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Human-specific subcellular compartmentalization of P-element induced wimpy testis-like (PIWIL) granules during germ cell development and spermatogenesis

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STUDY QUESTION: What is the dynamics of expression of P-element induced wimpy testis-like (PIWIL) proteins in the germline during human fetal development and spermatogenesis?

SUMMARY ANSWER: PIWIL1, PIWIL2, PIWIL3 and PIWIL4 were expressed in a sex-specific fashion in human germ cells (GC) during development and adulthood. PIWILs showed a mutually exclusive pattern of subcellular localization. PIWILs were present in the intermitochondrial cement and a single large granule in meiotic GC and their expression was different from that observed in mice, highlighting species-differences.

WHAT IS KNOWN ALREADY: In mice, PIWIL proteins play prominent roles in male infertility. PIWIL mouse mutants show either post-meiotic arrest at the round spermatid stage (PIWIL1) or arrest at the zygotene-pachytene stage of meiosis I (PIWIL2 and PIWIL4) in males, while females remain fertile. Recent studies have reported a robust piRNA pool in human fetal ovary.

STUDY DESIGN, SIZE, DURATION: This is a qualitative analysis of PIWILs expression in paraffin-embedded fetal human male ($N = 8$), female gonads ($N = 6$) and adult testes ($N = 5$), and bioinformatics analysis of online available single-cell transcriptomics data of human fetal germ cells ($n = 242$).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human fetal gonads from elective abortion without medical indication and adult testes biopsies were donated for research with informed consent. Samples were fixed, paraffin-embedded and analyzed by immunofluorescence to study the temporal and cellular localization of PIWIL1, PIWIL2, PIWIL3 and PIWIL4.

MAIN RESULTS AND THE ROLE OF CHANCE: PIWIL1, PIWIL2 and PIWIL4 showed a mutually exclusive pattern of subcellular localization, particularly in female oocytes. To our surprise, PIWIL1 immunostaining revealed the presence of a single dense paranuclear body, resembling the chromatoid body of haploid spermatocytes, in meiotic oocytes. Moreover, in contrast to mice, PIWIL4, but not PIWIL2, localized to the intermitochondrial cement. PIWIL3 was not expressed in GC during development. The upregulation of *PIWIL* transcripts correlated with the transcription of markers associated with piRNAs biogenesis like the *TDRD*s and *HENMT1* in fetal GC.

LARGE SCALE DATA: Non-applicable.

LIMITATIONS, REASONS FOR CAUTION: This study is limited by the restricted number of samples and consequently stages analyzed.

WIDER IMPLICATIONS OF THE FINDINGS: In the germline, PIWILs ensure the integrity of the human genome protecting it from 'parasitic sequences'. This study offers novel insights on the expression dynamics of PIWILs during the window of epigenetic remodeling and meiosis, and highlights important differences between humans and mice, which may prove particularly important to understand causes of infertility and improve both diagnosis and treatment in humans.

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Key words: PIWIL / human / intermitochondrial cement / gametogenesis / meiosis / oocyte / chromatoid body / subcellular localization / spermatogenesis / development

Introduction

The human germline is responsible for passing on genetic information to the next generation, ensuring the continuation of our species. It is therefore important that our genome in the germline retains its integrity, particularly by avoiding the random insertion of transposable elements (TE) (Juliano *et al.*, 2011; Siomi *et al.*, 2011; Yang and Wang, 2016).

In the mammalian germline, the genome is most vulnerable to disruption during two time windows. The first is during and after epigenetic reprogramming, when germ cells (GC) undergo genome-wide DNA demethylation. The second time window is during and after the pachytene-phase of meiosis when synapsed sister chromatids exchange chromosome segments. PIWI (P-element induced wimpy testis)-like (PIWIL) proteins operate during these two time windows to ensure the genome integrity of the germline (Juliano *et al.*, 2011; Siomi *et al.*, 2011; Iwasaki *et al.*, 2015; Yang and Wang, 2016). PIWIL proteins not only associate with a germline-specific category of small non-coding RNAs, the PIWI-interacting (pi)RNAs, forming a functional complex; but are also involved in their biosynthesis (Aravin *et al.*, 2008; Kuramochi-Miyagawa *et al.*, 2008; Siomi *et al.*, 2011).

In adult male mice, the 'pachythene'-type of piRNAs associate with PIWIL1 (MIWI) and PIWIL2 (MILI) in a single compact granule, known as satellite body in pachytene-stage spermatocytes and chromatoid body in haploid round spermatids (Deng and Lin, 2002; Kotaja and Sassone-Corsi, 2007; Onohara *et al.*, 2010; Siomi *et al.*, 2011). In mice, the 'pre-pachytene'-type of piRNAs are expressed between embryonic day (E)15 and post-natal day (P)3 and are associated with PIWIL2 and PIWIL4 (MIWI2) in mitotically-arrested prospermatogonia (Aravin *et al.*, 2008; De Fazio *et al.*, 2011; Kuramochi-Miyagawa *et al.*, 2008).

In prospermatogonia, PIWIL2-piRNA and PIWIL4-piRNA complexes are present in pi-bodies (also known as intermitochondrial cement) and piP-bodies (or discrete bodies), respectively (Eddy, 1974; Siomi *et al.*, 2011; van der Heijden *et al.*, 2010). They contain a distinct composition of proteins and are found in adjacent locations just outside nuclear pores (Aravin *et al.*, 2009; Shoji *et al.*, 2009; Siomi *et al.*, 2011; van der Heijden *et al.*, 2010). PIWIL4 can also be nuclear, where *de novo* DNA methylation of TE takes place, between E15-P3 (Aravin *et al.*, 2008).

In mice, the deletion of each PIWIL results in male infertility due to either post-meiotic arrest at the round spermatid stage (PIWIL1) (Deng and Lin, 2002) or arrest at the zygotene-pachytene stage of meiosis I (PIWIL2 and PIWIL4) (Carmell *et al.*, 2007; Kuramochi-Miyagawa *et al.*, 2004); whereas female mice remain fertile. Therefore, the role of PIWIL and piRNAs in females is less well understood. As in males, PIWIL2 granules have been described in oocytes in intermitochondrial cement from E12.5 until adulthood (from primordial to antral follicles) (Aravin *et al.*, 2008; Kabayama *et al.*, 2017; Lim *et al.*, 2013). By contrast, PIWIL4 is not expressed in mouse oocytes (Aravin *et al.*, 2008; Kabayama *et al.*, 2017). PIWIL1 was only observed in oocytes during adulthood (Ding *et al.*, 2013; Kabayama *et al.*, 2017), but its expression in satellite- or chromatoid body-like granule, as in males, has not been reported.

Compared to mice, humans have one extra PIWIL, PIWIL3, which is expressed in maturing oocytes in the adult ovary (Roovers *et al.*, 2015). Specific haplotypes in *PIWIL4* and *PIWIL3* as well as DNA hypermethylation of the *PIWIL2* promoter have been associated with increased risk of oligozoospermia and azoospermia (Gu *et al.*, 2010; Heyn *et al.*, 2012; Munoz *et al.*, 2014). Moreover, deregulation of PIWIL and piRNAs has been detected in several types of cancer, including epithelial ovarian cancer (Chen *et al.*, 2013; Lim *et al.*, 2014) and testicular germ cell tumors, such as seminoma, non-seminoma and mixed tumors (Ferreira *et al.*, 2014; Gainetdinov *et al.*, 2014; Hempfling *et al.*, 2017).

The expression of the different PIWIL proteins during gametogenesis has not been systematically investigated in humans. Here, we investigated the expression pattern of PIWIL1, PIWIL2, PIWIL3 and PIWIL4 in human female and male first (7–12 weeks of gestation, W7-12) and second (W17-22) trimester gonads, and adult testes. We show that, in contrast to mice, human oocytes in primordial follicles contain PIWIL4-enriched intermitochondrial cement (or pi-bodies), whereas PIWIL2 granules were widely expressed in the cytoplasm, but largely excluded from the (PIWIL4-enriched) intermitochondrial cement. Additionally, we provide evidence that PIWIL1 concentrates in a compact large granule in the cytoplasm of human oocytes in primordial follicles, reminiscent of the satellite body or chromatoid body.

Materials and Methods

Ethical approval for use of human fetal tissue

The Medical Ethical Committee of the Leiden University Medical Center (P08.087) approved all procedures regarding the collection and use of

human fetal material. The gestational age in weeks (W) and days was calculated from the crown-rump length (CRL) of the fetus, before the medical procedure, by obstetric ultrasonography. Human fetal tissue (Supplementary Table SI) was donated for research with informed consent from elective abortions without medical indication.

The Basque Ethics Committee for Clinical Research (CEIC-E PI2014205) approved the procedures regarding the collection and use of testicular biopsies from adult patients (Supplementary Table SI), obtained during a testicular biopsy performed in vasectomized patients in search of spermatozoa to be cryopreserved for a later intracytoplasmic sperm injection. Only biopsies positive for spermatozoa were studied. Fully signed written informed consent was obtained from those patients.

Collection of the fetal gonads

Gonads were isolated in cold 0.9% w/v NaCl (Fresenius Kabi), fixed in 4% paraformaldehyde (PFA) (MERCK) overnight (o/n) at 4°C, washed in phosphate-buffered saline (PBS) and transferred to a 70% ethanol solution followed by paraffin embedding using a Shandon Excelsior tissue processor (Thermo Scientific, Altrincham, UK). The material was sectioned (5 µm) using a RM2065 microtome (Leica Instruments GmbH, Wetzlar, Germany) onto StarFrost slides (Waldemar Knittel). Sex genotype was performed by PCR amplification of *AMELX* and *AMELY*, with fragments of 977 bp from X chromosome and 790 bp from Y chromosome. PCR conditions and primers were as previously described (Heeren *et al.*, 2015).

Immunofluorescence and imaging

Immunofluorescence was performed as previously described (Heeren *et al.*, 2016) and information regarding primary and secondary antibodies used is provided in Supplementary Table SII. Paraffin sections of mouse testes (gift from M. de Ruiter) were used as positive controls for PIWIL1, PIWIL2 and PIWIL4 (Supplementary Fig. S1A,B); and of adult ovary (approved by Medical Ethical Committee of the Leiden University Medical Center CME 05/03 K/YR) were used for positive control for PIWIL3 (Supplementary Fig. S1C). Negative controls were performed by omitting the primary antibodies (Supplementary Fig. S1D).

The immunostained slides were scanned on a 'Pannoramic' MIDI digital scanner (3DHISTECH, Budapest, Hungary) and analyzed with the software 'Pannoramic Viewer' (3DHISTECH). Imaging was also performed on a Leica TCS SP8 upright confocal microscope (Leica Microsystems, Wetzlar, Germany) using the Leica Application Suite Advanced Fluorescence software (LAS AF, Leica, Wetzlar, Germany). Figures were prepared in Adobe Photoshop (Adobe Systems, San Jose, CA, USA) and the image-processing software, Fiji (Schindelin *et al.*, 2012).

Fluorescence-activated cell sorting analysis and statistics

Fetal gonads were minced and treated with a mix of 0.1 mg/ml Dispase (17105-041, Life Technologies), 0.1 mg/ml Collagenase IV (LS004186, Worthington Biochemical Corporation), 9 µg/ml Hyaluronidase (0210074080, MP Biomedicals) and 27 Units Rnase-free DNase I (79254, Qiagen) for 1 h at 37°C. The cell suspension was filtered through a 100 µm cell strainer (734-0004, Falcon), fixed in 4% PFA for 5 min at room temperature (RT), permeabilized in 0.05% Saponin (47036-50g-F, Merck) for 10 min at RT. Cells were incubated with primary antibodies in blocking solution (1% Bovine Serum Albumin (BSA, A8022-50G, Merck), 1% Fetal Calf Serum, 0.05% Saponin) 1 h at RT, washed, incubated with secondary antibody in blocking solution 1 h at RT, resuspended in fluorescence-activated cell sorting (FACS) buffer (1% BSA, 10 µm EDTA in PBS) and analyzed on a BD FACSAria III (BD Biosciences, Erembodegem, Belgium). Part of each sample was used as negative control. Antibodies and isotype controls used are described in Supplementary Table SII. Statistical analysis of the GC populations was performed using the Mann–Whitney *U* Test, with *P*-value <0.05 for significance.

Bioinformatic analysis of single-cell transcriptomics from human germ cells

Unsupervised hierarchical clustering of online available single-cell transcriptomics data from human gonadal cells ($n = 93$ female germ cells, $n = 149$ male germ cells, $n = 38$ female somatic cells, $n = 48$ male somatic cells) of first and second trimester and 43 genes of interest is depicted in two different heat maps generated with the R package gplots. Data in reads per kilobase of transcript per million (RPKM) were downloaded from the Gene Expression Omnibus (GEO) database (GEO: GSE63818) (Guo *et al.*, 2015).

Results

PDPN and DDX4 distinguished different human GC during development

To understand the localization of PIWIL proteins in human germ cells (GC), we first distinguished their phases of development using immunostaining for POU5F1 (or OCT4), PDPN (or podoplanin) and DDX4 (or VASA) (Anderson *et al.*, 2007; Heeren *et al.*, 2016). Female and male W7-12 GC showed low or no DDX4 expression, but co-localization of nuclear POU5F1 and cell-surface PDPN (Fig. 1A,B). In W17-22 ovaries, we still observed POU5F1+/PDPN+/DDX4− GC, but a population of DDX4 +/POU5F1−/PDPN− GC, that included pre-meiotic, meiotic and those encapsulated in primordial follicles, became prominent (Fig. 1C). In the seminiferous tubules of W17-22 testes, both POU5F1+/PDPN+/DDX4− and DDX4+/POU5F1−/PDPN− GC were observed side-by-side (Fig. 1D). Quantification by FACS-analysis showed that between W18-22, GC made up 75% of the ovaries, but only 4% of the testis (Fig. 1E). The relative composition of GC in the gonads is 47 ± 11% POU5F1+ and 53 ± 11% DDX4+ GC in ovaries ($N = 4$); and significantly different ($P < 0.05$) from 14 ± 8% POU5F1+ and 86 ± 8% DDX4+ GC ($N = 4$) in testes (Fig. 1F).

PIWIL1 localized to a single large dense satellite-like body in meiotic female GC

Between W7-12, cytoplasmic PIWIL1 was detected in PDPN+/DDX4− female GC in the periphery of the gonad (Fig. 2A–C and A'). Between W17-22, the peripheral rim of PDPN+/DDX4− female GC continued to show cytoplasmic PIWIL1 (Fig. 2D,E and D',E'; Supplementary Fig. 2A). Surprisingly, DDX4+/PDPN− GC showed enrichment of PIWIL1 in a single large dense satellite-like granule just outside the nuclear envelope. This granule seemed to disappear during the pachytene stage, reappearing thereafter in oocytes in primordial follicles (green arrows in Fig. 2F', H'; Supplementary Fig. S2). Moreover, in oocytes in primordial follicles small nuclear foci of PIWIL1 were observed (green asterisks in Fig. 2H'; Supplementary Fig. S2), but those were not necessarily in close proximity of the single cytoplasmic PIWIL1-positive granule (Supplementary Fig. S1B).

In males, PIWIL1 was neither detected in PDPN+/DDX4- GC, nor in DDX4+/PDPN- GC (or prospermatogonia) (Fig. 2I–N). However, faint expression of PIWIL1 was detected in somatic cells outside the seminiferous tubules (Fig. 2I–N). In adult testes, (mitochondria-rich TUFM-positive) spermatogonia (Ramalho-Santos *et al.*, 2009) did not

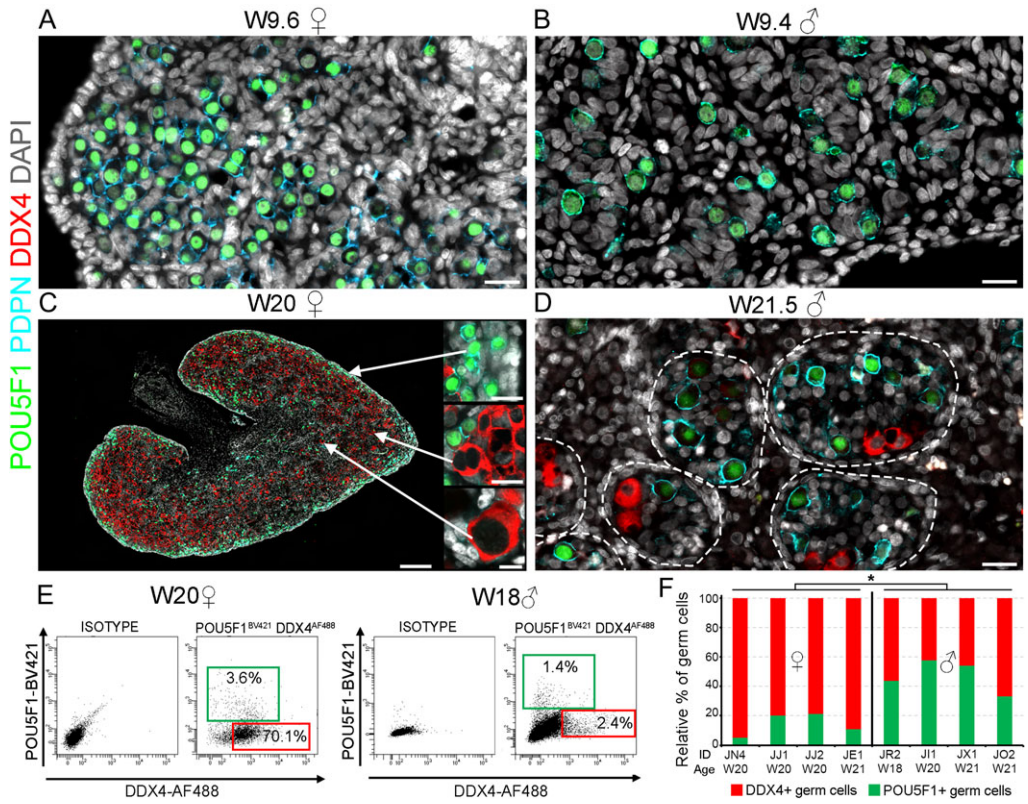

Figure 1 Expression of PDPN, POU5F1 and DDX4 in human gonads. (**A–B**) Female gonad (W9.6) (A) and male gonad (W9.4) (B) showing a homogeneous population of double positive POU5F1 and PDPN GC. (**C**) Female gonad (W20) showing in addition to POU5F1+/PDPN+ GC, a distinct population of DDX4+/POU5F1- GC (pre-meiotic, meiotic and those encapsulated in primordial follicles). (**D**) Male gonad (W21.5) showing distinct populations of POU5F1+/PDPN+ GC and DDX4+pre-meiotic GC in the seminiferous tubules (dashed line). (**E**) FACS dot-plots showing female and male gonads immunostained for POU5F1 and DDX4, as well the isotype controls. Gatings show the percentage of POU5F1-positive (green) and DDX4-positive (red) GC. (**F**) Quantification of relative percentage (%) of POU5F1-positive (green) and DDX4-positive GC per gonad. Identification (ID) and age (weeks) are provided for each gonad. Both the % POU5F1-positive and DDX4-positive GC were statistically different between males and females (*$P < 0.05$). Scale bars are 20 μm in A, B, D and inserts in C; and 200 μm C.

express PIWIL1, but PIWIL1 was expressed in the cytoplasm of spermatocytes and spermatids, particularly in the chromatoid body (Fig. 2O–S and O'–S').

PIWIL4 associated with intermitochondrial cement in oocytes in primordial follicles and spermatogonia

We observed PIWIL4 in the cytoplasm of PDPN+/DDX4- female GC (Fig. 3A–E and A'–E'; Supplementary Fig. S3A), but also in somatic cells in the mesonephros (Fig. 3A). In (pre-)meiotic DDX4+/PDPN- GC, PIWIL4-positive cytoplasmic granules became localized to the outer surface of the nuclear envelope (Fig. 3F,G and F',G').

In DDX4+/PDPN- oocytes in primordial follicles, PIWIL4 was still observed in small cytoplasmic granules, close to the nuclear envelope, in one (or several) characteristic broad crescent-like structures (Fig. 3H and H'; Supplementary Fig. S3), most probably the intermitochondrial cement, also known in female cells as Balbiani body (Hertig and Adams, 1967), where mitochondria are known to accumulate.

We next investigated the localization of (GM130-positive) Golgi apparatus and (TUFM-positive) mitochondria in primordial follicles and confirmed that the broad crescent-like structure in the cytoplasm where PIWIL4 accumulated was indeed (TUFM-positive) intermitochondrial cement (Fig. 4A).

As in females, in males cytoplasmic PIWIL4 was observed in PDPN+/DDX4– GC (Fig. 3I–N). PIWIL4 in DDX4+/PDPN- (pre-meiotic prospermatogonia) male GC from W17-22 showed strong localization to (TUFM-positive) intermitochondrial cement, suggesting active TE silencing at that stage (green arrow in Figs 3M and 4B). In adults testes, strong co-localization between TUFM-positive intermitochondrial cement and PIWIL4 remained (Figs 2O–S and 3O'–S').

PIWIL2 was depleted from intermitochondrial cement in oocytes in primordial follicles

Cytoplasmic PIWIL2 expression was detected in the majority of PDPN+/DDX4- female GC (Fig. 5A–E and A'–E'; Supplementary Fig.

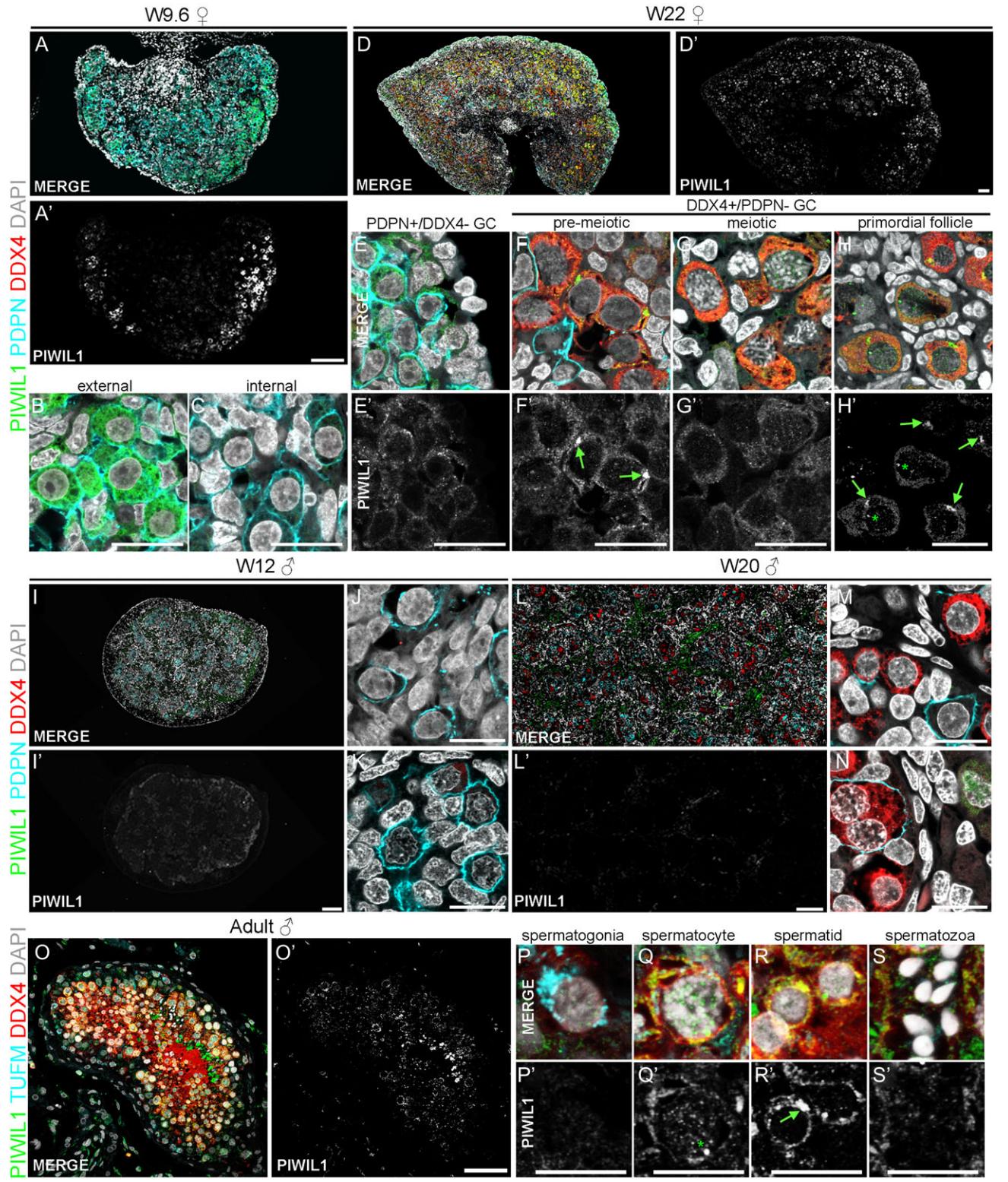

Figure 2 PIWIL1 expression in the female and male germ cells. (**A–N**) Histological sections immunostained for PIWIL1 (green), PDPN (cyan) and DDX4 (red) and counterstained with DAPI (white) of female gonad (W9.6) (A–C), female gonad (W22) (D–H), male gonad (W12) (I–K) and male gonad (W20) (L–N). (**O–S**) Histological sections immunostained for PIWIL1 (green), TUFM (cyan) and DDX4 (red) and counterstained with DAPI (white) of adult human testis. Green arrows point to the single dense paranuclear granule; green asterisks mark nuclear foci of PIWIL1. Scale bars are 500 μm in D, D'; 100 μm A, A', I, I', L, L'; 50 μm in H, H', O, O'; 20 μm in B, C, E–G, E'–G', J, K, M, N and 10 μm in P–S and P'–S'.

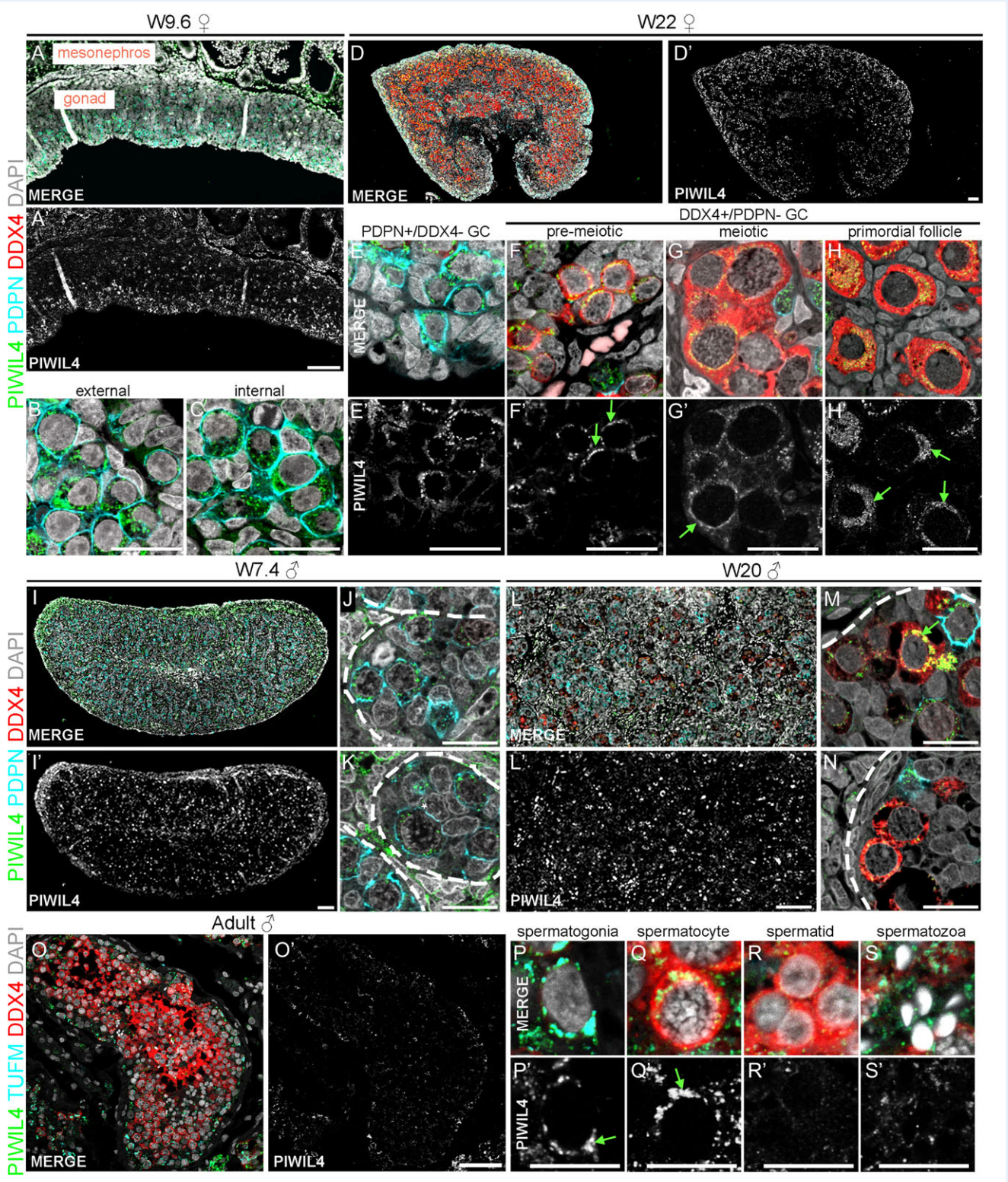

Figure 3 PIWIL4 expression in the female and male germ cells. (**A–N**) Histological sections immunostained for PIWIL4 (green), PDPN (cyan) and DDX4 (red) and counterstained with DAPI (white) of female gonad (W9.6) (A–C), female gonad (W22) (D–H), male gonad (W7.4) (I–K) and male gonad (W20) (L–N). (**O–S**) Histological sections immunostained for PIWIL4 (green), TUFM (cyan) and DDX4 (red) and counterstained with DAPI (white) of adult human testis. Green arrows point to intermitochondrial cement. Scale bars are 500 µm in D, D'; 100 µm A, A', I, I', L, L'; 50 µm in H, H', O, O'; 20 µm in B, C, E–G, E'–G', J, K, M, N and 10 µm in P–S and P'–S'.

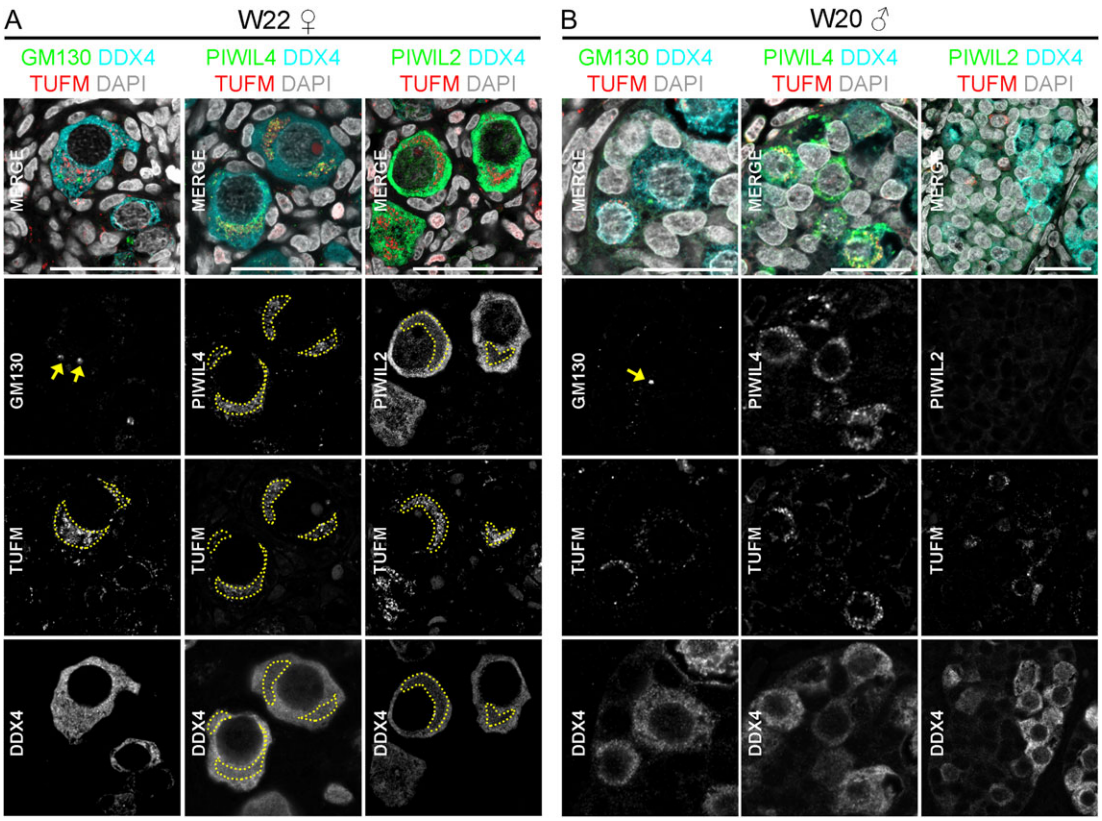

Figure 4 Localization of PIWILs relative to the Golgi apparatus and mitochondria. (**A–B**) Histological sections of human female (W22) (A) and male (W20) (B) gonads immunostained for the Golgi-marker GM130 (green), PIWIL4 (green) or PIWIL2 (green) in combination with DDX4 (cyan), mitochondria-marker TUFM (red) and counterstained with DAPI (white). Yellow arrows point to complexes of Golgi apparatus; yellow dashed areas in oocytes mark the concentration of TUFM+ mitochondria (intermitochondrial cement). Scale bars are 20 μm.

S3A). As DDX4+/PDPN- GC entered meiosis, the cytoplasmic expression of PIWIL2 decreased transiently and increased again in oocytes in primordial follicles (Fig. 5F–H and F'–H'; Supplementary Fig. S3). Surprisingly, in oocytes in primordial follicles, PIWIL2 was depleted from (TUFM-positive) intermitochondrial cement (Fig. 4A; green arrows in Fig. 5H'; Supplementary Fig. S3), in a seemingly opposite or complementary expression pattern to that observed for PIWIL4 (Fig. 4A).

PIWIL2 expression in PDPN+/DDX4- male GC was similar to that in females (Fig. 5I–N). Between W17-22, cytoplasmic and nuclear PIWIL2 granules were detected in some DDX4+/PDPN- GC (Fig. 5L–N). Interestingly, in adult testes strong nuclear accumulation was observed specifically in round spermatids (Fig. 5O–S and O'–S').

PIWIL1 and *PIWIL2* are enriched in meiotic female GC

Next, we used online available single-cell transcriptomics data from human male and female gonadal somatic and GC from first and second trimester (Guo *et al.*, 2015) to correlate the expression of *PIWIL* with 39 selected genes known to be functionally associated with PIWIL and or expressed in the germline (Chuma *et al.*, 2006; Juliano *et al.*, 2011; Siomi *et al.*, 2011; van der Heijden *et al.*, 2010; Yang and Wang, 2016).

First, we observed that *PIWIL3* was absent from both female and male GC in first and second trimester both at the RNA level (Fig. 6) and at the protein level between W7-12 and W17-22 (Supplementary Fig. S5). Moreover, we confirmed that PIWIL3 is expressed in human maturing oocytes during adult oogenesis (Supplementary Fig. S1C).

Hierarchical clustering showed that the somatic cells and different stages of GC clustered separately (Fig. 6). *HENMT1*, a methyltransferase that adds a 2′-O-methyl group at the 3′-end of (pre-pachytene and pachytene) piRNAs (Lim *et al.*, 2015) and *MAEL*, important for the biosynthesis of piRNA (Soper *et al.*, 2008), but also known to associate with unsynapsed chromosomes during meiosis (Costa *et al.*, 2006), were expressed in both female and male GC (Fig. 6).

In females, other genes associated with the PIWIL/piRNA pathway and biosynthesis machinery (gray area in Fig. 6A) were expressed and showed three distinct clusters: (i) either lowly (but not absent) expressed in female GC, including *PIWIL4* and piP-body markers *DDX6*, *XRN1* and *DCP1A*; (ii) moderately expressed in female GC, including *PIWIL1*, *PIWIL2* and *DICER1* and (iii) specifically expressed in meiotic GC, such as *ASZ1* and *MOV10L1*.

In males, *PIWIL2* and *TDRD9* clustered together and were expressed in GC (Fig. 6B). Most other genes associated with the PIWIL/piRNA pathway and biosynthesis machinery (gray area in Fig. 6B) were (i) lowly expressed (but not absent) in GC; or (ii) specifically (lowly)

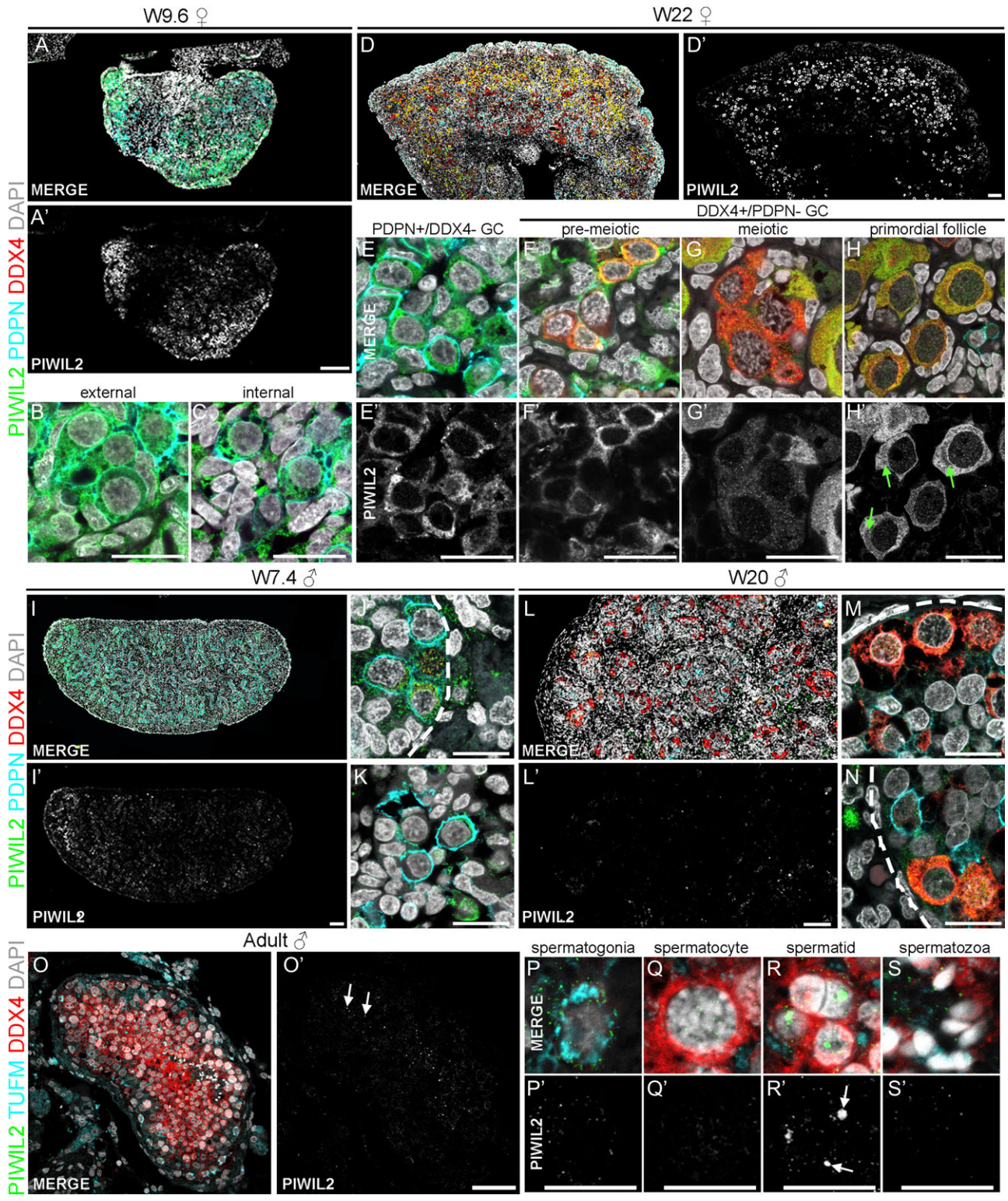

Figure 5 PIWIL2 expression in the female and male germ cells. (**A–N**) Histological sections immunostained for PIWIL2 (green), PDPN (cyan) and DDX4 (red) and counterstained with DAPI (white) of female gonad (W9.6) (A–C), female gonad (W22) (D–H), male gonad (W7.4) (I–K) and male gonad (W20) (L–N). (**O–S**) Histological sections immunostained for PIWIL2 (green), TUFM (cyan) and DDX4 (red) and counterstained with DAPI (white) of adult human testis. Green arrows point to intermitochondrial cement; white arrows point to nuclear foci of PIWIL2. Scale bars are 500 μm in D, D'; 100 μm A, A', I, I', L, L'; 50 μm in H, H', O, O'; 20 μm in B, C, E–G, E'–G', J, K, M, N and 10 μm in P–S and P'–S'.

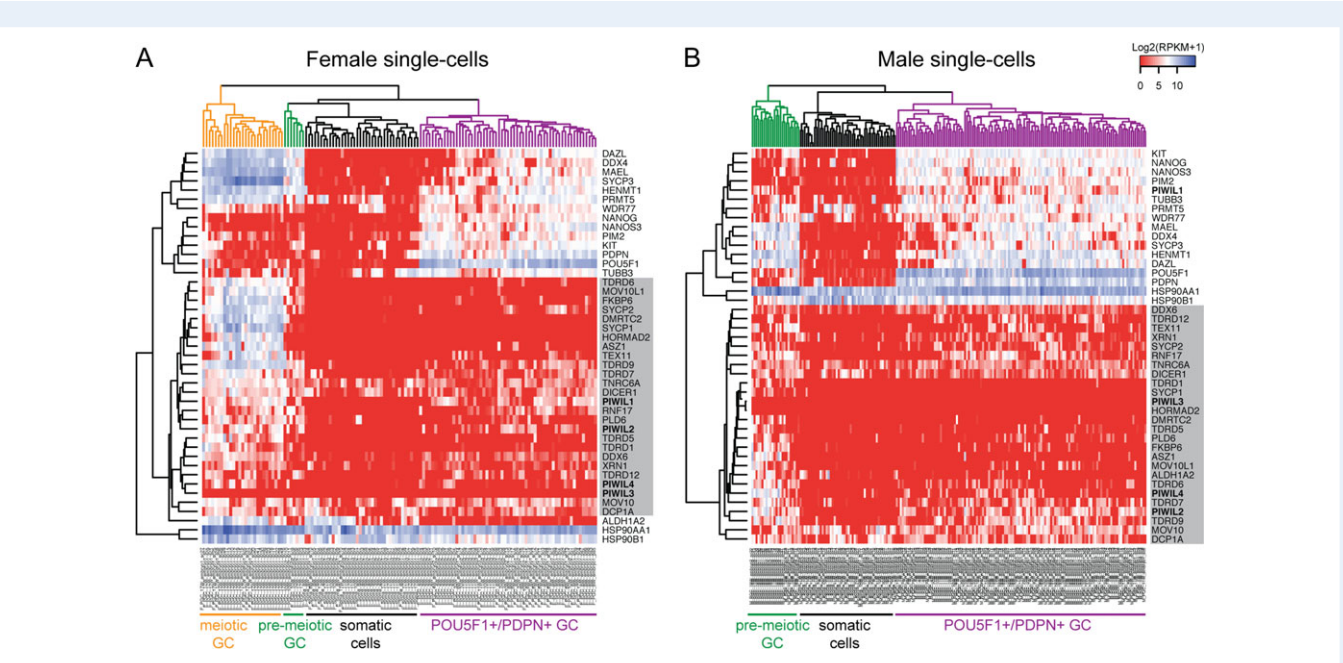

Figure 6 Unsupervised hierarchical clustering of single-cell human female and male germ cells. (**A–B**) Heat maps depict the expression of 43 selected germline-specific genes and PIWI/piRNA associated genes (cluster in gray box) in female (n = 93 germ cells and n = 38 somatic cells) and male (n = 149 germ cells and n = 48 somatic cells) single cells from nine different donors from first and second trimester (Guo *et al.*, 2015). Note that the single cells cluster into somatic (black), POU5F1+/PDPN+ germ cells (GC) (violet), pre-meiotic GC (green) and meiotic GC (orange). This dataset contained no oocytes in primordial follicles.

expressed in pre-meiotic GC. Together, our analysis suggests that the PIWIL/piRNA biosynthesis machinery is in place in the different stages of human GC.

Discussion

PIWILs were expressed in PDPN+/DDX4− positive GC

Human GC develop differently than mouse GC in several aspects, such as the pronounced asynchronicity regarding the upregulation of DDX4 and timing of meiotic entry (Anderson *et al.*, 2007; Heeren *et al.*, 2016; Kurilo, 1981). Interestingly, PDPN+/DDX4- female GC in first trimester do not synthesize piRNAs (Roovers *et al.*, 2015). Therefore, the observation that all PIWILs studied were expressed in the cytoplasm of PDPN+/DDX4− female GC, suggests that PIWILs are either stored in the cytoplasm to become functional later in development; or they have a piRNA-independent function in PDPN+/DDX4− GC.

In humans, PIWIL4 but not PIWIL2 is present in intermitochondrial cement

Surprisingly, PIWIL4 in male DDX4+/PDPN- GC, (but neither PIWIL2 nor DDX4), is present in intermitochondrial cement. By contrast, in mice it is PIWIL2 that resides in intermitochondrial cement (or pi-bodies), whereas PIWIL4 is in the larger piP-bodies (Aravin *et al.*, 2009; Eddy, 1974; van der Heijden *et al.*, 2010).

In addition, whereas PIWIL4 was not previously detected in the mouse female germline (Aravin *et al.*, 2008; Kabayama *et al.*, 2017), in female DDX4+/PDPN- GC the localization of PIWIL4 is unmistakably to intermitochondrial cement (Hertig and Adams, 1967). It is noteworthy that Roovers and colleagues were able to predict expression of PIWIL4 from the piRNAs detected in female GC (Roovers *et al.*, 2015).

PIWIL2 and PIWIL4 seem to occupy mutually exclusive cytoplasmic compartments in oocytes in primordial follicles, PIWIL2 being largely excluded from the intermitochondrial cement. Similar to PIWIL2, DDX4 was also depleted from the intermitochondrial cement, but the DDX4-positive granules distributed throughout the cytoplasm appear to be adjacent to PIWIL2-positive granules.

PIWIL4: is symmetry breaking needed in mammalian oocytes?

During zygotene, the telomeres of the pairing chromosomes gather at one nuclear pole ('bouquet') to facilitate pairing and that determines the position of the intermitochondrial cement (or Balbiani body) (Elkouby *et al.*, 2016).

In animals with a 'preformation mode' of germline formation, including Xenopus, zebrafish and fruit fly, the position of intermitochondrial cement in the maturing oocytes determines the animal-vegetal axis of the future embryo and marks the cells fated for the germline (Bertocchini and Chuva de Sousa Lopes, 2016). The segregation of intermitochondrial cement and its components, such as homologs of PIWIL and DDX4, involved in silencing transcription (Siomi *et al.*,

2010; Yang and Wang, 2016) to the future GC ensures that only GC undergo the necessary transcriptional silencing/regulation, but not the other cells of the embryo.

By contrast, in mammals with 'induction mode' of GC formation symmetry breaking via the localization of intermitochondrial cement may be less critical and even undesirable. This may explain the presence of multiple 'crescents' in oocytes and broad localization of PIWIL4 and PIWIL2. As all cells in the early mammalian embryo can still be induced to a GC fate, the strategy may be to initially distribute intermitochondrial cement and its components of each cell of the embryo, to prime but at the same time prevent germline lineage segregation until later in development through inductive signals, such as BMPs (Bertocchini and Chuva de Sousa Lopes, 2016).

Human PIWIL1 accumulated in single large dense paranuclear granules in meiotic oocytes

In male adult mice, PIWIL1 accumulated in the cytoplasm as a single compact granule, known as satellite body in pachytene-stage spermatocytes and chromatoid body in haploid round spermatids (Deng and Lin, 2002; Kotaja and Sassone-Corsi, 2007; Onohara *et al.*, 2010; Siomi *et al.*, 2011). As in mice, PIWIL1 was not observed in human fetal testes; but in adult testes it accumulated in the large cytoplasmic foci in spermatocytes and in the chromatoid body.

In female meiotic GC, we describe for the first time the accumulation of PIWIL1 in a single condensed granule that bears similarities in terms of shape and localization to the male-specific satellite body and chromatoid body. However, we did not observe co-localization of PIWIL1 and DDX4, characteristic of the chromatoid body, at least until W22. Interestingly, Castrillon and colleagues reported the presence of single large dense paranuclear DDX4-positive granules in oocytes at W35, but not at W17 (Castrillon *et al.*, 2000). This suggests that in meiotic oocytes the co-localization between DDX4 and PIWIL1 in paranuclear dense granules, as observed in the male chromatoid body (Kotaja and Sassone-Corsi, 2007; Parvinen, 2005), may only occur later in development.

PIWIL1 in meiotic female GC: a link between cytoplasm and nucleus?

In mice, PIWIL1 co-precipitates with MAEL in adult testes (Costa *et al.*, 2006). MAEL is present in the cytoplasm in intermitochondrial cement and chromatoid body, but also in the nucleus where it specifically associates with unsynapsed chromatin of autosomes and the XY-body during meiosis (Costa *et al.*, 2006). We also observed nuclear foci of PIWIL1 in meiotic oocytes in primordial follicles (as well as male spermatocytes). It remains to be investigated whether PIWIL1/piRNA could, perhaps together with MAEL, ensure silencing of unsynapsed chromatin during meiosis in the human germline.

The meiotic silencing of unsynapsed chromatin (MSUC) may be less crucial in females as all chromosomes, including the XX chromosomes, have homologs and pair during meiosis. By contrast, the non-homologous male X and Y chromosomes undergo transcriptional silencing or meiotic sex chromosome inactivation (MSCI) at the zygotene to pachytene transition (Turner, 2007). Defects in MSCI lead to male meiotic-derived sterility in mice and to a certain extent in humans (de Vries *et al.*, 2012; Turner, 2007), and that is, not surprisingly, the phenotype observed in PIWIL1 deficient mice (Deng and Lin, 2002).

Conclusion

Our data shows a dynamic, sex-specific and mutually exclusive expression of four different PIWILs during human gametogenesis. The pattern of PIWIL4 and PIWIL2 in humans is opposite to that in mice, with localization of PIWIL4, and not PIWIL2, to intermitochondrial cement. PIWIL3 was only expressed in growing oocytes. The pattern of PIWIL1 revealed the presence of a dense single granule in oocytes in primordial follicles. We speculate on a putative role for (nuclear) PIWIL1 ensuring silencing of unsynapsed chromosomes during meiosis; and the function of PIWIL4 and PIWIL2 regulating GC identity through transcriptional silencing during the life cycle. Together, the expression of PIWIL and of certain piRNA types (Roovers *et al.*, 2015; Williams *et al.*, 2015) suggests that PIWIL/piRNA may have functions independent of TE silencing in human gametogenesis.

In humans, regulation by PIWIL/piRNA seems in place to ensure genome integrity during epigenetic reprogramming and chromosome pairing during meiosis; however, mice and humans differ substantially regarding the localization of PIWILs. These novel insights will help optimize methods for *in vitro* gametogenesis in humans, and hopefully bypass the pachytene-checkpoint the current ones encounter (Handel *et al.*, 2014; Chuva de Sousa Lopes and Roelen, 2015). Finally, investigating gametogenesis in humans is crucial to increase our understanding of causes and develop better treatments for infertility in humans.

Supplementary data

Supplementary data are available at *Human Reproduction* Online.

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Authors' roles

M.G.F. and S.M.C.d.S.L. designed the study. M.G.F., N.H., F.W., L.v.I., C.E, R.M. B.A.J.R. and S.M.C.d.S.L. conducted experiments and analyzed data. M.G.F., N.H., B.A.J.R. and S.M.C.d.S.L. wrote the manuscript. All authors approved the last version of the manuscript.

Conflict of interest

The authors declare no competing or financial interests.

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References

Anderson RA, Fulton N, Cowan G, Coutts S, Saunders PT. Conserved and divergent patterns of expression of DAZL, VASA and OCT4 in the germ cells of the human fetal ovary and testis. *BMC Dev Biol* 2007;**7**:136.

Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Toth KF, Bestor T, Hannon GJ. A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol Cell* 2008;**31**:785–799.

Aravin AA, van der Heijden GW, Castaneda J, Vagin VV, Hannon GJ, Bortvin A. Cytoplasmic compartmentalization of the fetal piRNA pathway in mice. *PLoS Genet* 2009;**5**:e1000764.

Bertocchini F, Chuva de Sousa Lopes SM. Germline development in amniotes: a paradigm shift in primordial germ cell specification. *BioEssays* 2016;**38**:791–800.

Carmell MA, Girard A, van de Kant HJ, Bourc'his D, Bestor TH, de Rooij DG, Hannon GJ. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Dev Cell* 2007;**12**:503–514.

Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. *Proc Natl Acad Sci USA* 2000;**97**:9585–9590.

Chen C, Liu J, Xu G. Overexpression of PIWI proteins in human stage III epithelial ovarian cancer with lymph node metastasis. *Cancer Biomark* 2013;**13**:315–321.

Chuma S, Hosokawa M, Kitamura K, Kasai S, Fujioka M, Hiyoshi M, Takamune K, Noce T, Nakatsuji N. Tdrd1/Mtr-1, a tudor-related gene, is essential for male germ-cell differentiation and nuage/germinal granule formation in mice. *Proc Natl Acad Sci USA* 2006;**103**:15894–15899.

Chuva de Sousa Lopes S, Roelen BA. Current status of in vitro differentiation of stem cells into gametes. *Anim Reprod* 2015;**12**:46–51.

Costa Y, Speed RM, Gautier P, Semple CA, Maratou K, Turner JM, Cooke HJ. Mouse MAELSTROM: the link between meiotic silencing of unsynapsed chromatin and microRNA pathway? *Hum Mol Genet* 2006;**15**:2324–2334.

De Fazio S, Bartonicek N, Di Giacomo M, Abreu-Goodger C, Sankar A, Funaya C, Antony C, Moreira PN, Enright AJ, O'Carroll D. The endonuclease activity of Mili fuels piRNA amplification that silences LINE1 elements. *Nature* 2011;**480**:259–263.

de Vries M, Vosters S, Merkx G, D'Hauwers K, Wansink DG, Ramos L, de Boer P. Human male meiotic sex chromosome inactivation. *PloS one* 2012;**7**:e31485.

Deng W, Lin H. miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. *Dev Cell* 2002;**2**:819–830.

Ding X, Guan H, Li H. Characterization of a piRNA binding protein Miwi in mouse oocytes. *Theriogenology* 2013;**79**:610–615 e611.

Eddy EM. Fine structural observations on the form and distribution of nuage in germ cells of the rat. *Anat Rec* 1974;**178**:731–757.

Elkouby YM, Jamieson-Lucy A, Mullins MC. Oocyte polarization is coupled to the chromosomal bouquet, a conserved polarized nuclear configuration in meiosis. *PLoS Biol* 2016;**14**:e1002335.

Ferreira HJ, Heyn H, Garcia del Muro X, Vidal A, Larriba S, Munoz C, Villanueva A, Esteller M. Epigenetic loss of the PIWI/piRNA machinery in human testicular tumorigenesis. *Epigenetics* 2014;**9**:113–118.

Gainetdinov IV, Skvortsova YV, Stukacheva EA, Bychenko OS, Kondratieva SA, Zinovieva MV, Azhikina TL. Expression profiles of PIWIL2 short isoforms differ in testicular germ cell tumors of various differentiation subtypes. *PloS one* 2014;**9**:e112528.

Gu A, Ji G, Shi X, Long Y, Xia Y, Song L, Wang S, Wang X. Genetic variants in Piwi-interacting RNA pathway genes confer susceptibility to spermatogenic failure in a Chinese population. *Hum Reprod* 2010;**25**:2955–2961.

Guo F, Yan L, Guo H, Li L, Hu B, Zhao Y, Yong J, Hu Y, Wang X, Wei Y *et al*. The Transcriptome and DNA methylome landscapes of human primordial germ cells. *Cell* 2015;**161**:1437–1452.

Handel MA, Eppig JJ, Schimenti JC. Applying 'gold standards' to in-vitro-derived germ cells. *Cell* 2014;**157**:1257–1261.

Heeren A, van Iperen L, Klootwijk DB, de Melo Bernardo A, Roost MS, Gomes Fernandes MM, Louwe LA, Hilders CG, Helmerhorst FM, van der Westerlaken LA *et al*. Development of the follicular basement membrane during human gametogenesis and early folliculogenesis. *BMC Dev Biol* 2015;**15**:4.

Heeren AM, He N, de Souza AF, Goercharn-Ramlal A, van Iperen L, Roost MS, Gomes Fernandes MM, van der Westerlaken LA, Chuva de Sousa Lopes SM. On the development of extragonadal and gonadal human germ cells. *Biol Open* 2016;**5**:185–194.

Hempfling AL, Lim SL, Adelson DL, Evans J, O'Connor AE, Qu ZP, Kliesch S, Weidner W, O'Bryan MK, Bergmann M. Expression patterns of HENMT1 and PIWIL1 in human testis: implications for transposon expression. *Reproduction* 2017;**154**:363–374.

Hertig AT, Adams EC. Studies on the human oocyte and its follicle. I. Ultrastructural and histochemical observations on the primordial follicle stage. *J Cell Biol* 1967;**34**:647–675.

Heyn H, Ferreira HJ, Bassas L, Bonache S, Sayols S, Sandoval J, Esteller M, Larriba S. Epigenetic disruption of the PIWI pathway in human spermatogenic disorders. *PloS one* 2012;**7**:e47892.

Iwasaki YW, Siomi MC, Siomi H. PIWI-interacting RNA: its biogenesis and functions. *Annu Rev Biochem* 2015;**84**:405–433.

Juliano C, Wang J, Lin H. Uniting germline and stem cells: the function of Piwi proteins and the piRNA pathway in diverse organisms. *Annu Rev Genet* 2011;**45**:447–469.

Kabayama Y, Toh H, Katanaya A, Sakurai T, Chuma S, Kuramochi-Miyagawa S, Saga Y, Nakano T, Sasaki H. Roles of MIWI, MILI and PLD6 in small RNA regulation in mouse growing oocytes. *Nucleic Acids Res* 2017;**45**:5387–5398.

Kotaja N, Sassone-Corsi P. The chromatoid body: a germ-cell-specific RNA-processing centre. *Nat Rev Mol Cell Biol* 2007;**8**:85–90.

Kuramochi-Miyagawa S, Kimura T, Ijiri TW, Isobe T, Asada N, Fujita Y, Ikawa M, Iwai N, Okabe M, Deng W *et al*. Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. *Development* 2004;**131**:839–849.

Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, Asada N, Kojima K, Yamaguchi Y, Ijiri TW *et al*. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev* 2008;**22**:908–917.

Kurilo LF. Oogenesis in antenatal development in man. *Hum Genet* 1981;**57**:86–92.

Lim AK, Lorthongpanich C, Chew TG, Tan CW, Shue YT, Balu S, Gounko N, Kuramochi-Miyagawa S, Matzuk MM, Chuma S *et al*. The nuage mediates retrotransposon silencing in mouse primordial ovarian follicles. *Development* 2013;**140**:3819–3825.

Lim SL, Qu ZP, Kortschak RD, Lawrence DM, Geoghegan J, Hempfling AL, Bergmann M, Goodnow CC, Ormandy CJ, Wong L *et al*. HENMT1 and piRNA stability are required for adult male germ cell transposon repression and to define the spermatogenic program in the mouse. *PLoS Genet* 2015;**11**:e1005620.

Lim SL, Ricciardelli C, Oehler MK, Tan IM, Russell D, Grutzner F. Overexpression of piRNA pathway genes in epithelial ovarian cancer. *PloS one* 2014;**9**:e99687.

Munoz X, Navarro M, Mata A, Bassas L, Larriba S. Association of PIWIL4 genetic variants with germ cell maturation arrest in infertile Spanish men. *Asian J Androl* 2014;**16**:931–933.

Onohara Y, Fujiwara T, Yasukochi T, Himeno M, Yokota S. Localization of mouse vasa homolog protein in chromatoid body and related nuage structures of mammalian spermatogenic cells during spermatogenesis. *Histochem Cell Biol* 2010;**133**:627–639.

Parvinen M. The chromatoid body in spermatogenesis. *Int J Androl* 2005; **28**:189–201.

Ramalho-Santos J, Varum S, Amaral S, Mota PC, Sousa AP, Amaral A. Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Hum Reprod Update* 2009;**15**:553–572.

Roovers EF, Rosenkranz D, Mahdipour M, Han CT, He N, Chuva de Sousa Lopes SM, van der Westerlaken LA, Zischler H, Butter F, Roelen BA *et al*. Piwi proteins and piRNAs in mammalian oocytes and early embryos. *Cell Rep* 2015;**10**:2069–2082.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B *et al*. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012;**9**:676–682.

Shoji M, Tanaka T, Hosokawa M, Reuter M, Stark A, Kato Y, Kondoh G, Okawa K, Chujo T, Suzuki T *et al*. The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. *Dev Cell* 2009;**17**:775–787.

Siomi MC, Mannen T, Siomi H. How does the royal family of Tudor rule the PIWI-interacting RNA pathway? *Genes Dev* 2010;**24**:636–646.

Siomi MC, Sato K, Pezic D, Aravin AA. PIWI-interacting small RNAs: the vanguard of genome defence. *Nat Rev Mol Cell Biol* 2011;**12**: 246–258.

Soper SF, van der Heijden GW, Hardiman TC, Goodheart M, Martin SL, de Boer P, Bortvin A. Mouse maelstrom, a component of nuage, is essential for spermatogenesis and transposon repression in meiosis. *Dev Cell* 2008;**15**:285–297.

Turner JM. Meiotic sex chromosome inactivation. *Development* 2007;**134**: 1823–1831.

van der Heijden GW, Castaneda J, Bortvin A. Bodies of evidence—compartmentalization of the piRNA pathway in mouse fetal prospermatogonia. *Curr Opin Cell Biol* 2010;**22**:752–757.

Williams Z, Morozov P, Mihailovic A, Lin C, Puvvula PK, Juranek S, Rosenwaks Z, Tuschl T. Discovery and characterization of piRNAs in the human fetal ovary. *Cell Rep* 2015;**13**:854–863.

Yang F, Wang PJ. Multiple LINEs of retrotransposon silencing mechanisms in the mammalian germline. *Semin Cell Dev Biol* 2016;**59**:118–125.