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Regulation of *Hox* orthologues in the oyster *Crassostrea gigas* evidences a functional role for promoter DNA methylation in an invertebrate

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ABSTRACT

DNA methylation within promoter regions (PRDM) controls vertebrate early gene transcription and thereby development, but is neglected outside this group. However, epigenetic features in the oyster *Crassostrea gigas* suggest functional significance of PDRM in invertebrates. To investigate this, reporter constructs containing in vitro methylated oyster *Hox* gene promoters were transfected into oyster embryos. The influence of in vivo methylation was studied using bisulfite sequencing and DNA methyltransferase inhibition during development. Our results demonstrate that methylation controls the transcriptional activity of the promoters investigated, unraveling a functional role for PRDM in a lophotrochozoan, an important finding regarding the evolution of epigenetic regulation.

1. Introduction

DNA methylation is a highly prevalent epigenetic mark throughout evolution. Metazoan organisms exhibit methyl marks on cytosines, however, a great variability exists in both the amount of methylcytosines (meCs) and their distribution across genomes. In vertebrates, DNA is highly methylated and ca. 70–80% of cytosines within CpG dinucleotides bear a methyl group. Overall, vertebrate promoters can be divided into 2 groups depending on their CpG dinucleotide content, thereby displaying distinct methylation profiles with critical outcomes on the expression level of the downstream genes. The high-CpG promoters are hypomethylated and govern widely expressed genes, whereas the low-CpG promoters are associated with tissue-specific genes [9]. Such low-CpG promoters often display relatively CpG-rich regions called CpG islands, whose methylation is important for transcriptional control, as is the case for the *ace-1* gene in humans [21]. Promoter regions DNA methylation (PRDM) mostly inhibits gene transcription [13], through a direct steric hindrance of transcriptional machinery binding sites [1,7] and/or an indirect recruitment of chromatin remodeling proteins (review in [31]). The bimodal distribution of PRDM in vertebrates, which is set up during cell differentiation, noticeably affects genes of early development like *Hox* genes [14,16], which are strongly biased in terms of CpG overrepresentation in mice and humans [4]. As an example, the mouse *HoxA6* and *HoxA7* genes are silenced via PRDM and recruitment of polycomb proteins [29]. Therefore PRDM is of extreme significance in gene regulation in vertebrates, especially during early developmental processes.

In invertebrates, DNA is far less methylated, and DNA methylation in ecdysozoan models is rare [25,30]. For instance, less than 0.2% of the CpG cytosines are methylated in the silkworm *Bombyx mori*, and meCs in the genome of the fruitfly *Drosophila melanogaster* are so rare that their actual presence has long remained discussed, thereby making DNA methylation in *Drosophila* likely non-functional. Furthermore, another ecdysozoan model, the nematode *Caenorhabditis elegans*, even lacks conserved DNA methylation machinery. In addition to being weakly represented, methylcytosines within invertebrate genomes are not

Abbreviations: EtBr-AGE, ethidium bromide agarose gel electrophoresis; GBM, gene body DNA methylation; meC, methylcytosine; MeS-PCR, methylation-specific polymerase chain reaction; PRDM, promoter region DNA methylation

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evenly distributed and lie mostly within gene bodies [24], but not promoters or transcription regulatory regions like in vertebrates. However, DNA methylation in ecdysozoans, i.e. gene body methylation (GBM), has consequences for gene expression. Indeed, GBM controls exon selection during transcription and alternative splicing [10]. DNA methylation does not contribute to the control of early genes in *D. melanogaster*, where *Hox* genes are regulated by a network of cis-regulatory elements and transcription factors including polycomb and trithorax complexes, and non coding RNAs (lnc- and miRNAs) (review in [19]). However, in hymenopteran insect models, GBM governs important developmental outcomes, like caste differentiation in the honeybee *Apis. mellifera* [8,18] and in ants [3], and developmental gene expression in the wasp *Nasonia vitripennis* [28,33]. This highlights discrepancies in the epigenetic regulation of development between vertebrates and ecdysozoans, which reflects the evolutionary divergence between those groups, although the role of histone marks and of polycomb/trithorax proteins seems conserved (review in [26]). Thus, animal genomes are thought to have evolved towards an overall loss of (PRDM) DNA methylation in protostomes [24]. As a consequence, PRDM has remained largely neglected in invertebrates, and it remains unknown whether PRDM has significant functional outcomes outside deuterostomes.

However, recent studies in lophotrochozoans (that include mollusks and annelids), the protostome sister clade of ecdysozoans (that include insects and nematodes), suggest a more complex situation than previously depicted. Indeed, mollusks display higher levels of methylcytosines than other invertebrate taxa, although their DNA is similarly mostly methylated within gene bodies. Thus, insects display ca. 0.15% of methylcytosines [30] whereas this value reaches ca. 2% in the snail *Biomphalaria glabrata* [11], and ca. 7% in the gills of the oyster *Crassostrea gigas* [12]. In this bivalve, DNA methylation, which affects the 5′-regions of *Hox* gene orthologues, is a critical feature of early development [22]. Furthermore, oyster methylomes indicate that, although gene body methylation seems an important pathway in transcriptional regulation, putative promoters exhibit significant methylation [12]. Such methylation is associated with mRNA content in oyster male gametes [20]. These data strongly suggest, in a surprising fashion for an invertebrate, a putative role for PRDM in gene expression in the oyster [23].

However, such functional significance has, to the best of our knowledge, never been clearly demonstrated outside deuterostomes, despite being critical for our understanding of both the development processes in distant organisms and the evolution of epigenetic regulation of gene expression. To explore this issue, we characterized and cloned putative promoter regions of four oyster *Hox* genes. We inserted those fragments after in vitro methylation into luciferase reporter constructs and transfected them into developing oyster embryos. In addition, we investigated the influence of a DNA methyltransferase (DNMT) inhibitor on their in vivo mRNA expression, and mapped their methylation pattern across developmental stages using bisulfite sequencing.

2. Methods

2.1. In silico analyses

The four genes examined (*Engrailed 2* (*En2*) [GenBank Accession numbers EKC23209, GI:405956969], *HoxC11* (*Hox*) [EKC29599, GI:405964077], *Orthopedia* (*Otp*) [EKC38378, GI:405973681] and *Ultrabithorax* (*Ubx*) [EKC39601, GI:405974999]), were annotated regarding their homology with their putative vertebrate orthologues [22]. Proximal promoters were defined as the 1 kb 5′-upstream region regarding the putative transcription start site (when identified using the promoter 2.0 algorithm (www.ebi.ac.uk/emboss/promoter2.0)), or the putative translation start site [12] (see Fig. 1) in the present build of the oyster genome (v.9, [32]). CpG islands were determined using CpGPlot (www.ebi.ac.uk/emboss/cpgplot), as previously described [21].

2.2. Biological material, in vitro fecundations, DNA methyltransferase inhibitor treatment and embryolarval development

These procedures were realized as previously described [22]. Briefly, mature gonads of conditioned broodstock *C. gigas* specimens were scarified and sperm (passing fraction on a 10 μm mesh) and oocytes (remaining fraction on a 30 μm mesh) were harvested in filtered sterile seawater (FSW). Fecundations were triggered by the addition of ca. 100 spermatozoïds/oocyte (500 oocytes.10^{-3} L FSW). Embryos were left unattended at 23–25 °C until sampling (ca. 2 million embryos/sample) at ca. 3 h post-fecundation (hpf) (2–8 cells), 4.5 hpf (morula), 6 hpf (blastula), 9 hpf (gastrula), 16–17 hpf (trochophore), and 24 hpf (D larvae). Five aza-cytidine (Sigma) (10^{-5} mol L^{-1}) treated embryos were sampled at 6 and 24 hpf. Embryos were harvested by filtration as the remaining fraction through a 30 μm mesh and slight centrifugation (80×*g*, 3 min.), immediately frozen in liquid nitrogen (DNA extraction) or resuspended in TRI-reagent (Sigma) (1×10^{-3} L/10^6 embryos) (RNA extraction).

2.3. Reporter constructs

The *En2*, *Hox*, *Otp* and *Ubx* putative promoters (En2p, Hoxp, Otpp and Ubxp respectively) were amplified by PCR from genomic DNA (100 ng, Nucleospin DNA tissue kit, Macherey Nagel) using Phusion DNA polymerase (New England Biolabs). When necessary, nested PCRs were performed with primers containing restriction enzyme sites used for downstream subcloning (SacI for En2p and XmaI for Otpp). PCR products (En2p, 841 bp; Hoxp, 1158 bp; Otpp, 551 bp; Ubxp, 737 bp), were resolved by agarose gel electrophoresis stained with ethidium bromide (EtBr-AGE) and purified by affinity chromatography (Nucleospin Gel and PCR Cleanup, Macherey Nagel). The purified fragments were digested using SacI (En2p), XmaI (Otpp) or SmaI (Hoxp and Ubxp) and respectively subcloned using T4 DNA ligase (Promega) into the SacI, XmaI or EcoICRI site of the pGL2' vector (p3TP-lux plasmid [2] after PAI-1 promoter excision using SacI). The resulting constructs containing the firefly luciferase coding sequence under the control of the *C. gigas Engrailed2*, *HoxC11*, *Orthopedia* and *Ultrabithorax* genes putative proximal promoter, named pEn2p, pHoxp, pOtpp and pUbxp vectors, respectively (see Fig. 1) were assessed by PCR, restriction enzyme digestions and sequencing. Large amounts of the constructs were purified free of endotoxins from bacterial cultures using affinity chromatography (Nucleobond Xtra Maxi EF, Macherey Nagel).

2.4. In vitro methylation

The constructs pEn2p, pHoxp, pOtpp and pUbxp (10 μg each) were double-digested using KpnI and BclI, KpnI and NsiI, KpnI and SacI, or KpnI (Promega) and BstZ17I (New England Biolabs), respectively. The fragments corresponding to the promoter regions and the plasmid backbone were resolved by EtBr-AGE and purified separately. Promoter fragments were methylated in vitro using M. SssI (New England Biolabs) and fresh S-adenosyl methionine. Methylated fragments were then purified and re-ligated into their respective digested vector backbones using T4-DNA ligase (Promega) ('PRDM-only' constructs). In vitro methylation was also carried out on the undigested constructs using M.SssI, M.HhaI or M.HpaII DNA methylases ('fully-methylated' constructs).

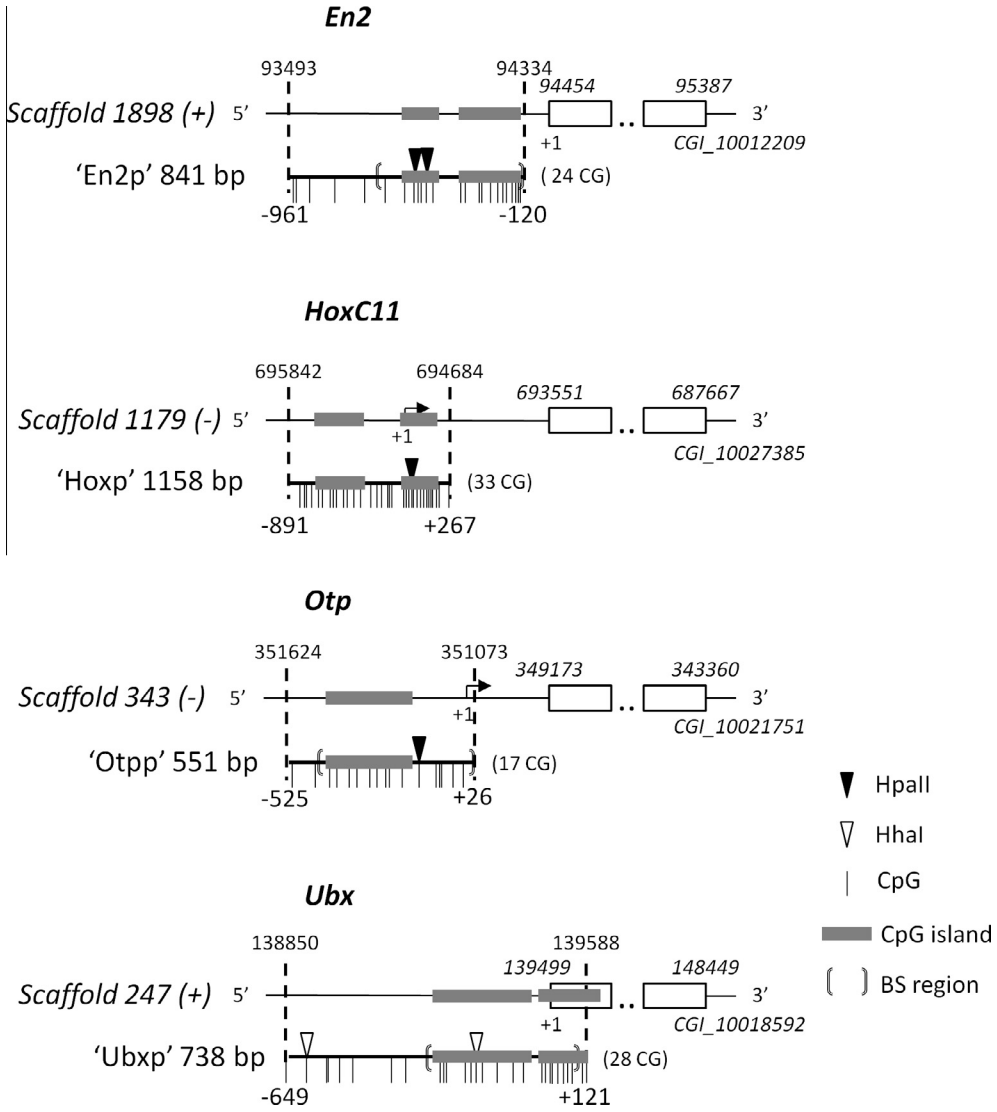

Fig. 1. Schematic representation of the *En2*, *HoxC11*, *Otp* and *Ubx* promoter regions examined in this study. Upper part, thin line: genomic context; scaffold number, strand, position on the scaffold, coding sequence (white boxes), translation start and stop positions, and accession number are indicated. Lower part, thick line: cloned sequence referred to as 'promoter region' in this study; fragment length, base numbering regarding the putative transcription (+1, arrow) or translation (+1) start sites are given, and reported onto the genomic context representation. The number of CpG dinucleotides corresponds to the number of M.SssI methylation sites. CpG islands (grey boxes), M.HpaII (arrowhead, black) and M.HhaI (arrowhead, white) sites are also marked.

Methylation efficiency was verified by enzymatic digestion (37 °C, 30 min.) using the methyl-sensitive AciI, HhaI or HpaII restriction enzymes (New England Biolabs), respectively. Mock methylations and digestions were performed for all conditions by replacing enzymes by water.

2.5. Transfections

Oyster embryos (30 min post-fecundation) were transfected in L15 medium. CHO-K1 cells (ATCC® CCL61™) were transfected in DMEM F12, 10% FCS (PAN Biotech GmbH), penicillin (100 U/10^{-3} L) and streptomycine (100 µg/mL) (Dominique Dutscher) at 37 °C under a 5% CO_2 atmosphere. Embryos and cells were transfected using FuGENE HD transfection reagent (Promega) with mock-methylated, PRDM-only methylated or fully methylated constructs and co-transfected with a normalization plasmid encoding the renilla luciferase, pGRL4-74 (Promega). The transcriptional activity was determined as the relative luciferase activities (firefly/renilla) within cell lysates at 16 (oyster embryos) or 36 (CHO cells) hours post-transfection using the Dual-Luciferase Reporter Assay system (Promega). The overall efficiency of the procedure was assessed in parallel by examining the fluorescence after transfection of a CMV-GFP plasmid.

2.6. Quantification of Hox gene expression

Hox gene mRNAs were quantified using RT-qPCR as previously described [22].

2.7. Bisulfite sequencing of genomic DNA

Two micrograms of genomic DNA from each development stage were converted using the Premium Bisulfite kit (Diagenode) according to the manufacturer's instructions. After conversion, DNA was purified using affinity chromatography, and amplified by MeS-PCR (primers were designed using methprimer at www.urogene.org/methprimer [17]). Nested reactions were performed when required. PCR products were resolved by EtBr-AGE,

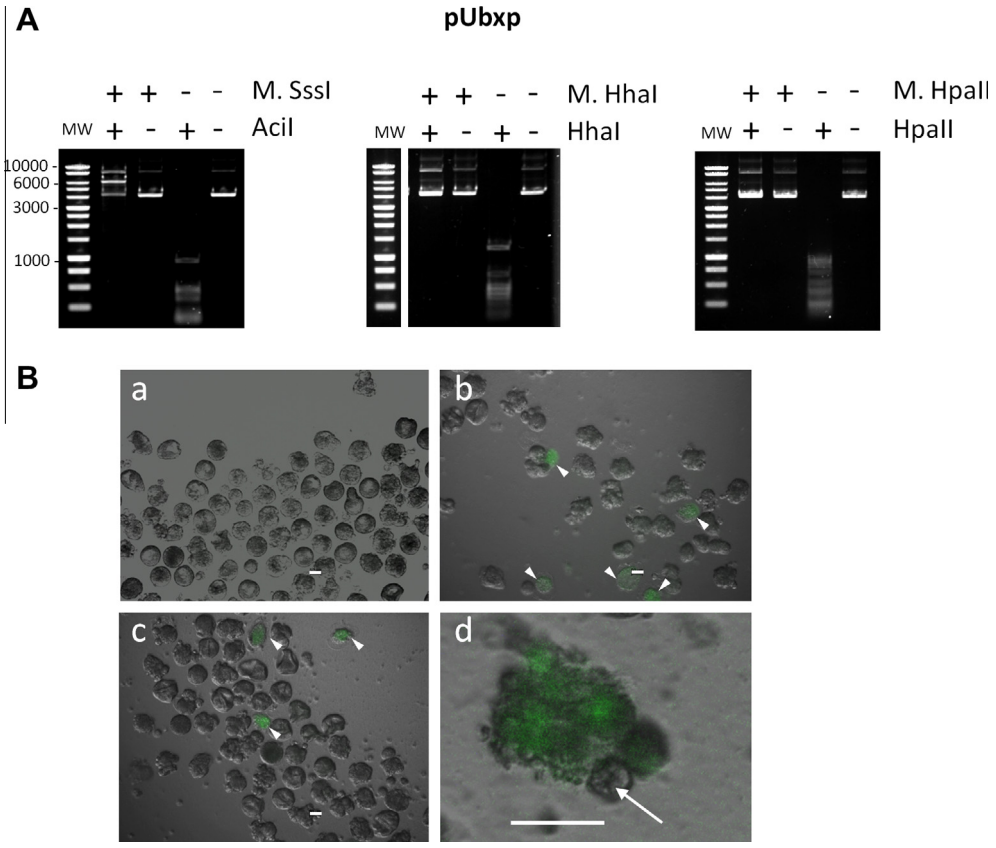

Fig. 2. In vitro methylation and transfection of oyster embryos. (A) Methyl-sensitive restriction assays. In vitro methylated plasmids using AciI (left), HhaI (middle) and HpaII (right) were incubated with the methyl-sensitive restriction enzyme corresponding to the methyltransferase used for in vitro methylation (M.SssI, M.HhaI and M.HpaII, respectively). Agarose gel electrophoreses of the pUbxp construct is shown as a representative example. Absence of digestion confirms complete methylation (please note that some SssI constructs could display a faint signal reflecting sub-complete methylation probably due to CpG proximity (left panel lane 1)). MW: molecular weight marker, fragment sizes are given in base pairs. (B) Cultured oyster embryos at 16hpf, untransfected (a) or transfected with a CMV-GFP plasmid. Three representative experiments are shown (b, c and d). Phase contrast and green fluorescence fields were merged for all pictures; white arrowheads, fluorescent transfected cells; white arrow in d, untransfected cell within a transfected embryo indicating a mosaïc phenotype. Note that transfected cells display an altered morphology. Scale bar (white): 50 μm.

purified, subcloned into the pCRII vector (TOPO TA Cloning kit, Invitrogen) and sequenced. Six to eleven clones were analysed to assess the methylation status of each cytosine within the examined regions. A position was considered methylated (score = 1) when more than half of the sequences showed an untransformed cytosine after bisulfate conversion.

2.8. Statistical analyses

All results are given as the mean ± S.E.M. (standard error of the mean) of at least triplicate independent experiments. Results were analysed at 95% confidence intervals using two-way ANOVA with Bonferroni's post hoc tests (luminescence in CHO cells, 5-aza cytidine effect on gene expression), one-way ANOVA (Kruskall–Wallis test) with Dunn's post hoc tests (expression of *Hox* genes). Mann–Whitney rank sum test was used when required (luminescence in oyster embryos). P (or U) values under 0.05 were considered significant ($^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$ et $^{***}P \leqslant 0.001$). Statistical analyses and plots were performed using R (v. 3.0.3) and Graphpad Prism (v. 5.0) softwares.

3. Results and discussion

3.1. Constructs, in vitro methylation and transfection of oyster embryos

The strategy led to the successful construction of the unmethylated, promoter-only methylated and fully methylated pCgEn2p, pCgHoxp, pCgOtpp and pCgUbxp plasmids. After in vitro methylation, the constructs were protected from digestion by a methyl-sensitive restriction enzyme, whereas mock-methylated constructs were degraded, indicating that in vitro methylations were successful (Fig. 2A). The transfection of all mock-methylated, PRDM-only methylated and fully methylated constructs was effective in all the culture systems used, including oyster developing embryos and CHO cells. Oyster embryos were successfully chosen as a homologue system as closely related to physiological conditions as possible, and CHO cells to provide a comparison basis with transcription mechanisms in vertebrates (see below). Nevertheless, the transfection of oyster embryos (estimated up to ca. 10% of cells) was less efficient than the transfection of CHO cells, strongly compromised the survival of transfected embryos, and could result in mosaic phenotypes (Fig. 2B).

3.2. The regions investigated are functional oyster promoters

The luminescence assays revealed a strong relative luciferase activity of the unmethylated constructs, when compared to the empty pGL2' backbone which never led to a significant luciferase signal, in both oyster embryonic and CHO cells (Fig. 3A and B). Therefore, the investigated regions were able to drive the transcription of the reporter gene in the homologue and the heterologue systems used, as well. This demonstrates that these regions correspond to functional oyster promoters, and that mollusk and vertebrate promoters share some common functional features, as illustrated by the presence of CpG islands in oyster promoters

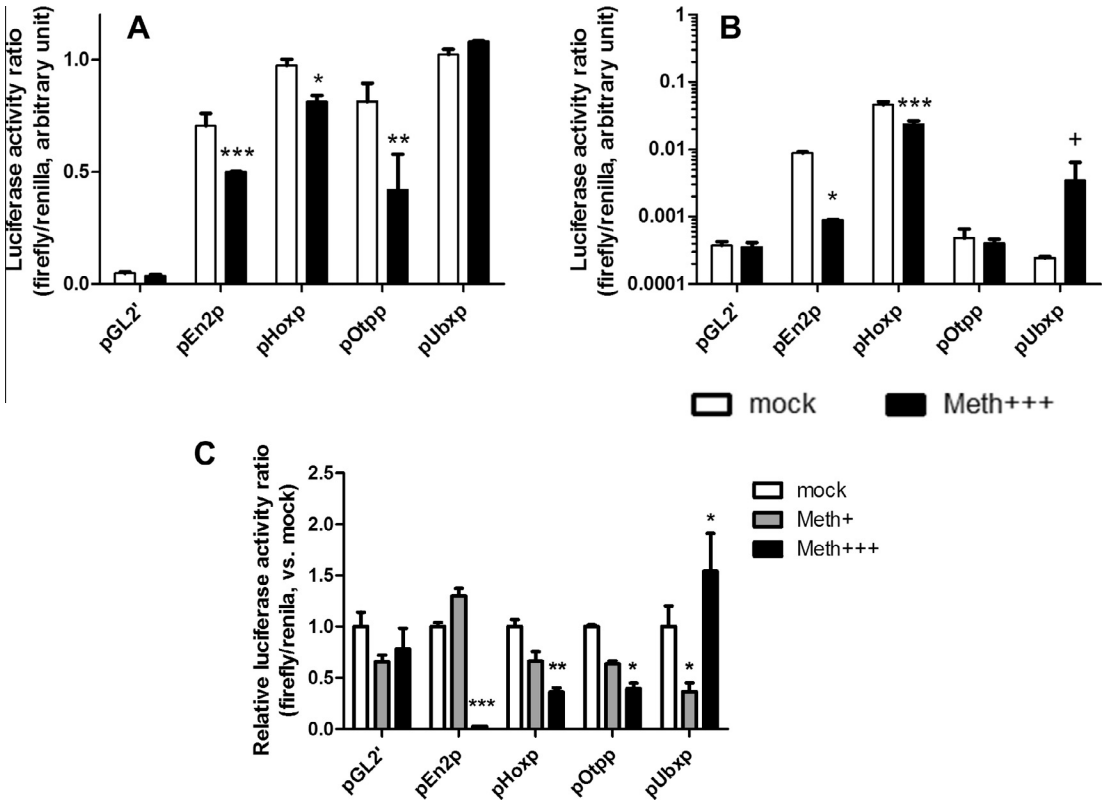

Fig. 3. Influence of methylation on the transcriptional activity of oyster *Hox* genes promoters. Relative luciferase activity in oyster embryos (A) and CHO cells (B) transfected with pEn2p, pHoxp, pOtpp and pUbxp methylated solely in the putative promoter regions ('PRDM-only' constructs). (C) Relative luciferase activity in CHO cells transfected with differentially methylated pEn2p, pHoxp, pOtpp, pUbxp ('fully methylated' constructs). Constructs were methylated at all CpG sites using M.SssI (Meth+++), or partially methylated using M.HpaII (pEn2p, pHoxp and pOtpp) or M.HhaI (pUbxp) (Meth+). The pGL2' plasmid (promoterless vector backbone) was used as a control (because no significant difference could be observed between the M.HpaII and M.Hhai values for pGL2', only the M. HpaII value is shown for clarity). $^{+}P < 0.1$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, *T*-test or Mann–Whitney rank sum test (A and B), or two-way ANOVA followed by Bonferroni's post hoc test (C).

(Fig. 1), somewhat similar to low CpG 'methylation-regulated' promoters in vertebrates regulated by methylation [6,13,15]. This finding should help characterize a consensus promoter in bivalve mollusks that remains elusive until now and constitutes a key step toward functional studies in lophotrochozoans.

3.3. DNA methylation influences the transcriptional activity of oyster Hox gene promoters

When compared to their mock-methylated counterpart, all 'PRDM-only' constructs transfected in oyster embryos display a significantly reduced luciferase activity, except for the *Ubx* promoter construct whose methylation does not lead to a significant shift in activity (Fig. 3A). Overall similar observations arise from CHO cells, despite slight differences: the reduction in the luciferase activity is more important for the pEn2p and pHoxp constructs and there is no significant effect of methylation on the activity of the pOtpp construct (Fig. 3B). 'Full-methylation' of the constructs give rise to similar observations in CHO cells (Fig. 3C, Meth+++). The ' partially-methylated' constructs displayed intermediate transcriptional activities for pHoxp and pUbxp, but not for pEn2p and pUbxp (Fig. 3C, Meth+). This may reflect the importance of the position of meCs regarding potential interactions with activating or repressive transcription factors. However, despite oyster and CHO cells sharing some common transcriptional pathways (see above), further interpretation is speculative considering the current knowledge in oysters. Nevertheless, these results suggest that the specific methylation of oyster promoters influences their transcriptional activity, which is to our knowledge the first demonstration of this in a lophotrochozoan species. This finding is consistent with the association between the methylation of putative promoter regions and the mRNA content of sperm in the oyster [20], despite such an association being hard to interpret because of the weak, if present, transcriptional activity in mature spermatozoa due to high chromatin compaction. Interestingly, PRDM represses the activity of promoters of the three genes with high expression (*En2, HoxC11, Otp*), but not of the weakly expressed *Ubx* (see Fig. 4). This could be reminiscent in the oyster of the relationship between the bimodal distribution of vertebrate PRDM and transcription, where very high and very low methylation is associated to weak expression, and moderate methylation to higher and more variable mRNA levels.

3.4. Treatment with a DNA methyltransferase inhibitor modifies the mRNA expression of the investigated Hox genes during embryogenesis

Treatment of developing embryos with 5-aza-cytidine, a DNA-methyltransferase inhibitor, has already been demonstrated to induce a strong decrease in DNA methylation as soon as 6 hpf [22]. Accordingly, no significant protection of cytosines could be detected after 5-aza treatment in the regions investigated (data not shown). This resulted in significant changes in the variation of the mRNA expression of the genes examined between 6 and 24 hpf when compared to untreated embryos, except *Ubx* (Fig. 4). The observed expression patterns in controls fit their expected profiles [22,32]. Furthermore, when considering these profiles with respect to the duration of the embryolarval stages, the 5-aza-C phenotypes match with a delayed cell differentiation already

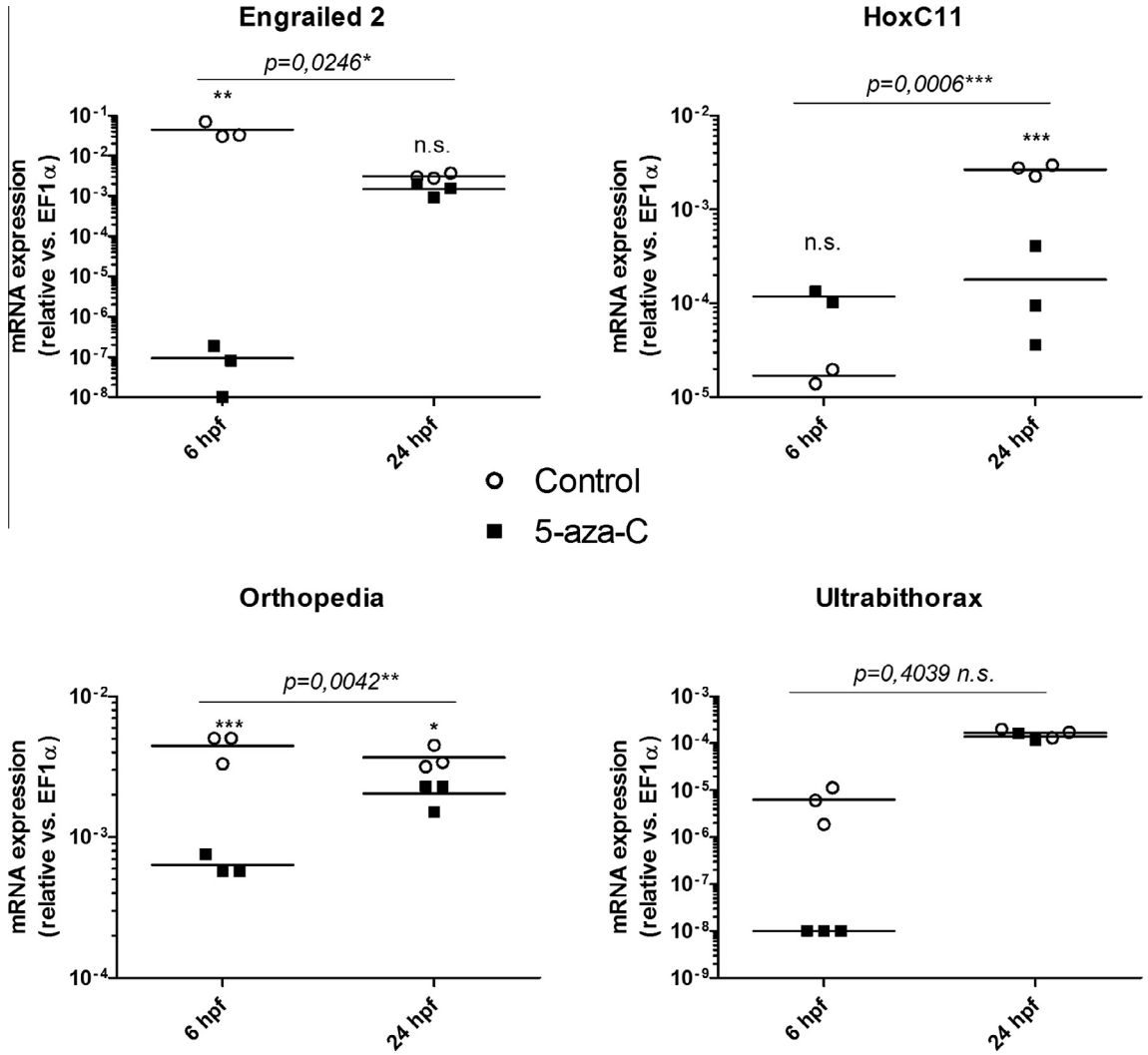

Fig. 4. Influence of a DNMT inhibitor on the mRNA expression level of oyster *Hox* genes. Influence of 5-aza-cytidine treatment on the mRNA expression of *Engrailed2*, *HoxC11*, *Orthopedia* and *Ultrabithorax* between 6 and 24 hpf. *P* values are given for two way ANOVA (influence of 5-aza-C on mRNA expression variation between 6 and 24 hpf); Control (white circle) and 5-aza-C (black squares) values were then analyzed using Bonferroni's post hoc test; n.s., not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

described as a consequence of DNA demethylation during oyster development [22] and, when considered on a per-gene basis with respect to the different durations of each larval stage, the mRNA levels observed herein interestingly fit this hypothesis. This result suggests that the transcription of the target genes is influenced by DNA methylation in vivo. However, because 5-aza-C induces a global demethylation of DNA, caution should be taken with the interpretation of a specific effect (at the respective promoter of each gene like on CpG binding protein recruitment [1,7,27]) versus or in addition to an indirect cis- or trans-effect (i.e. influence of DNA methylation on distant transcription factors expression and/or chromatin conformation [6], reviewed in [5,31]). Nevertheless, the mRNA expression changes upon DNMT treatment are consistent with an influence of PRDM in the oyster, in a similar fashion to the situation in vertebrates [7,13].

3.5. Variations of the investigated Hox genes PRDM during oyster embryolarval development

Bisulfite sequencing indicates that PRDM patterns of the *Hox* promoters investigated exhibit changes during embryolarval development in the oyster (Fig. 5). There is not a direct relationship between those patterns and the changes in the mRNA expression of the downstream gene. However, this observation does not necessarily rule out the role of PRDM in the transcriptional regulation of oyster *Hox* genes in vivo. Indeed, although the regions investigated by bisulfite sequencing encompass most the CpG dinucleotides of the cloned promoters (i.e. 22/23; 16/17 and 19/28 for En2p, Otpp and Ubxp, respectively), they unlikely include the complete promoter of the genes. Therefore, other regulatory regions including transcription factor binding sites and/or chromatin remodeling proteins likely span outside the examined sequences that influence transcription, and were not examined here. In this context, the changes in methylcytosine patterns observed here should be considered as only partly reflecting the precise in vivo influence of PRDM, and do not exclude a putative role of PRDM in *Hox* genes transcriptional control in vivo consistent with the situation in vitro.

3.6. Evolution of epigenetic regulation of gene expression by DNA methylation

In the oyster *C. gigas*, a marine bivalve representative of lophotrochozoans, like in other invertebrates, GBM is predominant [12]. However, we bring here the functional evidence that oysters (i) also present PRDM (ii) which influences promoter

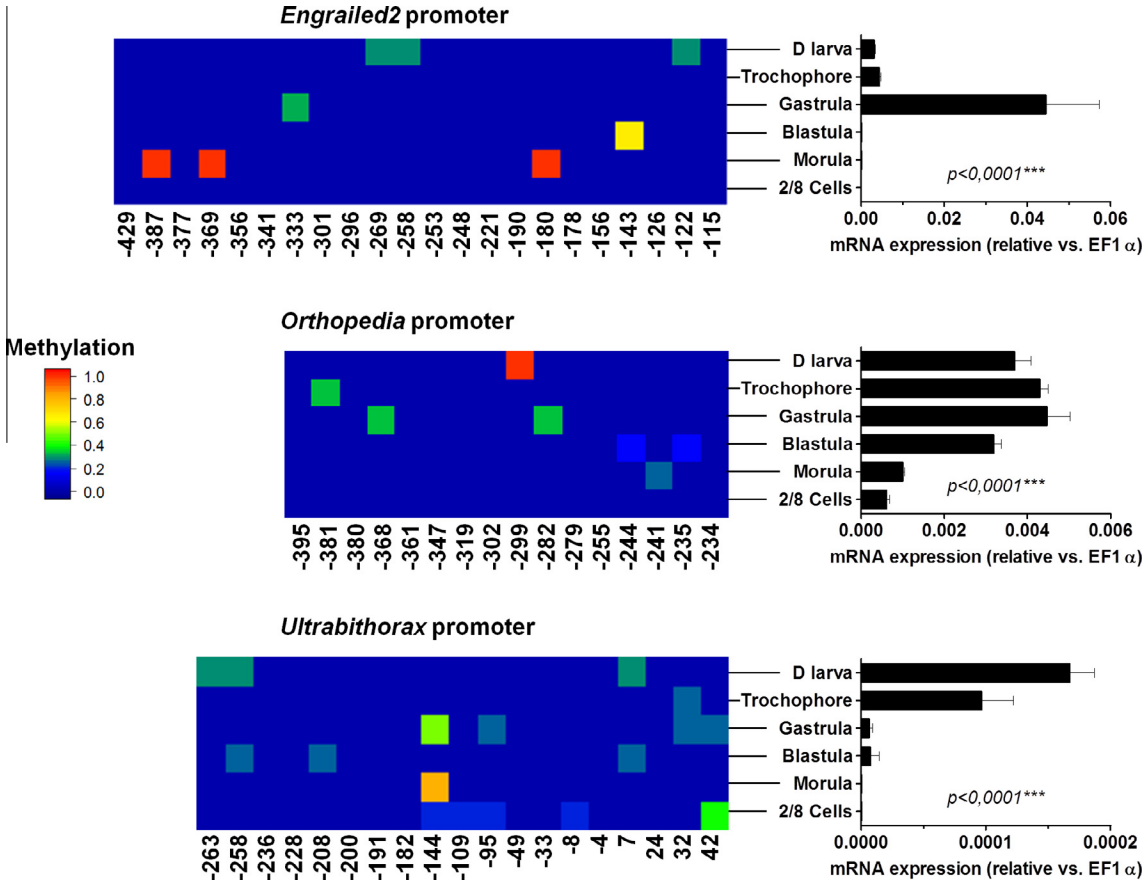

Fig. 5. In vivo Methylation maps of *Engrailed2*, *Orthopedia* and *Ultrabithorax* proximal promoters and corresponding mRNA expression during oyster development. The methylation map (left) mentions the position of the cytosine (below) regarding the putative start site (see Fig. 1). The development stage is indicated as well as the corresponding mRNA expression level (right). The color key corresponds to the methylation level (1, methylated; 0, unmethylated). *P* values correspond to the variation of the mRNA expression of the corresponding gene (one way ANOVA) between developmental stages; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

transcriptional activity (iii) and could thereby participate in the epigenetic control of development, possibly through the regulation of early developmental genes. These characteristics are surprisingly highly reminiscent of vertebrate features of epigenetic regulation by DNA methylation. Such functional significance was generally unexpected in invertebrates but was suspected from previous studies [20,22,23]. Therefore, it is demonstrated that lophotrochozoans, the sister clade of ecdysozoans within protostomes, can display an intermediate profile in DNA methylation level, distribution, and function, suggestive of a more complex evolution of DNA methylation patterns and epigenetic regulation than previously considered [24]. These observations support the emergence of PRDM before the divergence of bilaterians and/or a high versatility of this epigenetic mark between species that could depend on environmental interactions and life traits. The elucidation of whole developmental methylomes in oysters and other lophotrochozoan species under different environmental conditions will bring new insights into these fundamental questions.

4. Conclusion

Our results demonstrate that the methylation of DNA within promoter regions influences the transcription of *Hox* genes in vitro, and possibly in vivo, in a lophotrochozoan species, the bivalve mollusk *C. gigas*. To our knowledge, this study constitutes the first evidence of a functional role of PRDM outside of deuterostome animals. Such a finding clearly opens new perspectives in our understanding of not only the epigenetic control of transcription during development in distant organisms, but more generally in the evolution of epigenetic processes governing gene expression.

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