterized midichlorianae Candidatus taxa were used as query sequences to search the global collection of the microbial tag-sequence library of the IMNGS service12 with a minimal alignment length of 200 bp and a minimal identity of 99%. Identified amplicon libraries were grouped according to their deposited metadata. For the top 10% of libraries with the highest number of sequences from ‘G. incantans’, the habitat type (limnic or marine) and geolocation were manually collected from the deposited metadata and related publications. The detected 16S rRNA reads were aligned to the Rickettsiales dataset using MAFFT—addfragments and the evolutionary placements in the tree were performed using raxml v8.2.1268.

**Evolutionary model for amino acid substitutions.** The evolutionary model for amino acid substitutions and visualized and analysed using iTOL.

**Data availability.** The metagenomic and metatranscriptomic raw reads and assembled symbiont genomes are available in the European Nucleotide Archive under Study Accession Number PRJEB30343. The mass spectrometry proteomics data and protein sequence databases were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset PXD012106. The TEM 3D reconstruction data were deposited in figshare; the aligned tomography slices used for the reconstruction shown in Fig. are available at https://figshare.com/s/886698e9a9a0264f62ff (ref. 27).

**Code availability.** The script used for the assembly-based binning is available at https://github.com/HVRG/tools_and_scripts.

Received: 31 December 2018; Accepted: 26 April 2019; Published online: 10 June 2019

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**FISH.** We used ARB—SILVA database 128 (ref. 37) and the ARB PROBE DESIGN tool (the ARB software package v6.0.6) to design two FISH probes for each symbiont that were specific to their 16S rRNA sequences (Supplementary Table 2). We combined the specificities of the probes by sequencing their sequences to all available sequences in the ARB—SILVA database and Ribosomal Database Project release 11.5 (ref. 3). The most specific probe for *R. ludens* had two mismatches to first non-target hit sequences; the most specific probe for *G. incantans* also matched the six most closely related Grellia sequences; detailed results are presented in Supplementary Table 2.

**Serial sectioning.** Specimens were fixed on coverslips with 2% formaldehyde and 0.1% glutaraldehyde in 1.5x PIPES, HEPES, EGTA and MgCl2 (PHEM) buffers modified from Montanaro et al.38 at 4°C for 12 h. After three washing steps in 1.5x PHEM buffer, the samples were stored in 70% ethanol until use. Samples were rehydrated in PBS and hybridization was performed according to Manz et al.39. Mono-labelled, DOP-340, or MIL342 probes (Supplementary Table 2) at a concentration of 8.4 pmol µL−1 were diluted with hybridization buffer containing 35% formamide, 900 mM NaCl, 20 mM Tris/Cl, and 0.01% SDS at a ratio of 15:1. Whole animals were incubated in 30 µl of the probe/hybridization buffer mix at 46°C in 250 µl PCR tubes for 3–4 h, followed by a 30-min-long washing step in washing buffer containing 700 mM NaCl, 20 mM Tris/Cl, 5 mM EDTA and 0.1% SDS. After a 10-min-long washing step in PBS, the animals were stained with DAPI for 30 min, washed twice again in PBS and mounted on glass slides in Vectashield mounting medium.

To test the probes designed for this study, 30 clonal individuals of Trichoplax H2 were cooled, fixed as described above, homogenized by sonicating applied to a filter. The parts of the filter were then tested with different formamide concentrations and the optimal formamide concentration was determined. Fluorescence images were taken with a Zeiss LSM 780 equipped with a GaAsP detector or an Airyscan detector and a Plan-Apochromat 63x/1.4 and a Plan-Apochromat 100x/1.46 oil immersion objective using the ZEN software (black edition, 64bit, v4.1.0.210; Carl Zeiss Microscopy GmbH).

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Acknowledgements
This study was funded by the Max Planck Society, a Gordon and Betty Moore Foundation Marine Microbial Initiative Investigator Award (grant no. GBMF3811 to N.D.), grants to M.G.H. from the Gordon and Betty Moore Foundation (no. 5089) and the US Office of Naval Research (no. N00014-15-1-2658), the German Academic Exchange Service DAAD (T.H.) and the NC State Chancellor’s Faculty Excellence Program Cluster on Microbiomes and Complex Microbial Communities (M.K.). We thank M. Strous for access to proteomic equipment and A. Kouris for LC−MS/MS operation. The purchase of the proteomics equipment was supported by a grant from the Canadian Foundation for Innovation to M. Strous. We thank the Electron Microscopy Facility of the MPI-CBG, the Max Planck Genome Centre and the Electron Microscopy Facility at the MPI-PZ Cologne and the Cell Imaging and Ultrastructure Research Core Facility of the University of Vienna for technical support. We thank C. Peters for nucleic acid extractions, C. Peters, M. Meyer and W. Ruschmeier for support with Trichoplax cultivation, B. Nedved for support in the field and G. Bennett, T. Erb, L. Schada von Borzyskowski and P. A. Chakkiath for discussions on symbiont physiology.

Author contributions
M.G.H., N.D., M.M.-N. and H.R.G.-V. conceived the study. H.R.G.-V. sampled and cultivated the organisms and performed the assemblies, genome and transcriptome analyses, tag-sequencing analyses and phylogenetic analyses. H.R.G.-V. reconstructed the symbiont physiology with the help of M.L. and M.K. M.K. and T.H. generated the proteomic data and H.R.G.-V. and M.K. analysed the proteomic data. N.L. performed the fluorescence microscopy, electron microscopy and electron tomography, subsequent data analysis and 3D reconstruction. H.R.G.-V. and N.L. wrote the manuscript with support from N.D., M.M.-N. and M.G.H. All authors revised the manuscript and approved the final version.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41564-019-0475-9.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- Sequencing data were produced on an Illumina HiSeq 2500 instrument with manufacturer’s software. For the 16S tag sequencing analysis, the IMNGS web-platform was used to gather data; see https://www.imngs.org/
- Tomography tilt-series were acquired using SerialEM. Transmission electron micrographs were acquired with the xT microscope control software ver. 6.2.6.3123

Data analysis

- PhyloFlash 3.0 beta1: https://github.com/HRGV/phyloFlash
- BBmap suite v37.9 from Bushnell B. - sourceforge.net/projects/bbmap
- BayesHammer 3.6.2
- SPAdes 3.62
- MetaBAT 1.0
- Bandage 0.8.1
- QUAST 5.0.2
- checkM 1.0.11
- Rast http://rast.nmpdr.org/
- ANI/AAI matrix calculator - http://enve-omics.ce.gatech.edu/g-matrix/
- PATRIC https://www.patricbrc.org
- Pathway Tools 22.0
- EffectiveDB https://effectors.csb.univie.ac.at
- kallisto v0.45.0
- Proteome Discoverer version 2.2.0.388
- Geneious R11 http://www.geneious.com
- iTOL https://itol.embl.de
- arb software package 6.0.6.
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For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers.

The metagenomic and metatranscriptomic raw reads and assembled symbiont genomes are available in the European Nucleotide Archive under Study Accession Number PRJEB30343.

The mass spectrometry metaproteomics data and protein sequence database were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset PXD012106 (access for reviewers: https://www.ebi.ac.uk/pride/archive/login, email/Username: reviewer71196@ebi.ac.uk, password: RSqmy9cK).

The aligned tomography slices used for the 3D-reconstruction shown in Figure 4 and the Supplementary video were deposited in figshare and are available at https://figshare.com/s/886b869a9ada0264ffb2 or under the doi 10.6084/m9.figshare.7429793 with the dataset PXD012106 (access for reviewers: https://www.ebi.ac.uk/pride/archive/login, email/Username: reviewer71196@ebi.ac.uk, password: RSqmy9cK).

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf.
Data collection
Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection.

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Data exclusions
If no data were excluded from the analyses, state so OR if data were excluded, provide the exact number of exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.

Non-participation
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Randomization
If participants were not allocated into experimental groups, state so OR describe how participants were allocated to groups, and if allocation was not random, describe how covariates were controlled.

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description
Placozoans were isolated from a coral tank to study their symbiotic association with intracellular bacteria

Research sample
Placozoans were isolated from a coral tank at the Kewalo Marine Laboratory, University of Hawaii‘i at Mānoa, Honolulu, Hawaii‘i by placing glass slides mounted in cut-open plastic slide holders into the tank for 10 days. Placozoans were identified under a dissection microscope, transferred to 400 ml glass beakers with 34.5 % artificial seawater (ASW) and fed weekly with 2x10^6 cells ml-1 of Isochrysis galbana from a log-phase culture. At 25°C in 34.5 % ASW and with a 16:8 hour light/dark regime, doubling times were 2-3 days.

Sampling strategy
All individuals used in the experiments originated from an asexually dividing clonal lineage of Trichoplax H2, which was established from a single individual. Individual specimens were sampled from the cultures at chance for all experiments conducted. Only intact, healthy-looking animals were used in the experiments. Single individuals were used for metagenomic, metatranscriptomic and imaging analyses. Samples were pooled (10-30) for metaproteomic analyses. Sample size was chosen according to sizes used in comparable studies.

Data collection
The gene expression data were generated by DNA and RNA sequencing at the Max Planck Genome Centre in Cologne under the supervision of Dr. B. Huetter. Metaproteomics data were generated by mass spectrometry at the University of Calgary by Dr. M. Kleiner and Torsten Hinzke. Imaging data were collected at the M Ps for Marine Microbiology (Bremen) and of Molecular Cell Biology and Genetics (Dresden) by Dr. Nikolaus Leisch.

Timing and spatial scale
Placozoan specimens were collected in Hawaii‘i in October 2015 and cultivated in the lab until used for this study. Animal specimens for metagenomics and metatranscriptomics were taken from the lab cultures in February 2016, for proteomics 2017 and for microscopy between January 2016 and September 2018

Data exclusions
none

Reproducibility
DNA metagenomic libraries were constructed from five specimens, transcriptomic libraries from 3 of the five metagenomic specimens. Proteomic data were generated from 3 separate pools of 10 to 30 specimens. Microscopy data were generated from three to nine separate specimens as indicated in the text and materials and methods. Using the deposited raw sequencing, proteomic and imaging data, the data analyses that were performed in this study can be easily and repeatedly reproduced. All attempts to repeat the experiments were successful.

Randomization
All experiments were performed on a clonal lineage and specimens were randomly chosen for experiments.

Blinding
Blinding was not performed because it was not relevant to this study. This study was an exploratory survey of microbial diversity without a priori expectations that would influence the analyses.

Did the study involve field work?  Yes  No

Field work, collection and transport

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Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).

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State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).

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#### Methods

| Cancer research | n/a | Involved in the study | n/a | Involved in the study |
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#### Animals and other organisms

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| Laboratory animals | Wild animals | Field-collected samples |
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| A clonal lineage of haplotype H2 placozoans was isolated from a coral tank at the Kewalo Marine Laboratory, University of Hawai‘i at Mānoa, Honolulu, Hawai‘i. Initial specimens were sampled by placing glass slides mounted in cut-open plastic slide holders into the tank for 10 days. Placozoans were identified under a dissection microscope, and single individuals were transferred to 400 ml glass beakers with 34.5 ‰ artificial seawater (ASW). The cultures were fed weekly with 2x10^6 cells ml-1 of Isochrysis galbana from a log-phase culture. At 25°C in 34.5 ‰ ASW and with a 16:8 hour light/dark regime, doubling times were 2-3 days. The culture is available upon request. | An initial sample of placozoans was used to isolate a clonal lineage that was then used for all experiments - see section on laboratory animals above. The culture is available upon request. | No field collected samples were used. |
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ChIP-seq

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Software
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  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

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| Diffusion MRI   | Used | Not used |

#### Preprocessing

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#### Models & analysis

| n/a | Involved in the study |
|-----|-----------------------|
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|     | □ Graph analysis |
|     | □ Multivariate modeling or predictive analysis |

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