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Developmental origins of transgenerational sperm DNA methylation epimutations following ancestral DDT exposure

Millissia Ben Maamar[a], Eric Nilsson[a], Ingrid Sadler-Riggleman[a], Daniel Beck[a], John R. McCarrey[b], Michael K. Skinner[a,*]

[a] Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA
[b] Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249, USA

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ABSTRACT

Epigenetic alterations in the germline can be triggered by a number of different environmental factors from diet to toxicants. These environmentally induced germline changes can promote the epigenetic transgenerational inheritance of disease and phenotypic variation. In previous studies, the pesticide DDT was shown to promote the transgenerational inheritance of sperm differential DNA methylation regions (DMRs), also called epimutations, which can in part mediate this epigenetic inheritance. In the current study, the developmental origins of the transgenerational DMRs during gametogenesis have been investigated. Male control and DDT lineage F3 generation rats were used to isolate embryonic day 16 (E16) prospermatogonia, postnatal day 10 (P10) spermatogonia, adult pachytene spermatocytes, round spermatids, caput epididymal spermatozoa, and caudal sperm. The DMRs between the control versus DDT lineage samples were determined at each developmental stage. The top 100 statistically significant DMRs at each stage were compared and the developmental origins of the caudal epididymal sperm DMRs were assessed. The chromosomal locations and genomic features of the different stage DMRs were analyzed. Although previous studies have demonstrated alterations in the DMRs of primordial germ cells (PGCs), the majority of the DMRs identified in the caudal sperm originated during the spermatogonia stages in the testis. Interestingly, a cascade of epigenetic alterations initiated in the PGCs is required to alter the epigenetic programming during spermatogenesis to obtain the sperm epigenetics involved in the epigenetic transgenerational inheritance phenomenon.

1. Introduction

Numerous environmental factors have been shown to promote the epigenetic transgenerational inheritance of disease such as the agricultural fungicide vinclozolin [1] and the pesticide DDT (dichlorodiphenyltrichloroethane) [2]. Caloric restriction, high fat diets, stress and a variety of different toxicants have also been linked to the transgenerational epigenetic inheritance phenomenon [3–5]. This non-genetic form of inheritance involves epigenetic modifications of the germline (sperm and egg) to pass an altered epigenome to the early embryo that can then impact the transcriptomes and epigenetics of all subsequently derived somatic cells [1,5]. Different epigenetic processes are involved in the transgenerational germline transmission where the environment can impact the health and evolution of species [3,4,6].

Epigenetics is defined as “molecular factors and processes around the DNA that regulate genome activity independent of DNA sequence and that are mitotically stable” [7]. Transgenerational epigenetic inheritance requires the germline transmission of epigenetic information. Different epigenetic processes have been shown to be involved in the transgenerational phenomenon such as DNA methylation [8–10], non-coding RNA [11,12], and histone modifications and retention [10,13,14]. Recently, concurrent alterations in all three of these processes have been observed in transgenerational sperm transmission after exposure to DDT and vinclozolin [15,16]. Most previous studies have been conducted in sperm due to the ability to isolate large numbers of cells and the inability to isolate large numbers of eggs, but experiments have shown that the female germline also has the capacity to transmit epigenetic inheritance [2,17]. The current study investigated the transgenerational sperm transmission of DNA methylation alterations after DDT exposure.

The primordial germ cells (PGCs), which give rise to spermatogenic (or oogenic) cells, develop and migrate down the genital ridge to colonize the indifferent gonad [18]. Upon sex determination and depending on the chromosomal sex, the PGCs differentiate into the

* Corresponding author.
E-mail address: skinner@wsu.edu (M.K. Skinner).

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male or female germline lineage [19]. In the rat gonad, the early developing testis germ cells will become prospermatogonia cells during the male gonadal sex determination period by embryonic day 16 (E16) [20,21]. By postnatal day 10 (P10) the germline develops into spermatogonia. Following the onset of puberty the initial wave of spermatogenesis begins in the testis where the spermatocytes, including the meiotic stage of pachytene spermatocytes, develop. After meiosis the round spermatid stage differentiates, then in the final spermatozoa stage the cells are released into the lumen of the seminiferous tubules [22], Fig. 1A. After entering the caput region of the epididymis the spermatozoa undergo further differentiation. During the transit in the epididymis the spermatozoa mature. When the spermatozoa reach the final caudal stage of the epididymis, the sperm have developed the capacity for motility and fertility [23,24]. Prior to ejaculation or degradation the mature caudal epididymal sperm will be stored in the vas deferens. In the current study we used several male germline developmental periods (E16 prospermatogonia, P10 spermatogonia, adult pachytene spermatocytes, round spermatids, caput epididymal spermatozoa, and caudal epididymal sperm), Fig. 1A, to investigate the developmental origins of the transgenerational sperm DMRs following DDT exposure in the F0 generation.

Epigenetic programming occurs during these male germline developmental periods. The migrating PGCs have an erasure of DNA methylation that then re-methylate during gonadal sex determination to create the male germline and prospermatogonia [25]. In the adult testis, during spermatogenesis some epigenetic programming events occur [26,27]. The first examples of this male germline epigenetic programming were observed with imprinted genes [28,29]. Imprinted genes can develop either early in the embryonic period or develop during spermatogenesis. Although previous studies have discussed the

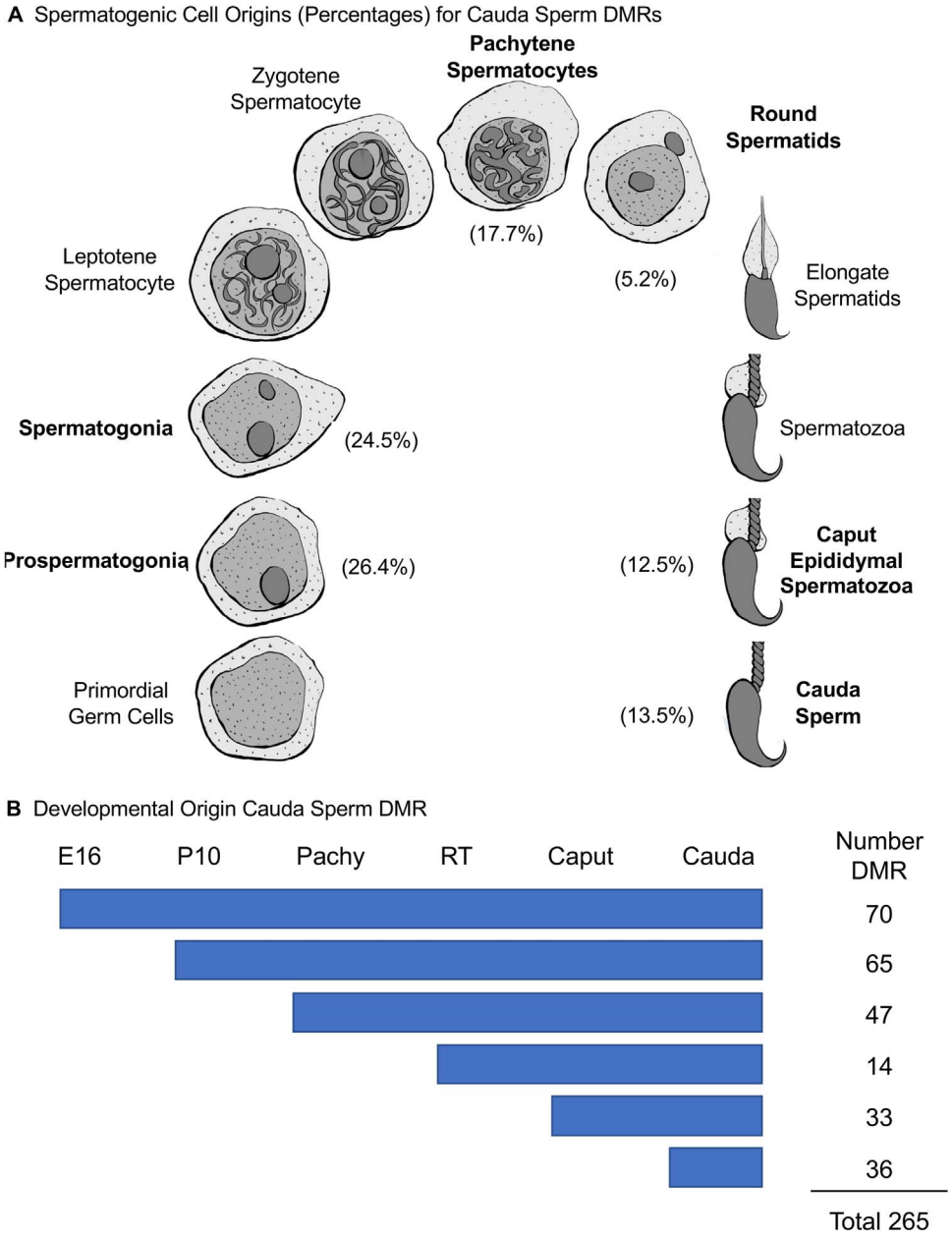


Fig. 1. Sperm DMR developmental origins. (A) Spermatogenic cell origin percentages for cauda sperm DMRs. The schematic of each developmental cell morphology listed during gametogenesis and spermatogenesis and epididymal maturation. The percentage DMR in brackets is presented for the developmental stages analyzed. (B) Developmental origins of cauda sperm DMRs for each stage of development with number DMRs at specific stages listed. The prospermatogonia (E16), spermatogonia (P10), pachytene spermatocytes (Pachy), round spermatids (RT), caput spermatozoa (Caput) and cauda sperm (Cauda) are presented.

germline developmental origins of epigenetic inheritance [5,30–34], none of these studies identified the developmental programming origins of the transgenerational epigenetic alterations observed in sperm.

The current study was designed to investigate the developmental origins of the DDT induced transgenerational sperm DMRs termed epimutations. The hypothesis tested is that a cascade of epigenetic changes occurs during development such that the majority of DMRs develop throughout gametogenesis and spermatogenic stages. It has been previously demonstrated that epigenetic alterations occur in the PGC [35]. However, due to this being the earliest stem cell stage of the germline, the PGCs DMRs were found to be different from the epimutations observed in sperm [35]. These observations suggest that the epimutations in sperm develop at later stages of epigenetic programming. The present study shows the majority of sperm DMRs originate during spermatogonia development and spermatogenesis, but some also do originate during epididymal maturation. This is one of the first genome-wide analyses of epigenetic programming during gametogenesis for transgenerational sperm epimutations [36].

2. Results

F3 generation control and DDT lineage male rats were used in this experimental design to isolate various germ cell stages for epigenetic analysis. The F0 generation gestating female rats at 90 days of age were transiently exposed to DDT or a vehicle control during gestational days 8–14. This stage of fetal development corresponds to when the PGCs migrate to colonize the indifferent gonad through the early stage of gonadal sex determination as previously described [18,25,35]. The F1 generation offspring were obtained and when they reached 90 days of age they were bred within the control and DDT lineages to generate the F2 generation (grand offspring) which were then bred at 90 days of age to obtain the transgenerational F3 generation for each control and DDT lineage. No cousin or sibling breedings were used to avoid any inbreeding artifacts. Only the F0 generation gestating females were exposed to DDT, which also directly exposed the F1 generation fetus and the germline within the F1 generation fetus that will generate the F2 generation. Therefore, the F3 generation is the first transgenerational generation not directly exposed [37] and was used to study the developmental origins of the transgenerational sperm epimutations. The F3 generation control and DDT lineage male rats were aged to the embryonic day 16 (E16) fetal stage for prospermatogonia cell isolation, to postnatal day 10 (P10) for spermatogonial cell isolation, and to 120 days of age for pachytene spermatocyte isolation, round spermatid isolation, caput spermatozoa isolation, and cauda epididymal sperm isolation. A gravity sedimentation StaPut protocol, as described in the Methods [38,39], was used to isolate the prospermatogonia, spermatogonia, pachytene spermatocytes and round spermatids cells. Three different pools each with different individual sets of animals from different litters were obtained with 5–6 males per pool for the E16 stage, 6–7 males per pool for the P10 stage, 3 adult males per pool for the adult stages. Therefore, 3–7 different males were used in the analysis within each of the three different pools analyzed for both the control and DDT lineages. Genomic DNA was isolated from each purified germ cell stage pool and from individual animals for epididymal caput spermatozoa and cauda sperm [8,13] and then three pools generated for subsequent epigenetic analysis.

The analysis of differential DNA methylation regions (DMRs) was performed with a methylated DNA immunoprecipitation (MeDIP) procedure followed by next generation sequencing for an MeDIP-Seq protocol described in the Methods [8]. The F3 generation control versus DDT lineage pools were compared to identify the DMRs at each developmental stage of the male germline. The DMRs at various p-value thresholds are presented at each developmental stage in Fig. 2. An EdgeR p-value of $p < 1e-05$ was selected at all stages for further analysis of the DMRs. This correlated with an FDR (false discovery rate) value of < 0.1 for all except the E16 prospermatogonia which was more variable. The All Windows is all DMRs with at least one 100 bp region (window) with statistical significance and the Multiple Windows being ≥2 significant 100 bp windows. The number of DMRs with multiple windows is also presented in Fig. 2. Most developmental stages showed approximately 100–300 DMRs. The epigenetic alterations were higher at the pachytene spermatocyte and round spermatid stages which showed more than 300 DMRs. The chromosomal locations of the DMRs at each developmental stage are presented in Fig. 3. The red arrowheads indicate the location of a DMR with the black boxes indicating clusters of DMRs. In most stages, all the chromosomes, except the Y and mitochondrial DNA (MT), were found to contain DMRs. The genomic features of the DMRs at each stage of development were investigated. The CpG density of the DMRs at all stages was 1–5 CpG per 100 bp with 1 CpG per 100 bp being predominant, Fig. 4. This is characteristic of a low density CpG desert [40] which has been observed with previous transgenerational DMRs. The length of the DMRs at all the developmental stages were between 1 and 4 kb with 1 kb length being predominant, Fig. 5. Therefore, the DMRs are generally 1 kb in size with around 10 CpGs as previously observed [40]. The overlap between the various developmental stage DMRs was investigated and is presented in Fig. 6A. The DMRs were found to be primarily stage specific with a very low number of DMRs overlapping between the stages with a $p < 1e-05$. The cauda sperm and the caput spermatozoa had the highest level of overlap with 88 DMRs, Fig. 6B and S7 Table, comprising over 45% of the caput epididymal spermatozoa DMRs. These observations suggest a cascade of epigenetic programming occurs throughout male germline development.

The DNA methylation alterations of the DMRs at each stage of development were investigated. For the developmental time-course the top 100 statistically significant DMRs at each stage of development were examined regarding the scaled mean read depth between the control versus DDT lineage DMR data. The scaled mean read depths at each DMR was separated into control greater than DDT data (i.e. decrease in DNA methylation) and DDT greater than control data (i.e. increase in DNA methylation) which is presented as a scaled mean read depth, Figs. 7 and 8. The E16 prospermatogonia DMRs had 28 DMR with a decrease in methylation and 72 DMR with an increase in methylation, Figs. 7A & 8A. For both data sets the DMR methylation alterations generally dropped by the P10 spermatogonia stage and became mixed with no distinct patterns in later stages. Although a few of both populations stayed elevated, the majority had an alteration in DNA methylation at later stages of development. The P10 spermatogonia DMRs had 38 DMRs with a decrease in methylation and 62 with an increase in methylation in regard to % mean read depth, Figs. 7B & 8B. A dramatic change in methylation between the prospermatogonia to spermatogonia stage and from the spermatogonia to later stages of development were observed. Therefore, the E16 prospermatogonia and P10 spermatogonia stage DMRs are for the most part unique at these stages of development. The pachytene spermatocyte DMRs had predominantly a decrease in methylation with 63 DMRs and 37 DMRs with an increase, Figs. 7C & 8C. Therefore, DNA methylation was primarily decreased in the pachytene spermatocyte DMRs. The round spermatid DMRs had a higher number of 72 DMR with an increase and 28 DMR with a decrease, Figs. 7D & 8D. The caput epididymal spermatozoa DMRs had 46 DMR with a decrease and 54 DMR with an increase in methylation, Figs. 7E & 8E. The cauda epididymal sperm DMRs had 49 DMR with a decrease and 51 DMR with an increase, Figs. 7F & 8F. The caput spermatozoa and cauda epididymal sperm have relatively consistent DNA methylation characteristics for both data sets. Therefore, the cauda sperm show more consistent DNA methylation profiles during spermatogenesis and epididymal sperm maturation, but are distinct in the spermatogonial stem cell stages.

Genes associated with the DMRs were identified and compared at each of the developmental stages. The DMRs that had a gene within 10 kb distance, so the promoter is considered, were identified and the

A Prospermatogonia (E16)

P-value	All Window	Multiple Window
0.001	4011	265
1e-04	522	25
1e-05	**94**	**11**
1e-06	27	5
1e-07	12	4

Number Windows	1	2	3	4	5
Number of DMR	83	7	2	1	1

B Spermatogonia (P10)

P-value	All Window	Multiple Window
0.001	4728	464
1e-04	722	107
1e-05	**195**	**47**
1e-06	86	32
1e-07	48	19

Number Windows	1	2	3	4	5	20
Number of DMR	148	35	4	5	2	1

C Pachytene Spermatocytes

P-value	All Window	Multiple Window
0.001	11776	891
1e-04	1917	78
1e-05	**340**	**13**
1e-06	71	5
1e-07	20	2

Number Windows	1	2	3
Number of DMR	327	9	4

D Round Spermatids

P-value	All Window	Multiple Window
0.001	11006	1276
1e-04	1816	131
1e-05	**323**	**17**
1e-06	60	0
1e-07	7	0

Number Windows	1	2
Number of DMR	306	17

E Caput Epididymal Spermatozoa

P-value	All Window	Multiple Window
0.001	6644	616
1e-04	1146	148
1e-05	**284**	**72**
1e-06	117	52
1e-07	74	38

Number Windows	1	2	3	4	≥5
Number of DMR	212	39	13	5	15

F Cauda Sperm

P-value	All Window	Multiple Window
0.001	4611	478
1e-04	809	143
1e-05	**265**	**82**
1e-06	140	55
1e-07	87	40

Number Windows	1	2	3	4	≥5
Number of DMR	183	39	20	10	13

Fig. 2. DMR identification and numbers. The number of DMRs found using different p-value cutoff thresholds. The All Window column shows all DMRs. The Multiple Window column shows the number of DMRs containing at least two significant windows (100 bp each). The number of DMRs with the number of significant windows (100 bp per window) at a p-value threshold of 1e-05 is presented. (A) E16 prospermatogoina. (B) P10 spermatogonia. (C) Pachytene spermatocytes. (D) Round Spermatids. (E) Caput Spermatozoa. (F) Cauda Sperm.

associated genes and gene functional categories determined, S1–S6 Tables. The DMRs with a p < 1e-05 were used in this analysis at all the developmental stages. The number of genes associated with specific gene functional categories is presented in Fig. 9A. Each of the developmental stages are presented to compare the major gene categories. The signaling, metabolism, transcription and receptor gene categories were the major categories present in the different developmental stages. Additional categories were cytoskeleton, development, transport and immune system, that were present in all the developmental stages, Fig. 9A. The DMR associated genes at each developmental stage were also used in a gene pathway analysis applying the KEGG pathway association as described in the Methods. The number of DMR associated genes involved with the major pathways is presented in Fig. 9B. The most predominant pathways present in at least two different developmental stages are shown. The E16 prospermatogonia, round spermatids, caput spermatozoa and cauda sperm had DMR associated genes in the metabolic pathways. The E16 prospermatogonia, P10 spermatogonia, pachytene spermatocytes had DMR associated genes in the pathways in cancer in common. Therefore, the only pathways that were present in nearly all the stages are metabolism and pathways in cancer. The caput epididymal spermatozoa and cauda sperm were more consistent than the others which were more distinct.

The final aspect of the study involved an investigation of the developmental origins of the cauda sperm DMRs, meaning when the differential DNA methylation alterations appeared within the developmental stages examined. In the cauda sperm there are 265 DMRs at p < 1e-05. For the analysis of developmental origins the 265 DMRs were examined individually with a reduced statistical stringency of p < 0.05 to see the first stage the DMR appears or originates. Fig. 1B demonstrates 70 DMRs were developed in the E16 prospermatogonia stage, 65 DMRs in the P10 spermatogonia stage, 47 DMRs in the spermatocyte stage, 14 DMRs in the round spermatid stage, 33 DMRs in the caput epididymal spermatozoa stage, and 36 DMRs in the cauda sperm stage, S8 Table. The cauda sperm had a total of 265 DMRs at p < 1e-05 with the majority of DMRs originating in the prospermatogonia and spermatogonia. Fewer were observed in the round spermatids and caput epididymal spermatozoa stages, Fig. 1B. The percentage of the total DMR origins and schematic of the different

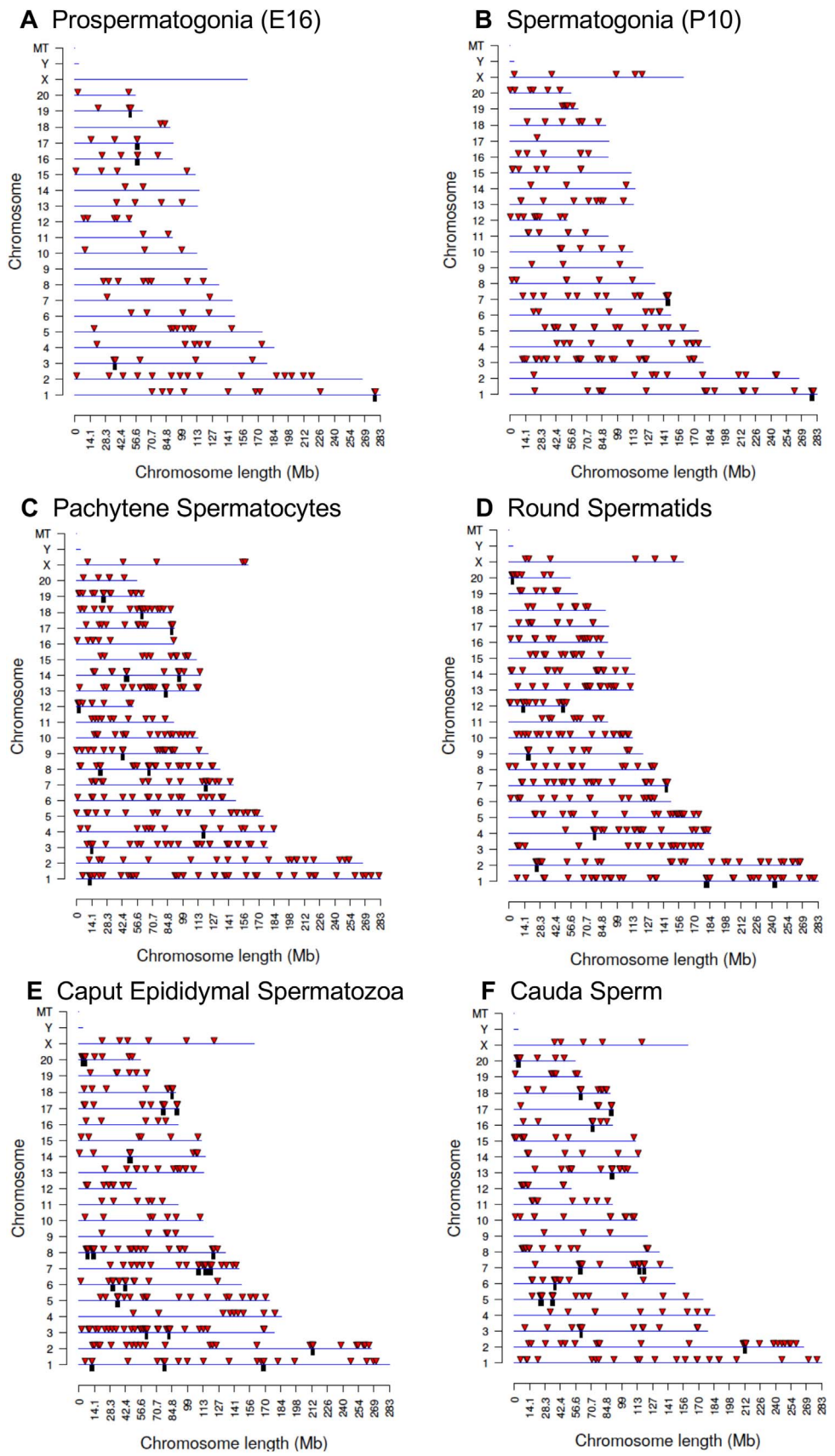


Fig. 3. DMR chromosomal locations. The DMR locations on the individual chromosomes is represented with an arrowhead and a cluster of DMRs with a black box. All DMRs containing at least one significant window at a p-value threshold of 1e-05 are shown. (A) E16 prospermatogoina. (B) P10 spermatogonia. (C) Pachytene spermatocytes. (D) Round Spermatids. (E) Caput Spermatozoa. (F) Cauda Sperm.

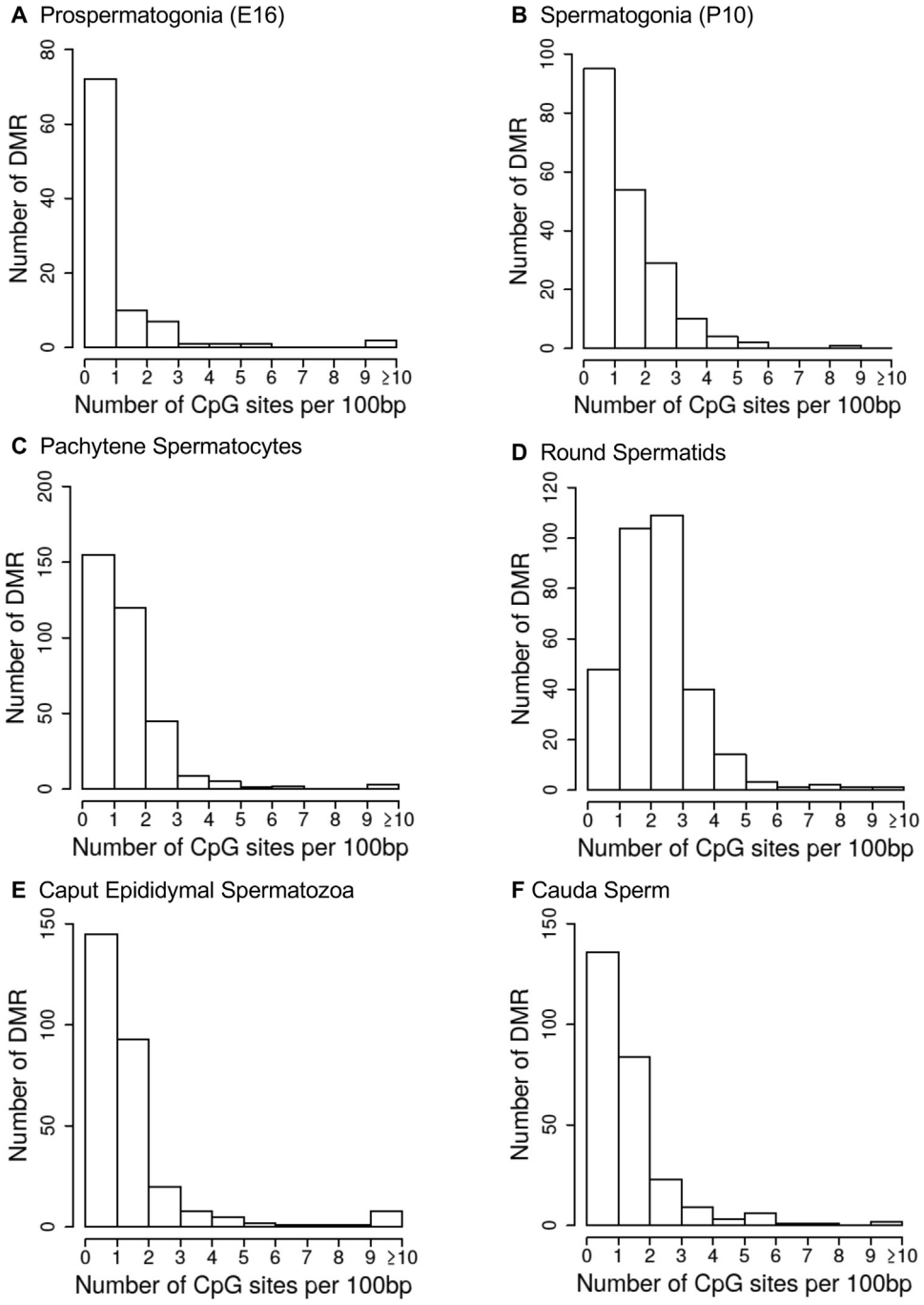


Fig. 4. DMR genomic features. The number of DMRs at different CpG densities. All DMRs at a p-value threshold of 1e-05 are shown. (A) E16 prospermatogoina. (B) P10 spermatogonia. (C) Pachytene spermatocytes. (D) Round Spermatids. (E) Caput Spermatozoa. (F) Cauda Sperm.

stage cells during development is presented in Fig. 1A. Therefore, the majority of the transgenerational sperm DMRs were developed during the prospermatogonia and spermatogonia stages with a small number developed in the epididymal maturation stages. This correlated to a cascade of epigenetic and transcriptome changes during these stages of male germline development to generate the transgenerational sperm epimutations, Fig. 1A.

3. Discussion

The aim of the current study was to identify the developmental origins of the cauda sperm transgenerational DMRs that transmit the epigenetic transgenerational inheritance of disease and phenotypic variation. During embryonic day 8–14 in the rat, when the primordial germ cells (PGCs) migrate to colonize the indifferent fetal gonad, the F0 generation gestating female exposure to DDT was performed [18,25,35]. Five different stages of male germ cell development were investigated: E16 prospermatogonia, P10 spermatogonia, and adult pachytene spermatocytes, round spermatids, caput epididymal spermatozoa and cauda sperm, Fig. 1A. The functional considerations of these cell populations involve the prospermatogonia precursor stem cell population, spermatogonia stem cell population in the testis, the meiotic pachytene spermatocyte cell population, the post-meiotic spermatid population and the spermatozoa present in the caput epididymis, and mature sperm in the cauda epididymis. The caput spermatozoa and cauda epididymal sperm were directly isolated as described in the Methods then sonicated and washed to remove any contaminating somatic cells, so are pure sperm cell preparations. For

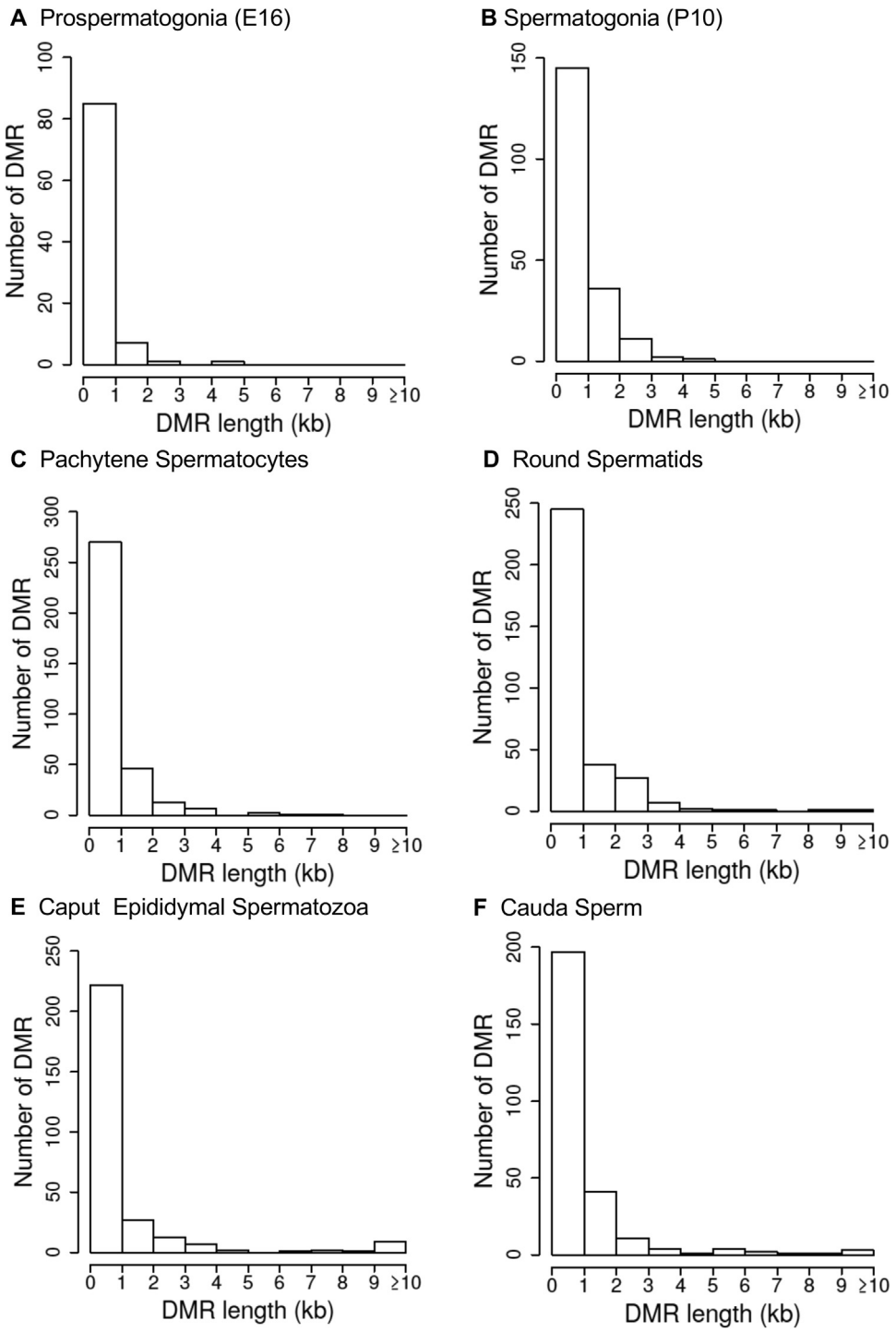


Fig. 5. DMR lengths. All DMRs at a p-value threshold of 1e-05 are shown. (A) E16 prospermatogoina. (B) P10 spermatogonia. (C) Pachytene spermatocytes. (D) Round Spermatids. (E) Caput Spermatozoa. (F) Cauda Sperm.

the other cell populations a gravity sedimentation on a StaPut apparatus procedure was used to isolate the specific cell populations as previously described [39,40]. The E16 prospermatogonia and P10 spermatogonia were found to have a greater than 85% purity. The pachytene spermatocytes were greater than 95% spermatocytes with two-thirds of these cells pachytene spermatocytes and one-third other spermatocyte stages. This is due to the longer developmental life span or developmental period of pachynema compared to the other stages of first meiotic prophase [41] and to the efficiency of the StaPut gradient protocol. For the isolated round spermatids 95% of them were spermatids with 90% of them being round spermatids and 10% being elongate spermatids and other spermatogenic cell stages [42]. Despite obtaining a high purity for these developmental stage male germline cell populations, cell purity needs to be considered in the data interpretations. The embryonic day 16 (E16) prospermatogonia were isolated from the fetal gonad, the postnatal day 10 (P10) from the early pubertal age, the pachytene spermatocytes, round spermatids, caput epididymal spermatozoa and cauda sperm were all isolated from postnatal day 120 (P120) age adult male rats. DDT has been previously shown to induce transgenerational inheritance of disease between 6 and 12 months of age [5]. Therefore, by choosing the postnatal day 120 or earlier with negligible disease present, no disease artifacts are

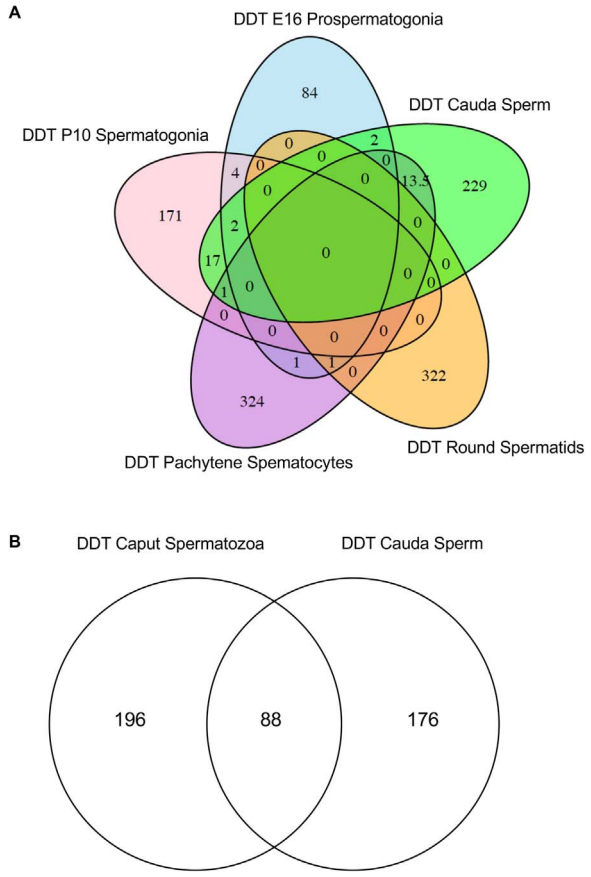


Fig. 6. Developmental stage DMR overlaps. (A) Different developmental stage DMRs overlap. (B) Epididymal caput spermatozoa and caudal sperm DMR overlap.

anticipated in the current study. This is consistent with the primary objective of the study to investigate the developmental origins of the sperm DMR, but future studies can now consider disease correlations.

The transgenerational F3 generation control and DDT lineage germline samples were compared at each of the developmental stages. The F1 and F2 generations have somatic cell and germline epigenetic alterations due to the F1 generation fetal direct exposure and F1 generation fetal germline direct exposure that will generate the F2 generation. Therefore, the F3 generation is the first transgenerational generation not having any direct exposures, so was the focus of the current study [37]. A comparison of the F1 generation direct exposure sperm DMR development with the transgenerational F3 generation observations will be an interesting future study to consider. All F3 generation developmental samples had differential DNA methylation regions (DMRs). Excluding the caput spermatozoa and cauda epididymal sperm with common DMRs, all the other stage DMRs were found to be primarily distinct from each other, Fig. 6. The majority of the epigenome was not altered (i.e. potentially millions of CpG regions) and the current study focused on the transgenerational alterations at each stage of development. The distinct DMRs at each stage indicate a cascade of epigenetic changes occur to program the sperm transgenerational epigenome.

A comparison of the different developmental stage germline populations was conducted in regard to DNA methylation alterations for the top 100 most statistically significant DMRs at each developmental stage separately. Analysis of these DNA methylation alterations for the different developmental stage DMRs revealed that the E16 prospermatogonia and P10 spermatogonia presented distinct patterns of DNA methylation compared to the other stages, Figs. 7 and 8. The statistically significant top 100 DMRs at both these developmental stages altered greatly with adjacent stages and often lost their statistical significance. In contrast, the pachytene spermatocytes, round spermatids, caput epididymal spermatozoa and caudal sperm were more consistent between each other regarding the DMRs maintaining similar DNA methylation alterations and statistical significance, Figs. 7 and 8. This suggests that the developing stem cells and spermatogonia have principally unique DNA methylation profiles associated with the cascade of epigenetic programming of the cells. When the spermatogonia initiate the spermatogenesis process there is a cascade of epigenetic programming and DNA methylation observed between the spermatogenic cell populations, as well as between spermatozoa undergoing epididymal maturation. Observations suggest a dynamic epigenetic programming of the male germline in the testis and epididymis. Epididymal maturation has been shown to promote structural and molecular alterations in the developing sperm, which also appear to be involved in the transgenerational sperm DMR programming [32].

Significant epigenetic reprogramming in the primordial germ cells (PGCs) has already been described. DNA methylation erasure during migration and colonization of the fetal gonad has been shown to occur [18]. This process enables the germline stem cell population to facilitate the generation of the male or female germ lines following gonadal sex determination [18,25]. A stable epigenetic cascade of events occurs to produce the spermatogonial stem cell population in the adult testis. Studies have shown that environmental toxicants have the ability to promote epigenetic DNA methylation alterations in the PGCs and prospermatogonia [35]. These transgenerational DMRs observed in the PGCs were found to be distinct from the caudal epididymal sperm DMRs [35]. The current study supports this observation and determines that sperm DMRs not only originate in the fetal period, but trigger a cascade of epigenetic alterations that eventually impact the mature sperm epigenome, Fig. 1B. Therefore, the developmental cascade of epigenetic programming initiated by fetal exposure induces a number of DMRs that are also maintained throughout development.

In the cauda sperm, the origins of the DMRs were identified. The majority of them originate throughout the development of prospermatogonia, spermatogonia and spermatocytes, Fig. 1B and S9 Table. A smaller number of DMRs also arose in the round spermatids and cauda epididymal sperm. The highest number of DMRs was observed in the prospermatogonia, the stage of development during which the initial F0 generation gestating female and F1 generation developing fetus were exposed. The DMRs in the cauda sperm originated in all the earlier developmental spermatogenic stages and through epididymal maturation. These observations correlated with the DNA methylation alteration data for DMRs between the different developmental stages shown in Figs. 7 and 8. Thus, the primary origin of the transgenerational sperm DMR epimutations/DMRs does not occur during early PGC development or in the embryonic stem cell population, but throughout development and spermatogenesis in the testis, as well as during epididymal maturation. Although the induction of a cascade of epigenetic programming in the PCGs is critical as previously suggested [7,35], there is a continual cascade of epigenetic alterations throughout spermatogenesis to give rise to the transgenerational sperm DMRs, Fig. 1A. Another recent study investigating vinclozolin induced sperm DMR origins found similar observations, but the highest level of DMRs were developed at the pachytene spermatocyte stage [36]. Therefore, an exposure specificity may exist in the developmental epigenetics observed.

The DMRs associated genes were identified for DMRs at $p < 1e\text{-}05$ at each stage of development. The analysis of associated gene functional categories identified signaling, transcription, metabolism and receptor as the major categories at all stages of development. The pathway analysis also identified a number of stage specific pathways with negligible overlap except in metabolism and pathways in cancer. Gene pathways more specific to testis development such as meiosis or the pyruvate pathway did not contain more than a few DMR associated

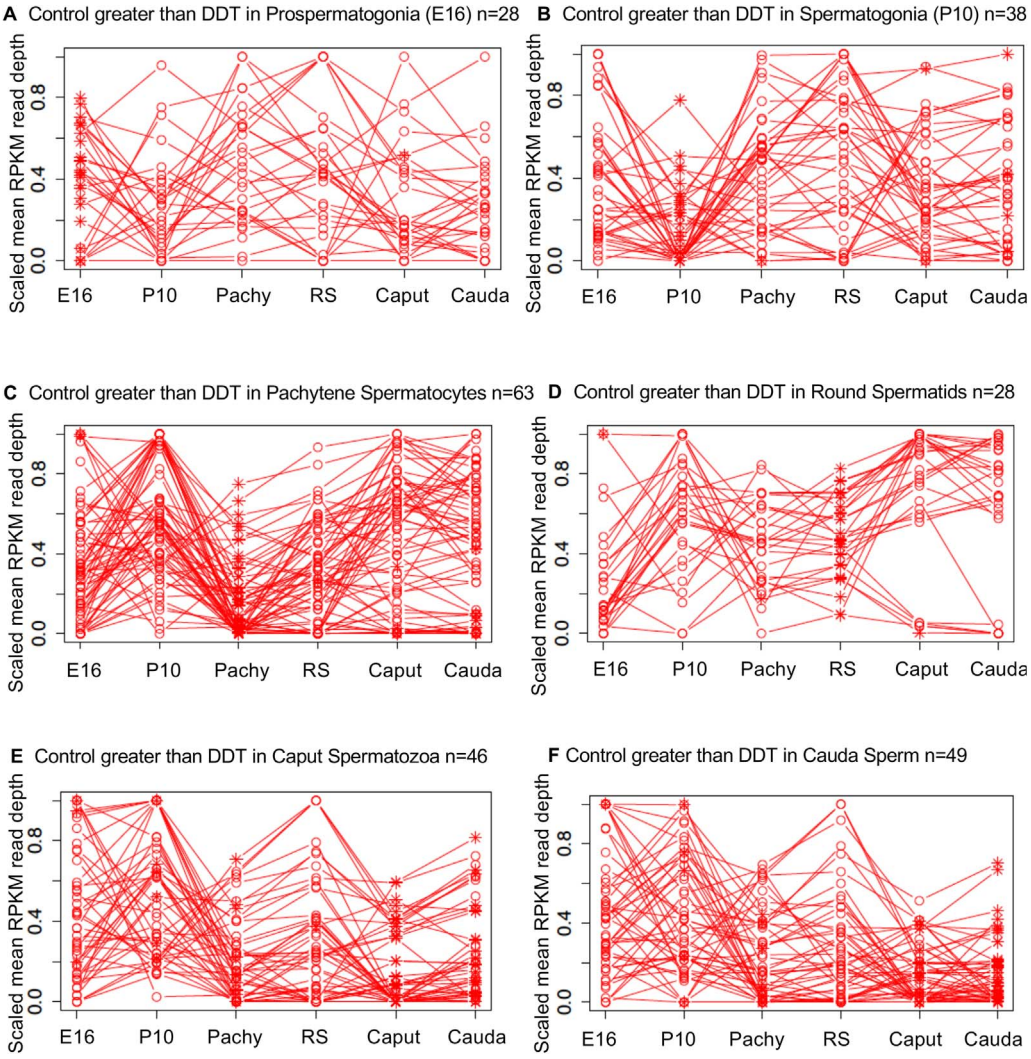


Fig. 7. Timeline DMR development. Top 100 statistically significant DMR developmental alterations for control greater than DDT in read depths (decrease in DNA methylation). Genomic windows with an edgeR p-value < 10^{-5} are indicated by astrisks and windows separated showing only those with an RPKM read depth elevated in the control. (A) E16 prospermatogoina. (B) P10 spermatogonia. (C) Pachytene spermatocytes. (D) Round spermatids. (E) Caput spermatozoa. (F) Cauda sperm.

genes. Therefore, for all the stages of development for the DMR-associated genes involved similar gene categories, but different pathways. Future studies are needed to correlate the DMRs with data identified from transcriptome and altered non-coding RNA expression to provide a better understanding as to how the DMRs may regulate genome activity.

The caput epididymal spermatozoa displayed unique DMRs and others that were in common with the cauda sperm. Interestingly, the cauda epididymal sperm did acquire DMRs that were not present in earlier stages of development. Even though the majority of the cauda epididymal sperm DMRs were obtained during prior developmental stages, 36 DMRs were acquired at the cauda epididymal sperm stage of maturation only, S7 and S8 Tables. Two studies have previously suggested that epididymal maturation (epididymosomes) may be involved with transgenerational sperm maturation regarding epigenetic alterations, but no direct data have been provided [43–45]. The current study supports a role for epididymal maturation altering sperm DMRs. However, the majority of DMRs originated during earlier developmental stages and spermatogenesis in the testis and not the epididymis, Fig. 1B. A combination of early development, spermatogenesis and epididymal mutation appears to be involved in the development of the transgenerational sperm epimutations. Further investigation is needed to elucidate the molecular mechanisms in the epididymis that alter sperm epigenetics.

4. Conclusions

The current study was designed to investigate the developmental origins of transgenerational sperm DMRs induced by the pesticide DDT. Results show that each stage of male germ cell development examined, including the E16 prospermatogonia, P10 spermatogonia, pachytene spermatocytes, round spermatids, caput epididymal spermatozoa and cauda sperm, has distinct and unique DMRs when the DDT lineage and control lineage F3 generation samples are compared. Both the prospermatogonia and spermatogonia display unique DNA methylation alterations in comparison with later stages that are more consistent. The origins of the cauda sperm DMRs developed throughout the earlier developmental stages with the highest number during prospermatogonia, spermatogonia and pachytene spermatocytes, and fewer originating during epididymal maturation. In conclusion, a developmental cascade of epigenetic programming occurs from the PGCs to the sperm with epimutations developing throughout the different stages of development, Fig. 1A. The initial speculation that the DMRs may develop in the PGCs is not the case, even though the developmental cascade initiated is important. Recently, sperm carrying transgenerational epimutations were found to have alterations in DNA methylation, ncRNAs and histone retention, such that all epigenetic factors are involved in the epigenetic transgenerational inheritance phenomenon [15,16]. Future studies will now need to investigate these

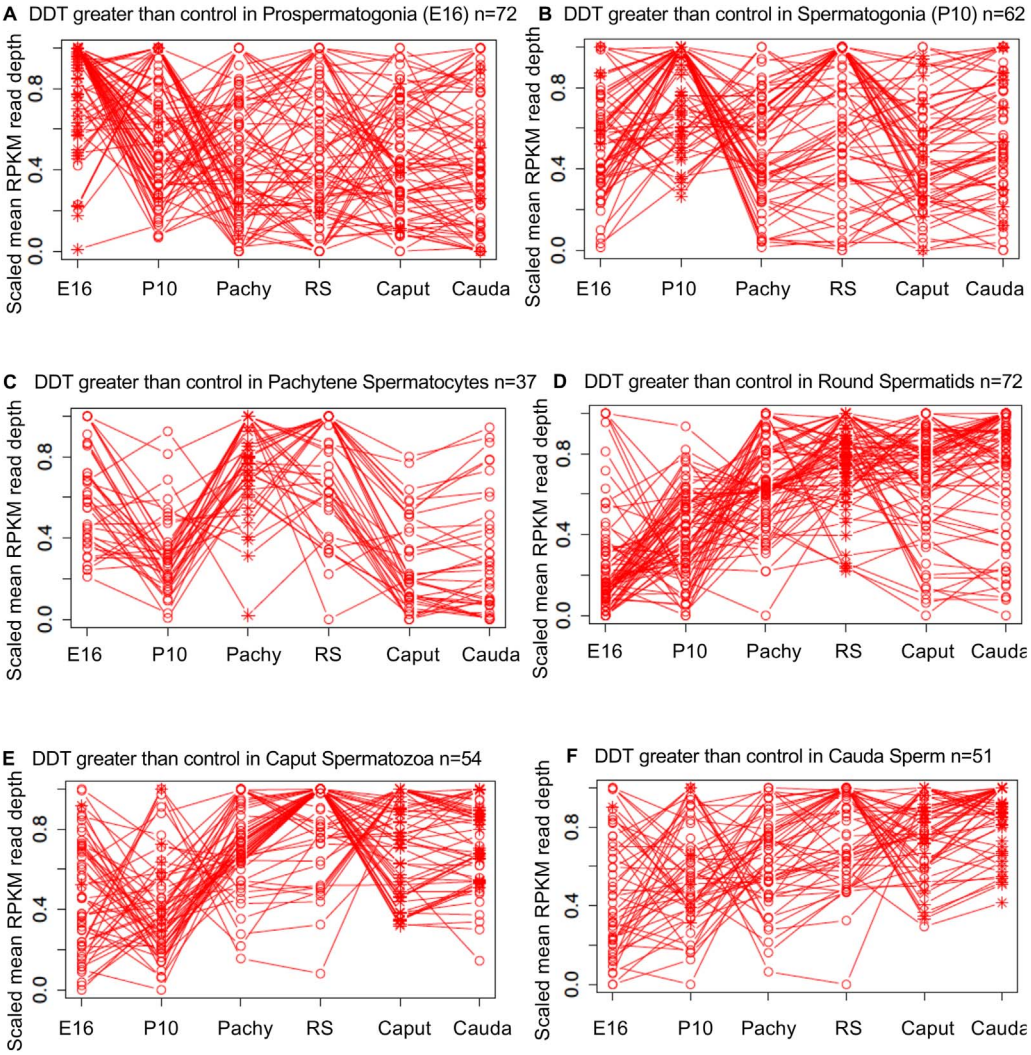


Fig. 8. Timeline DMR development. Top 100 statistically significant DMR developmental alterations for DDT greater than control in read depths (increase in DNA methylation). Genomic windows with an edgeR p-value < 10^{-5} are indicated by astrisks and windows separated showing only those with an RPKM read depth elevated in the DDT over control. (A) E16 prospermatogoina. (B) P10 spermatogonia. (C) Pachytene spermatocytes. (D) Round spermatids. (E) Caput spermatozoa. (F) Cauda sperm.

other epigenetic processes and correlated gene expression to develop a more systems biology assessment of the molecular mechanism involved.

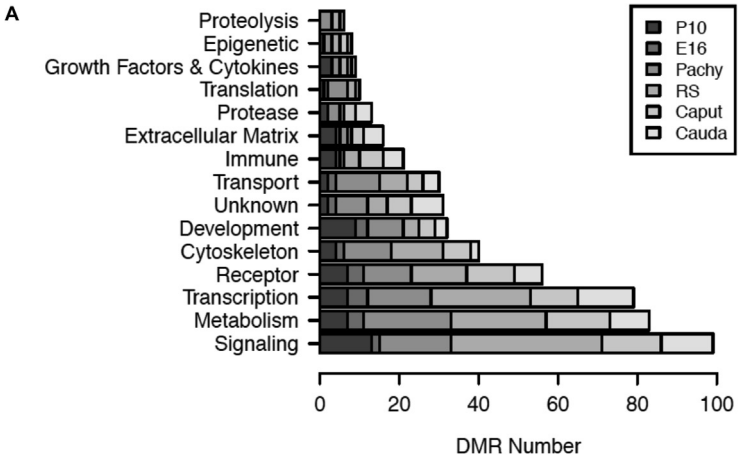
5. Methods

5.1. Animal studies and breeding

Female and male rats of an outbred strain Hsd: Sprague Dawley®™SD®™ obtained from Harlan/Envigo (Indianapolis, IN) at about 70–100 days of age were maintained in ventilated (up to 50 air exchanges/hour) isolator cages (cages with dimensions of 10 ¾″ W×19 ¼″ D×10 ¾″ H, 143 square inch floor space, fitted in Micro-vent 36-cage rat racks; Allentown Inc., Allentown, NJ) containing Aspen Sani chips (pinewood shavings from Harlan) as bedding, and a 14 h light: 10 h dark regimen, at a temperature of 70 F and humidity of 25–35%. The mean light intensity in the animal rooms ranged from 22 to 26 ft-candles. Rats were fed ad lib with standard rat diet (8640 Teklad 22/5 Rodent Diet; Harlan) and ad lib tap water for drinking. To obtain time-pregnant females, the female rats in proestrus were pair-mated with male rats. The sperm-positive (i.e. sperm plug present) (day 0) rats were monitored for diestrus and body weight. On days 8 through 14 of gestation [46], the females received daily intraperitoneal injections of DDT (25 mg/kg body weight/day) or dimethyl sulfoxide (DMSO). The DDT (dichlorodiphenyltrichloroethane) was obtained from Chem Service Inc. (West Chester, PA) and reported to have a purity of 98.2%. DDT were dissolved and injected in DMSO vehicle as previously described [47]. Treatment lineages are designated 'control' or 'DDT' lineages. The treated gestating female rats were designated as the F0 generation. The offspring of the F0 generation rats were the F1 generation. Non-littermate females and males aged 70–90 days from the F1 generation of control or DDT lineages were bred to obtain F2 generation offspring. The F2 generation rats were bred to obtain F3 generation offspring. The F1–F3 generation offspring were not themselves treated directly with DDT. The control and DDT lineages were housed in the same room and racks with lighting, food and water as previously described [47–49]. All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 6252).

5.2. Epididymal sperm collection and DNA isolation

Testis and epididymis were collected from 12 month old rats for germ cell collection. The epididymis was dissected free of connective tissue and divided into caput and cauda halves with a cut in the mid-corpus. A small cut made to the cauda and to the caput and each half was placed in 3 ml of PBS for up to 2 h at 4 °C. Caput and cauda epididymal tissue were each coarsely minced and the liquid with the



B DMR Gene Association Pathways

DDT Prospermatogonia E16
rno05160 Hepatitis C (2)
rno04913 Ovarian steroidogenesis (2)
rno04217 Necroptosis (2)
rno04976 Bile secretion (2)
rno01100 Metabolic pathways (2)

DDT Spermatogonia (P10)
rno05200 Pathways in cancer (6)
rno05224 Breast cancer (4)
rno05226 Gastric cancer (4)
rno04928 Parathyroid hormone synthesis, secretion and action (3)
rno04380 Osteoclast differentiation (3)

DDT Pachytene Spermatocytes
rno01100 Metabolic pathways (10)
rno04014 Ras signaling pathway (6)
rno05206 MicroRNAs in cancer (6)
rno05200 Pathways in cancer (6)
rno05165 Human papillomavirus infection (6)

DDT Round Spermatids
no01100 Metabolic pathways (11)
rno05200 Pathways in cancer (8)
rno04010 MAPK signaling pathway (7)
rno04360 Axon guidance (6)
rno04151 PI3K-Akt signaling pathway (6)
rno04144 Endocytosis (6)

DDT Caput Spermatozoa
rno05168 Herpes simplex infection (9)
rno04514 Cell adhesion molecules (CAMs) (9)
rno04612 Antigen processing and presentation (8)
rno05165 Human papillomavirus infection (8)
rno01100 Metabolic pathways (7)

DDT Cauda Sperm
rno01100 Metabolic pathways (5)
rno04514 Cell adhesion molecules (CAMs) (4)
rno04650 Natural killer cell mediated cytotoxicity (4)
rno04145 Phagosome (4)
rno04510 Focal adhesion (4)

Fig. 9. Sperm DMR associated gene categories and pathways. (A) DMR associated gene functional categories for each stage (color insert) versus DMR number. (B) DMR associated gene pathways for each stage of development with number of DMR associated genes in pathway in brackets. Bolded pathways show overlap in at least three developmental stages.

released sperm collected. For each sample the released sperm was centrifuged at 6000×*g*, then the supernatant removed, and the pellet resuspended in NIM buffer, to be stored at −80 °C until further use. One hundred µl of sperm suspension was sonicated to destroy somatic cells and tissue, spun down at 6000×*g*, the sperm pellet washed with 1x PBS once, and then combined with 820 µl DNA extraction buffer and 80 µl 0.1 M DTT. The sample was incubated at 65 °C for 15 min. Following this incubation 80 µl proteinase K (20 mg/ml) was added and the sample incubated at 55 °C for at least 2 h under constant rotation. Then 300 µl of protein precipitation solution (Promega, A7953) was added, the sample mixed thoroughly and incubated for 15 min on ice. The sample was centrifuged at 12,500×*g* for 30 min at 4 °C. One ml of the supernatant was transferred to a 2 ml tube and 2 µl of glycoblue and 1 ml of cold 100% isopropanol were added. The sample was mixed well by inverting the tube several times then left in −20 °C freezer for at least one hour. After precipitation the sample was centrifuged at 12,500×*g* for 20 min at 4 °C. The supernatant was taken off and discarded without disturbing the (blue) pellet. The pellet was washed with 70% cold ethanol by adding 500 µl of 70% ethanol to the pellet and returning the tube to the freezer for 20 min. After the incubation the tube was centrifuged for 10 min at 4 °C at 12,500×*g* and the supernatant discarded. The tube was spun again briefly to collect residual ethanol to bottom of tube and then as much liquid as possible was removed with gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 min). Pellet was then resuspended in 100 µl of nuclease free water.

5.3. Developing germ cell stage isolation and DNA preparation

Harlan Sprague-Dawley rats (Harlan Inc., Indianapolis IN) were used for all experiments. The rats were kept in a temperature controlled environment and given food and water ad libitum. Estrous cycles of female rats were monitored by cellular morphology from vaginal smears. Rats in early estrus were paired with males overnight and mating confirmed by sperm-positive smears, denoted day 0 of pregnancy. Pregnant rats were euthanized at embryonic day 16 (E16) of gestation, and fetal gonads were collected for germ cell preparations. Sex was determined on the basis of gonadal morphology. Germ cells were isolated exclusively from males.

Purified populations of male PGCs type T1 prospermatogonia (at E16) were prepared using a mini StaPut gradient method as previously described [50,51]. Briefly, fetal testes were pooled and dissociated by incubation in 0.25% trypsin-EDTA (Sigma) with vigorous pipetting using a 1000 microliter pipette tip, and the resulting cell solution was filtered through 100 µm nylon mesh to yield a single cell suspension. This cell suspension was then loaded onto a 50 ml 2–4% bovine serum albumen (BSA) gradient prepared in KREBS buffer, and the cells were allowed to sediment at unit gravity at 4 °C for two hours as described [50,51]. The gradient was then fractionated and aliquots of the fractions were examined under phase optics to identify those enriched for the appropriate cell types on the basis of morphological characteristics. The enriched fractions were pooled to yield the final sample which was ≥85% pure for the desired male germ cell type in each case.

Three pools of prospermatogonia were prepared for each treatment group, with each pool derived from testes of 5–6 rats from different litters.

A similar mini StaPut gradient method [50,51] was used to isolate spermatogonia from testes of 10-day old rats, with the addition of incubation of the testes in 0.5 mg/ml collagenase (Sigma C1639) at 33 °C for 20 min with agitation to dissociate the seminiferous tubules. Three pools of spermatogonia from 10-day old rats were prepared for each treatment group, with each pool derived from testes of 6–7 rats from different litters.

To isolate pachytene spermatocytes and round spermatids, testes were collected from 12 month old rats suspended in F-12 culture medium (Gibco-Life Technologies, USA. Ref 11765-054) and shipped overnight on ice to Dr. John McCarrey. A StaPut gradient method was used to isolate the developing germ cell stages as previously described [50,51]. Three pools of cells of each cell type were prepared for each treatment group, with each pool derived from testes of three rats from different litters. DNA was isolated from prospermatogonia, spermatogonia, pachytene spermatocytes and round spermatids using the same procedure as was used for sperm, with the omission of sonication and DTT treatments.

5.4. Methylated DNA immunoprecipitation MeDIP

Methylated DNA Immunoprecipitation (MeDIP) with genomic DNA was performed as follows: rat DNA pools were generated using the appropriate amount of genomic DNA from each individual for 3 pools each of control and DDT lineage animals. Genomic DNA pools were sonicated using the Covaris M220 the following way: the pooled genomic DNA was diluted to 130 µl with TE buffer into the appropriate Covaris tube. Covaris was set to 300 bp program and the program was run for each tube in the experiment. 10 µl of each sonicated DNA was run on 1.5% agarose gel to verify fragment size. The sonicated DNA was transferred from the Covaris tube to a 1.7 ml microfuge tube and the volume measured. The sonicated DNA was then diluted with TE buffer (10 mM Tris HCl, pH7.5; 1 mM EDTA) to 400 µl, heat-denatured for 10 min at 95 °C, then immediately cooled on ice for 10 min. Then 100 µl of 5X IP buffer and 5 µg of antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added to the denatured sonicated DNA. The DNA-antibody mixture was incubated overnight on a rotator at 4 °C.

The following day magnetic beads (Dynabeads M-280 Sheep anti-Mouse IgG; 11201D) were pre-washed as follows: The beads were resuspended in the vial, then the appropriate volume (50 µl per sample) was transferred to a microfuge tube. The same volume of Washing Buffer (at least 1 ml PBS with 0.1% BSA and 2 mM EDTA) was added and the bead sample was resuspended. Tube was then placed into a magnetic rack for 1–2 min and the supernatant discarded. The tube was removed from the magnetic rack and the beads washed once. The washed beads were resuspended in the same volume of IP buffer (50 mM sodium phosphate ph7.0, 700 mM NaCl, 0.25% TritonX-100) as the initial volume of beads. 50 µl of beads were added to the 500 µl of DNA-antibody mixture from the overnight incubation, then incubated for 2 h on a rotator at 4 °C.

After the incubation the bead-antibody-DNA complex was washed three times with IP buffer as follows: The tube was placed into magnetic rack for 1–2 min and the supernatant discarded, then washed with IP buffer 3 times. The washed bead-DNA solution is then resuspended in 250 µl digestion buffer with 3.5 µl Proteinase K (20 mg/ml). The sample was then incubated for 2–3 h on a rotator at 55 °C and then 250 µl of buffered Phenol-Chloroform-Isoamylalcohol solution was added to the supe and the tube vortexed for 30 s then centrifuged at 12,500×*g* for 5 min at room temperature. The aqueous supernatant was carefully removed and transferred to a fresh microfuge tube. Then 250 µl chloroform were added to the supernatant from the previous step, vortexed for 30 s and centrifuged at 12,500×*g* for 5 min at room temperature. The aqueous supernatant was removed and transferred to a fresh microfuge tube. To the supernatant 2 µl of glycoblue (20 mg/ml), 20 µl of 5 M NaCl and 500 µl ethanol were added and mixed well, then precipitated in −20 °C freezer for 1 h to overnight.

The precipitate was centrifuged at 12,500×*g* for 20 min at 4 °C and the supernatant removed, while not disturbing the pellet. The pellet was washed with 500 µl cold 70% ethanol in −20 °C freezer for 15 min then centrifuged again at 12,500×*g* for 5 min at 4 °C and the supernatant discarded. The tube was spun again briefly to collect residual ethanol to bottom of tube and as much liquid as possible was removed with gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 min) then resuspended in 20 µl H2O or TE. DNA concentration was measured in Qubit (Life Technologies) with ssDNA kit (Molecular Probes Q10212).

5.5. MeDIP-Seq analysis

The MeDIP pools were used to create libraries for next generation sequencing (NGS) using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, San Diego, CA) starting at step 1.4 of the manufacturer's protocol to generate double stranded DNA. After this step the manufacturer's protocol was followed. Each pool received a separate index primer. NGS was performed at WSU Spokane Genomics Core using the Illumina HiSeq. 2500 with a PE50 application, with a read size of approximately 50 bp and approximately 30 million reads per pool. Five to six libraries were run in one lane.

5.6. Statistics and bioinformatics

For the DMR analyses, the basic read quality was verified using summaries produced by the FastQC program http://www.bioinformatics.babraham.ac.uk/projects/fastqc/. The raw reads were trimmed and filtered using Trimmomatic. The reads for each MeDIP sample were mapped to the Rnor 6.0 rat genome using Bowtie2 [52] with default parameter options. The mapped read files were then converted to sorted BAM files using SAMtools [53]. To identify DMRs, the reference genome was broken into 100 bp windows. Genomic windows with no CpG or ambiguous base within 1000 bp were identified in the reference genome and used as control genes to perform RUVg normalization [54]. The MEDIPS [55] and edgeR [56] R packages were used to calculate differential coverage between control and exposure sample groups. The edgeR p-value was used to determine the relative difference between the two groups for each genomic window. Windows with an edgeR p-value less than 10–5 were considered DMRs. The FDR adjusted p-values were also calculated. The DMR edges were extended until no genomic window with an edgeR p-value < 0.1 remained within 1000 bp of the DMR. CpG density and other information was then calculated for the DMR based on the reference genome. DMRs were annotated using the biomaRt R package [57] to access the Ensembl database [58]. The genes that fell within 10kbp of the DMR edges were then input into the KEGG pathway search [59,60] to identify associated pathways. The associated genes were then sorted into functional groups by consulting information provided by the DAVID [61], Panther [62], and Uniprot databases incorporated into an internal curated database (www.skinner.wsu.edu under genomic data). All molecular data has been deposited into the public database at NCBI (GEO # GSE121585).

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Declaration of interests

The authors declare no competing interests.

CRediT authorship contribution statement

Millissia Ben Maamar: Writing - original draft, Writing - review & editing, Formal analysis, Investigation, **Eric Nilsson:** Writing - review & editing, Formal analysis, Investigation, **Ingrid Sadler-Riggleman:** Writing - review & editing, Formal analysis, Investigation, Validation, **Daniel Beck:** Writing - review & editing, Data curation, Investigation, Validation, **John R. McCarrey:** Writing - review & editing, Investigation, Supervision, **Michael K. Skinner:** Writing - original draft, Writing - review & editing, Conceptualization, Funding acquisition, Investigation, Project administration, Supervision

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.11.016.

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