DNA methylation regulates transcriptional homeostasis of algal endosymbiosis in the coral model Aiptasia

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Abstract

The symbiotic relationship between cnidarians and dinoflagellates is the cornerstone of coral reef ecosystems. Although research is focusing on the molecular mechanisms underlying this symbiosis, the role of epigenetic mechanisms, which have been implicated in transcriptional regulation and acclimation to environmental change, is unknown. To assess the role of DNA methylation in the cnidarian-dinoflagellate symbiosis, we analyzed genome-wide CpG methylation, histone associations, and transcriptomic states of symbiotic and aposymbiotic anemones in the model system Aiptasia. We find methylated genes are marked by histone H3K36me3 and show significant reduction of spurious transcription and transcriptional noise, revealing a role of DNA methylation in the maintenance of transcriptional homeostasis. Changes in DNA methylation and expression show enrichment for symbiosis-related processes such as immunity, apoptosis, phagocytosis recognition and phagosome formation, and unveil intricate interactions between the underlying pathways. Our results demonstrate that DNA methylation provides an epigenetic mechanism of transcriptional homeostasis during symbiosis.

Introduction

Coral reefs are ecologically important marine ecosystems, which cover less than 0.2% of our oceans but sustain an estimated ~25% of the world's marine species and 32 of 33 animal phyla (Spalding and Grenfell 1997; Davidson 2002; Sylvain 2006). Coral reefs are also economically important by providing food and livelihood opportunities to at least 500 million people; worldwide, they have a net present value of almost USD 800 billion, and they generate USD 30 billion in net economic benefits annually (Sylvain 2006). Unfortunately, these

34 ecosystems are under severe threat from anthropogenic stressors including global warming and
35 water pollution, among others, which can cause coral bleaching (loss of intracellular
36 endosymbionts from coral) and overall coral reef decline. Despite increasing efforts on studying
37 the mechanisms underlying the regulation and environmental stress related breakdown of this
38 symbiotic association (Davy et al. 2012; Meyer and Weis 2012), we still lack knowledge on
39 basic molecular processes, for instance whether epigenetic mechanisms are involved in
40 symbiosis regulation and could potentially contribute to increased resilience in response to
41 environmental stress as reported in other organisms (Rando and Verstrepen 2007; Lämke and
42 Bäurle 2017).

43 DNA methylation plays an important role in many biological processes of plants and
44 animals (Bird 2002; Suzuki and Bird 2008; He et al. 2011; Jones 2012). It has been proposed as a
45 mechanism for organisms to adjust their phenotype in response to their environment in order to
46 optimize organismal response to changing environmental conditions (Richards 2006; Rando and
47 Verstrepen 2007). For instance, recent findings in mice show an important function for DNA
48 methylation in inhibiting spurious transcription along the gene body, allowing for reduction of
49 nonsense transcripts from highly expressed loci (Neri et al. 2017). Similar functions have also
50 been proposed in plants, suggesting a general role of DNA methylation in the maintenance of
51 transcriptional homeostasis (Zilberman 2017).

52 Several studies on DNA methylation in cnidarians have been published recently (Dixon
53 et al. 2016; Putnam et al. 2016), however, its role and function in cnidarians is, at present, mainly
54 unknown (Torda et al. 2017). The sea anemone Aiptasia is an emerging model to study the
55 cnidarian-dinoflagellate symbiosis. Like corals, it establishes a stable but temperature sensitive
56 symbiosis with dinoflagellates of the genus *Symbiodinium* but, unlike corals, can also be

57 naturally maintained in an aposymbiotic state. This, compounded with its ease of culture,
58 provides a tractable system to study the molecular mechanism underlying symbiosis without the
59 impeding stress responses associated with coral bleaching stress (Voolstra 2013; Baumgarten et
60 al. 2015).

61 Using the model system Aiptasia (strain CC7, sensu *Exaiptasia pallida*), we obtained
62 whole-genome CpG DNA methylation, ChIP-Seq and RNA-Seq data from aposymbiotic (Apo)
63 and symbiotic (Sym) individuals to study the function of DNA methylation in transcriptional
64 regulation and its role in the cnidarian-dinoflagellate symbiosis.

65

66 **Results**

67 **Aiptasia DNA Methylation patterns change with symbiotic states**

68 To assess changes in DNA methylation in response to symbiosis, we performed whole-
69 genome bisulfite sequencing with an average coverage of 53× per individual on 12 anemones,
70 providing 6 biological replicates per treatment (symbiotic vs. aposymbiotic). Methylation calling
71 using the combined dataset identified 710,768 CpGs (6.37% of all CpGs in Aiptasia genome),
72 i.e. methylated sites in the Aiptasia genome. Notably, the percentage of CpGs is much lower than
73 in mammals (60–90%) (Tucker 2001), but comparable to the coral *Stylophora pistillata* (7%)
74 (Liew et al. 2017). We identified 10,822 genes (37% of all 29,269 gene models identified in the
75 Aiptasia genome) with at least 5 methylated positions that were subsequently defined as
76 methylated genes. On average, these genes had 18.4% CpGs methylated, 3-fold higher than the
77 average methylation density across the entire genome (Chi-squared test p value $< 2.2 \times 10^{-16}$)
78 and 167-fold higher than the methylation levels in non-coding regions. These findings indicate

79 that the distribution of CpG methylation is non-random and mainly located in gene bodies,
80 similar to corals (Dixon et al. 2017; Liew et al. 2017) and other invertebrate species (Feng et al.
81 2010; Gavery and Roberts 2010; Wang et al. 2013; Gonzalez-Romero et al. 2017).

82 To analyze the relationship between methylation density (percentage of CpGs) and gene
83 density (the number of genes per 10,000 bp), we ran a sliding window (window size: 40 kb, step:
84 30 kb) and visualized the results in a Circos plot (Fig. S1) (Krzywinski et al. 2009). The
85 correlation of CpG content and distribution of methylation showed a negative correlation
86 (Pearson correlation coefficient: $r = -0.31$, p value $< 2.2 \times 10^{-16}$) suggesting that methylation
87 tends to preferentially occur in CpG-poor regions (Fig. S2). Gene density had a positive
88 correlation with methylation density ($r = 0.21$, p value $< 2.2 \times 10^{-16}$) consistent with the finding
89 that methylation is predominantly located in gene bodies (Fig. 1). We also observed that within
90 gene bodies, introns showed significantly higher methylation densities than exons (Fig. 1B).

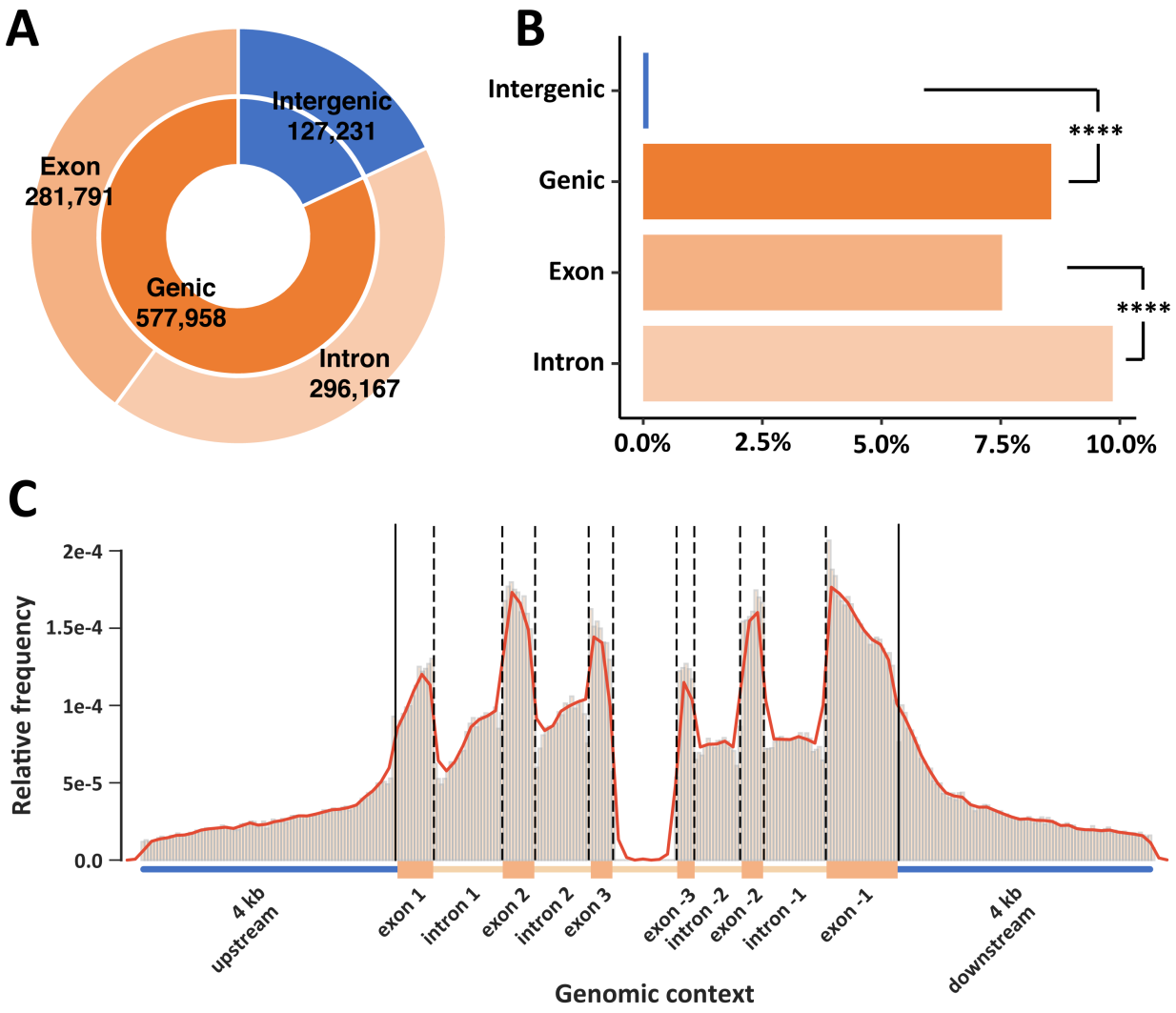

Fig. 1. DNA methylation landscape

(**A**) Distribution of methylated CpG across intergenic (18%), genic (82%), intronic (42%) and exonic (40%) regions in the Aiptasia genome. (**B**) Normalized percentage of methylated CpGs in different regions. Chi-squared test shows significant differences between intergenic and genic regions, and between exons and intron (****$p<0.0001$). (**C**) Relative frequencies of methylated positions across a normalized gene model.

99 **Methylated genes are marked by H3K36me3**

100 Analysis of methylation patterns (see above) within gene bodies showed rapidly
101 increasing methylation levels after the transcription start site (TSS) that are maintained before
102 slowly decreasing towards the transcription termination site (TTS) (Fig. S3A). Interestingly, we
103 found that gene body methylation in Aiptasia is positively correlated with expression (Fig. 2A),
104 suggesting that DNA methylation either increases the expression of genes or that DNA
105 methylation is increased as a consequence of transcription whereby increased expression results
106 in methylation of the respective loci. The latter interpretation would be in line with recent
107 findings in mouse embryonic stem cells (Neri et al. 2017), which demonstrated that gene body
108 methylation is established and maintained as a result of active transcription by RNA polymerase
109 II (Pol II) and recruitment of the histone modifying protein SetD2 that trimethylates histone H3
110 at lysine 36 (H3K36me3). This histone mark is specifically bound via the PWWP domain
111 present in the DNA methyltransferase Dnmt3b, which in turn methylates the surrounding DNA
112 accordingly, resulting in the inhibition of transcription initiation from cryptic promoters within
113 the gene body and thus a significant reduction of spurious transcription.

114 Analysis of the Aiptasia gene set identified a DNMT3 gene (AIPGENE24404) that also
115 encodes a PWWP domain as reported for the mouse homolog. In order to test if the mechanism
116 previously described in mice is conserved in Aiptasia, we performed a ChIP-Seq experiment
117 using a validated antibody against H3K36me3 (Fig. S4-S5). As predicted, our analysis confirmed
118 a significantly higher association of H3K36me3 with methylated genes ($p = 2.48 \times 10^{-20}$ for
119 highly methylated genes and all methylated genes, Fig. 2B and C) suggesting that DNA
120 methylation in Aiptasia might indeed be a consequence of expression. We then analyzed if
121 methylated genes also exhibited significantly lower levels of spurious transcription in Aiptasia.

122 Analysis of transcriptional profiles of methylated and unmethylated genes indeed showed
123 significantly lower levels of spurious transcription along the gene body of methylated genes ($p <$
124 2×10^{-6}, Fig. 3A).

125 A dampening effect of DNA methylation on transcription was also observed with regard
126 to transcriptional noise similar to findings in the coral *Stylophora pistillata* (Liew et al. 2017).
127 Regression analysis of median methylation levels and the coefficient of transcriptional variation
128 of genes showed that, given the same expression level, methylated genes always exhibited lower
129 levels of transcriptional variation (Fig. 3B).

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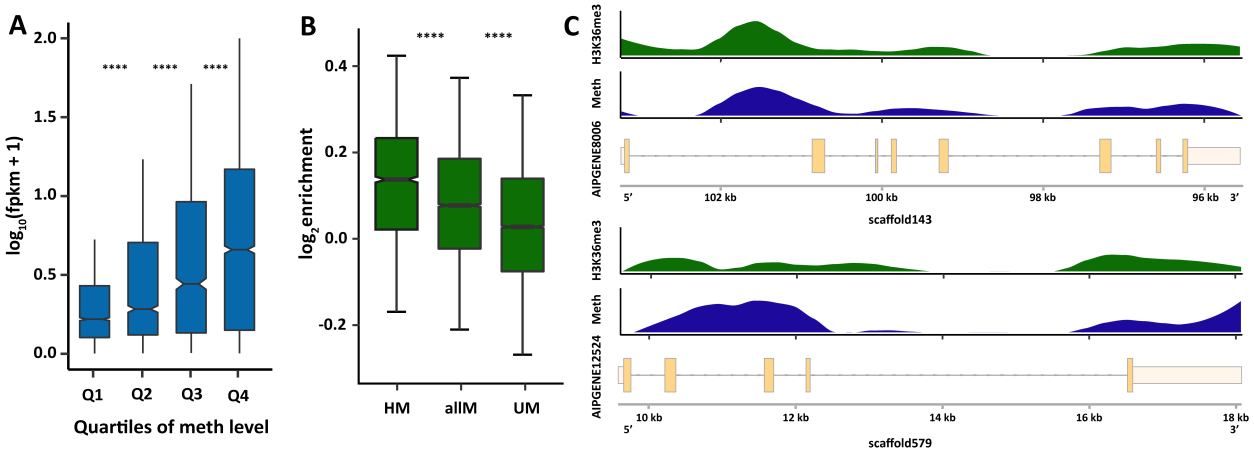

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132 **Fig. 2. DNA methylation is associated with higher expression**

133 (**A**) Gene expression is positively correlated with median methylation levels, *t*-test *p* values are
134 7.65×10^{-21}, 3.75×10^{-14} and 1.75×10^{-13} for the first quartile (Q1) and the second quartile (Q2)
135 of methylation levels, Q2 and Q3, and Q3 and Q4, respectively. (**B**) ChIP-Seq analysis of
136 H3K36me3 signals show significant enrichment in methylated genes (*t*-test *p* values: 2.48×10^{-20}
137 for highly methylated genes (HM) and all methylated genes (allM), and 1.06×10^{-72} for
138 unmethylated genes (UM) and allM). Highly methylated genes show the strongest enrichment

139 with H3K36me3 followed by all methylated genes. In contrast unmethylated genes show only
140 weak enrichment of H3K36me3 over input controls. (**C**) Distribution of H3K36me3 enrichment
141 and DNA methylation levels across two exemplary gene models. H3K36me3 and DNA
142 methylation show coinciding distribution patterns over genes.
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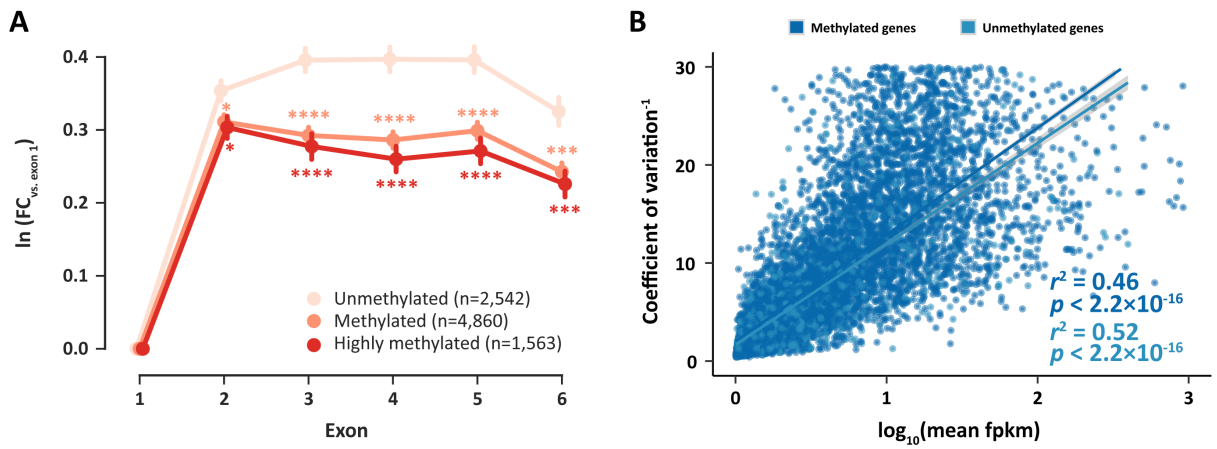

145

146 **Fig. 3. DNA methylation regulates transcriptional homeostasis**

147 (A) Spurious transcription in gene bodies is significantly lower in methylated and highly
148 methylated genes. The *y*-axis shows the natural logarithm of the coverage fold change of exons
149 1–6 vs. exon 1. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. (B) There is a linear
150 relationship between the inverse of transcriptional noise (CV^{-1}) and log expression level
151 ($\log_{10}$fpkm). Given same expression level, methylated genes always show lower levels of
152 transcriptional noise. For methylated genes, n = 8,561, $r^2 = 0.46$, $p < 2.2 \times 10^{-16}$, for
153 unmethylated genes, n = 2,491, $r^2 = 0.52$, $p < 2.2 \times 10^{-16}$.
154
155

156 **DNA methylation regulates transcriptional homeostasis during symbiosis**

157 Based on our previous findings, we investigated if DNA methylation might also be
158 involved in the regulation of symbiosis by identifying differentially methylated genes (DMGs)
159 between symbiotic and aposymbiotic Aiptasia. Comparison of DNA methylation patterns using
160 Principal Component Analysis (PCA) clearly separated symbiotic and aposymbiotic individuals
161 by the first principal component, which accounted for ~18% of the variance (Fig. 4 and Fig. S6).
162 This analysis echoed the findings from a PCA analysis on gene expression where symbiosis state
163 was separated by the second principal component accounting for ~25% of the variance (Fig. 4B)
164 (Venables and Ripley 2002) and highlighted that specific changes in DNA methylation patterns
165 occurred in response to symbiosis. Analysis of DNA methylation and expression profiles using
166 correlation analyses further confirmed this finding, providing additional evidence that the
167 observed changes were indeed treatment specific (Fig. S6).

168 Subsequently we analyzed changes in DNA methylation and gene expression between
169 symbiotic and aposymbiotic Aiptasia to assess their correlation on potential biological functions
170 in symbiosis. We determined differentially methylated genes using a generalized linear model
171 from Foret *et al*. (Foret et al. 2012) that was modified to allow for replicate-aware analysis. This
172 approach identified 2,133 DMGs (FDR ≤ 0.05, Supplement Table S1) that specifically changed
173 their methylation status in response to symbiosis. To verify these results, we sequenced a subset
174 of 14 DMGs using bisulfite PCRs. The results show a strong correlation ($r^2 = 0.815$ and $p =$
175 1×10^{-5} for Apo, $r^2 = 0.922$ and $p = 5.2 \times 10^{-8}$ for Sym) to our WGBS and confirm the observed
176 methylation changes within these loci (Fig. S7).

177 Analysis of gene expression changes in the same 12 samples (i.e., 6 symbiotic and 6

178 aposymbiotic anemones) identified 1,278 differentially expressed genes (DEGs, FDR ≤ 0.05,
179 Supplement Table S2), of which 14 genes were subsequently confirmed via qPCR (Fig. S8).
180 However, analysis of the overlap between DMGs and DEGs showed only 103 genes that were
181 shared, suggesting that differentially expressed genes are not necessarily the same cohort of
182 genes that are differentially methylated. Functional enrichment analyses based on Gene Ontology
183 (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of all DMGs and
184 DEGs identified several symbiosis relevant functions and pathways in both groups (Supplement
185 Table S3-S10).

186 Based on the finding that gene body DNA methylation is likely a consequence of active
187 transcription, we hypothesized that changes in DNA methylation patterns might also provide a
188 record of transcriptional activity over longer periods of time. We therefore tested if differential
189 methylation and acute transcriptional changes, obtained from our RNA-Seq analysis, provide a
190 complementary view of the processes underlying symbiosis. For this we compared enrichment of
191 symbiosis-specific pathways across the sets of 2,133 DMGs, 1,278 DEGs, and the combined set
192 of both DMGs and DEGs (3,308 DEMGs). Interestingly, we observed that the combined data set
193 (DEMGs) provided significantly lower p-values for previously identified symbiosis-related
194 pathways, including apoptosis, phagosome formation, nitrogen metabolism, and arginine
195 biosynthesis, among others (paired *t*-test: DEMG vs. DEG $p = 0.015$; DEMG vs. DMG $p = 0.009$)
196 (Fig. 4C and Supplement Table S11). This suggested that changes in methylation and
197 transcription indeed provide complementary information with regard to transcriptional
198 adjustments in response to symbiosis.

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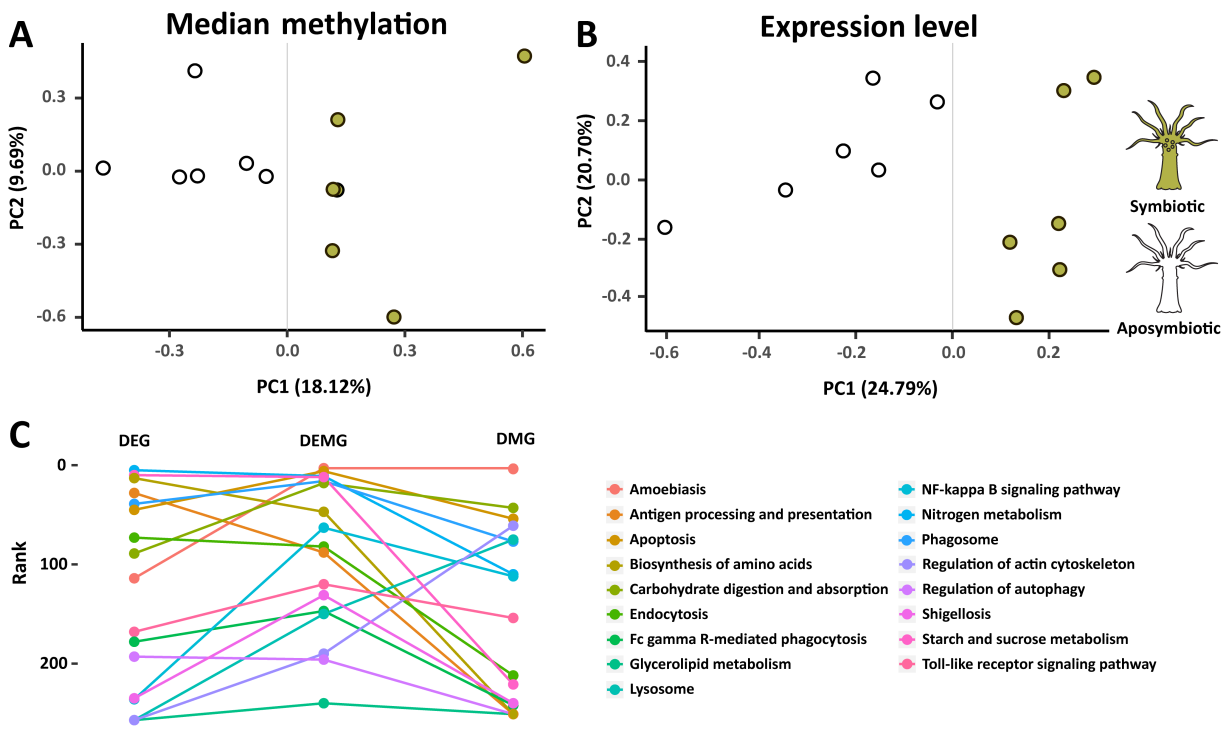

Fig.4. PCA and KEGG pathway enrichment analysis

(**A**, **B**) PCA (Principal Component Analysis) of gene expression and median methylation levels of Aiptasia genes. Both gene expression and DNA methylation separate samples by symbiosis state. (**C**) KEGG pathway enrichment analysis. The combined sets of differentially expressed and differentially methylated genes (DEMG) provides significant lower *p* values (front ranks) for symbiosis related pathways.

DMGs and DEGs are involved in all stages of symbiosis

Analysis of the combined DMG and DEG gene set showed significant enrichment of genes involved in the distinct phases of symbiosis, that is symbiosis establishment, maintenance, and breakdown (Davy et al. 2012). Using an integrated pathway analysis based on known

212 molecular interactions between proteins we found that these processes are linked through several
213 DMGs and/or DEGs (Fig. S9 and Fig. S10, and see Supplement Table S11-S12 and
214 Supplementary discussion).

215 For instance, we found numerous symbiosis-related receptors to respond to symbiosis on
216 a transcriptional and/or methylation level (Fig. S9), including C-type lectins (Fig. S9.3), Toll-like
217 receptors (Fig. S9.5), and the scavenger receptor SRB1 (Fig. S9.2) that has previously been
218 implicated in symbiont recognition in the sea anemone *Anthopleura elegantissima* (Rodriguez-
219 Lanetty et al. 2006; Neubauer et al. 2016). Following symbiont recognition, we also found
220 several known engulfment and sorting-related genes to change in methylation and/or expression
221 such as Rab5 (Fig. S9.10), sorting nexin (Fig. S9.17), Rac1 (Fig. S9.6), the lysosomal-associated
222 membrane protein 1/2 (Fig. S9.22), and many genes related to the cytoskeleton and movement
223 (Fig. S9.33-39).

224 As expected in a metabolic symbiosis (Muscatine 1990; Davy et al. 2012) we also identified a
225 large number of genes involved in nutrient exchange. These included genes involved in the
226 provision of inorganic carbon in the form of CO_2 or bicarbonate (HCO_3^-) to fuel symbiont driven
227 photosynthesis (Rädecker et al. 2017) (Fig. S10.1) as well as genes involved in the exchange of
228 fixed carbon in the form of lipids (Fig. S10.11), sugars and amino acids (Fig. S10.10, S10.4)
229 (Oakley et al. 2016). Concordantly, we also found that genes involved in nitrogen acquisition,
230 such as ammonium transporter (Fig. S10.2) and genes involved in glutamate metabolism (Fig.
231 S10.5-7), respond to symbiosis.

232 Finally, our analysis also highlighted genes putatively involved in the expulsion or
233 degradation of symbionts in response to environmental stress or as a means to control symbiont

234 densities. Autophagy is of interest in this regard because it links to other membrane trafficking
235 pathways and to apoptosis, and evidence suggests that autophagy also plays a role in removal of
236 symbionts during bleaching (Dunn et al. 2007; Downs et al. 2009). Intracellular degradation of
237 the symbiont is a result of reengagement of the phagosomal maturation process or autophagic
238 digestion of the symbiont by the host cell (Davy et al. 2012), and we find both apoptosis- and
239 autophagy-related genes to significantly change in their methylation and/or expression level.
240 These include the apoptosis genes RAC serine/threonine-protein kinase (Fig. S9.25), Caspase 7
241 (Fig. S9.31), Caspase 8 (CASP8) (Fig. S9.30), Nitric oxide synthase (Fig. S9.21) and Bcl2 (Fig.
242 S9.27), as well as the Autophagy proteins 5 and 10 (Fig. S9.14-15), among others.

243

244 **Discussion**

245 To assess the role of CpG methylation in the cnidarian-dinoflagellate symbiosis, we
246 undertook a global analysis of changes in the DNA methylomes and transcriptomes of
247 aposymbiotic and symbiotic Aiptasia. In contrast to their vertebrate counterparts, only 6.37% of
248 the CpGs in the Aiptasia genome are methylated, but their distribution is highly non-random ($p <$
249 3×10^{-300}) and that methylated CpGs are most highly localized in gene bodies (18.4% of CpGs).
250 Analysis of the distribution of the histone modification H3K36me3 further showed significant
251 enrichment of this epigenetic mark in methylated genes, echoing findings in mammals and
252 invertebrates (Nanty et al. 2011). More importantly, we find that methylated genes show
253 significant reduction of spurious transcription and transcriptional noise (Fig. 2B), suggesting that
254 both the underlying mechanism of epigenetic crosstalk as well as the biological function of DNA
255 methylation is evolutionarily conserved throughout metazoans. These results highlight a tight
256 interaction of transcription and epigenetic mechanisms in optimizing gene expression in response

257 to changing transcriptional needs (Neri et al. 2017). Further support for such a role is provided
258 by the analysis of differentially methylated and differentially expressed genes, which, when
259 combined, showed significant increase in enrichment of symbiosis relevant processes. This
260 suggests that DNA methylation and transcriptome analyses provide complementary views of
261 cellular responses to symbiosis whereby methylation changes provide a transcriptional record of
262 longer-term transcriptional adjustments.

263 While our analysis identified several genes, processes, and pathways previously reported
264 to be involved in symbiosis, it further highlights their intricate molecular interactions. Symbiosis
265 recognition, sorting and breakdown are interconnected processes, which is reflected in the
266 observed changes in methylation and expression. The molecular machinery involved in
267 phagosome maturation is tightly linked to autophagy and apoptosis enabling the host to respond
268 to potential pathogen invasion but also to degrade and remove dead or unsuitable symbionts.
269 This is strongly supported by immunofluorescence examinations of *Aiptasia pulchella*
270 gastrodermal cell macerates, showing that Rab5 appears around healthy, newly ingested and
271 already established *Symbiodinium*, but is replaced by Rab7 in heat-killed or DCMU-treated
272 newly ingested *Symbiodinium*. Conversely, Rab7 is absent from untreated newly infected or
273 already-established *Symbiodinium* (Chen et al. 2003; Chen et al. 2004).

274 Rab5 is also required for the exosomal release of CD63 (Baietti et al. 2012), which
275 mediates the endocytotic sorting process and transport to lysosomes (Latysheva et al. 2006). This
276 process is further regulated by Rac1 (Anitei et al. 2010) in conjunction with sorting nexin and the
277 GTPase Rho, all of which were also identified in our analyses. The sorting of phagocytosed
278 *Symbiodinium* is critical to symbiosis establishment as *Symbiodinium* cells are phagocytosed at
279 the apical end and transported to the base of the cell, where they are protected from digestion. In

280 contrast, *Symbiodinium* staying at the apical end of the cell are degraded (McAuley and Smith
281 1982).

282 Similar to the processes of symbiosis initiation and breakdown, we also found significant
283 enrichment of genes involved in nutrient exchange and many of these transporters have
284 previously been implicated in symbiosis maintenance (Davy et al. 2012; Lin et al. 2015).
285 Notably, this also included genes involved in the transport and assimilation of ammonium.
286 Nitrogen is a main limiting nutrient in coral reefs (Cook et al. 1992; Grover et al. 2008; Radecker
287 et al. 2015), and the coral-dinoflagellates symbiosis has been proposed to increase the efficiency
288 of nitrogen utilization by both partners (Wang and Douglas 1998b) whereby the underlying
289 nature of this mechanism is currently debated (Wang and Douglas 1998a; Aranda et al. 2016).

290

291 **Conclusions**

292 This study provides the first analysis of the function and role of DNA methylation in a symbiotic
293 anthozoan. Our results show that the epigenetic crosstalk between the histone mark H3K36me3
294 and gene body methylation is conserved in cnidarians and reveal a role of gene body methylation
295 in reducing of spurious transcription and transcriptional noise. Furthermore, we show that
296 changes in DNA methylation patterns are specific to symbiosis and imply a functional in the
297 establishment, maintenance, and breakdown of this important symbiotic association. Our
298 findings therefore provide evidence for a role of DNA methylation as an epigenetic mechanism
299 involved in the maintenance of transcriptional homeostasis during the cnidarian-dinoflagellate
300 symbiosis. The premise that epigenetic mechanisms play a role in organismal acclimation
301 warrants future experiments targeted to investigate if DNA methylation could also contribute to

302 resilience through the epigenetic adjustment of transcription in response to environmental stress
303 in Aiptasia and corals.
304

305 **Data availability**

306 Sequencing data of Bis-Seq, RNA-Seq and ChIP-Seq were deposited in NCBI Sequence Read
307 Archive (SRA) under BioProject codes PRJNA415358.
308

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314

315 **Author contributions**

316 M.A. conceived and coordinated the project. Y.L., G.C., M.J.C. and N.Z. performed
317 experiments. M.A., C.R.V. and Y.J.L. provided tools and/or data. C.T.M. constructed libraries
318 for whole genome bisulfite sequencing, ChIP-Seq and RNA-Seq. Y.L., Y.J.L. and M.A. analyzed
319 expression, methylation and ChIP-Seq data. M.A. and Y.L. wrote the manuscript with input from
320 Y.J.L. and C.R.V. All authors read and approved the final manuscript.
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411

412 Supplementary Materials for

413

414 DNA methylation regulates transcriptional homeostasis of algal endosymbiosis 415 in the coral model Aiptasia

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422

423 Materials and methods

424 *Exaiptasia pallida* Culture and DNA/RNA Extraction

425 *Exaiptasia pallida* of the clonal strain CC7 (Sunagawa et al. 2009) was used for this study. 426 Anemones were maintained in polycarbonate tubs with autoclaved seawater at ~25 °C on a 12 h: 427 12 h light: dark cycle at 20-40 µmol m^{-2} s^{-1} light intensity and fed freshly hatched *Artemia* 428 *nauplii* (brine-shrimp) approximately twice per week. To generate aposymbiotic anemones, 429 animals were subjected to multiple cycles of cold-shock treatment and the photosynthesis 430 inhibitor diuron (Sigma-Aldrich, St. Louis, MO) as described in Baumgarten (2015) 431 (Baumgarten et al. 2015). Aposymbiotic anemones were kept individually in 15 ml autoclaved

432 seawater in 6-well plates and inspected by fluorescence stereomicroscopy to confirm complete
433 absence of dinoflagellates. In order to exclude potential batch effects as source of DNA
434 methylation changes we first generated four separate batches of aposymbiotic anemones and
435 maintained them for a period of 1 year before beginning of the experiment described below.

436 To generate symbiotic anemones, we then separately infected aposymbiotic CC7 individuals
437 from each of the four aposymbiotic cultures described above using the compatible Clade B
438 *Symbiodinium* strain SSB01, originally isolated from Aiptasia strain H2 (Xiang et al. 2013;
439 Baumgarten et al. 2015). The four batches of symbiotic anemones were maintained for further 12
440 months under regular culture condition as described above. The corresponding four aposymbiotic
441 cultures were maintained in darkness until 3 months before collection. For the last 3 months
442 individuals from these aposymbiotic cultures were subjected to the same culture conditions as
443 the symbiotic cultures in order to monitor for unwanted spontaneous re-establishment of
444 symbiosis under light.

445 After the 12-month experimental period, we collected six biological replicates from each of
446 the four aposymbiotic and symbiotic cultures (one additional replicate was taken from batches 1
447 and 2 of each treatment) for subsequent DNA and RNA extraction as described below.

448 For each treatment, 6 biological replicates, weighing 20-28 mg (wet weight), were extracted
449 using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). The
450 manufacturer's protocol was followed with the omission of the optional step 4 (temporal storage
451 at 4°C if not performing DNA purification immediately). DNA concentrations were determined
452 using a Qbit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). RNA
453 concentrations and integrity were determined using a Bioanalyzer Nano RNA Kit (Agilent
454 Technologies, Santa Clara, CA).

455

456 **RNA-Seq and Bisulfite Sequencing**

457 Directional mRNA libraries were produced using the NEBNext® Ultra™ Directional RNA
458 Library Prep Kit for Illumina® (NEB) following the manufacturer's protocol.

459 Bisulfite DNA libraries were prepared following a modified version of the NEBNext®
460 Ultra™ II DNA Library Prep Kit for Illumina® (NEB). Methylated TruSeq Illumina® adapters
461 (Illumina) were used during the adapter ligation step followed by bisulfite conversion with the
462 EpiTect Bisulfite kit (QIAGEN), with the following cycling conditions (95°C – 5 min, 60°C – 25
463 min, 95°C – 5 min, 60°C – 85 min, 95°C – 5 min, 60°C – 175 min, and 3 cycles of 95°C – 5 min,
464 60°C – 180 min, hold at 20°C ≤ 5 hours) (reference is Illumina Bisulfite).

465 The final libraries were enriched with the KAPA HiFi HotStart Uracil+ ReadyMix (2X)
466 (KAPA Biosystems) following the standard protocol for bisulfite-converted NGS library
467 amplification. Final libraries were quality checked using the Bioanalyzer DNA 1K chip
468 (Agilent), and quantified using Qubit 2.0 (Thermo Fisher Scientific), and then pooled in
469 equimolar ratios and sequenced on the HiSeq2000.

470

471 **Identification of methylated CpGs**

472 Sequencing of the 12 libraries (2 conditions, 6 biological replicates each) resulted in 819
473 million read pairs from 8 lanes of the Illumina HiSeq2000 platform. Adapters were trimmed
474 from the raw sequences using cutadapt v1.8 (Martin 2011). Subsequently, trimmed reads were
475 mapped to the *Exaiptasia pallida* genome (Baumgarten et al. 2015) using Bowtie2 v2.2.3
476 (Langmead and Salzberg 2012), and methylation calls was performed using Bismark v0.13

477 (Krueger and Andrews 2011).

478 Three filters were used to reduce false positives. Firstly, for each position with k methylated
479 reads mapping to it, the probability of it occurring through sequencing error (i.e. unmethylated
480 position appearing as methylated) was modelled using a binomial distribution $B(n, p)$, where n is
481 the coverage (methylated + unmethylated reads) and p the probability of sequencing error (set to
482 0.01). We kept positions with k methylated reads if $P(X \geq k) < 0.05$ (post-FDR correction).
483 Secondly, retained methylated positions had to have ≥ 1 methylated read in all six biological
484 replicates of at least one growth condition. Finally, median coverage of retained positions across
485 all 12 samples had to be ≥ 10.

486

487 **Assignment of genomic context to methylated cytosines**

488 Based on the gene annotation of the *Exaiptasia pallida* genome (GFF3 file) (Baumgarten et
489 al. 2015) and the positional coordinates of the methylated cytosines produced by Bismark, we
490 annotated every methylated cytosine based on the genomic context, including whether the
491 methylated position resides in a genic or intergenic region, and the distances to the 5' and 3' end
492 of each genomic feature (gene/intergenic region/exon/intron).

493

494 **CpG bias**

495 Methylated cytosines are frequently spontaneously deaminated to uracil which can be
496 subsequently converted to thymine after DNA repair. As a result of this process, methylated
497 CpGs are expected to decrease in abundance over evolutionary time, and the ratio of observed to
498 expected CpGs (CpG O/E) has previously been used to predict putatively methylated and

499 unmethylated genes (Suzuki et al. 2007; Wang and Leung 2008). CpG O/E of *Exaiptasia pallida*
500 protein coding genes were calculated according to J. Zeng *et al* (Zeng and Yi 2010).

501

502 **Identification of differentially methylated genes**

503 Using the methylation level of aposymbiotic genes as a control, generalized linear models
504 (GLMs) (Hastie and Pregibon 1992) were implemented in R (R Core Team 2016) to identify
505 genes that were differentially methylated in the symbiotic treatment. The general formula used
506 was:

507 glm(methylated, non_methylated ~ treatment * position, family="binomial")

508 where "methylated, non_methylated" was a two-column response variable denoting the
509 number of methylated and non-methylated reads at a particular position. For predictor variables,
510 "position" denoted relative position of the methylated site in the gene, while "treatment" denoted
511 symbiotic or aposymbiotic conditions. Data from individual replicates were entered separately to
512 assign equal weightage to each replicate, as pooling results in a disproportionate skew towards
513 the replicate with the highest coverage. Genes with < 5 methylated positions were filtered out to
514 reduce type I errors; and genes with FDR ≤ 0.05 were considered as differentially methylated
515 genes (DMGs).

516

517 **Identification of differentially expressed genes**

518 RNA-Seq generated 889 million raw read pairs from six lanes on the Illumina Hiseq2000
519 platform. Adaptors, primers and low quality bases were removed from the ends of raw reads
520 using Trimmomatic v0.33 (ILLUMINACLIP:TruSeq2-PE.fa:4:25:9 LEADING:28

521 TRAILING:28 SLIDINGWINDOW:4:30 MINLEN:50). The resulting trimmed reads were
522 mapped to the *Exaiptasia pallida* genome using HISAT v2.0.1 (Kim et al. 2015) and transcripts
523 were assembled based on the *Exaiptasia pallida* gene models (GFF3 file) using StringTie v1.2.2
524 (Pertea et al. 2015). Trinity (align_and_estimate_abundance.pl – Bowtie2 v2.2.7/RSEM
525 v1.2.22/edgeR v3.10.5) (Robinson et al. 2010; Grabherr et al. 2011; Li and Dewey 2011;
526 Langmead and Salzberg 2012; Haas et al. 2013) was run against the transcripts using trimmed
527 reads for expression abundance estimation, then differentially expressed genes (DEGs) were
528 identified with FDR $\leq$ 0.05.
529
530 **PCA and correlation matrices**
531 Median methylation levels and log FPKM (base 2) of genes were shifted to be zero
532 centered and analyzed by Principal Component Analysis (PCA) using the prcomp function in R.
533 Using the same data we calculated correlation matrices (Pearson correlation coefficient)
534 and clustered samples with hclust implemented in R using complete linkage and euclidean
535 distance .
536
537
538 **Spurious transcription analysis**
539 Trimmed reads were mapped to the *Exaiptasia pallida* genome using HISAT2 v2.1.0 and
540 mapping coverage per position was extracted using BEDtools v2.17.0. Coverage per exon was
541 calculated and normalized across all 6 replicates (assuming every replicate had 1 million
542 coverage in total), then average coverage ratios of exon 2 to 6 versus exon 1 per gene were
543 calculated to determine spurious transcription levels.

544

545 **GO enrichment of DMGs and DEGs**

546 GO (Gene Ontology) (Ashburner et al. 2000) annotation was based on the previously
547 published *Exaiptasia pallida* genome (Baumgarten et al. 2015). Functional enrichment of DMGs
548 and DEGs was carried out with topGO respectively (Adrian Alexa 2016) using default settings.
549 GO terms with $p \leq 0.05$ were considered significant, and the occurrence of at least ≥ 5 times in
550 the background set was additionally required for DMGs. Multiple testing correction was not
551 applied to the resulting *p*-values as the tests are considered to be non-independent (Adrian Alexa
552 2016).

553

554 **KEGG enrichment of DMGs and DEGs**

555 KEGG (Kyoto Encyclopedia of Genes and Genomes)(Kanehisa and Goto 2000; Kanehisa et
556 al. 2016) orthology (KO) annotation was carried out by combining the KEGG annotations
557 provided in the original *Exaiptasia pallida* genome publications and a separate set of annotations
558 based on the KAAS (KEGG Automatic Annotation Server, http://www.genome.jp/tools/kaas/)
559 (parameters: GHOSTZ, Eukaryotes, Bi-directional Best Hit) (Moriya et al. 2007). A KEGG
560 pathway enrichment analysis of both DMGs and DEGs was carried out using Fisher's exact test
561 and pathways with $p \leq 0.05$ were considered significant.

562

563 **Validation of gene expression changes from RNA-Seq by qPCR**

564 Three randomly picked RNA libraries per treatment were used for qPCR validation of
565 RNA-Seq results. cDNA was synthesized using Invitrogen SuperScript III First-Strand Synthesis
566 SuperMix kit. A total of 14 genes were validated for differential expression using qPCR
567 (Supplement Table S13-S15). RPS7, RPL11 and NDH5 were used as internal reference
568 standards (Lehnert et al. 2014). qPCR was carried out using Invitrogen Platinum SYBR Green
569 qPCR SuperMix-UDG kit on Applied Biosystems 7900HT Fast Real-Time PCR System. All
570 protocols were strictly followed.

571

572 **Validation of methylation changes using bisulfite PCR**

573 Three randomly picked DNA libraries per treatment were used for methylation validation.
574 Bisulfite conversion was done using the EZ-96 DNA Methylation-Gold Kit (Zymo Research). 18
575 genes were used to design primers, 14 of 18 obtained effective amplifications (Supplement Table
576 S16), then the fragments were enriched by PCR amplification using Promega PCR Master Mix.
577 Sequencing indices were added to enriched fragments using Illumina Nextera XT Index Kit.
578 Enriched fragments were sequenced on the Illumina MiSeq platform. All protocols were strictly
579 followed. 1,870x data per replicate were obtained, methylated CpGs were identified using
580 Bismark as described above. The correlations between whole genome bisulfite conversion and
581 bisulfite PCR were calculated using generalized linear model.

582

583 **Chromatin Immunoprecipitation – ChIP**

584 We used the Zymo-Spin ChIP Kit to conduct histone bound chromatin extraction, with
585 minor adjustments to manufacturer's protocol. Briefly, three biological replicates, each

586 consisting of two symbiotic anemones, were used for this experiment. Each anemone was first
587 washed with PBST (phosphate-buffered saline with 0.1% triton). Anemones were then fixed in
588 1X PBS with 1% formaldehyde for 15 minutes. To stop cross-linking reactions glycine was
589 added to the solution and left to rest for 10 more minutes. Following manufacturer's protocol, we
590 centrifuged and washed whole anemones. We prepared the Nuclei Prep Buffer according to
591 protocol and crushed the two anemones of each replicate together using a douncer for
592 homogenization. Samples were then sonicated for 15 cycles on ice (15 sec ON, 30 sec cooling)
593 to ensure fragmentation to 200-500 bp. Thereafter the protocol was followed without further
594 modifications.

595 Immunoprecipitation was achieved using a target specific antibody to histone 3 lysine 36
596 tri-methylation (H3K36me3) (ab9050, Abcam), which has been validated in many eukaryotic
597 species, including mouse(Soboleva et al. 2017), *Arabidopsis thaliana*(Wollmann et al. 2017),
598 yeast(Janke et al. 2017) and zebrafish(Vastenhouw et al. 2010; Wu et al. 2011) *et al*. Comparison
599 of Aiptasia histone H3 to the respective homologs from several species for which this antibody
600 has been previously validated showed high conservation of the N-terminal tail containing
601 position H3K36 (Fig. S4) whereby 100% conservation to the zebrafish homolog was observed.

602 Corresponding input controls for each of the 3 replicates were generated as suggested by
603 the manufacturer. DNA fragment quality and quantity were confirmed using High Sensitivity
604 DNA Reagents (Agilent Technologies, California, United States) on a bioanalyzer, after which
605 ChIP libraries were constructed using the NEBNext® ChIP-Seq Library Prep Master Mix Set
606 (#E7645, New England Biolabs, Massachusetts, United States).

607 Sequencing resulted in 10M read pairs per replicate. These read pairs were trimmed using
608 Trimmomatic and mapped to the *Exaiptasia pallida* genome using HISAT2 as described above.

609 H3K36me3 enrichments were calculated as $\log_2$(average signal/average input control) for all
610 genes, unmethylated genes and highly methylated genes (methylation level > 70 and methylation
611 density > 40). P-values were calculated using t-test.
612

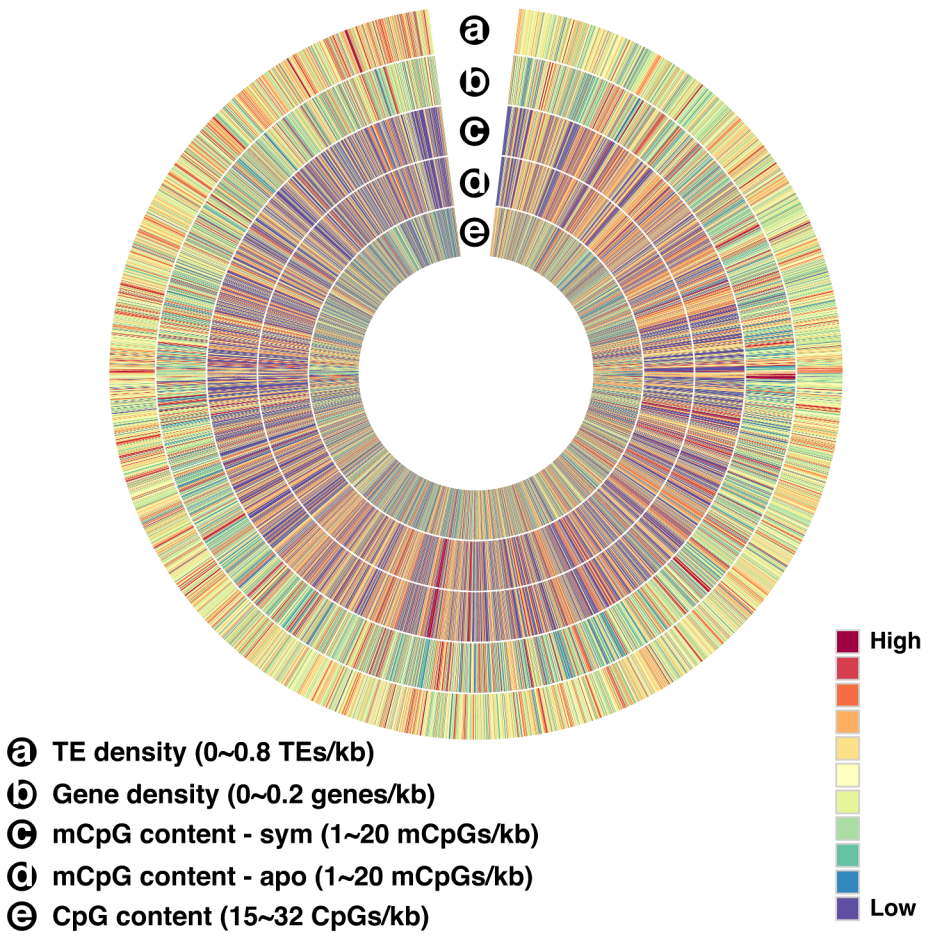
613 **Antibody affinity validation through Western blotting**

614 Total protein was extracted from a snap-frozen anemone crushed in 10% TCA
615 (Trichloroacetic acid). The homogenized sample was left to incubate overnight at -20 °C to allow
616 proteins to precipitate. The solution was centrifuged at 20,000g at 4 °C for 20 minutes to collect
617 suspended proteins. The pellet was then washed three times in 80% acetone and then spun down
618 again as previously. The final pellet was then air-dried for 10-15 minutes to remove residual
619 acetone. The final protein was suspended in urea lysis buffer (7 M urea, 2 M thiourea, pH 7.5) by
620 vortexing for 2 hours.

621 Samples were then prepared for western blot by adding 4x sampling buffer (0.38 M Tris
622 base, 8% SDS, 4mM EDTA, 40% glycerol, 4mg/ml bromphenol blue) to a final concentration of
623 1X. After a 2 minute incubation at 90°C samples were ready to be run on a gel at 10-12 mA. The
624 gel was transferred to a PVDF nitrocellulose membrane, rinsed with TBS buffer (150mM NaCl,
625 25mM Tris pH7.4, 0.1% Triton X-100) and blocked for 30 min at RT in TBS containing 5% fat-
626 free powder milk. The primary antibody was diluted in TBS/milk and incubated on an undulating
627 orbital shaker overnight at 4 °C. After three washes in TBS for 10 minutes each, the membrane
628 was again blocked in TBS/milk for 20 minutes at RT before proceeding with secondary antibody
629 staining. The horseradish peroxidase-linked-antibody (Anti-Rabbit IgG HRP conjugate W4011
630 and Anti-Mouse IgG HRP conjugate W4031, Promega. Wisconsin, United States) was diluted in

TBS/milk (1:10000) and incubated for 2 hours at RT. After final triplicate 10 minute washes in TBS, membranes were developed.

Supplementary Figures



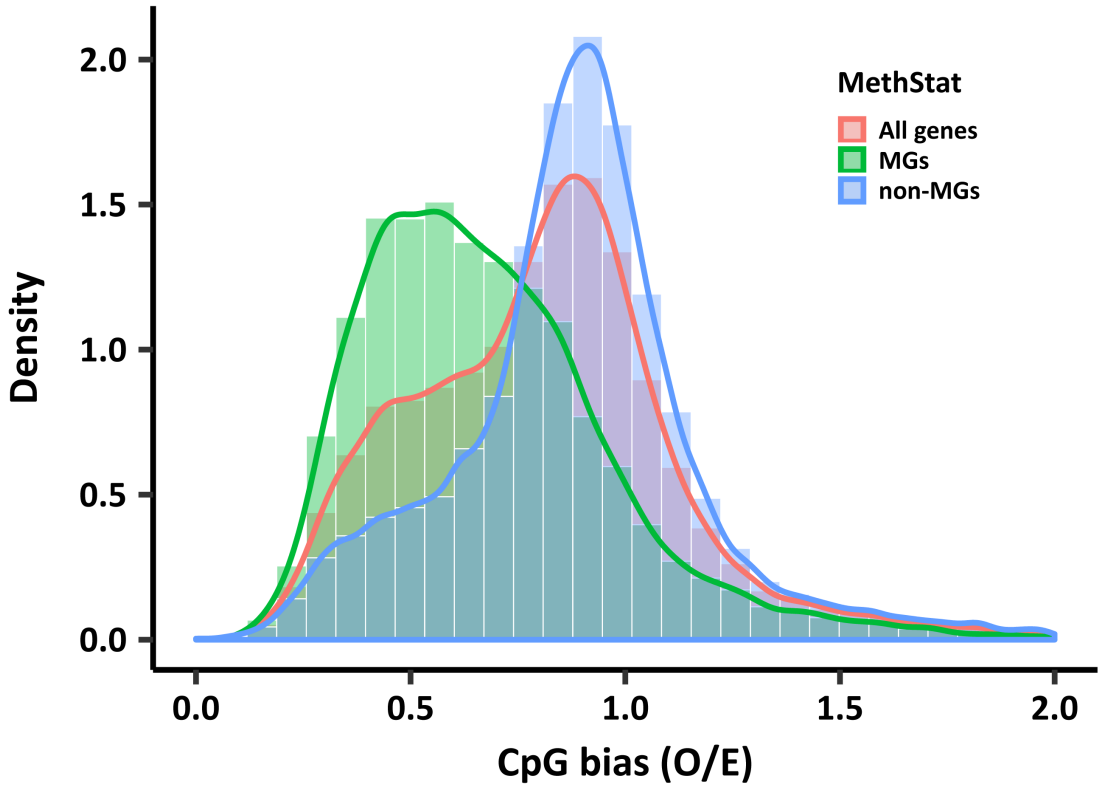
638 **Fig. S1. Circos visualization of different data at the genome-wide level**

639 (**a**) TE density. (**b**) Gene density. (**c**) Fraction of methylated CpGs in symbiotic treatment. (**d**)

640 Fraction of methylated CpGs in aposymbiotic treatment. (**e**) CpG content.

641

642



643

644 **Fig. S2. Methylated genes in Aiptasia have lower CpG O/E**

645 CpG distribution of methylated genes (represented by red curve) peaks at around 0.5, which is

646 lower than in unmethylated genes (represented by green curve) peaking at around 0.9. mC to T

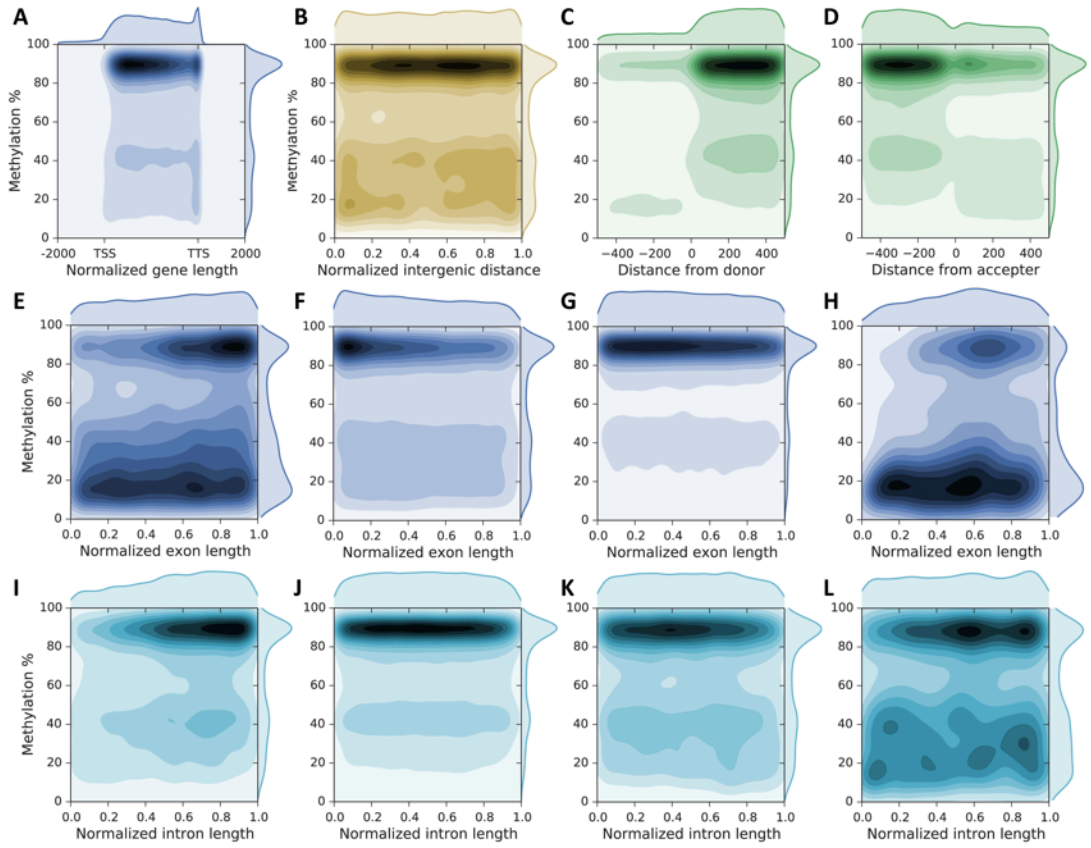
647 conversion skews the CpG O/E distribution of all genes as expected (represented by blue curve),

648 but methylated and unmethylated genes still show a large overlap of their CpG O/E distributions.

649 These results indicate that gene body methylation cannot be accurately inferred from CpG O/E in

650 Aiptasia.

651



652

653 **Fig. S3. Methylation patterns**

654 (**A**) DNA methylation is mainly located in the proximal part of gene bodies with slightly

655 decreasing levels towards the end. (**B**) Methylation pattern over intergenic regions. (**C**)

656 Methylation pattern around splice donor sites show increasing levels immediately after donor

657 sites. (**D**) Methylation pattern around acceptor sites show decreasing levels immediately after

658 splice acceptor sites. (**E**) Methylation pattern over initial exons show increasing methylation

659 levels (3,147 exons with 35,885 methylation sites were used). (**F**) Methylation pattern over

660 internal exons show decreasing methylation levels (7,977 exons with 139,009 methylation sites

661 were used). (**G**) Methylation pattern over terminal exons show decreasing methylation levels
662 (7,905 exons with 102,162 methylation sites were used). (**H**) Methylation pattern over introns
663 from single-exon genes follow a similar trend as observed for multi exon genes with increasing
664 methylation levels in the proximal and decreasing levels in the posterior part of the exon (298
665 exons with 4,735 methylation sites were used). (**I**) Methylation pattern over initial introns show
666 increasing methylation levels (3,381 introns with 39,262 methylation sites were used). (**J**)
667 Methylation pattern over internal introns maintain stable methylation levels (7,371 introns with
668 211,950 methylation sites were used). (**K**) Methylation levels over terminal introns decrease
669 slightly (3,959 introns with 34,246 methylation sites were used). (**L**) Methylation levels over
670 introns from one-intron genes change gently with initial increase followed by a decrease (1,055
671 introns with 10,709 methylation sites).
672

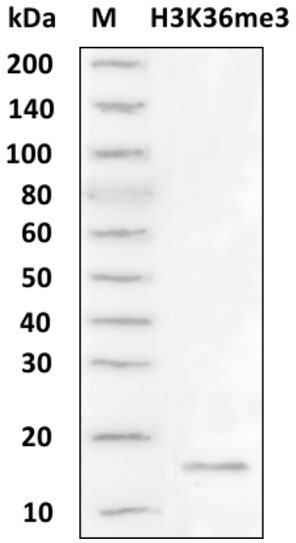
```
                            **********************:********:*********::*******
aiptasia_AIPGENE3063        MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALR    50
zebrafish_XP_002666951.1    MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALR    50
mouse_AAA37813.1            MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALR    50
human_CAB02546.1            MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALR    50
Arabidopsis_AAA32809.1      MARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRFRPGTVALR    50
yeast_PJP07865.1            MARTKQTARKSTGGKAPRKQLASKAARKSAPSTGGVKKPHRYKPGTVALR    50
                            1.......10........20........30........40........50

                            ***::***********************************: ****: **
aiptasia_AIPGENE3063        EIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAY   100
zebrafish_XP_002666951.1    EIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAY   100
mouse_AAA37813.1            EIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEACEAY   100
human_CAB02546.1            EIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEACEAT   100
Arabidopsis_AAA32809.1      EIRKYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVAALQEAAEAY   100
yeast_PJP07865.1            EIRRFQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAIGALQESVEAY   100
                            ........60........70........80........90.......100
```

Fig. S4. Sequence conservation of histone H3 homologs

Sequence conservation of Aiptasia histone H3 protein and histone H3 homologs from species for which antibody (ab9050, Abcam) has previously been validated. The N-terminal tail of Aiptasia H3 is identical to the fragment from the zebrafish *Danio rerio* (the first 100 amino acid fragment from human was used to produce this antibody).

682



683

684 **Fig. S5. Western blot**

685 Western blot result for antibody affinity validation, target band is 15kDa in size as expected from

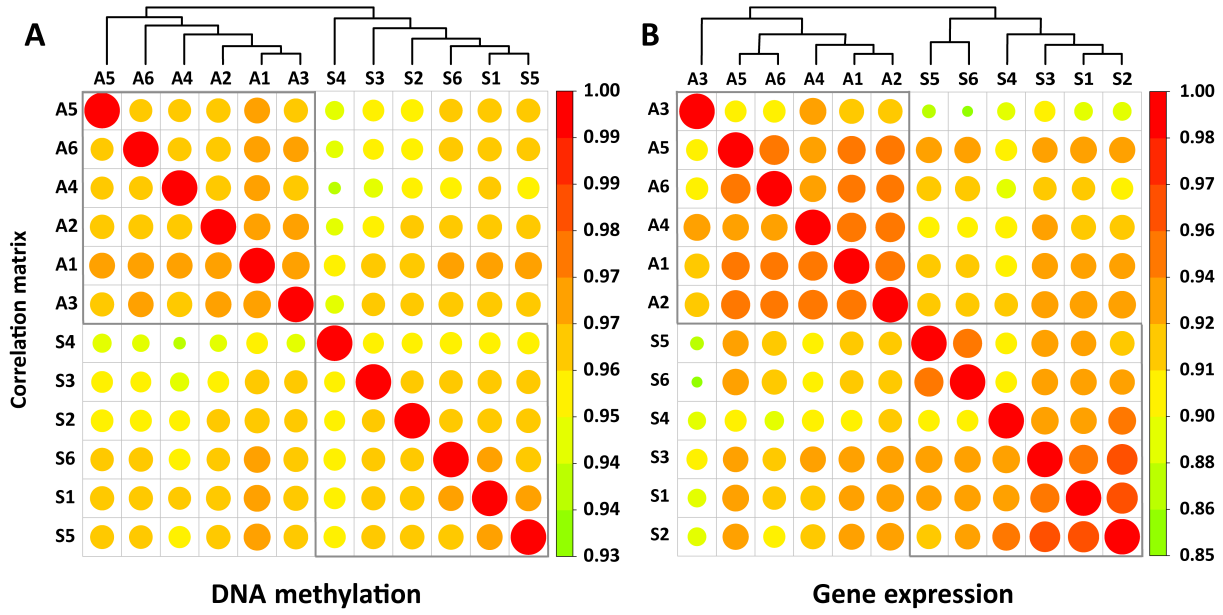
686 molecular weight analysis.

687

688

689

690



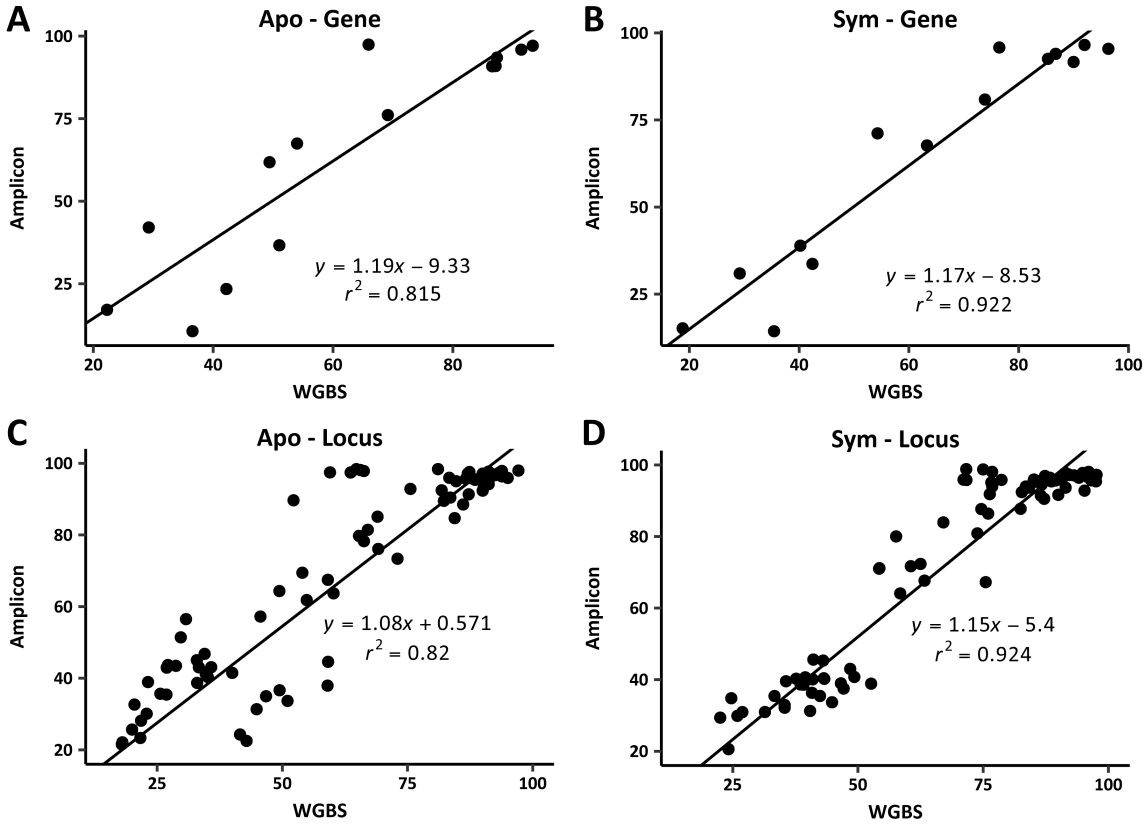
691

692 **Fig. S6. Correlation matrices of replicates**

693 Correlation matrices of replicates based on median DNA methylation level of genes (**A**) and log
694 gene expression values (base 2) (**B**). Replicates from the same treatments showed higher
695 correlation and clustered together both based on DNA methylation as well as gene expression
696 profiles, further supporting the findings obtained from the PCA analyses (figure 4) that changes
697 in DNA methylation and expression are treatment specific.

698

699



700

701 **Fig. S7. Validation of methylation level**

702 Validation of methylation level using bisulfite PCR on selected genes. (**A**, **B**) validation of
703 methylation level on genes (median methylation levels of methylated CpGs were used to
704 represent genes). (**C**, **D**) validation of methylation level on locus (methylated CpGs). WGBS:
705 whole genome bisulfite sequencing; Amplicon: MiSeq sequencing results of bisulfite PCR
706 amplicons on selected genes.

707

708

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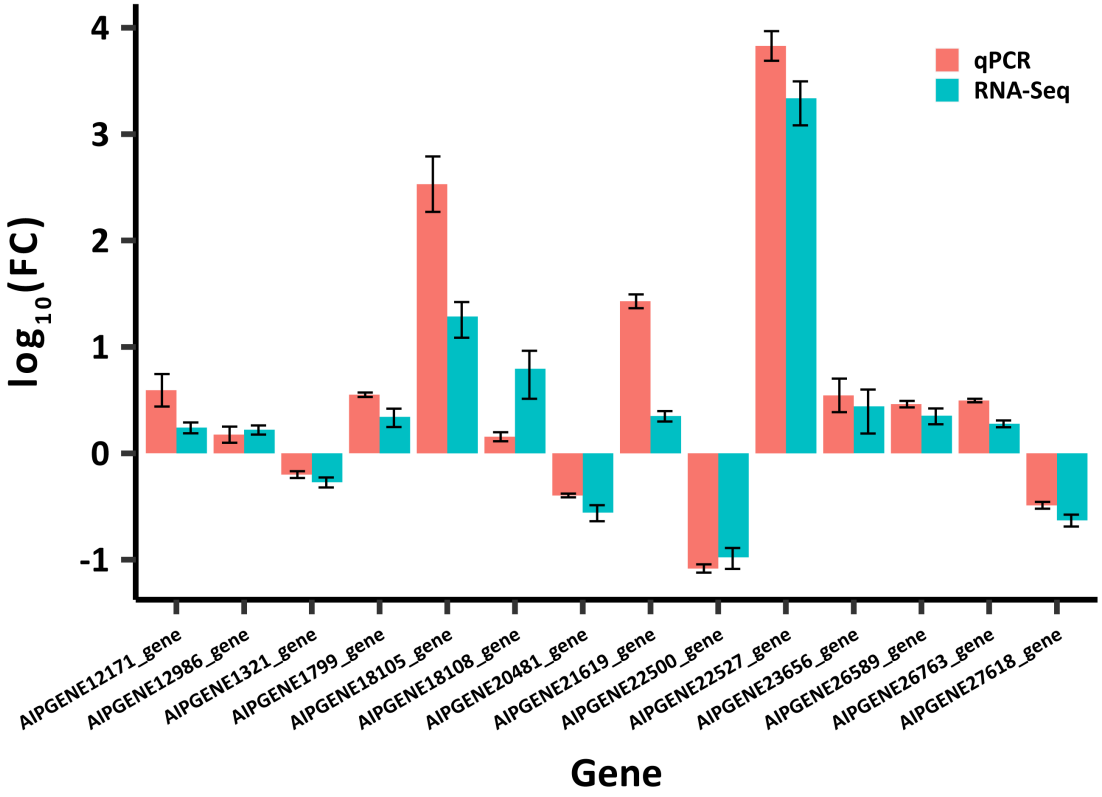
710



711

712 **Fig. S8. qPCR validation of gene expression levels**

713 Validation of gene expression changes using qPCR. Expression levels are shown as $\log_{10}$(fold

714 change). All genes show similar expression changes as determined by RNA-seq and q-PCR.

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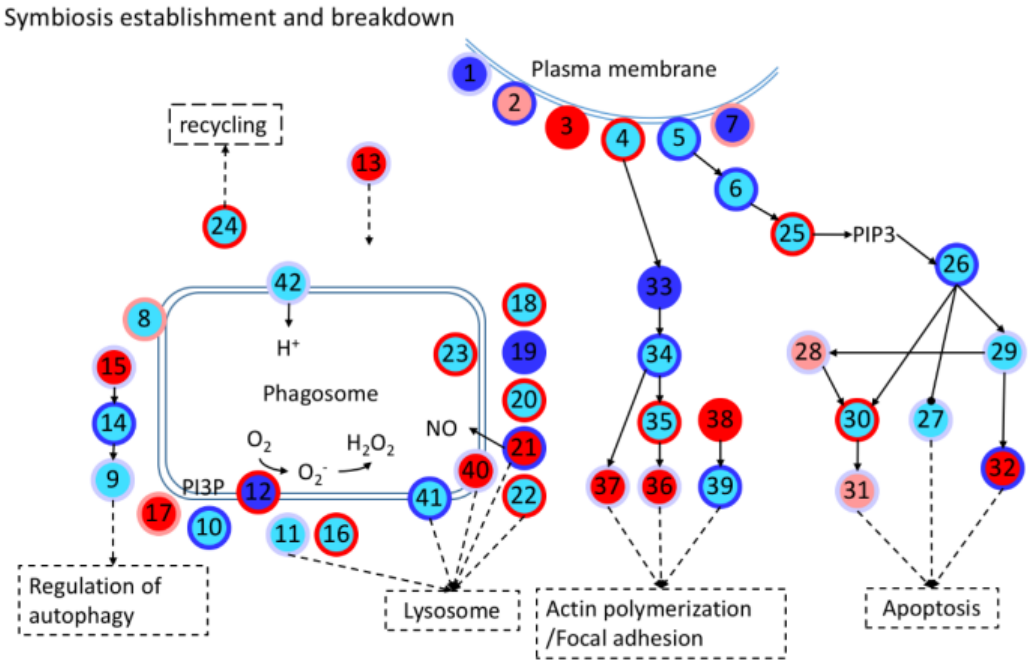


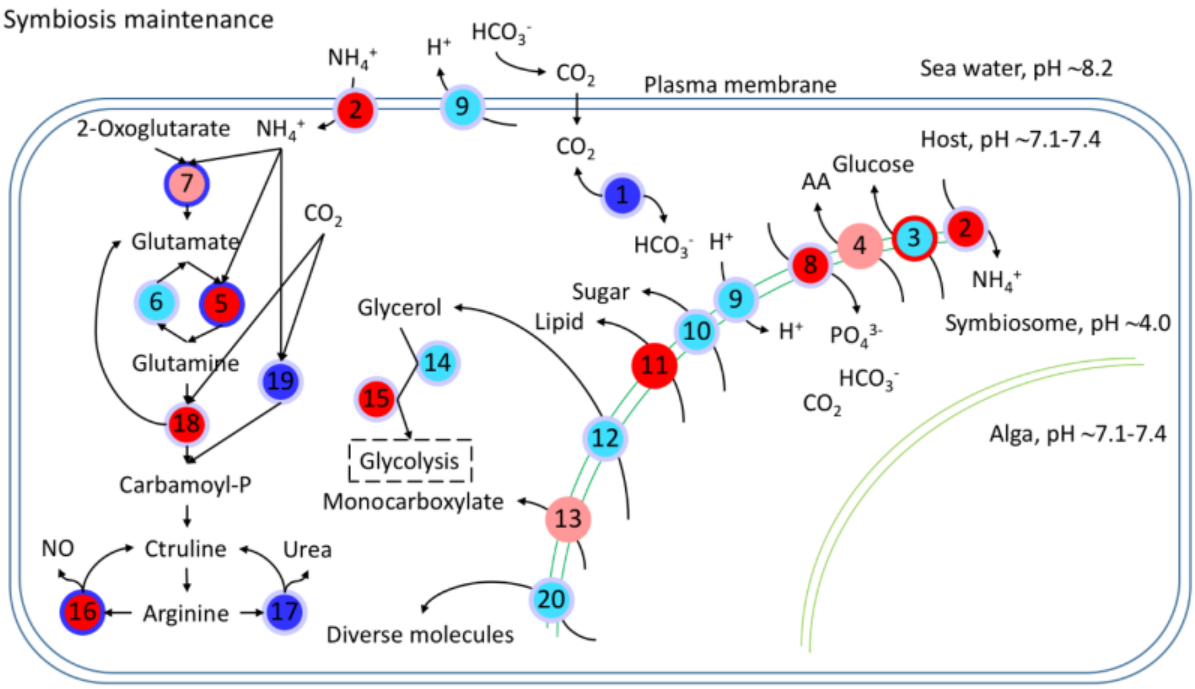
Fig. S9. Schematic diagram of symbiosis establishment and breakdown associated genes. Every node represents a category of genes, and generally has multiple corresponding genes. The inside colors of nodes represent the expression changes of corresponding genes, including non-DEGs (cyan), up-regulated (red), down-regulated (blue) and up- and down-regulated DEGs (light red). The colors of node edges represent the methylation level changes of corresponding genes, including non-DMGs (light blue), hypermethylated (red), hypomethylated (blue) and hyper- and hypo-methylated DMGs (light red). Numbers in circles denote genes/proteins as detailed below.

1. Complement receptor
2. Scavenger receptor
3. C-type lectin
4. Integrin
5. Toll-like receptor
6. Ras-related C3 botulinum toxin substrate 1 - rho family (RAC1)
7. Collagen
8. Vesicle-associated membrane protein (VAMP)
9. Autophagy-related protein 16 (ATG16)
10. Ras-related protein 5 (Rab5)
11. Ras-related protein 7 (Rab7)
12. NADPH oxidase (NOX)
13. Syntaxin 12
14. Autophagy-related protein 5 (ATG5)
15. Autophagy-related protein 10 (ATG10)
16. Programmed cell death 6-interacting protein
17. Sorting nexin (SNX)
18. Cytoplasmic dynein
19. Tubulin alpha chain (TUBA)
20. Tubulin beta chain (TUBB)
21. Nitric oxide synthase (NOS)
22. Lysosome-associated membrane glycoprotein/Cluster of differentiation (LAMP/CD)
23. Cathepsin L
24. Kinesin
25. Phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)
26. RAC serine/threonine-protein kinase (AKT)
27. Bcl-2-antagonist of cell death (BAD)
28. TNF receptor-associated factor (TRAF)
29. Nuclear factor of kappa light polypeptide gene (NFKB)
30. Caspase 8 (CASP8)
31. Caspase 7 (CASP7)
32. Apoptosis regulator/Bcl-2 (BCL2)
33. Ras homolog (RHO)
34. Rho-associated protein kinase (ROCK)
35. Phosphatidylinositol 4-phosphate 5-kinase / Phosphatidylinositol 5-phosphate 4-kinase / Phosphatidylinositol 3-phosphate 5-kinase (PI4P5K/ PI5P4K/ PI3P5K)
36. Vinculin
37. Radixin
38. Profilin
39. Actin
40. CD63
41. Lysosomal-associated transmembrane protein
42. V-type proton ATPase

PI3P: phosphatidylinositol-3-phosphate

PIP3: Phosphatidylinositol (3,4,5)-trisphosphate

715



716

717 **Fig. S10. Schematic diagram of symbiosis maintenance associated genes.** Every node
718 represents a category of genes, and generally has multiple corresponding genes. The inside
719 colors of nodes represent the expression changes of corresponding genes, including non-DEGs
720 (cyan), up-regulated (red), down-regulated (blue) and up- and down-regulated DEGs (light red).
721 The colors of node edges represent the methylation level changes of corresponding genes,
722 including non-DMGs (light blue), hypermethylated (red), hypomethylated (blue) and hyper- and
723 hypo-methylated DMGs (light red).

724

1. Carbonic anhydrase (CA)
2. Ammonium transporter
3. Glucose transporter
4. Amino acid transporter
5. Glutamine synthetase (GS)
6. Glutamate synthase
7. Glutamate dehydrogenase (GDH)
8. Phosphate transporter
9. V-type proton ATPase
10. Sugar transporter
11. Lipid transfer protein
12. Aquaporin 3 (Glycerol transporter)
13. Monocarboxylate transporter
14. Alcohol dehydrogenase
15. Aldehyde dehydrogenase
16. Nitric oxide synthase (NOS)
17. Arginase
18. Carbamoyl-phosphate synthase / Aspartate carbamoyltransferase / Dihydroorotase (CAD)
19. Carbamoyl-phosphate synthase (ammonia) (CPS1)
20. ABC transporter

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