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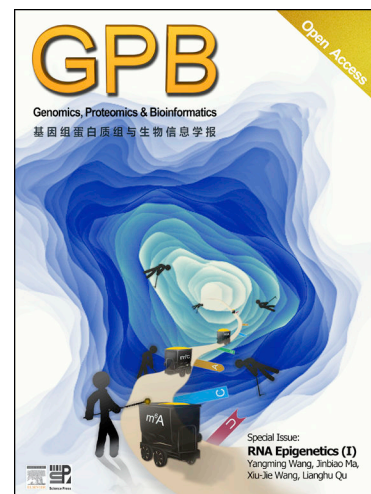
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***N*⁶-Methyladenosine Profile Dynamics Indicates Regulation of Oyster Development by m⁶A-RNA Epitranscriptomes**

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Abstract

The *N*⁶-methylation of RNA adenosines (*N*⁶-methyladenosine, m⁶A) is an important regulator of gene expression with critical implications in vertebrate and insect development. However, the developmental significance of epitranscriptomes in lophotrochozoan organisms remains unknown. Using methylated RNA immunoprecipitation sequencing (MeRIP-seq), we generated transcriptome-wide m⁶A-RNA methylomes covering the whole development of the oyster from oocytes to juveniles. Oyster RNA classes display specific m⁶A signatures, with messenger RNAs (mRNAs) and long non-coding RNAs (lncRNAs) exhibiting distinct profiles and being highly methylated compared to transposon transcripts. Epitranscriptomes are dynamic and correspond to the chronological steps of development (cleavage, gastrulation, organogenesis, and metamorphosis), with a minimal mRNA and lncRNA methylation at the morula stage followed by a global increase. mRNA m⁶A levels are correlated to transcript levels and shifts in methylation profiles correspond to expression kinetics. Differentially methylated transcripts cluster according to embryo-larval stages and bear the corresponding developmental functions (cell division, signal transduction, morphogenesis, and cell differentiation). The m⁶A level of transposon transcripts is also regulated and peaks during the gastrulation. We demonstrate that m⁶A-RNA methylomes are dynamic and associated to gene expression regulation during oyster development. The putative epitranscriptome implication in the cleavage, maternal-to-zygotic transition, and cell differentiation in a lophotrochozoan model brings new insights into the control and evolution of developmental processes.

KEYWORDS: RNA methylation; Transcription; Embryo; Metamorphosis; Evo-devo

Introduction

The success of development of metazoan organisms is conditioned by the precise temporo-spatial regulation of gene expression. The *N*⁶-methylation of RNA adenosines (*N*⁶-methyladenosine, m⁶A) has recently emerged as a critical layer of the gene expression regulatory network. Indeed, m⁶A-RNA methylation is required in vertebrates for the proper regulation of developmental processes such as cell differentiation [1,2], X chromosome inactivation [3,4], maternal-to-zygotic transition (MZT) [5,6] and neurogenesis [7–9]. Besides, the invalidation of enzymes which deposit m⁶A on RNA is lethal during the early development [10,11].

The m⁶A-RNA is regulated by an enzymatic machinery comprising writers and erasers. The METTL3/METTL14/WTAP writer core complex [12] deposits methyl marks at the consensus sequence DRACH (D = A/G/T, R = A/G, H = A/C/T) [13–16]. Erasers like ALKBH5 [17] and the controversial FTO [18] remove methylation. The combined action of m⁶A writers and erasers makes m⁶A profiles highly dynamic during the development of investigated species [6,19,20]. The biological effects of RNA methylation are mediated by reader proteins able to bind m⁶A. For example, the YTH protein family of readers is involved in RNA stability, translation level [21–24], splicing [25], and nuclear export [26]. Other readers like hnRNPA2B1 [27], IGF2BP [28], and Prrc2a [8] mediate m⁶A influence on RNA stability, and eIF3a guide cap-independent translation of m⁶A-RNAs [29]. This whole machinery allows to guide the methylation on specific RNA targets and modulates their cellular fate. As a result, m⁶A is a chemical modification that influences gene expression without modifying the associated transcript nucleic acid sequence and is therefore referred to as “epitranscriptomic” [30].

Many RNA classes are subjected to m⁶A methylation, which is the most abundant internal modification in eukaryotic messenger RNAs (mRNAs). m⁶A in mRNAs are mostly found in the 3' untranslated transcribed region (UTR) at the vicinity of the stop codon [13,14,31]. However, m⁶A is also present at lower levels in 5' UTRs [29,32], long internal exons [15,31] and introns [16,33].

While m⁶A modification is well described in mRNA targets, it also affects most non-coding RNA classes (ncRNAs) including microRNAs (miRNAs) [27], circular RNAs (circRNAs) [34], small nuclear RNAs (snRNAs) [35] and small nucleolar RNAs (snoRNAs) [36]. In vertebrates, more than 300 long non-coding RNAs (lncRNAs) are

m⁶A-methylated [14]. Such modification alters the RNA structure towards an increased protein accessibility [37], as well as subcellular localization [38]. The m⁶A of lncRNAs has critical developmental outcomes, as indicated by the requirement of m⁶A of the *Xist* lncRNA for the transcriptional silencing of the inactivated X chromosome [4]. Besides, the m⁶A in RNA encoded by transposable element genes (repeat RNAs) increases their stability and promotes chromatin compaction [39].

Aside from vertebrates, the m⁶A was described in a wide diversity of organisms, such as insects [40–42], yeasts [43], and plants [44]. However, despite the developmental significance of m⁶A being conserved across the evolution, several differences exist between animal groups. Indeed, in fruit flies, m⁶A is mostly present in the 5' UTR of mRNAs throughout head and embryo development [42,45], whereas methylation enrichment at 5' UTRs promotes cap-independent translation for transcript selection during the stress response in vertebrates [29,32,46]. In the silkworm *Bombyx mori*, mRNA is mostly methylated in coding sequences (CDS) but not UTRs, and a high methylation is associated with a high gene expression [47]. Besides, a higher m⁶A content and gene expression regulation is found upstream the diapause [41], a development phase which is reflective of a highly plastic phenotype. Finally, in *Caenorhabditis elegans*, where only few actors of the m⁶A machinery are present, methylation is mostly found in ribosomal RNAs (rRNAs) [48]. This situation opens crucial questions about the evolution of m⁶A-related molecular pathways, target genes, and developmental significance that require investigations in divergent models. However, despite being of utmost importance for our understanding of the evolution of the molecular control of developmental processes, there is a critical lack of knowledge in lophotrochozoan organisms where epitranscriptomes were not investigated to date to our knowledge.

The Pacific oyster *Crassostrea gigas* (i.e., *Magallana gigas*) is a bivalve mollusk whose great ecological and economical significance allowed it to emerge as a model species within lophotrochozoan organisms. As such, an important amount of genetic, transcriptomic, and epigenetic data has been generated in this model [49–51]. Besides, the embryo-larval development of *C. gigas* is under the strong epigenetic influence of DNA methylation [52–54] and histone marks [55,56]. Moreover, it has recently been demonstrated that m⁶A and its complete associated machinery are conserved in oysters, with features strongly indicating an epitranscriptomic regulation of development [57]. Furthermore, oysters develop as pelagic larvae in sea water before they metamorphose

into fixed adult specimen. Therefore, the early life stages of oysters are directly subjected to the variations of external environmental conditions such as temperature changes, ultraviolet (UV) exposure, or endocrine disruptor contamination, which are described to influence m⁶A-RNA in investigated species [29,46,58–60]. These elements are highly suggestive of a functional significance of m⁶A-RNA in oyster development, which remains unknown to date.

To investigate these questions, we characterized the transcriptome-wide dynamics of m⁶A-RNA methylomes across the entire development of the pacific oyster from the oocytes to the completion of the organogenesis. We used methylated RNA immunoprecipitation sequencing (MeRIP-seq) to investigate and monitor the dynamics of m⁶A levels, localization, target RNA subsets, and functional implications in a lophotrochozoan model to provide a better understanding of the evolution of developmental mechanisms and their epigenetic regulation.

Results

Oyster RNA classes display specific m⁶A signatures

In developing oysters, m⁶A methylation affects a vast majority of RNA classes, and is detected in 91.0% of mRNAs, 65.7% of lncRNAs, 87.0% of RNA transposable elements (TEs) (retrotransposons), and 99.4% of DNA TEs. In contrast, oyster transfer RNA (tRNA) are mostly unmethylated (rRNA were depleted during library preparation and not investigated here). In addition, 35.8% of mRNAs and 11.1% of lncRNAs are significantly m⁶A-enriched over background levels (**Figure 1A**). The methylation pattern depends on the RNA class considered (Kolmogorov–Smirnov [KS] test $P < 7.55 \times 10^{-11}$). While m⁶A in mRNA is found from the 5' UTR to the 3' UTR, and is more abundant around the stop codon, it is more randomly spread along lncRNAs (Figure 1B). Methylation affects adenosines located within the “AGUGA*C” sequence (where * marks the modified adenosine) (Figure 1C). The mean methylation level differs between RNA classes and DNA TE transcripts are less methylated than mRNAs, lncRNAs, and RNA TE (Figure 1D). The MeRIP-seq procedure was validated by investigation of the status of five transcripts exhibiting contrasted m⁶A vs. mRNA profiles in other species (*c-myc*, *klf*, *mettl3*, *hnrnpa2b1*, and *oct4*) by targeted methyl–RNA immuno-precipitation followed by quantitative polymerase chain reaction (MeRIP-qPCR) [15,28,32,61]. All the examined candidates displayed similar

patterns between the two techniques and those patterns are consistent with the literature (Figure S1).

Oyster m⁶A epitranscriptomes are dynamic and correspond to defined steps of the development chronology

The principal component analysis (PCA) of MeRIP-seq data indicates that the samples group together according to the embryo-larval stages and are overall organized chronologically along the X-axis (PC1) (**Figure 2A**). The PCA plot can be divided into four areas defining four developmental phases: cleavage (from oocytes to 2- to 8- cell stage), gastrulation (blastula and gastrula stages), tissue differentiation (trochophore and D-larvae stages), and metamorphosis (pediveliger and spat stages). The morula stage is clearly individualized along the PC2, suggesting peculiar m⁶A methylation and development features. This segregation is confirmed by the pairwise correlation matrix (Figure 2B). The chronological segregation of developmental stages according to m⁶A methylation indicates that m⁶A-RNA epitranscriptomes are an important component of the developmental process.

The m⁶A epitranscriptomes are dynamic during oyster development. There is an important decrease in the number of methylated transcripts at the morula stage followed by a general shift towards increased m⁶A mRNAs and lncRNAs onwards, which is particularly noticeable at the late development stages (*i.e.*, pediveliger and spat stages) (Figure 2C). The same tendency is also observed, although to a lesser extent, for RNA TE, whose unmethylated population becomes reduced in the late developmental phases (Figure 2D).

mRNA m⁶A profiles are regulated and correlated to gene expression level and kinetics throughout oyster development

The mRNA m⁶A methylation is highly dynamic during oyster development. Indeed, among the significantly methylated mRNAs (analysis of variance (ANOVA) $P < 0.05$, $n = 8404$), 17.8% of m⁶A-mRNA ($n = 1494$) display a significant variation in their m⁶A level (**Figure 3A**), and this m⁶A level is significantly correlated to their expression level (Figure 3B). The differentially methylated (DM) m⁶A-mRNAs define 4 clusters according to their methylation (and expression) profiles, corresponding to the developmental steps defined previously. Cluster1 includes mRNAs with a strong methylation and transcript content in the early stages (up to the morula stage) which

decreases afterwards. By contrast, the mRNAs within the three other clusters display poor methylation during the early stages, which strongly increases from the gastrulation (cluster 2), the trochophore stage (cluster 3), and the pediveliger stage (cluster 4), and remain strongly methylated afterwards. Overall, there is a marked decrease of m⁶A methylation at the morula stage.

Besides the level of methylation, the localization of m⁶A within mRNAs can also vary between clusters (KS test, $0.31 < P < 5.11 \times 10^{-12}$ depending on cluster pairwise comparison). Indeed, cluster 1 mRNAs, which are essentially maternal mRNAs, are methylated mostly around their stop codon in oocytes and thereafter. By contrast, mRNAs within the three other clusters, that are essentially expressed from the zygotic genome, display a marked biphasic profile, with an increased 5' m⁶A content at the vicinity of the CDS start (Figure 3C). However, this pattern shifts towards a less biphasic profile with m⁶A becoming relatively more dominant around the stop codon in the later development stages when the methylation and expression levels increase (KS test, $5.87 \times 10^{-7} < P < 5.35 \times 10^{-10}$ depending on the cluster). Furthermore, these m⁶A-mRNAs have their methylation and expression dynamics strongly correlated (clusters 2, 3, and 4), whereas early DM transcripts without 5' CDS methylation do not (cluster 1) (Figure 3D). This indicates that mRNA expression dynamics are correlated with m⁶A profile dynamics after, but not before, the zygotic genome activation. Together these results bring to light a regulation of gene expression by both the level and localization of m⁶A methylation across oyster developmental stages.

Differentially m⁶A methylated mRNAs bear the corresponding developmental functions

Gene Ontology (GO) analyses of DM m⁶A-mRNAs shows an overall term enrichment related to developmental processes, like morphogenesis and mesoderm development (Figure 4; Table S1). More specific functions correspond to each methylation cluster. Cluster 1 (cleavage) is enriched in terms related to the cell division whereas later clusters (gastrulation, tissue differentiation, and metamorphosis) bear terms that may be more a reflection of signal transduction associated to cell differentiation such as the TGF β /SMAD pathway. These results point to a functional implication of the m⁶A in the regulation of oyster developmental processes.

ncRNA transcript content is also associated to m⁶A profiles

Similar to mRNAs, lncRNA can be grouped into clusters according to the developmental chronology of their m⁶A dynamics, with the highest methylation levels during the early stages for a cleavage cluster (cluster 1), during the gastrulation and tissue type differentiation (cluster 2), and at the pediveliger and spat stages for a metamorphosis cluster (cluster 3), respectively. A marked decrease of m⁶A levels at the morula stage is also observed for lncRNAs (**Figure 5A**). The methylation and expression levels are positively correlated overall (Figure 5B), although the methylation and expression dynamics are correlated only for the “late” clusters 2 and 3, but not for the “early” cluster 1 (Figure 5C).

RNA TE m⁶A methylation is regulated across oyster development

The methylation of RNATE is mostly represented by the methylation of long terminal repeat (LTR) and long interspersed nuclear elements (LINE) groups (62.92% and 31.46%, respectively). With 85.5% and 100% of transcripts being methylated, respectively. Their methylation levels display a peak at the blastula and trochophore stages for LTR and LINE, respectively (**Figure 6A**). Both DNA and retrotransposon methylation levels were dynamic during oyster development (ANOVA $P < 0.05$). Terminal inverted repeat (TIR) (37.13%), Helitron (32.50%), and Crypton (20.25%) are the three main DNA TE types whose transcripts are the most methylated and represented 92.6%, 88.5%, and 100% of transcripts in each type, respectively. Their m⁶A levels are the highest around the gastrulation, and Helitron TEs exhibit an additional peak in pediveliger larvae (Figure 6B). These results show that the m⁶A methylation of transposon transcripts displays class-specific dynamics during oyster development that are different from mRNAs and lncRNAs.

Discussion

This work presents the profiling of m⁶A epitranscriptomes during the development of the oyster *C. gigas* from the egg to the completion of organogenesis. We demonstrate that the methylation of RNA is dynamic throughout the oyster’s early life and specific to the RNA population considered. The methylation kinetics of mRNAs defines clusters corresponding to the chronology of development, which is correlated to gene expression level and dynamics, and the genes whose transcripts display m⁶A regulation bear developmental functions. lncRNAs display similar profiles while TE transcripts

have a specific signature with a peak in m⁶A levels during gastrulation. Our study provides evidence for a developmental significance of m⁶A epitranscriptomes at various levels in oysters.

The distinct oyster RNA classes present specific m⁶A signatures and vary in terms of methylation level and location. Indeed, while mRNAs are m⁶A-modified from the CDS start but mostly around the stop codon, lncRNAs do not present a biased methylation enrichment at their 3' end. This result, suspected from our previous measurements of global m⁶A content in polyA vs. total RNAs [57], is more consistent with what is observed in human embryonic cell types [16] than in insects where methylated adenosines are mostly lying in the 5' UTR and CDS [19,42,47]. This finding is rather surprising because insects are ecdysozoans which are considered the “sister clade” of lophotrochozoans within protostomes. Nevertheless, this assumption is increasingly moderated by the closer resemblance of molecular features in mollusks and annelids to vertebrates, *i.e.*, deuterostomes, rather than to ecdysozoan features [54,62,63]. Overall, m⁶A are found in oysters within the motif “DRA*C” which contains the GAC consensus sequence which is widely conserved across the evolution [14,19,40,43,44]. The repetitive nature of TE sequences only allows a less precise analysis of their m⁶A features compared to other RNA classes. However, we show that oysters' DNA TE transcripts are significantly less methylated than RNA TEs, which is in line with observations made in *Arabidopsis* [64].

Oyster epitranscriptomes are dynamic and associated to defined successive developmental steps, namely cleavage, gastrulation, tissue differentiation, and metamorphosis. The similarity of m⁶A profiles in eggs before and after fertilization suggests a poor contribution of sperm to embryonic m⁶A methylomes, which is consistent with the sperm scarce RNA content regarding the oocyte stock. Epitranscriptomes clearly shift during the developmental chronology, with a marked decrease of m⁶A levels in mRNA and lncRNA transcripts at the end of cleavage followed by a gradual increase, and a maximum methylation reached at the pre-metamorphosis stage that remains high afterwards in juveniles. The morula stage constitutes a pivot point where the methylation of mRNA and lncRNA, but not of RNA TEs, is strongly depleted. Interestingly, based on transcriptomic [65] and 5mC-DNA methylation data [52], this stage could be considered an important window of MZT in the oyster, when most maternal RNA resources are consumed and early transcriptional events occur [66]. Interestingly, the marked epitranscriptomic features at cleavage and

metamorphosis may remind of the important shifts in 5mC-DNA profiles (decrease during cleavage and important levels prior to metamorphosis) [52] and could suggest an interplay between the epigenetic layers in the regulation of oyster development, possibly through competition for methyl donor [67] or histone-related pathways [68,69]. This observation is reminiscent of the crosstalk between the 5mC-DNA status of the Pou5f1 pioneer stem cell factor promoter and the m⁶A methylation of its transcript in humans [16], hence raising the question of m⁶A-modified transcripts in the oyster development.

The m⁶A content and transcript abundance of DM mRNAs are positively correlated across oyster development, which surprisingly contrasts with the case of the honeybee where highly methylated transcripts exhibit downregulated expression [19], thus further indicating an evolutionary divergence between mollusks and insects regarding epitranscriptomic mechanisms. Overall, oyster DM mRNAs have functions related to developmental processes, which further confirms the developmental significance of the epitranscriptomic regulation in the oyster. More precisely, DM mRNA transcripts group into 4 clusters that match the development steps described above, whose GO annotations assess the functional relevance in the cognate developmental processes (cluster 1, cleavage; cluster 2, gastrulation; cluster 3, tissue differentiation; cluster 4, metamorphosis). However, these clusters do not always display similar methylation profiles neither correlations between m⁶A and expression level dynamics.

Cluster 1 mRNA transcripts are highly methylated and abundant during the cleavage. They are present in oocytes and therefore correspond to maternal transcripts. While their expression and methylation levels are dramatically reduced at the onset of MZT (morula stage), their m⁶A and transcript content dynamics are not significantly correlated, and they display predominant methylation around the stop codon throughout oyster development. Such m⁶A profiles promote mRNA degradation via interaction with readers such as YTHDF proteins during cleavage in other species [5,70], and we showed that an oyster YTHDF orthologue is present at high levels in this window [57]. Besides, cluster 1 DM mRNAs bear functions in the cytoskeleton processes of cytokinesis. Together, these results indicate that the oyster MZT may be triggered by YTHDF-mediated decay of maternal mRNAs, whose translation is important for cell proliferation during cleavage. We hypothesize that the depletion of the cytokinesis machinery mRNA templates of maternal origin below a certain threshold, and/or the putative associated slowdown of cell divisions, may participate in triggering the zygotic

genome activation. It remains to be deciphered whether and how such signals could be interpreted in terms of cell fate decisions.

Clusters 2, 3, and 4 respectively correspond to the gastrulation, the setting up of differentiated tissues, and the metamorphosis. In contrast to cluster 1, their m⁶A pattern is dynamic and shifts from bimodal profiles in early stages to a late mostly unimodal m⁶A distribution around the stop codon. Such 5'-enriched m⁶A profiles are observed in plants [44], *Xenopus* [71], mice [2], and fruit flies [45], and may be associated to increased RNA turnover and fast transcript processing depending on the positioning of methylation in the CDS or 5' UTR. Consistently, in developing oysters, the early bimodal m⁶A distribution is associated with a low transcript abundance. The 3' patterns are associated to the recruitment of readers such as YTHDC1 [25,72,73], IGF2BP [28], or Prrc2a [8], and mediate translation initiation during gastrulation, as well as splicing and RNA trafficking during cell type differentiation. In oysters, the later sequential shift of clusters towards more 3'-dominant methylation is correlated to an increased expression level of the DM mRNA transcripts. Furthermore, those transcripts that become successively methylated and expressed bear the cognate developmental functions such as mesoderm specification, morphogenesis, and signal transduction related to cell differentiation. In addition, readers including YTHDCs, IGF2BP, and Prrc2a are functional in oysters and present in stage-specific sets after the MZT [57], and METTL3 expression is also dramatically increased after the MZT and sustained later on. Together, this strongly suggests that m⁶A regulates gene expression towards cell differentiation upon the zygotic genome activation, possibly by promoting translation via transcript selection and increased mRNA stability. As suggested by GO annotation, m⁶A likely participates in mesoderm formation during gastrulation via a METTL3-dependent regulation of epithelial to mesenchymal transition, as described in human cancer cells [74,75]. Our findings (Table S1) are also consistent with the METTL3/YTHDF2-dependent osteoblast differentiation by SMAD inhibitor downregulation in mammalian cells [76,77]. Regarding the conservation of the TGF β pathway in oyster development [78,79], these results may also suggest feedback loops between TGF β signaling and m⁶A regulation in the control of (pluri)potency during differentiation [80].

lncRNAs methylation levels display similar developmental dynamics to mRNAs, although m⁶A are not preferentially located at their 3'-end. Like mRNAs, the methylation levels in lncRNAs are also correlated to their transcript content and

dynamics after the MZT. How lncRNA epitranscriptomes might participate in oyster development is less clear because oyster lncRNAs are poorly conserved and annotated. Besides, their limited number compared to mRNAs in addition to the limited resolution of MeRIP-seq did not allow the detection of m⁶A peak localization shifts. Nevertheless, the vertebrate writer complex cofactor RBM15/15B and the hnRNPA2B1 nuclear complex are conserved in oysters [57]. Therefore, we speculate that oyster lncRNA epitranscriptomes could regulate the chromatin state and transcription in an m⁶A-dependent manner, which is somewhat reminiscent of the *Xist*-mediated silencing [3,4], MALAT1-mediated stimulation [81], and/or chromosome-associated regulatory RNA (carRNA) switches in mammals [39]. However, *Xist* is not conserved in oysters and further work is required to test this hypothesis.

Transposon transcripts display class-specific dynamics during oyster development that are different from mRNAs and lncRNAs. Both RNA TEs and DNA TEs, which are poorly m⁶A modified during cleavage, exhibit higher methylation during gastrulation and cell differentiation. Although the role of epitranscriptomes in transposon control remains largely unknown, this kinetics may suggest the participation of m⁶A within the widely conserved RNA mechanisms of TE silencing required for cell differentiation [82–84].

Altogether, our results demonstrate that oyster m⁶A epitranscriptomes are dynamic and RNA-class specific, and reveal their implication in oyster MZT and sequential expression of genes required for gastrulation, cell differentiation, and metamorphosis. Although additional studies would be required to determine the interplay of m⁶A-RNA with the epigenetic network and the underlying mechanisms, this first evidence of an epitranscriptomic regulation of development in a lophotrochozoan species allows for a better understanding of developmental processes and their evolution.

Materials and methods

Animals

Broodstock oysters, embryos, larvae, and spat were obtained at the Ifremer marine facility (Argenton, France) as previously described [52,85]. Briefly, gametes of mature broodstock oysters were obtained by stripping the gonads and filtering the recovered material on a 60 µm mesh to remove large debris. Oocytes were collected as the remaining fraction on a 20 µm mesh and spermatozoa as the passing fraction on a 20

μm mesh. Oocytes were pre-incubated in 5 l of UV-treated and 1 μm mesh filtered sterile sea water (SSW) at 21 °C until germinal vesicle breakdown. Fertilization was triggered by the addition of *ca.*10 spermatozooids per oocyte. After the expulsion of the second polar body was assessed by light microscopy, embryos were transferred in 150 l tanks of oxygenated SSW at 21 °C. The embryonic stages were determined by light microscopy observation. The embryonic stages collected were oocytes (E, immediately before sperm addition), fertilized oocytes (FE, immediately before transfer to 150 l tanks), two to eight cell embryos (2–8 C, *ca.* 1.5 h post fertilization [hpf]), morula (M, *ca.* 4 hpf), blastula (B, *ca.* 6 hpf), gastrula (G, *ca.* 10 hpf), trochophore (T, *ca.* 16 hpf), and D larvae (D, *ca.* 24 hpf). For pediveliger (P) and spat (S) stages, D-larvae were collected and reared in a flow-through rearing system at 21.5 °C in SSW. At the end of the pelagic stage, competent larvae were collected on a 100 μm mesh to allow the larval settlement. At 20 days post fertilization (dpf) the pediveliger stage was sampled as the remaining fraction on a 400 μm mesh. The post-larvae were maintained in a downwelling system. Then, at 25 dpf the spat stage was sampled after metamorphosis as the remaining fraction on a 400 μm mesh.

For each embryonic stage, 3 million embryos were collected as the remaining fraction on a 20 μm mesh and centrifuged at 123 g for 5 min at room temperature. The supernatant was discarded and samples of 1 million embryos were then snap-frozen in liquid nitrogen directly after resuspension in TRI Reagent (Catalog No. T9424, Sigma-Aldrich, St Louis, MO) (1 ml/10⁶ embryos). For pediveliger and spat samples, 100 mg of each stage were resuspended in TRI Reagent (Catalog No. T9424, Sigma-Aldrich, St Louis, MO) (1 ml/100 mg) and snap-frozen in liquid nitrogen. All samples were stored at –80 °C. Two distinct experiments were realized (February and March 2019) using the gametes of 126 and 140 broodstock animals, respectively (Figure S2).

RNA extraction and fragmentation

RNA of each development stage was extracted using phenol-chloroform followed by affinity chromatography as previously described [86]. Briefly, embryos were ground in TRI Reagent (Catalog No. T9424, Sigma-Aldrich, St Louis, MO) and RNA was purified using affinity chromatography with NucleoSpin RNA Clean Up Kit (Catalog No. 740948, Macherey-Nagel, Duren, Germany). Potential contaminating DNA was removed by digestion with rDNase (Catalog No. 740963, Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions for 15 min at 37 °C and then

RNA was purified using Agencourt AMPure XP solid-phase reversible immobilization (SPRI) paramagnetic beads (Catalog No. A63880, Beckman Coulter, Brea, CA) according to the manufacturer's instructions. Briefly, paramagnetic beads and RNAs were mixed slowly and incubated 5 min at room temperature followed by 2 min on a magnetic rack. The cleared supernatant was removed, and beads were washed three times with 70% ethanol. After 4 min of drying at room temperature, RNAs were mixed slowly with RNase free water and incubated for 1 min at room temperature on the magnetic rack. The eluted total RNA was stored at -80°C .

The RNA was heat-fragmented as previously described [87]. Briefly, for each RNA sample (development stages and pools of development stages), 5 μg of total RNA was suspended in 18 μl of RNase free water. The RNA was fragmented by the addition of 2 μl of fragmentation buffer (100 mM Tris-HCl pH 7.0, 100 mM ZnCl_2 , diethyl-pyrocabonate-treated (DEPC) water) and incubated for 2 min at 70°C . After the incubation, 2 μl of ethylenediaminetetraacetic acid (EDTA) 0.5 M was immediately added and RNAs were incubated on ice for 2 min to stop the reaction. Then, RNAs were purified using Agencourt AMPure XP SPRI paramagnetic beads (Catalog No. A63880, Beckman Coulter, Brea, CA) as previously described and eluted in 50 μl of RNase free water. The fragment size (*ca.* 100 nucleotides) was verified by fluorescent capillary electrophoresis using an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Pico Kit (Catalog No. 5067-1513, Agilent, Santa Clara, CA) according to the manufacturer's instruction.

MeRIP

The MeRIP-seq of total RNA was performed on fragmented RNA. Of the 50 μl fragmented RNA solution, 5 μl (*i.e.*, corresponding to 500 ng of starting RNA before fragmentation) was non immunoprecipitated and used as the input fraction (Input), and 44 μl (*i.e.*, corresponding to 4.5 μg of starting RNA before fragmentation) were subjected to m^6A -immunoprecipitation using m^6A antibody-coupled PierceTM Protein A/G Magnetic Beads (Catalog No. 88803, Thermo Fisher Scientific, Waltham, MA) following the low/high salt procedure by Zeng and collaborators [87]. For each sample, 30 μl of protein A and 30 μl of protein G magnetic beads were incubated on a magnetic rack and washed twice with IP buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, IGEPAL 0.1%, DEPC water) and resuspended in 500 μl of IP buffer. The magnetic

beads were incubated overnight at 4 °C under gentle shaking with 5 µg of anti-m⁶A antibody (Catalog No. ABE572, Millipore, Burlington, MA). Then magnetic beads were washed twice with IP buffer and resuspended in RNA mixture composed of 100 µl of 5× IP buffer, 44 µl of fragmented RNAs, and 200 units of RNasin Plus Ribonuclease Inhibitor (Catalog No. N2611, Promega, Madison, WI), and the mixture was completed to 500 µl with IP buffer. The obtained MeRIP solution containing magnetic beads coupled to antibody and fragmented RNAs was incubated for 2 h at 4 °C with gentle shaking. After incubation, the MeRIP solution was incubated on a magnetic rack and the supernatant was removed. Beads were washed twice in IP buffer, twice in low salt buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.1% IGEPAL, DEPC water), and twice in high salt buffer (500 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.1% IGEPAL, DEPC water) for 10 min at 4 °C with gentle shaking for each washing step. After extensive washing with IP buffer, RNAs hybridized to the antibody-coated magnetic beads were resuspended in 200 µl of RA1 buffer supplied in the NucleoSpin RNA Clean Up Kit (Catalog No. 740948, Macherey-Nagel, Duren, France) and incubated 2 min at room temperature then 1 min on a magnetic rack. The supernatant containing immunoprecipitated RNA was collected and mixed with 400 µl of 100% ethanol. Then the immunoprecipitated RNA was purified using affinity chromatography with NucleoSpin RNA Clean Up Kit (Catalog No. 740948, Macherey Nagel, Duren, France) and eluted twice with 14 µl of RNase free water. These immunoprecipitated RNAs correspond to the immunoprecipitated fraction (IP).

Reverse transcription-quantitative polymerase chain reaction

To validate the immunoprecipitation of m⁶A methylated RNA, a Reverse transcription-quantitative polymerase chain reaction (RT-Qpcr) was performed on immunoprecipitated RNA pools. These pools are composed of equal amounts of RNA from each developmental stage for the two distinct experiments. Input and IP fractions were obtained as described above. The equivalent amount of 1.75 ng of Input and IP RNAs were used as starting template for the RT-qPCR protocol previously described [86]. Targets included one reference transcript (*ef1α*) and five conserved genes exhibiting distinct transcript m⁶A methylation in vertebrates (*c-myc*, *klf*, *mettl3*, *hnrnpa2b1*, and *oct4*). Briefly, RNAs were reverse-transcribed using 200 U of M-MLV Reverse Transcriptase (Catalog No. M1701, Promega, Madison, WI) and 100 ng random hexamers. Resulting cDNAs were assayed for target gene expression using the

Input expression as a reference. SYBR Green quantitative PCR was performed on a CFX96 apparatus (Catalog No. 1845096, Bio-Rad, Marnes-la-Coquette, France). Gotaq qPCR Master Mix (Catalog No. A6002, Promega, Madison, WI) was used in 40 cycles (95 °C/15 s and 60 °C/15 s) reactions. Target genes included one reference gene and 5 target genes with distinct transcript m⁶A methylation in vertebrates, and were amplified with the following primers: *Cg-efl α* (CGI_10012474, LOC105338957; forward: 5'-ACCACCCTGGTGAGATCAAG-3', reverse: 5'-ACGACGATCGCATTTCTCTT-3') as reference transcript of gene expression in *C.gigas* [51], genes described as methylated in vertebrates: *Cg-c-myc* (CGI_10002799, LOC105341025; forward: 5'-CGGTCTCTCCCAAATTCTCCC-3', reverse: 5'-TGCTACTTCCACTTGCCCTG-3') [15,28], *Cg-klf* (CGI_10019173, LOC105329817; forward: 5'-GAAATCTCCGATGTTGCTGG-3', reverse: 5'-CTTTCCACGGTATTTGCGAG-3') [15], *Cg-mettl3* (CGI_10023713, LOC105329074; forward: 5'-TGGAACCAAAGAAGAGTGTCAGA-3', reverse: 5'-AGAAATGAACAGTCTCCAAGGGA-3') [32], and *Cg-hnrnpa2b1* (CGI_10028469, LOC105333434; forward: 5'-CCAGGGAGGCTACAATGAAGG-3', reverse: 5'-ACACCACCACCAAAGCTGTT-3') [61], and a gene described as not methylated in vertebrates: *Cg-oct4* (XM_034479449, LOC117680409; forward: 5'-GTGAAAGGTGCGCTAGAAAA-3', reverse: 5'-GGACCACTTCTTTCTCCAGT-3') [15]. The methylation level was calculated as the normalized ratio of the IP and the Input signals by the formulae: $2^{(-\Delta(\text{Ct IP}-\text{Ct Input}))}$. The gene expression level was assayed on the Input fractions, normalized on the reference transcript *efl α* [51], and given by the formula $2^{(-\Delta\text{Ct})}$.

Library preparation and sequencing

Amounts equivalent to 1.125 μg of starting RNA of the IP fraction (*i.e.*, 3.5 μl of the eluted IP fraction) and to the 50 ng of starting RNA of the Input fraction for each sample, respectively, were used for library construction using the SMARTer Stranded Total RNA-Seq Kit v.2 (Catalog No. 634418, Pico Input Mammalian, Takara/Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's protocol without RNA fragmentation. The ribosomal cDNA depletion step and a final cDNA amplification of 16 cycles were performed. Paired-end 150-bp sequencing of Input and IP cDNA libraries of each sample were conducted on an Illumina HiSeq 4000 platform

(Catalog No. SY-401-4001, Illumina, San Diego, CA) at the Genome Quebec Innovation Center (McGill University, Montréal, Canada).

MeRIP-seq data analyses

Read quality was evaluated using FastQC (v.0.11.7) and MultiQC (v.1.7) and adaptor sequences and low-quality reads were removed using Trimmomatic (v.0.38). The remaining reads were aligned to the oyster genome v.9 (GCF_000297895.1) and uniquely mapped reads were counted using STAR (v.2.7.3a) with the parameter “-quantMode GeneCounts” [88]. mRNA and ncRNA were identified from the gene annotation and TEs were identified using the RepeatMasker annotation output provided with assembly data. The expression level in all Input samples was expressed in transcripts per million (TPM) [89]. The identification of m⁶A-enriched peaks was performed on uniquely mapped reads of the IP samples using SAMtools (v.1.9) and MeTPeak R package [90] [false discovery rate (FDR) < 5%] with the cognate Input samples as controls. The methylation level of these m⁶A-enriched RNAs corresponds to the mean of the IP/Input fold change provided by MeTPeak from the two development experiments. The distribution of m⁶A across mRNAs and ncRNAs was visualized using Guitar plots [91]. The methylation level of TEs was assessed as the ratio of IP/Input with reads per gene expressed in TPM. Transcript variants were pooled for each gene and only the transcripts that were present in the two distinct development experiments were considered expressed or methylated in Input and IP data, respectively. The gene expression and m⁶A level are expressed as the mean of the two replicates of development stages from the independent experiments

The m⁶A motif was searched in the 1000 m⁶A peaks presenting the lowest FDR and the highest IP/Input fold change using HOMER (v.4.10.4). The motif length was restricted to 5–6 nucleotides. All peaks mapped on mRNAs and ncRNAs were used as the target sequences and the background sequences were constituted of 5% of the Input reads (pool of February development experiment sample) selected randomly using SAMtools (v.1.9).

GO analysis

The RNA sequences identified as DM across oyster development were identified using BLASTN [92–94] against the GigaTON reference transcriptome database [65] with default settings. GO analyses were carried out with the GO annotations obtained from

the GigaTON database gene universe [65]. GO term enrichment tests were performed using ClueGO plugin (v.2.5.7) [95] on Cytoscape (v.3.8.0). The hypergeometric test with a FDR < 5% was used to consider significant GO term enrichment.

Statistical analyses and graph production

The m⁶A level variation across oyster development was analyzed using one-way ANOVA (factor: development stage) followed by Bonferroni's post hoc test when required, unless otherwise stated. m⁶A levels variations were computed on significantly methylated mRNAs and lncRNAs (*e.g.*, “enriched” RNAs according to MeTPeak results) and on RNA TE displaying detectable methylation because of limitations in their accurate location within the genome due to their repetitive sequences. The values were log-centered and reduced for heatmap production when stated. Methylation profiles were compared using KS tests (CDS regions were divided into 20 bins, while start and stop codon intervals as well as 5' UTR and 3' UTR regions were left unmodified from the default MetPeak output). The correlation between methylation level and transcript content across oyster development, as well as methylation variation and transcript content variation between stages, were estimated using linear regression. A *P* value < 0.05 was considered significant. All bioinformatic analyses (unless otherwise stated) were performed using R (v.3.6.3) and RStudio (v.1.0.153) softwares. The R packages *eulerr* [96], *ComplexHeatmap* [97], *ggplot2* [98], *Guitar* [91], *PCAtools* [99], and Prism v.6 (GraphPad) software were used for figure production.

Data availability

All data relative to this study are publicly available at the National Center for Biotechnology Information Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) under the accession number GEO: GSE180388, and in the Genome Sequence Archive [100] at the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences / China National Center for Bioinformation (GSA: CRA007976), and are publicly accessible at <https://ngdc.cncb.ac.cn/gsa>.

CRedit author statement

Lorane Le Franc: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review and editing, Visualization. **Bruno Petton:** Investigation, Resources, Writing - review and editing. **Pascal Favrel:** Writing - review and editing, Funding acquisition. **Guillaume Rivière:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review and editing, Visualization, Supervision, Project administration, Funding acquisition. All authors have read and approved the final manuscript.

Competing interests

The authors have declared no competing interests.

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Figure legends

Figure 1 m⁶A signatures of oyster RNA classes

A. m⁶A distribution among RNA classes. Diagram diameter was normalized between RNA classes. The internal circles in diagrams represent the proportion of RNA displaying significant m⁶A methylation over background (light color) and significant m⁶A enrichment (dark color), respectively. **B.** m⁶A localization along mRNAs and ncRNAs (5' to 3'). The mean m⁶A density and confidence interval are represented. **C.** Consensus sequence of the m⁶A motif in the oyster identified by HOMER. **D.** Mean methylation level of the methylated transcripts of mRNA, lncRNA, DNA TE, and RNA TE classes during oyster development. Letters discriminate significantly different methylation levels (ANOVA followed by Bonferroni's post hoc test, $P < 0.05$). mRNA, messenger RNA; lncRNA, long non-coding RNA; DNA TE, DNA transposable elements; RNA TE, retrotransposons; misc_RNA, miscellaneous RNA; tRNA, transfer RNA; ncRNA, non-coding RNA; ANOVA, analysis of variance; UTR, untranslated transcribed region; CDS, coding sequence; kb, kilobase.

Figure 2 Epitranscriptome dynamics during oyster development

A. PCA of MeRIP-seq results. PCA plot of the first two principal components shown the MeRIP-seq results for each developmental sample of the two distinct experiments. **B.** Similarity (pairwise Pearson's correlation matrix) of m⁶A methylation between samples based on IP/Input signal (see methods). **C.** Dynamics of the m⁶A methylation level of the mRNA (purple) and lncRNA (blue) transcripts. All the transcripts found m⁶A-enriched over background using MetPeak in at least one development stage are represented. Areas under the curve are normalized for each RNA class, and undetectable methylation at certain stages was arbitrarily affected a -5 value for representation purpose. **D.** Dynamics of the m⁶A methylation level of the DNA TE (green) and RNA TE (yellow) transcripts. All the transcripts found m⁶A-enriched over background using MetPeak in at least one development stage are represented. Areas under the curve are normalized for each RNA class, and undetectable methylation at certain stages was arbitrarily affected a -5 value for representation purpose. **E.** egg (oocyte); FE, fertilized egg; 2-8C, two to eight cell-stage; M, morula; B, blastula; G, gastrula; T, trochophore; D, D-larva; P, pediveliger; S, spat/juvenile; PCA, principal

component analysis; MeRIP-seq, methylated RNA immunoprecipitation sequencing; PC, principal component; IP, the immunoprecipitated fraction; Input, the input fraction.

Figure 3 Dynamics of mRNA expression and m⁶A methylation during oyster development

A. Normalized expression (TPM, purple) and m⁶A content (IP/Input, pink) of significantly differentially methylated mRNA genes during oyster development (n =1494). The development stages are indicated below. **B.** Correlation between expression (Y-axis, TPM) and methylation (X-axis, IP/Input, divided into twelve quantiles). **C.** Methylation profiles of mRNAs displaying high (pink) or low (grey) expression within each cluster. The development stages in high and low expression groups are indicated (mean m⁶A density; confidence intervals were omitted for clarity). **D.** Correlation of expression vs. methylation dynamics throughout development. The linear correlation of the methylation variation vs. expression variation was assessed for each gene across oyster development per cluster and the results are given as surface plots (R-square, X-axis; *P* value, Y-axis; density of genes, Z-axis). Yellow color in the right bottom corner (*i.e.*, *P* value < 0.05 and *R*² > 0.5) indicates strong correlation. TPM, transcripts per million.

Figure 4 Functional annotation of differentially methylated mRNAs during oyster development

Enriched Gene Ontology terms (hypergeometric test, FDR < 0.05) associated to differential m⁶A mRNA methylation across oyster development. Enriched terms corresponding to specific clusters are colored, and common terms between clusters are black. Color intensity is relative to cluster specificity, with color limit set to 51% of genes inside the respective clusters. Circle diameter is inversely proportional to the *P* value of the enrichment. FDR, false discovery rate.

Figure 5 Dynamics of lncRNA expression and m⁶A methylation during oyster development

A. Normalized expression (blue) and m⁶A content (turquoise) of significantly methylated lncRNA genes during oyster development. Transcript variants were pooled for each gene. **B.** Correlation between expression (Y-axis) and methylation (X-axis) levels. The methylation level was divided into ten quantiles. **C.** Correlation plot of

expression vs. methylation dynamics throughout development. The linear correlation of the methylation vs. expression variation was assessed for each gene across oyster development per cluster and the results are given as surface plots (R-square, X-axis; *P* value, Y-axis; density of genes, Z-axis).

Figure 6 m⁶A dynamics of TE transcripts during oyster development

A. Relative representation of methylated RNA TE transcripts. The percentages in parenthesis indicate the proportion of the TE class that was found methylated, the initial proportion of the transposon class in the genome, and its proportion of the TEs investigated (top). Relative representation of methylation level variation during oyster development is also shown (bottom). **B.** Relative representation of methylated DNA TE transcripts. The percentages in parenthesis indicate the proportion of the TE class that was found methylated, the initial proportion of the transposon class in the genome, and its proportion of the TEs investigated (top). Relative representation of methylation level variation during oyster development is also shown (bottom). Values are given as the mean of the two independent experiments. Only TEs representing more than 20% of their class are represented and error bars were omitted for clarity. Reference methylation level is the mean oocyte methylation level of RNA TE and DNA TE, respectively. LTR, long terminal repeat; LINE, long interspersed nuclear elements; TIR, terminal inverted repeat; DIRS, *Dictyostelium* intermediate repeat sequence.

Supplementary material

Figure S1 Validation of the m⁶A immunoprecipitation procedure by MeRIP- and RT-qPCR on target transcripts

A. Relative methylation (left) and expression level (right) of targeted transcripts *c-myc*, *klf*, *mettl3*, *hnrnpa2b1*, and *oct4* quantified by methyl-RNA immunoprecipitation followed by quantitative polymerase chain reaction (MeRIP-qPCR) (left) and RT-qPCR (right) on a pool of RNA from oyster developmental stages (see methods). The dotted line represents the threshold for m⁶A-RNA enrichment. **B.** Methylation (left) and expression level in TPM (right) of targeted transcripts *c-myc*, *klf*, *mettl3*, *hnrnpa2b1*, and *oct4* quantified by MeRIP-seq (left) and RNA sequencing (RNA-seq) on a pool of RNA from oyster developmental stages. Error bars represent the standard deviation from two independent biological replicates each assayed in technical triplicates.

Figure S2 Overview diagram of the sampling procedure

UV, ultraviolet; SSW, sterile sea water.

Table S1 Complete list of significant GO terms in each cluster for m⁶A-mRNAs**CRedit author statement**

Lorane Le Franc: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review and editing, Visualization.

Bruno Petton: Investigation, Resources, Writing - review and editing. **Pascal Favrel:** Writing - review and editing, Funding acquisition. **Guillaume Rivière:**

Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review and editing, Visualization, Supervision, Project administration, Funding acquisition. All authors have read and approved the final manuscript.

Figure 1

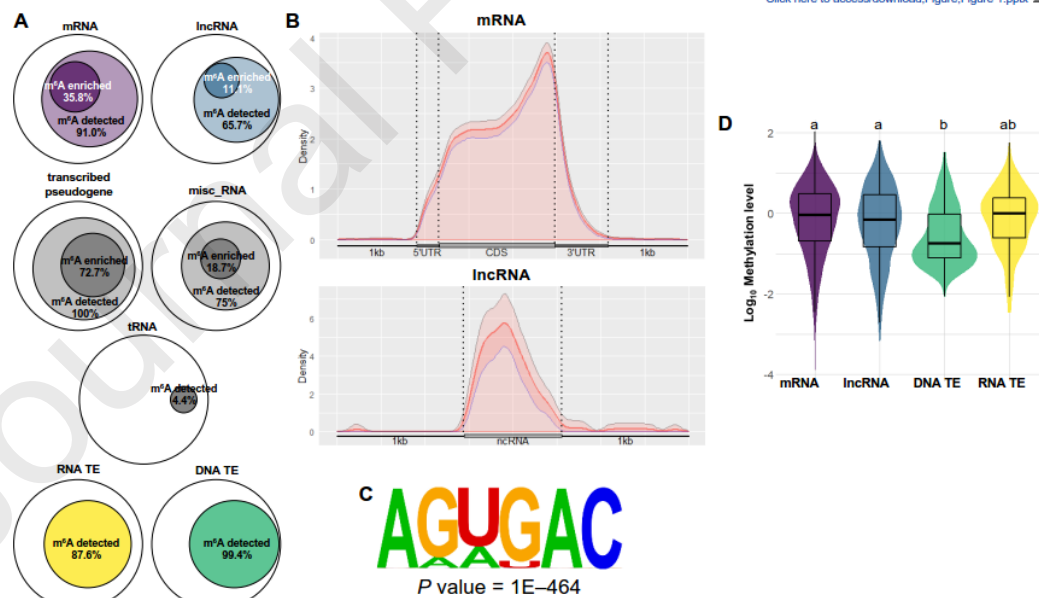


Figure 2

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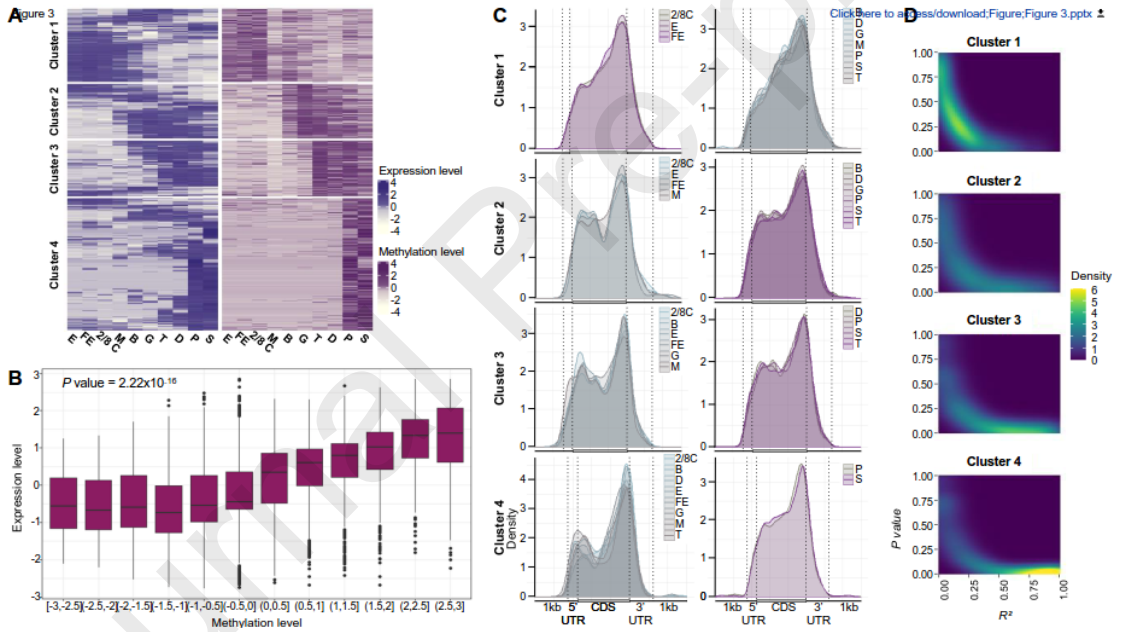
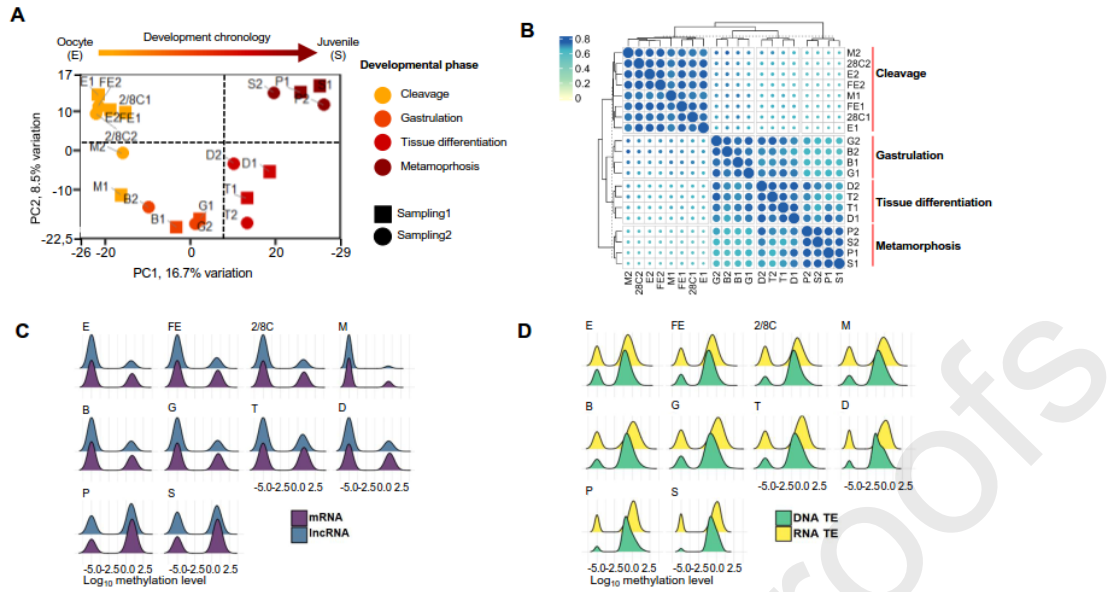


Figure 4

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Figure 5

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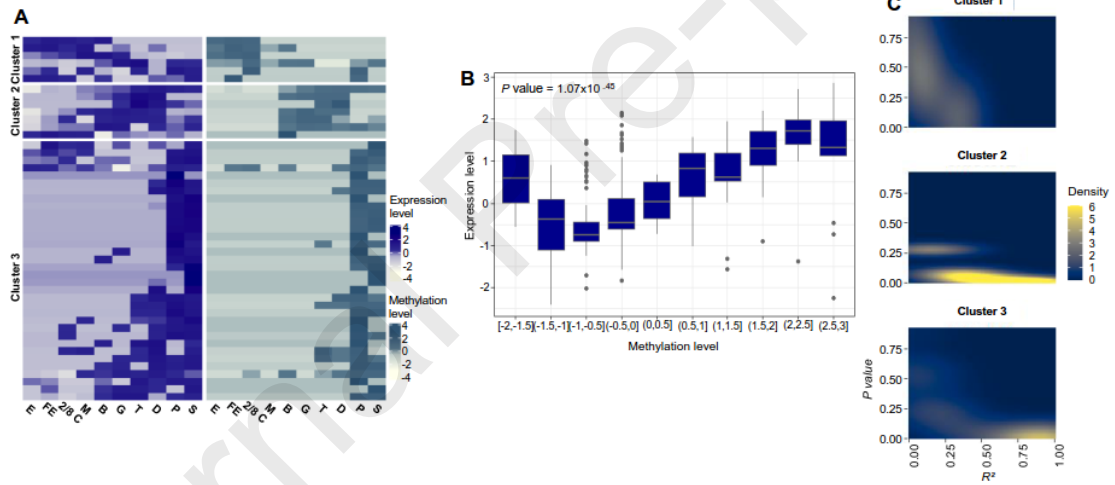
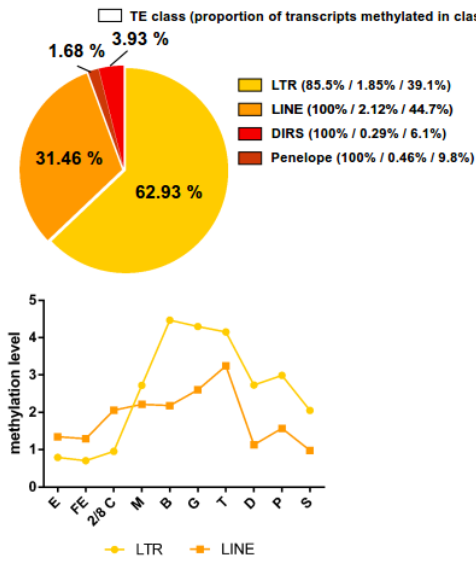


Figure 6 **A**



B

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