



Sperm is epigenetically programmed to regulate gene transcription in embryos

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1 **Title: Sperm is epigenetically programmed to regulate gene transcription in**
2 **embryos**

3

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1 **Running title:** Sperm is programmed for embryonic transcription

2

3 **Abstract**

4 For a long time it has been assumed that the only role of sperm at fertilization is to
5 introduce the male genome into the egg. Recently, ideas have emerged that the epigenetic
6 state of the sperm nucleus could influence transcription in the embryo. However, conflicting
7 reports have challenged the existence of epigenetic marks on sperm genes, and there are no
8 functional tests supporting the role of sperm epigenetic marking on embryonic gene
9 expression. Here we show that sperm is epigenetically programmed to regulate embryonic
10 gene expression.

11 By comparing the development of sperm- and spermatid-derived frog embryos we
12 show that the programming of sperm for successful development relates to its ability to
13 regulate transcription of a set of developmentally important genes. During spermatid
14 maturation into sperm, these genes lose H3K4me2/3 and retain H3K27me3 marks.
15 Experimental removal of these epigenetic marks at fertilization deregulates gene expression in
16 the resulting embryos in a paternal chromatin dependent manner. This demonstrates that
17 epigenetic instructions delivered by the sperm at fertilization are required for correct
18 regulation of gene expression in the future embryos.

19 The epigenetic mechanisms of developmental programming revealed here are likely to
20 relate to the mechanisms involved in transgenerational transmission of acquired traits.
21 Understanding how parental experience can influence development of the progeny has broad
22 potential for improving human health.

23

24

1 **Keywords:** *Xenopus*, programming, sperm, spermatid, histone post-translational
2 modifications, transcription, H3K27, H3K4, epigenetics, embryonic development

3

4 **Introduction**

5

6 Embryos obtained by fertilization develop to adulthood more frequently than those
7 obtained by other methods, such as nuclear transfer (Gurdon et al. 1958; Kimura and Yanagimachi
8 1995), suggesting that sperm is specially programmed to support normal embryonic
9 development. Several hypotheses were proposed to explain the nature of this programming,
10 including the idea that sperm is programmed for efficient replication after fertilization
11 (Lemaitre et al. 2005) or for supporting proper embryonic transcription (Suzuki et al. 2007; Hammoud
12 et al. 2009; Zheng et al. 2012). The latter hypothesis was proposed following the observation that
13 promoters of developmentally-important genes escape global replacement of histones by
14 protamines in mature sperm. In fact, these promoters retain post-translationally modified
15 histones, suggesting that epigenetic marks on sperm chromatin may be transmitted to the
16 embryo at fertilization and could subsequently pattern transcription of embryonic genes
17 (Suzuki et al. 2007; Hammoud et al. 2009; Brykczynska et al. 2010; Wu et al. 2011; Paradowska et al. 2012;
18 Zheng et al. 2012; Ihara et al. 2014; Siklenka et al. 2015). However, the validity of this hypothesis was
19 recently questioned (Carone et al. 2014; Samans et al. 2014).

20 In this work we compared the developmental potential of sperm to that of spermatids
21 in order to understand the nature of sperm programming for development in *Xenopus laevis*.
22 The use of spermatids, immediate precursors of sperm, suits such comparisons because: 1.
23 spermatids have completed meiosis and have the same DNA content as sperm, 2. spermatid
24 chromatin structure resembles that of somatic cells (Gaucher et al. 2010). Furthermore, in the
25 mouse, embryos derived from injection of spermatids into unfertilized oocytes develop to

1 adulthood less frequently than embryos derived from injection of sperm, suggesting that
2 developmentally important information is acquired during spermatid to sperm maturation
3 (Kimura and Yanagimachi 1995; Kishigami et al. 2004). Here, we demonstrate that sperm is
4 epigenetically programmed to regulate transcription of several developmentally important
5 embryonic genes.

6

7 **Results**

8

9 *Sperm-derived embryos develop better than spermatid-derived embryos*

10 We first compared the development of embryos obtained by transplanting somatic cell
11 nuclei (SCNT) with the development of sperm-derived embryos. To minimize the
12 experimental difference in the way the embryos were generated, both types of embryos were
13 generated by nuclear injection: cloned embryos were obtained by transplanting nuclei of
14 embryonic cells to enucleated eggs (Fig. 1A), and sperm-derived embryos were produced by
15 intracytoplasmic injection of sperm (ICSI) to eggs (Fig. 1C). We observed that cloned
16 embryos developed less efficiently to the swimming tadpole stage than sperm-derived
17 embryos (Fig. 1B). This illustrates the better developmental potential of sperm over that of a
18 somatic cell. In this experimental set up however, the way the embryos are generated is quite
19 different: the maternal genome is present in sperm-derived embryos whereas it has been
20 removed in the SCNT embryos. In order to better assess the developmental potential of
21 sperm, we aimed to compare embryos produced in the same way. For that purpose, we
22 generated embryos by injecting permeabilized purified sperm or spermatids (Supplemental
23 Fig. S1) to the cytoplasm of unfertilized eggs (ICSI, Fig. 1C). In that way both types of
24 embryos are obtained in the same manner and their development can be compared. The two
25 types of embryos reached the gastrula stage with a similar frequency. However, sperm-

1 derived embryos developed significantly better to the swimming tadpole stage than
2 spermatid-derived ones (p -value < 0.05) (Fig. 1D&1E). This is in agreement with
3 observations made previously in mouse (Kimura and Yanagimachi 1995; Kishigami et al. 2004).
4 Spermatid-derived embryos and cloned embryos exhibit a similar reduction in developmental
5 potential when compared to sperm-derived embryos (Fig. 1B-1E), suggesting that the
6 spermatid is as severely impaired to support development as a somatic cell.

7 In conclusion, embryos can be generated in the same way from sperm or spermatids,
8 and spermatids show reduced developmental potential compared to sperm. Therefore, the
9 comparison of sperm and spermatids was subsequently used to investigate why sperm support
10 better development than other cell types.

11

12 *Spermatids replicate their DNA as efficiently as sperm*

13 Since it has been shown that sperm as opposed to other cells replicate DNA more
14 efficiently (Lemaitre et al. 2005), we hypothesized that the poor embryonic development of
15 spermatid-derived embryos is due to inefficient DNA replication. Egg extracts from *Xenopus*
16 have been widely used to investigate mechanisms of replication (Hutchison et al. 1989; Lemaitre et
17 al. 2001). By incubating nuclei in egg extracts one can mimic replication events that occur
18 prior to the first embryonic cell division. We measured DNA replication efficiency in sperm
19 and spermatids incubated in egg extracts (Hutchison, 1989 #16) by molecular combing analysis
20 (Gaggioli et al. 2013). In this assay the egg extract is supplemented with modified nucleotide
21 precursors that will be incorporated into replicating DNA (Fig. 2A). Following replication,
22 DNA is stretched on a slide, and both the total (green) and replicated (red) DNA fibers are
23 fluorescently labeled (Fig. 2B). By measuring the extent of replication on several hundreds of
24 DNA fibers, we observed equally efficient DNA replication in both sperm and spermatids
25 (Fig. 2C). We concluded from this analysis that the nature of sperm programming is not

1 related to replication. We then tested whether spermatid-derived embryos are capable of
2 correctly initiating embryonic transcription.

3

4 ***Haploid sperm-derived embryos develop better than haploid spermatid-derived embryos***

5 To rigorously assess the developmental potential and transcriptional ability of sperm
6 and spermatids, and to eliminate the risk of any potential interference from the maternal
7 nucleus, we used haploid paternally-derived embryos generated by injection of permeabilized
8 sperm or spermatids into enucleated eggs (Narbonne et al. 2011) (Fig. 3A and Supplemental Fig.
9 S2). We first confirmed that haploid sperm-derived embryos developed significantly better to
10 the swimming tadpole stage than haploid spermatid-derived embryos (p -value < 0.05) (Fig.
11 3B), recapitulating the results from diploid embryos (Fig. 1E). Previous mammalian
12 experiments comparing developmental potential of sperm and spermatids used diploid,
13 biparental embryos (Kimura and Yanagimachi 1995; Kishigami et al. 2004). Our results with paternal
14 haploid embryos directly demonstrate that the sperm nucleus supports better development
15 than the spermatid nucleus, with or without the maternal genome. Furthermore, this
16 experimental setup allows a specific assessment of transcription originating exclusively from
17 sperm- or spermatid-derived chromatin at the time of embryonic gene activation.

18

19 ***Developmentally important genes are misregulated in spermatid-derived embryos***

20 Since experiments in the mouse suggested that sperm might be better than other cell
21 types at supporting mRNA transcription (Ziyyat and Lefevre 2001; Vassena et al. 2007; Ihara et al.
22 2014), we tested this hypothesis using haploid sperm- and spermatid-derived embryos.
23 Embryos were rigorously staged (see Supplemental experimental procedures) and collected at
24 gastrulation, before the onset of developmental defects. Gene expression was then analyzed
25 by RNA-seq, using a set of 39,384 transcripts (provided as Supplemental gene

1 annotation). Out of 18340 expressed genes, 255 were differentially expressed in spermatid-
2 derived embryos compared to sperm-derived embryos (FDR < 0.05) (Supplemental Table
3 S1). 100 of these transcripts showed consistent changes in at least 6 out of 7 experiments and
4 we will refer to them as ‘misregulated’ (Fig. 3C, Supplemental Table S1). The majority
5 (82/100) were upregulated in spermatid-derived embryos and they include transcriptional
6 regulators (e.g. *gata2*, *gata3*, *hes1* and *fos*) as well as morphogens (e.g. *bmp2*, *bmp7* or *dhh*)
7 essential for embryonic development. Accordingly, gene enrichment analysis revealed that
8 several development-related terms were significantly enriched in the list of misregulated
9 genes (p-value < 0.05) (Fig. 3D).

10 The fact that most of the misregulated genes were upregulated in spermatid-derived
11 embryos raised the possibility that these genes were actively transcribed in spermatids and
12 continued to be transcribed in embryos. Indeed the spermatid, as opposed to the sperm, is a
13 transcriptionally active cell and this difference might explain why genes are upregulated in
14 embryos originating from spermatids rather than sperm. To test this possibility we compared
15 the expression level of genes in spermatids and in spermatid-derived embryos. We did not
16 observe any correlation between the expression of misregulated genes in spermatid-derived
17 embryos and their expression in spermatids (Fig. 3E, $r = -0.17$, p-value < 0.05). This
18 suggests that the upregulation of these genes in spermatid-derived embryos is not the result of
19 transcript carry-over (or ongoing transcription) from spermatid chromatin. Because
20 permeabilised spermatids used to generate embryos are likely to contain additional RNAs, we
21 performed an additional control for the potential effect of spermatid-derived RNAs on
22 embryonic development. We purified total RNAs from testis, and injected a quantity
23 corresponding to the amount found in one spermatid (50pg) to embryos generated with
24 sperm. TRIzol® was used to isolate RNAs as it allows the recovery of RNA in a broad range
25 of sizes (El-Khoury et al. 2016). All embryos generated in that way developed normally,

1 indicating that testicular RNAs are not detrimental to embryonic development (Supplemental
2 Fig. S3). Lastly we have verified that the synthesis of rRNAs is not affected in spermatid-
3 derived embryos. Indeed, in mouse, defect in rRNAs synthesis has been proposed to explain
4 the developmental defect of nuclear transfer embryos (Zheng et al. 2012) (Suzuki et al. 2007) , and
5 could explain the difference in the bulk of RNA synthesis observed between sperm and
6 spermatid derived embryos (Bui et al. 2011). We observed that newly synthesized 18S and 28S
7 rRNA are equally well produced in sperm and spermatid derived embryos (Supplemental Fig.
8 S4).

9 We conclude that the developmental failure of spermatid-derived embryos is not
10 associated with carried over spermatid RNAs or with defects in rRNAs expression. Rather,
11 we observe a correlation between developmental defects and misexpression of a set of
12 developmentally important genes in spermatid-derived embryos. We hypothesised that
13 differences in gene expression between sperm- and spermatid-derived embryos might result
14 from epigenetic differences of sperm/spermatid chromatin.

15

16 ***Epigenetic differences between sperm and spermatid chromatin***

17 To investigate potential links between the epigenetic marking of paternal chromatin
18 and embryonic gene expression, we carried out epigenetic profiling of mononucleosomal
19 chromatin from sperm and spermatid using an extensive MNase digestion protocol applied by
20 others to probe for histones stably associated with chromatin in mature sperm in mouse and
21 human (Hammoud et al. 2009; Brykczynska et al. 2010) (Supplemental Table S2 and S3,
22 Supplemental Fig. S5). In *Xenopus*, the transition from spermatid to sperm is characterized by
23 histone H3 and H4 retention and partial loss of H2A and H2B (Risley and Eckhardt 1981). We
24 first compared nucleosome occupancy profiles in sperm and spermatids. Similarly to what
25 was observed in other vertebrates (Hammoud et al. 2009; Brykczynska et al. 2010), we observed

1 higher nucleosomal occupancy (MNase-seq, Fig. 4A) around TSSs (transcriptional start
2 sites) in sperm when compared to spermatids. In this context, the positioned nucleosomes
3 show a similar distribution in sperm and spermatids (Supplemental Fig. S6). Secondly, we
4 analysed DNA methylation profiles in sperm and spermatids by MBD-seq. DNA methylation
5 was higher around sperm TSSs than spermatid TSSs (Fig. 4B). These differences were
6 observed at the genome-wide level between sperm and spermatids, as well as on the set of
7 misregulated genes (Supplemental Fig. S6 and S7). A lower level of nucleosome occupancy
8 and DNA methylation in spermatids compared to sperm could therefore explain the
9 upregulation of genes in spermatid- compared to sperm-derived embryos.

10 To further characterize sperm and spermatid chromatin, we performed ChIP-seq
11 analysis of histone marks associated with activation (H3K4me2, H3K4me3) or repression
12 (H3K27me3, H3K9me3) of transcription. We looked for peaks (Fig. 4C, Supplemental Table
13 S4) as well as for the overall methylation levels (Fig. 4D, Supplemental Fig. S8,
14 Supplemental Table S5) around TSSs for each of these modifications. When compared to all
15 genes, the set of misregulated genes was significantly enriched for H3K27me3 in both sperm
16 and spermatids (Fig. 4C). However, since H3K27me3 was present in both sperm and
17 spermatids, it means that this repressive mark alone cannot explain the difference in gene
18 expression observed in sperm- and spermatid-derived embryos.

19 Interestingly, histone marks associated with active transcription (H3K4me2/3)
20 showed an enrichment at promoters of misregulated genes in spermatids but not in sperm
21 (Fig. 4C, Fig. 4D), providing a plausible explanation for the upregulation of these genes in
22 spermatid-derived embryos.

23

24 ***Co-existence of H3K4me2/3 and H3K27me3 in spermatids correlates with embryonic gene***
25 ***upregulation***

1 The epigenetic features analysed (histone marks, DNA methylation and nucleosome
2 occupancy) can individually provide a possible explanation for the observed differences in
3 expression in sperm- and spermatid-derived embryos. However, complex interactions
4 involving more than one epigenetic feature might better explain this differential embryonic
5 gene expression. In order to identify such interactions we have performed a partial correlation
6 analysis. In this analysis all the measured parameters are assessed simultaneously to produce
7 maps indicating the way epigenetic features associate with each other and with gene
8 expression. The aim of such analysis is to extract more general principles describing the
9 paternal epigenetic program underlying gene expression and identify which epigenetic
10 features are likely to have the strongest contribution to embryonic gene expression. We
11 applied the partial correlation analysis to identify links between the measured epigenetic
12 features in sperm and the expression of the misregulated genes in sperm-derived embryos
13 (Fig. 5A), or between the measured epigenetic features in spermatid and their expression in
14 spermatid-derived embryos (Fig. 5B). We observed that sperm H3K4me_{2/3} and embryonic
15 gene expression were positively associated, whereas sperm H3K27me₃, H3K9me₃ and DNA
16 methylation are negatively linked to embryonic gene expression (Fig. 5A). Interestingly,
17 activating H3K4me_{2/3} and repressive H3K27me₃ marks in spermatids were positively linked
18 to gene expression in spermatid-derived embryos and at the same time they were also
19 strongly associated with each other (Fig. 5B). These associations were also observed when
20 performing a similar analysis using an extended set of misregulated genes obtained by
21 relaxing the selection parameters from $FDR \leq 0.05$ (255 genes) to $FDR \leq 0.4$ and $|\log FC| \geq$
22 0.2 (1116 genes). The use of this extended set increased the predictive power of the analysis
23 and showed stronger links between the features tested within an overall similar network
24 (Supplemental Fig. S9). Therefore, the difference between sperm- and spermatid-derived
25 embryos is best explained by the fact that in contrast to sperm, where H3K27me₃ is

1 overrepresented at genes differentially expressed in haploid embryos, in spermatids
2 H3K27me3 co-exists with H3K4me2/3 on these genes, thereby contributing to their
3 upregulation in spermatid-derived embryos.

4 We checked whether the observed distribution of H3K4me2/3 and H3K27me3 on the
5 misregulated genes of *Xenopus* sperm was a conserved feature across species. For that
6 purpose we investigated how these histone marks were distributed in human sperm (Hammoud
7 et al. 2009), on human orthologues of the *Xenopus* misregulated genes. We observed that
8 similarly to *Xenopus* sperm, human sperm showed an enrichment for H3K27me3 on
9 misregulated genes (Fig. 5C). Additionally, misregulated genes did not exhibit any
10 enrichment over the genome-wide distribution for H3K4me2 in both species. This indicates a
11 conservation of sperm epigenetic features on these genes in the two species.

12 We next tested if paternally-derived H3K4me2/3 and H3K27me3 were indeed
13 involved in patterning embryonic gene expression.

14

15 ***Paternal H3K4me2/3 and H3K27me3 influence embryonic gene expression***

16 To test the function of epigenetic marks from sperm or spermatids chromatin on the
17 regulation of embryonic transcription, we experimentally removed these marks in embryos
18 (Fig. 6A and Supplemental Fig. S10). mRNAs encoding histone demethylases or control
19 mRNAs were first injected into immature oocytes. After allowing 24h for the enzymes to be
20 expressed, the oocytes were *in vitro* matured into eggs (IVM) and injected with sperm or
21 spermatids (ICSI). The resulting embryos were collected at gastrulation stage for RNA-seq
22 analysis. In this protocol histones from both maternal and paternal chromatins are
23 demethylated when the embryo is generated. By comparing embryos produced with different
24 paternal chromatins (sperm or spermatid) we can evaluate the effect of paternal epigenetic
25 mark removal on embryonic gene expression.

1 We first expressed the H3K4me_{2/3} demethylase, KDM5B. As expected, removal of
2 the activating H3K4me_{2/3} marks led to gene downregulation: 68% (1893 genes) and 80%
3 (1392 genes) of all differentially expressed (FDR < 0.05) genes were downregulated in
4 sperm- and spermatid-derived embryos, respectively (Fig. 6B, Supplemental Table S6).
5 Importantly, genes downregulated in sperm-derived embryos showed only limited overlap
6 with those downregulated in spermatid-derived embryos (Fig. 6C). This indicates a paternal
7 chromatin dependent effect of H3K4me_{2/3} removal on embryonic gene expression.
8 Additionally, among the genes affected by H3K4me_{2/3} removal in sperm- and spermatid-
9 derived embryos, the misregulated genes are overrepresented, indicating that paternal
10 H3K4me_{2/3} specifically regulates this set of genes (Fig. 6E). Interestingly, the genes
11 downregulated in spermatid-derived embryos are enriched for H3K4me_{2/3} in spermatids
12 (Fig. 6D). These observations are in agreement with the hypothesis that the loss of
13 H3K4me_{2/3} from H3K27me₃ marked genes during the spermatid to sperm maturation is
14 necessary for their proper expression in embryos.

15 To further validate this hypothesis we tested the influence of paternal H3K27me₃ by
16 overexpressing the H3K27me₃ demethylase KDM6B (Fig. 6A). In accordance with its
17 repressive function, removal of H3K27me₃ at fertilization led to upregulation of genes at
18 gastrulation in both sperm- and spermatid-derived embryos. 87% (487 genes) and 76% (173
19 genes) of differentially expressed genes (FDR < 0.05) were upregulated in sperm- and
20 spermatid-derived embryos, respectively (Fig. 6F, Supplemental Table S6). Again, there was
21 only a partial overlap between genes affected by KDM6B in sperm- and spermatid-derived
22 embryos (Fig. 6G), indicating that this effect is paternal chromatin dependent. The affected
23 genes were marked by H3K27me₃ in the corresponding paternal cells (Fig. 6H).
24 Additionally, upon H3K27me₃ demethylation about five times more genes were specifically
25 upregulated in sperm- than in spermatid-derived embryos (402 versus 88 genes). This

1 suggests that the programming of genes for embryonic expression in the paternal chromatin
2 relies on the establishment of an effective H3K27me₃-mediated repression at the spermatid to
3 sperm transition. Lastly, the misregulated genes are enriched among the genes affected by the
4 H3K27me₃ removal indicating that paternal H3K27me₃ specifically regulates this set of
5 genes (Fig. 6E).

6

7

8 **Discussion**

9 Previous works characterizing the epigenetic features of sperm in zebrafish, mouse
10 and human have revealed the presence of modified histones around genes involved in
11 embryonic development (Hammoud et al. 2009; Brykczynska et al. 2010; Wu et al. 2011). In these
12 species, the presence of activating (H3K4me₃) and repressive (H3K27me₃) histone marks in
13 sperm correlated with gene expression in the early embryos (Hammoud et al. 2009; Brykczynska et
14 al. 2010; Wu et al. 2011). In this work we have used a comparison of sperm and its immediate
15 precursor, the spermatid, to investigate the functional relationship between histone marks and
16 gene expression.

17 Our analysis shows that, similarly to what has been observed in mouse (Kimura and
18 Yanagimachi 1995; Kishigami et al. 2004), spermatids are not as good at supporting development as
19 sperm. Second, we tested several hypotheses proposed to explain the developmental
20 advantage of sperm over spermatids. We have ruled out the hypothesis that spermatids are
21 less efficient than sperm at supporting replication. Instead, we found evidence supporting the
22 hypothesis that sperm is programmed to support proper embryonic expression of genes
23 encoding important embryonic regulators (Fig. 3). Importantly, overexpression and knock
24 down studies of several of these genes have shown embryonic developmental defects
25 reminiscent of what is observed in spermatid-derived embryos (*sfrp2* (Lee et al. 2006); *tbx3*

1 (Weidgang et al. 2013), *foxa2* (Suri et al. 2004), *otx2* (Yasuoka et al. 2014)). These observations
2 suggests that misexpression of this set of genes is the cause of the developmental defect
3 observed in spermatid-derived embryos. We also showed that the developmental advantage
4 of sperm over spermatids is maintained in haploid, paternally derived embryos, indicating
5 that the effect observed is independent of the presence of the maternal genome. To our
6 knowledge this is the first time that these two hypotheses, developmental advantage related to
7 ability to support replication *versus* transcription, have been rigorously tested.

8 These analyses allowed us to conclude that sperm is not merely a carrier of DNA, but
9 that it also contributes epigenetic information required for proper embryonic gene expression.
10 We then focused our analysis on the sperm chromatin as it represents the most likely vector
11 of such epigenetic information.

12 During spermiogenesis in *Xenopus laevis* core histones H3 and H4 are retained
13 whereas ~90% of core histones H2A and H2B are lost (Risley and Eckhardt 1981). This leaves
14 *Xenopus* sperm with about 10% of the amount of nucleosomal content of a spermatid. This
15 level of histone retention in sperm is higher than that of mouse (~1%) (Brykczynska et al. 2010),
16 lower than that of zebrafish (~100%) (Wu et al. 2011), and similar to that of human (~10%)
17 (Brykczynska et al. 2010). Nucleosome retention in vertebrates therefore seems to show a degree
18 of variation among species. The epigenetic analysis of *Xenopus* sperm provided here extends
19 the repertoire of characterized higher vertebrate sperm chromatin and identifies conserved
20 chromatin features relevant to developmental programming. In that respect we observed that
21 the programming of sperm for embryonic gene expression entails a loss of H3K4me2/3
22 marking at H3K27me3 target genes during spermatid to sperm maturation (Fig. 6I). We
23 showed that the set of genes programmed for embryonic expression during *Xenopus* sperm
24 maturation had similar epigenetic features in *Xenopus* and human sperm (Fig. 4C and Fig.
25 5D). So, despite the existence of hugely variable degree of histone retention in sperm among

1 species, this points towards the existence of universal mechanisms preparing sperm for
2 participation in the normal development of embryos in vertebrates.

3 To functionally test the role of sperm epigenetic marks on embryonic gene expression
4 one would ideally like to erase these marks from the sperm nucleus immediately prior to the
5 generation of embryos. Chromatin of mature sperm is highly condensed and inaccessible,
6 making enzymatic treatments to alter the epigenetic marks inefficient. Alternative strategies
7 have been developed to use such enzymes either during the process of spermiogenesis, prior
8 to full maturation of sperm (Siklenka et al. 2015), or at fertilization when the sperm chromatin
9 becomes again accessible (used in this study). In mouse, the former strategy has been used to
10 overexpress the H3K4/H3K9 demethylase KDM1A in germ cells. Embryos generated with
11 sperm from animals overexpressing KDM1A exhibited developmental defects which were
12 transmitted for several generations in the absence of exogenous KDM1A. This analysis
13 demonstrated the existence of epigenetic instruction delivered by sperm to the embryos and
14 transmitted through several generations. However, the overexpression of KDM1A very early
15 in the process of sperm differentiation leads to numerous abnormalities in sperm, for example
16 the presence of additional mRNAs (Siklenka et al. 2015). These abnormalities are indirect effects
17 of the overexpression of KDM1A early in the process of sperm differentiation. For that
18 reason, it has been difficult to link the difference in gene expression and associated
19 developmental defects to particular epigenetic changes in sperm. The approach we describe
20 here complements and extends previous analyses. First, by comparing the epigenetic profiles
21 of sperm and spermatids to differential gene expression in sperm- and spermatid-derived
22 embryos we identified H3K4me_{2/3} and H3K27me₃ as candidate marks responsible for the
23 programming of genes. We then tested this hypothesis by demethylating the chromatin using
24 KDM6B (H3K27 demethylase) or KDM5B (H3K4 demethylase) in embryos generated with
25 sperm or spermatids. Importantly, in our experimental setup both sperm and spermatids used

1 had been through a normal differentiation process. Removal of H3K4me_{2/3} at fertilization
2 affects different set of genes in sperm- and spermatid-derived embryos. Genes affected in
3 sperm-derived embryos are enriched for H3K27me₃ whereas genes affected in spermatid-
4 derived embryos are enriched for H3K4me_{2/3}. This indicates the importance of H3K4me_{2/3}
5 dynamics at the transition from spermatid to sperm for patterning of the future embryonic
6 gene expression. One hypothesis to explain the sensitivity of sperm H3K27me₃ marked
7 genes to H3K4me_{2/3} removal would be that these genes acquire H3K4 methylation following
8 fertilization. Our analysis also suggests a conserved role for these marks in *Xenopus* and
9 mouse (Siklenka et al. 2015). Additionally, we also demonstrated that removal of H3K27me₃ at
10 fertilization affects the embryonic expression of genes that are marked by H3K27me₃ in
11 sperm/spermatids. Recent reports probing histone modifications distribution in mouse and
12 human sperm suggested that these epigenetic marks occurred mostly on repetitive regions of
13 the genome rather than genes (Carone et al. 2014; Samans et al. 2014). These observations put into
14 question the possibility that such marks would influence gene expression in embryos. By
15 providing functional test of the need for histone modifications for embryonic gene
16 expression, our analysis, together with that of Siklenka *et al*, clearly shows that, regardless of
17 their genomic location, sperm delivered modified histones are important regulators of
18 expression in future embryos (Siklenka et al. 2015).

19 Further investigations into the nature of sperm programming, especially the
20 requirement of other epigenetic marks and their cross-talk in the patterning of embryonic
21 expression, will provide a better understanding of the transgenerational inheritance of
22 epigenetic traits via gametes, and could shed light on the mechanisms underlying male
23 infertility and other diseases in humans.

24

25

1 **Materials and Methods**

2 All the experiments involving the use of animals were conducted according to the
3 regulatory standards of the funding bodies.

4

5 *Separation of sperm and spermatids*

6 For each round of sperm and spermatid purification, testes from 6 adult *Xenopus*
7 *laevis* males were isolated and manually cleaned from blood vessels and fat body in 1×MMR
8 (100 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES pH 7.4) using
9 forceps and paper tissues. It is crucial to clean the testes well from any non-testicular tissues,
10 as otherwise the cells released from the tissues may negatively affect the final purity of
11 isolated cells. Subsequently, testes were torn into small pieces with forceps and homogenized
12 with 2-3 strokes of a Dounce homogenizer (tissue from 1 testis at a time). The cell suspension
13 was then filtered to remove tissue debris and cell clumps (CellTrics, cat. 04-0042-2317) and
14 spun down at 800rcf, 4°C, 20 minutes. Supernatant was discarded and cell pellet resuspended
15 in 12ml of 1×MMR. If any red blood cells were visible at the bottom of the pellet (a result of
16 incomplete removal of blood vessels), only the uncontaminated part of the pellet was
17 recovered, taking extreme care not to disturb the red blood cells. Subsequently, step gradients
18 of iodixanol (Optiprep – Sigma, D1556, is 60% iodixanol in water) in 1×MMR final were
19 manually prepared in pre-chilled 14ml ultra-clear centrifuge tubes (Beckman Coulter,
20 344060) in the following order from the bottom to the top of the tube: 4ml of 30% iodixanol,
21 1ml of 20% iodixanol, 5ml of 12% iodixanol (all in 1×MMR) and 2ml of cell suspension in
22 1×MMR on top. Gradients were spun down in a pre-chilled SW40Ti rotor at 7500rpm
23 (10000g), 4°C, 15 minutes, deceleration without break (Beckman Coulter Ultra-centrifuge,
24 Optima L-100XP). The top interface fraction (between 1×MMR and 12% iodixanol),
25 containing spermatids, and the pelleted fraction, containing mature sperm, were collected.

1 Collected fractions were diluted six times with 1×MMR and collected by spinning first at 805
2 rcf, 4°C, 20 minutes and re-spinning at 3220 rcf, 4°C, 20 minutes to pellet remaining cells.
3 Pelleted cells were subjected to nuclei preparation (see below).

4

5 *Sperm and spermatid nuclei preparation, intra-cytoplasmic sperm injections (ICSI) to non-*
6 *enucleated and to enucleated eggs and embryo culture*

7 Sperm and spermatids nuclei were permeabilized as described before (Smith et al. 2006)
8 and stored at -80°C. Injections were performed using a Drummond Nanoject microinjector
9 (NanojectII Auto Nanolitre Injector, Biohit, 3-00-206A) and glass capillaries (Biohit, 3-00-
10 203-G/XL) pulled using a Flaming-Brown micropipette puller (settings: heat 700, pull 100,
11 velocity 100, time 10). Cell suspension was sucked into the injection needle filled with
12 mineral oil. Cells were injected in sperm dilution buffer (SDB) (Smith et al. 2006) and cell
13 concentration was adjusted by doing mock injections on a microscope slide to deliver 1 cell
14 per 4.6nl injection. The eggs were placed in batches of 20-25 on a blotting paper. If they were
15 to be enucleated, they were placed with animal pole facing upwards, whereas if they were not
16 subjected to enucleation, they were placed on a side (with the marginal zone upwards). For
17 enucleation, eggs were treated for 30s with a UV mineralite lamp (Gurdon 1960) (this step was
18 omitted for non-enucleated eggs). Jelly was removed by a 5s Hanovia lamp treatment. The
19 eggs were immediately injected with sperm or spermatid solution and moved to 1×MBS
20 (Gurdon 1976) supplemented with 0.2% bovine serum albumin (BSA). Cell suspension in the
21 needle was replaced every 20-25 batch of eggs injected. At 4-cell stage embryos were sorted
22 (all the non-cleaved embryos or those with irregular cleavage furrows were discarded) and
23 the culture media replaced with 0.1MBS, 0.2% BSA. Embryos were cultured in 0.1MBS,
24 0.2% BSA (changed daily) at 16-18°C incubator. Assessment of developmental stages was
25 performed according to Nieuwkoop and Faber (Nieuwkoop 1994). Using this table, matching

1 gastrula embryos from the various experimental groups were collected at stage 10^{1/2}-11, and
2 processed for gene expression analysis (see supplemental data procedures for details).

3

4 *Interphase egg extract preparation*

5 Eggs were collected in 1×MMR, dejellied with 0.2×MBS, 2% cysteine (pH 7.8-7.9)
6 (Sigma, W326305) and washed with 0.2×MMR. Subsequently, eggs were activated for 3
7 minutes at RT with 0.2×MMR supplemented with 0.2ug/ml calcium ionophore (Sigma,
8 C7522). Eggs were rinsed with 0.2×MMR and subsequently all abnormal or not activated
9 eggs were removed. Eggs were washed with 50ml of ice-cold extraction buffer (EB) (5mM
10 KCl, 0.5mM MgCl₂, 0.2mM DTT, 5mM Hepes pH 7.5) supplemented with protease
11 inhibitors (PI) (Roche, 11873580001), transferred into centrifugation tube (Thinwall, Ultra-
12 Clear™, 5 mL, 13 × 51 mm tubes, Beckman, 344057) and supplemented with 1ml of EB
13 buffer with PI and 100ug/ml of cytochalasin B (Sigma, C2743) and placed on ice for 10
14 minutes. Subsequently, eggs were spun briefly at 350g for 1 minute at 4°C (SW55Ti rotor,
15 Beckman Coulter Ultra-centrifuge, Optima L-100XP) and excess buffer was discarded. Eggs
16 were then spun at 18000g for 10 minutes at 1°C, the extract was collected with a needle,
17 transferred to a fresh, pre-chilled tube, supplemented with PI and 10ug/ml of cytochalasin B
18 and re-spun using the same conditions. Extract was collected with a needle and used fresh for
19 replication assay (see below).

20

21 *Replication in egg extracts and sample preparation for analysis of DNA fibres*

22 Replication on single DNA fibres was performed as described before (Gaggioli et al.
23 2013) with slight modifications. Freshly prepared egg extracts were supplemented with 20×
24 energy regeneration mix: 2mg/ml Creatine Kinase (Roche, 10127566001), 150mM Creatine
25 Phosphate (Roche, 10621714001), 20mM ATP (Roche, 10519979001), 2mM EGTA, 20mM

1 MgCl₂) and with 20 μ M biotin-16-dUTP (Roche, 11093070910). Permeabilized cells were
2 added to a final concentration of 200 nuclei/ μ l of extract and incubated at RT for 2h (tapping
3 every 10 minutes). Reaction was stopped by adding 10 volumes of ice-cold 1 \times PBS
4 (Phosphate Buffer Saline: 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄ \times 2H₂O, 2mM
5 KH₂PO₄) and cells were spun down at 1000g, 4°C, 7 minutes. Cells were resuspended in 50 μ l
6 of 1 \times PBS and mixed immediately with 50 μ l of melted (at 65°C) 2% low melting point
7 agarose (Invitrogen, 16520050) in 1 \times PBS. After solidification, the agarose plug was
8 incubated overnight (O/N) at 50°C with 1mL 0.5M EDTA pH8, 100 μ L 10% sarkosyl (Sigma,
9 L5125), 1mg/mL Proteinase K (New England Biolabs, P8102S) followed by three washes in
10 TE pH 6.5. Subsequently, the plug was incubated twice in TE supplemented with 0.1mM
11 PMSF (Sigma, 93482) for 30 minutes at 50°C and washed four times with 1ml of 50mM
12 MES (Sigma, 69889) pH 6.35, 1mM EDTA (1h at RT each wash). Then the solution was
13 removed; the plug was melted in 400 μ l of MES pH 6.35, 1mM EDTA at 68°C for 20 minutes
14 and the agarose was digested with 2 units of β -agarase (New England Biolabs, M0392S) O/N
15 at 42°C.

16 *Analysis of replication on single DNA fibres*

17 Silanized coverslips were prepared as described before (Labit et al. 2008). Thirty
18 microliters of replicated DNA solution was pipetted onto a silanized coverslip, covered with a
19 non-silanized coverslip and incubated for 5 minutes at RT. Subsequently, the top coverslip
20 was slid away to stretch DNA fibres and the silanized coverslip with stretched fibres was
21 fixed in 3:1 solution of Methanol:Glacial Acetic Acid for 10 minutes, RT. The fibres were
22 then denatured with 2.5M HCl (1h, RT) and dehydrated by washes in 70% ethanol, 90%
23 ethanol and 100% ethanol (1 minute for each wash). Subsequently, the coverslip was dried,
24 washed 3 times in PBS, 0.1% Tween (Sigma, P5927) (5 minutes for each wash) and blocked

1 in 3%BSA in PBS (1h, RT). All antibodies were diluted in PBS, 3%BSA, 0.1% Tween. Total
2 DNA was detected simultaneously to replicated DNA with primary antibodies: anti-DNA
3 antibody (Millipore, MAB3034) 1:300 dilution, and streptavidin-Alexa 594 antibody 1:50 to
4 detect biotin (Invitrogen, S-11227) for 30 minutes at 37°C. Primary antibodies were washed
5 away with PBS, 0.1% Tween (4 washes) and detected with secondary antibodies diluted 1:50:
6 chicken anti-mouse Alexa 488 (Invitrogen, A-21200) and biotinylated antibody anti-
7 streptavidin (Vector Labs, BA-0500) for 30 minutes, 37°C. After four washes in PBS, 0.1%
8 Tween, a tertiary detection was performed with antibodies diluted 1:50: goat anti-chicken
9 Alexa 488 (Invitrogen, A-11039) and streptavidin-Alexa 594 for 30 minutes, 37°C. The
10 coverslip was washed three times with PBS 0.1% Tween, three times in PBS and mounted on
11 a microscope slide with a mounting medium (50% glycerol in PBS) and sealed with a nail
12 polish. Images were acquired with a Zeiss 510 META confocal LSM microscope. Image
13 analysis was performed in ImageJ; the amount of replicated DNA and total DNA was
14 measured individually on single DNA fibres.

15 *RNA extraction and preparation of cDNA library for sequencing*

16 Spermatid (1 millions) or pool of 5 stage 10.5-11.5 embryos were collected and
17 frozen at -80°C. RNA extractions were performed using Qiagen RNeasy Mini kit according
18 to the manufacturer's protocol. RNA was eluted in 50ul of DEPC H₂O and used to generate
19 cDNA sequencing libraries using Illumina TrueSeq kit (RS-122-2001), according to the
20 manufacturer's protocol.

21

22 *mRNA Injection to 1-cell embryos*

23 Mouse KDM6B (aa1025-1642) or KDM5B (aa1-770) were cloned using p-Entry
24 cloning system (Invitrogen, K2400-20 and 11791-020) into pCS2+ plasmid with a C-terminal

1 HA-tag and NLS-tag. mRNA was synthesized *in vitro* using MEGAscript® SP6 Kit
2 (Ambion, AM1330M) following the manufacturer's instructions. Eggs were *in vitro* fertilized
3 and dejellied using 2% Cysteine solution in 0.1×MMR. Injections into 1-cell stage embryos
4 were performed in injection solution (Smith et al. 2006) using a Drummond Nanoject
5 microinjector, delivering 9.2ng of mRNA per injection (mRNA at 1mg/ml in DEPC H₂O).
6 Embryos were cultured at 18°C and collected for Western Blot analysis at stage 21 (Nieuwkoop
7 1994). Western blot analysis were performed on 12% polyacrylamide gels using antibodies
8 against H3K27me₃ (Cell Signalling, 9733), H3K9me₃/2 (Cell Signalling, 5327), H3K4me₂/3
9 (Abcam 8580), H4 (Abcam 31830), and against H3 (Abcam 18521)

10

11 *Preparation of ChIP-seq samples*

12 Sperm and spermatids were separated as described above. Chromatin fractionation
13 and chromatin immunoprecipitation (ChIP) were performed as described before (Erkek et al.
14 2013; Hisano et al. 2013) with slight modifications. Pre-treatment of sperm cells with DTT
15 was omitted and chromatin was digested with 2.5U of MNase/1 million of cells (Roche,
16 12533700) for 30 minutes at 37°C. The following antibodies against histone marks were used
17 in the study: anti-H3K4me₂ (Millipore, 07-030), anti-H3K4me₃ (Abcam, ab8580), anti-
18 H3K4me₃ (Millipore, CS200580), anti-H3K27me₃ (Millipore, 07-449), anti-H3K27me₃
19 (kind gift from Dr Thomas Jenuwein), and anti-H3K9me₃ (Abcam, ab8898). Before ChIP,
20 primary antibodies were bound to magnetic beads conjugated with secondary antibody
21 (Invitrogen, 11204D) according to manufacturer's protocol and all wash steps in the protocol
22 were carried with a magnet, instead of centrifugation. Bound DNA was isolated, separated by
23 electrophoresis and mononucleosomal bands from sperm and spermatids were excised and
24 subjected to library preparation with TruSeq DNA kit (Illumina, FC-121-2001). For the
25 generation of the input sample, 5-10% of the MNase digested chromatin was collected and

1 the same purification scheme was followed as with the immunoprecipitated chromatin prior
2 to library preparation with TruSeq DNA kit (Illumina, FC-121-2001).

3

4 *Preparation of MBD-seq samples*

5 Sperm and spermatids chromatin were separated as described above and 200ng of
6 digested genomic DNA was used to purify methylated DNA using the Methyl Collector TM
7 Ultra kit (Active Motif 55005). The purification was carried out according to the
8 manufacturer's instructions, using the low salt buffer to wash the bead-methyl DNA
9 complexes. The purified methylated DNA and the input DNA were then subjected to library
10 preparation with TruSeq DNA kit (Illumina, FC-121-2001).

11

12 *Sequencing data analysis*

13 Details of the sequencing data analysis methods used in this study are described in
14 Supplemental information section.

15 **Data access**

16 All ChIP-seq, RNA-seq , MBD-seq, and MNase-seq data sets have been submitted to the
17 NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession
18 number GSE75164.

19

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11 **Author Contributions:**

12 M.T. and J.J. conceived the study, M.T., A.S. and J.J. analysed the experiments and wrote the
13 paper, M.T. performed the experiments, V.G. performed and analysed replication
14 experiments, AS. performed all bioinformatics analysis, G.A. and A.S. analysed RNA-seq
15 data, K.M. and J.J. performed *in vitro* maturation/ICSI experiments, S.E. and A.H.F.M.P.
16 helped with ChIP-seq experiments, T.K. and E.M. helped to acquire RNA-seq data and
17 provided *Xenopus laevis* transcriptome and genome, C.R.B. customized *Xenopus laevis*
18 genome and transcriptome and performed homology searches, A.S., C.R.B. and G.A. wrote
19 the experimental procedures section for ChIP-seq and RNA-seq. K.M., V.G., A.S., P.Z. and
20 J.B.G. helped with the experimental design and paper writing. J.J. and J.B.G. supervised the
21 research.

22

23

1 **Figure legends:**

2

3 **Figure 1.** *Xenopus sperm is better at supporting development than a spermatid or a somatic*
4 *cell.*

5 **(A)** Experimental design for the generation of cloned embryos. The somatic nucleus of a
6 gastrula cell is transplanted to a UV-enucleated egg. The resulting embryos are scored at
7 gastrulation and tadpole stage. **(B)** Scoring of embryos as % of gastrulae and as % of
8 swimming tadpoles to the total number of cleaved embryos (average of n=6 independent
9 experiments (sperm ICSI), and n=3 independent experiments (embryo cell NT). The total
10 number of embryos analyzed is shown above the graph. Error bars: sem. * p-value < 0.05
11 (chi-square test). **(C)** Experimental design for the generation of sperm- and spermatid-derived
12 embryos. Permeabilized sperm or spermatids are injected to the cytoplasm (ICSI) of
13 unfertilized egg. The resulting embryos are scored at gastrulation and tadpole stage. **(D)**
14 Representative images of sperm- and spermatid-embryos. Scale bars = 1mm. **(E)** Scoring of
15 embryos as % of gastrula and as % of swimming tadpoles to the total number of cleaved
16 embryos (average of n=6 independent experiments). The total number of embryos analyzed is
17 shown above the graph. Error bars: sem. * p-value < 0.05 (chi-square test).

18

19 **Figure 2.** *Spermatids are as good as sperm at DNA replication.*

20 **(A)** Sperm and spermatids are separately incubated with egg extracts supplemented with
21 biotin-dUTP. Subsequently, DNA fibers are isolated and subjected to molecular combing,
22 which reveals replication on single DNA fibers. **(B)** Examples of DNA fibers after
23 immunostaining procedure. Antibody staining against DNA reveals the total length of the
24 fibre (green) and antibody staining against biotin reveals the replicated DNA (red). The
25 bottom panels show representative examples of replication staining from sperm and from

1 spermatids incubated in egg extracts. **(C)** Replication extent measured as the proportion of
2 DNA that incorporated biotin-dUTP to the total fibre length. Results are from at least 125
3 independent DNA fibres (22000 kb of DNA for each sample). Error bars: sem. Samples were
4 not significantly different (p-value=0.41, KS-test).

5

6 **Figure 3.** *Transcription of developmentally important genes is misregulated in spermatid-*
7 *derived embryos compared to sperm-derived embryos*

8 **(A)** Schematic representation of paternally-derived haploid embryos generation by UV
9 enucleation of eggs followed by intra-cytoplasmic sperm injection (ICSI). **(B)** Developmental
10 advantage of sperm over spermatid is maintained in haploid embryos. Embryos were scored
11 as the % of embryos reaching a gastrula stage and a swimming tadpole stage to the total
12 number of cleaved embryos (average of n=3 independent experiments). Numbers of embryos
13 analyzed are indicated above the bars. Error bars: sem. * indicates p-value < 0.05 (chi-square
14 test). **(C)** Genes important for development are misregulated (mostly upregulated) in
15 spermatid derived embryos. Heatmap representing log fold-change in expression levels of the
16 100 genes (rows) misregulated in spermatid versus sperm gastrula embryos (FDR < 0.05, red
17 – upregulated; blue – downregulated in spermatid) across seven independent experiments
18 (columns). **(D)** Developmentally-important gene ontology terms enriched in the list of
19 misregulated genes (p-value < 0.05). **(E)** Upregulation of genes in spermatid-derived
20 embryos does not correlate with their transcription in spermatid. Density scatter plot showing
21 gene expression in spermatid-derived embryos versus that in spermatids. No correlation is
22 observed between the two parameters for all genes (r=0.06) as well as for the misregulated
23 genes (red dots, r=-0.17).

24

1 **Figure 4.** *Genes that are misregulated in spermatid-derived embryos have different*
 2 *epigenetic features in sperm and spermatid.*

3 (A) Genome-wide average nucleosome occupancy at the TSS of sperm (blue) and spermatid
 4 (green) genes. (B) Boxplots showing genome-wide DNA methylation levels at the TSS±1kb
 5 of sperm (blue) and spermatid (green) genes. Inset shows correlation between the DNA
 6 methylation levels of sperm and spermatid ($R = 0.8$, $p\text{-value} < 0.05$), red line: regression,
 7 dotted line: diagonal. (C) Percentage of genes harboring H3K27me3, H3K4me3, H3K4me2
 8 or H3K9me3 peaks genome-wide (GW) and at misregulated genes (Mis). *: $p\text{-value} < 0.05$
 9 (chi-square test). (D) Heatmaps representing H3K27me3, H3K4me3, H3K4me2 and
 10 H3K9me3 overall levels (see Supplemental Information and Supplemental Fig. S8) at
 11 misregulated genes in sperm (first column) and spermatid (second column). Each map is
 12 sorted according to the signal in spermatid. Boxplots show the distribution of methylation
 13 levels across misregulated genes. *: $p\text{-value} < 0.05$ (KS-test, Supplemental Table S7).

14

15 **Figure 5.** *H3K27me3 target genes that lose H3K4me2/3 in sperm compared to spermatids*
 16 *are misregulated in spermatid-derived embryos.*

17 (A, B) Differential gene expression between sperm- and spermatid-derived embryos best
 18 correlates with differential H3K4me2/3 and H3K27me3 marking in sperm and spermatids.
 19 Partial correlation network between all tested epigenetic features of the paternal chromatin
 20 (A- sperm, B- spermatid) and gene expression in the corresponding embryos. Edges (lines)
 21 represent positive (red) or negative (blue) partial correlations. Edges thickness: strength of
 22 the partial correlations. (C) H3K4me2/3 and H3K27me3 marking on misregulated genes is
 23 conserved between *Xenopus* and human sperm. As compared to all orthologues, the
 24 misregulated orthologues are enriched for H3K27me3 marks over the genome-wide average

1 in human sperm (Chi-square test, *: p-value < 0.05). No statistical enrichment for H3K4me2
2 on misregulated genes as compared to the genome-wide average is observed in human sperm.

3

4

5 **Figure 6.** *Paternal genome marking by H3K4me2/3 and H3K27me3 is required for gene*
6 *expression in the embryos.*

7 **(A)** Histone demethylase expression assay. **(B)** MA plot showing log fold-change (logFC, y-
8 axis) in gene expression between *Kdm5b* (H3K4me2/3 demethylase) - versus control-mRNA
9 injected embryos, against log counts per million (logCPM, x-axis). Red dots: genes
10 differentially expressed (FDR < 0.05) ; N = 4 independent experiments. **(C)** Venn-diagram of
11 downregulated genes upon KDM5B expression in sperm- (blue) and spermatid-derived
12 (green) embryos. **(D)** Percentages of genes downregulated upon KDM5B expression in
13 embryos that show H3K4me2/3 and H3K27me3 promoter peaks in the paternal cell. *: p-
14 value < 0.05 (chi-square test), ↑: over-represented when compared to genome-wide
15 distribution. **(E)** Proportion of misregulated genes affected in each demethylase expression
16 assay. *: p-value < 0.05 (chi-square test). **(F)** same as **(B)** for KDM6B (H3K27me3
17 demethylase) expression. **(G)** same as **(C)** with genes upregulated upon KDM6B expression.
18 **(H)** same as **(D)** for genes upregulated upon KDM6B expression. **(I)** Model of epigenetic
19 programming of sperm for the regulation of embryonic transcription.

20

1

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3

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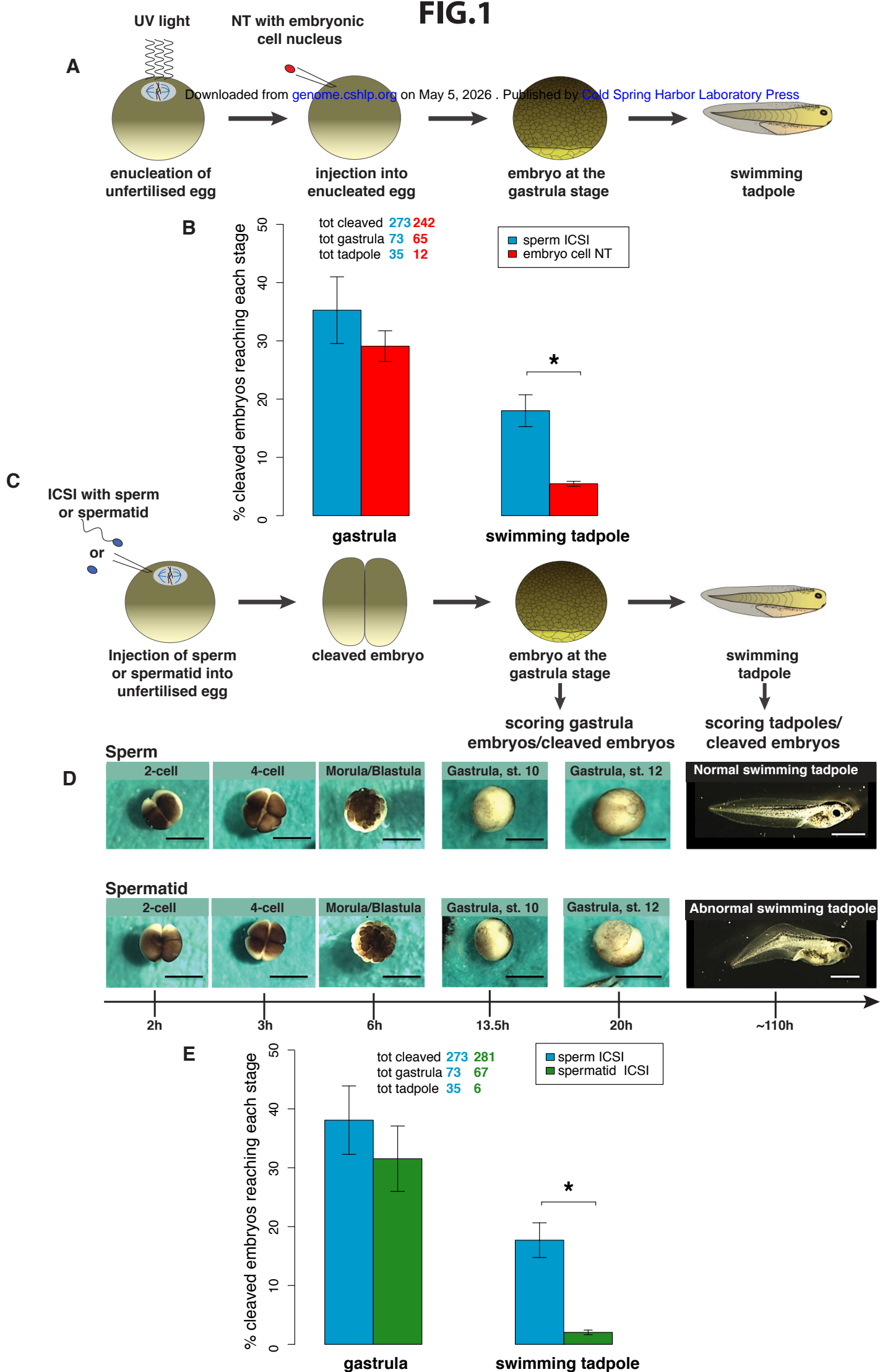
FIG.1

FIG.2

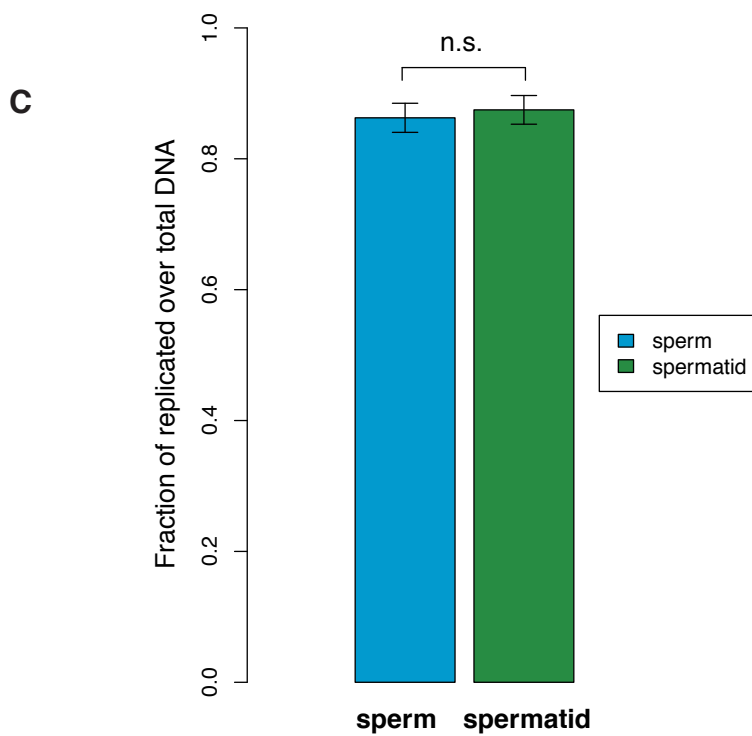
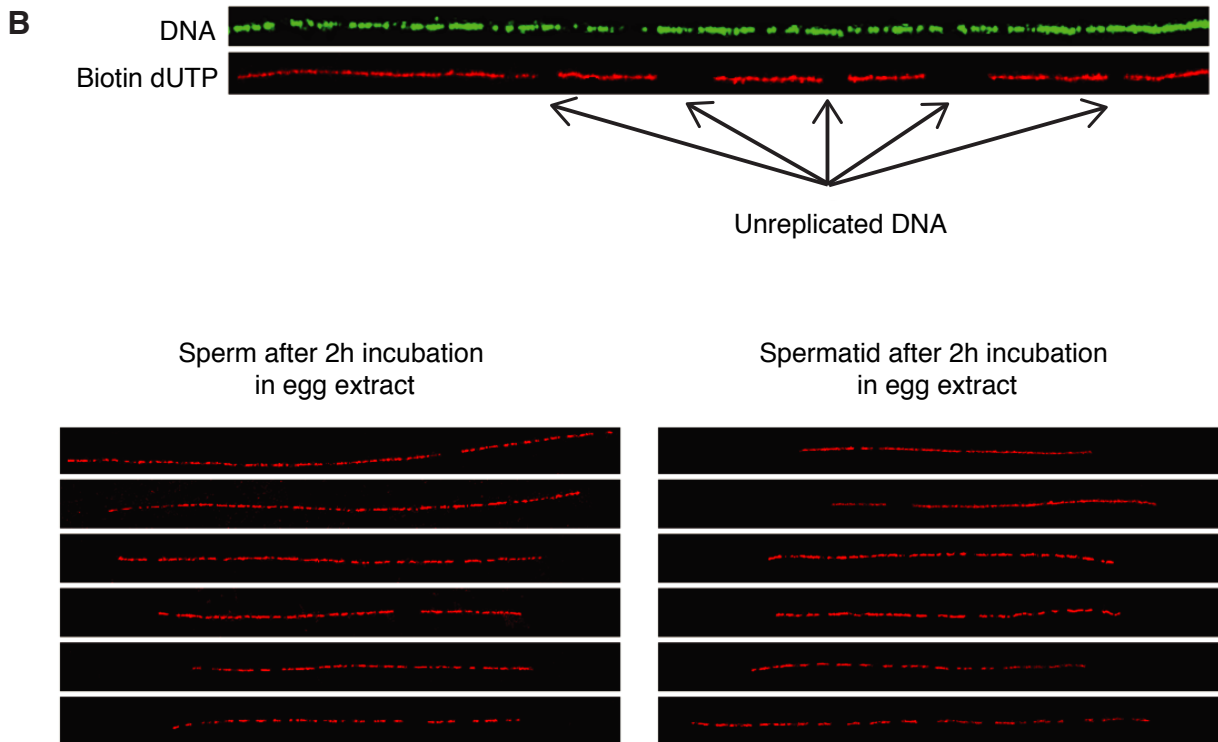
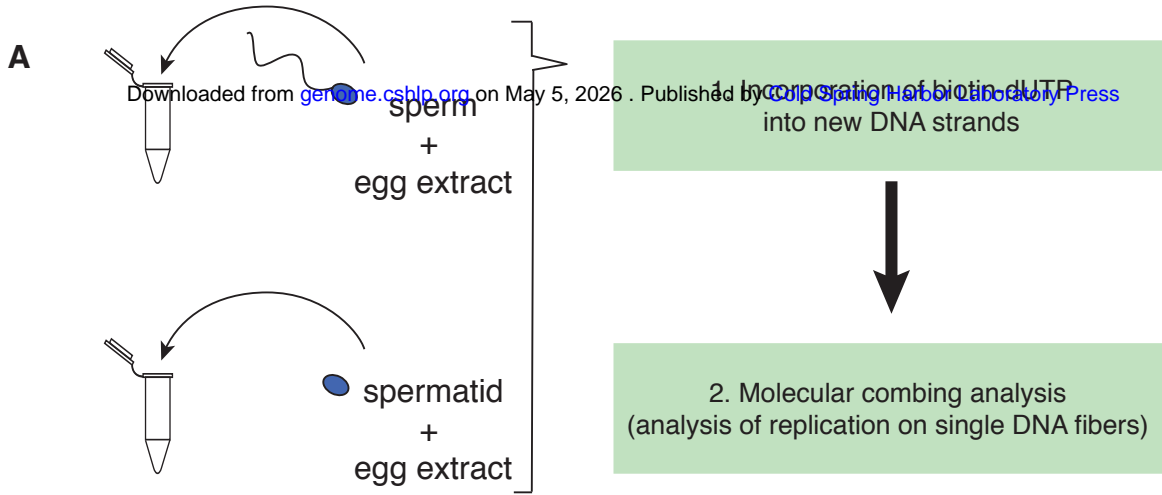


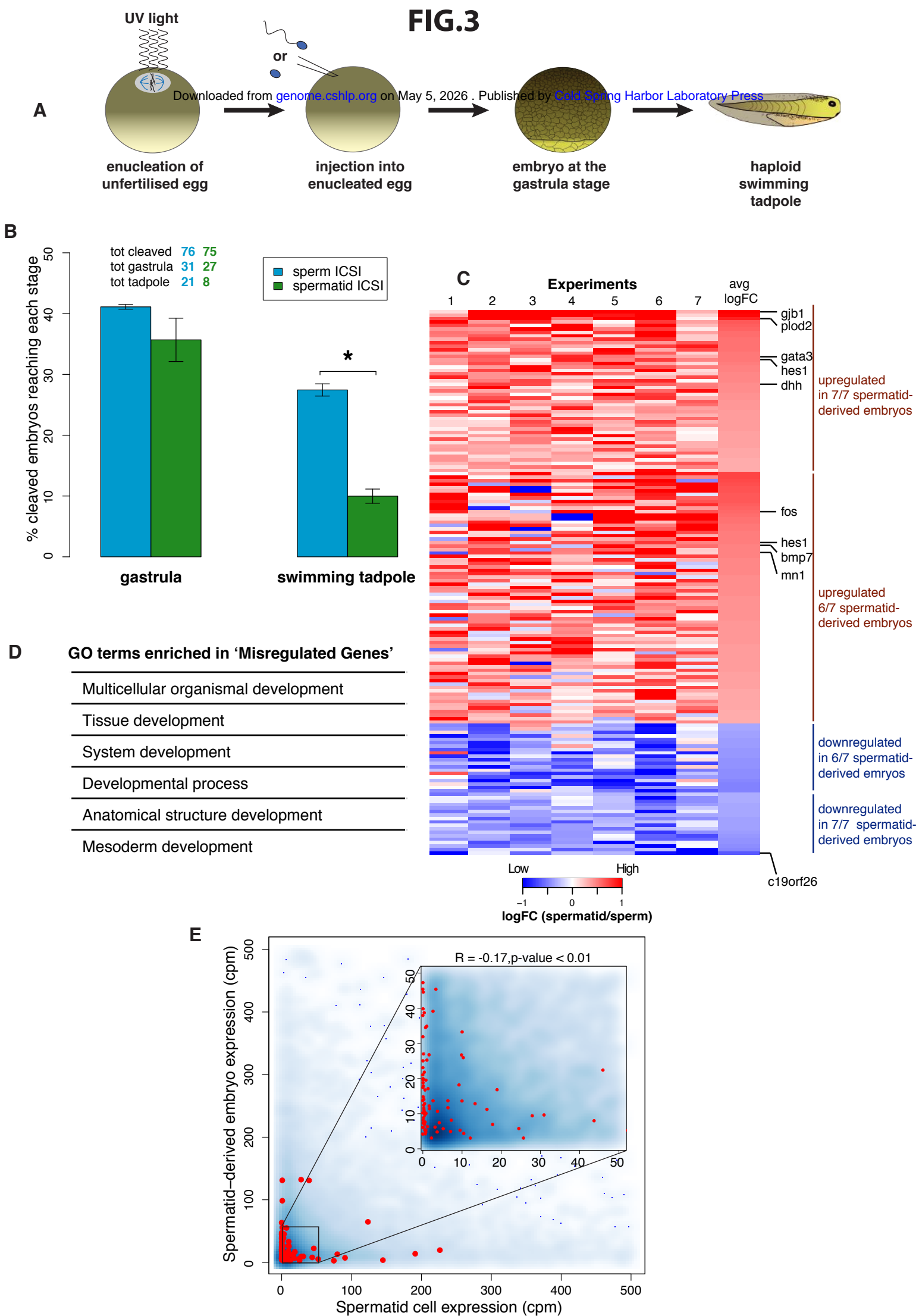
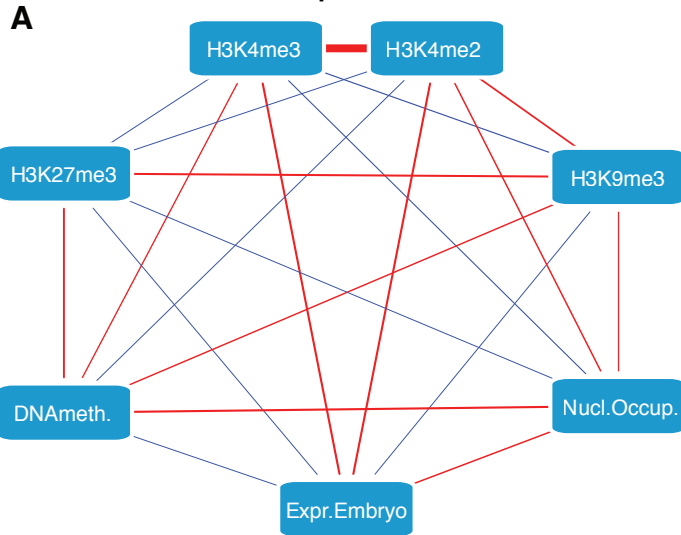
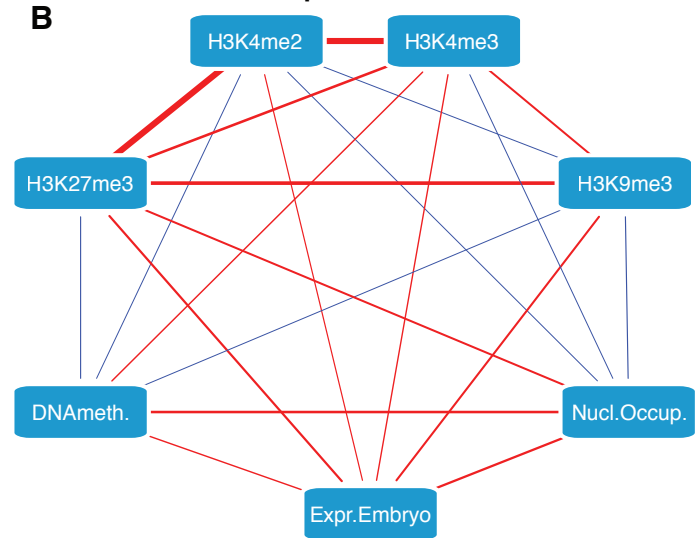
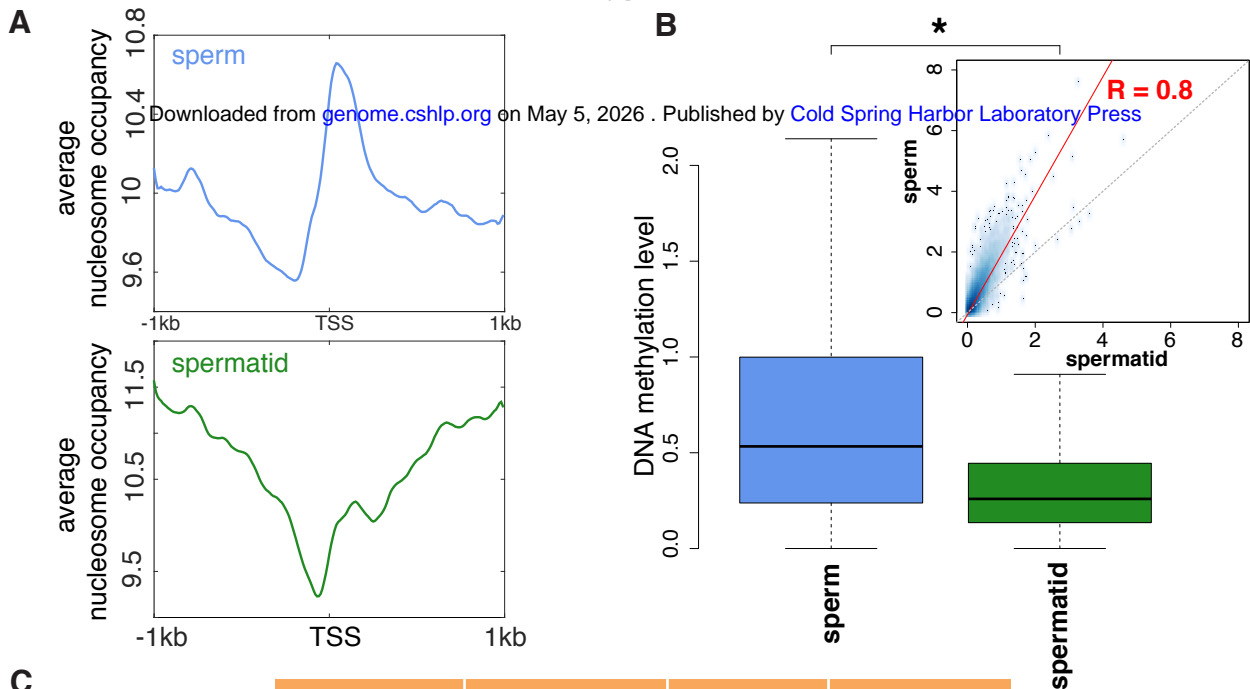
FIG.3

FIG.4**sperm****spermatid****C**

Number of *X. laevis* orthologues of human genes with H3K27me3 and H3K4me2 marks in sperm

	Number of genes	Number (%) of H3K27me3-positive genes	Number (%) of H3K4me2-positive genes
All orthologs (<i>X. laevis</i> to human)	8812	1140 (13%)	2469 (28%)
Misregulated genes orthologs	54	18 (33%) *	12 (22%)

FIG.5



C

	H3K27me3	H3K4me3	H3K4me2	H3K9me3
Sperm GW	16 %	6 %	61 %	2 %
Sperm Mis	35 %	3 %	65 %	5 %
Spermatid GW	23 %	58 %	72 %	8 %
Spermatid Mis	43 %	61 %	73 %	7 %

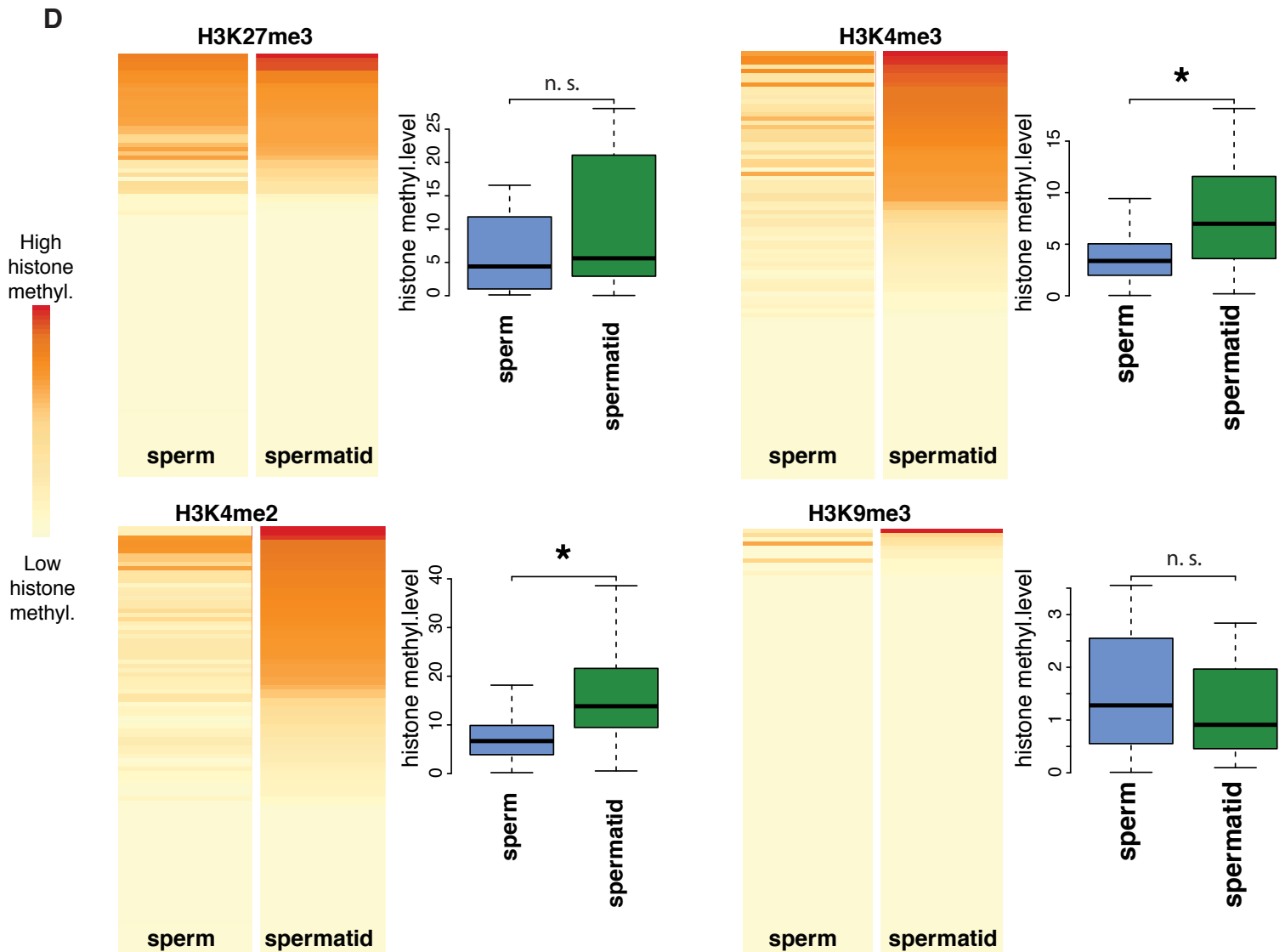
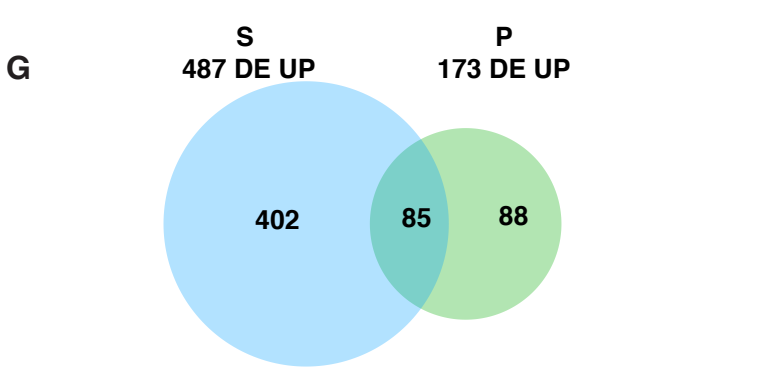
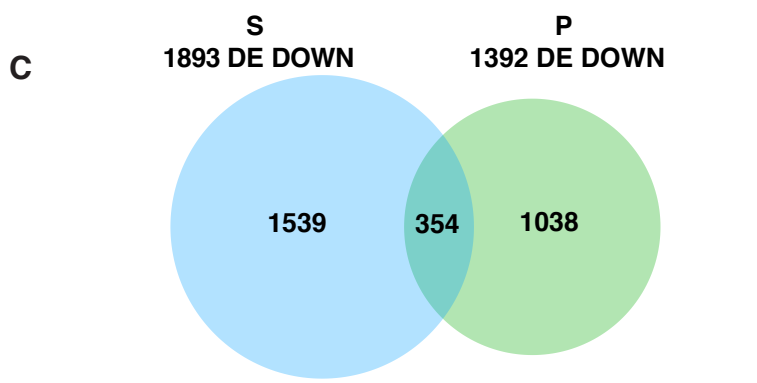
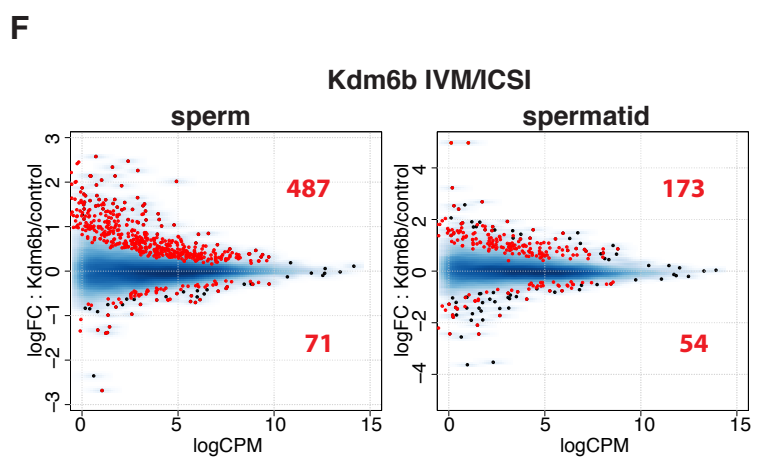
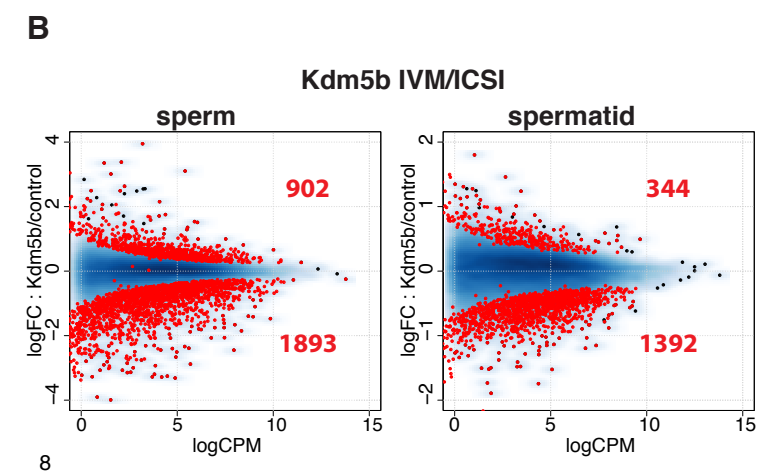
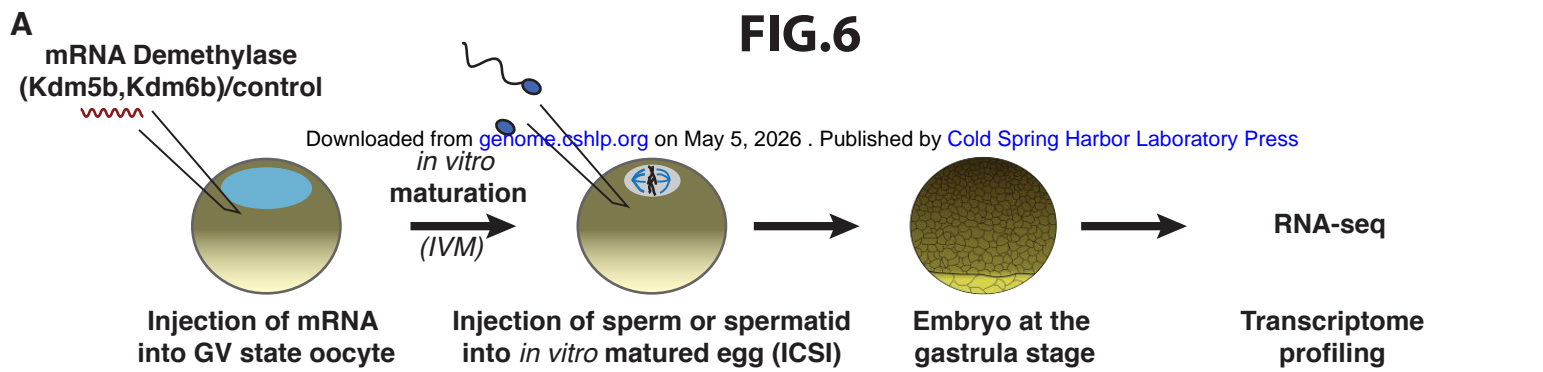


FIG.6



D

Promoter mark	sperm (DOWN)	spermatid (DOWN)
H3K4me3	3.4%	71% * ↑
H3K4me2	58.1%	82.2% * ↑
H3K27me3	21.3% * ↑	23%

H

Promoter mark	sperm (UP)	spermatid (UP)
H3K4me3	1.2%	50.3%
H3K4me2	50.7%	73.4%
H3K27me3	46.8% * ↑	46.82% * ↑

