Primordial germ cells and amnion development in the avian embryo

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3

Meiotic wave adds extra asymmetry to the development of female chicken gonads

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ABSTRACT

Development of female gonads in chicken is asymmetric. This asymmetry affects gene expression, morphology and germ cell development. As a result only the left ovary develops into a functional organ, whereas the right ovary remains vestigial. In males, both gonads develop into functional testes.

Here, we revisited the development of asymmetric traits in female (and male) chicken gonads between Hamburger Hamilton stage 16 (HH16) and hatching. At HH16, primordial germ cells (PGCs) migrated preferentially to the left gonad independently of gender, accumulating in the left coelomic hinge between the gut mesentery and developing gonad. Using the meiotic markers SYCP3 and (phosphorylated) H2AFX, we were able to identify a pronounced asymmetry regarding meiotic progression in the germ cells located in the central, lateral and extremity part of the cortex of the left female gonad from HH38 until hatching. This has not been previously described. Moreover, in contrast to the current view that medullary germ cells enter apoptosis during development in the right female gonad, we show that medullary germ cells are not apoptotic, but arrested in pre-leptotene until hatching.

We provide a systematic analysis of the asymmetric distribution of germ cells in female chicken gonads until hatching and propose an updated model suggesting that the localization of germ cells [(1) in the left or right gonad, (2) in the cortex or medulla of the left gonad and (3) in the central part or the extremities of the left cortex] has direct consequences for their development and functionality in reproduction.

INTRODUCTION

In chicken, primordial germ cells (PGCs), the progenitors of the gametes, are of extraembryonic origin and, at Hamburger and Hamilton stage (HH)10-12 [1], they are found in the developing blood islands in a region of the yolk sac anterior to the head. From there, they migrate axially through the bloodstream, concentrating in the sinus terminalis, and enter the embryo mainly through the anterior vitelline veins [2]. The PGCs then travel through the embryonic vasculature to reach the gonadal ridges. After the PGCs colonize both left and right gonadal ridges, those undergo sex differentiation, to become morphologically different according to their gender developing as ovaries or testes. In males, both gonads develop into functional testes but in females, as in most birds, it is only the left gonad that develops into a functional ovary, while the right gonad remains rudimentary [3,4]. Before any signs of sex differentiation, the number of germ cells present in the gonadal ridges shows a sex-independent asymmetrical distribution with a preference for the left side in both males and females at HH15-HH17 [5] and at HH22-26 [6]. This sex-independent left-right asymmetry in the number of germ cells is still present at HH35 [7]. In addition to the asymmetry in the number of germ cells, the thickness of surface epithelium of the gonadal ridges also has pronounced sex-independent left-right asymmetry, being consistently thicker in the left gonad until HH36 [8,9].

During sex differentiation, the differences between the female left and right gonads are enhanced [4]. The left female gonad develops a strong spatial asymmetry by forming a germ cell-rich "cortex" and germ cell-poor "medulla" from HH32 onwards [10,11]. By contrast, the right female gonad does not develop a "cortex" and seems to be formed only by germ cell-poor "medulla". The left "cortex" harbors the great majority of the PGCs that cluster in compact cords, whereas the lacunar medulla of both left and right female gonads contains single or small clusters of dispersed germ cells [10,11]. Here, we have examined the sequential steps in gonadogenesis from HH16 until hatching that lead to the asymmetric development of the female chicken gonads. We were particularly interested in the events leading to the "regression" of the right female gonad and wanted to determine the timing of apoptosis assumed to occur in the germ cells present in the germ cell-poor "medulla" in both left and right female gonads [12]. However, until HH45 and using immunostaining for both early and late markers of apoptosis we were unable to confirm the massive wave of apoptosis in medullary germ cells reported previously in the left or right female gonad. Instead, using immunostaining for (phosphorylated) H2AFX (also known as γH2A.X), considered a marker of both apoptosis and meiosis, and the meiotic marker SYCP3, we detected a pronounced spatial wave of meiosis progression in the cortex of the left chicken gonad from the central part of the cortex to its extremities. This previously overlooked wave in meiosis progression adds a novel layer of asymmetric development to germ cell development in chicken.

MATERIAL AND METHODS

Embryo collection and sexing

Fertilized White Leghorn chicken (*Gallus gallus*) eggs were incubated in a humidified atmosphere at 37°C. Eggs were windowed and embryos staged [1]. The sex of HH35 embryos until prior to hatching was determined by eye, whereas the sex of HH16-30 embryos was determined by genomic polymerase chain reaction (PCR) as described [37]. Embryos were isolated and used whole from HH16-HH30, whereas from HH35 until prior to hatching, the paired gonads were further dissected out of the embryo.

Immunofluorescence on whole mount and paraffin section

Embryos and gonads were fixed in 4% paraformaldehyde (PFA) (MERCK, Germany) in phosphate-buffered saline (PBS) overnight (o/n) at 4˚C. Immunofluorescence on HH16-HH19 embryos (n=7) was performed as whole amount and on HH25-HH42 embryos (n=14) on paraffin sections was performed essentially as described [2,38]. Primary antibodies used were rabbit anti-DDX4 (1:500, gift from T. Noce), mouse anti-PCNA (1:500, sc-56, Santa Cruz Biotechnology, USA), rabbit anti-cleaved CASP3 (Asp 175) (1:300, 9661S, Cell Signaling Technology, USA), mouse anti-H2AFX (Ser139) (1:500, 05-636, Millipore, USA) and rabbit anti-FASLG (1:100, sc-6237, Santa Cruz Biotechnology, USA). Secondary antibodies used were Alexa Fluor 568 goat anti-mouse (1:500, A-21124, Life Technologies, UK) and Alexa Fluor 488 donkey anti-rabbit (1:500, A11008, Life Technologies, UK). DNA fragmentation was determined by TUNEL assay with TMR red In Situ Cell Death Detection Kit (Roche Applied Science, Germany) for 1 hour at 37°C following manufacturer's instructions. Slides were counterstained with 1:1000 dilution of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies, UK) in PBS for 1 minute and mounted with ProLongGold antifade reagent (Life Technologies, UK).

Germ cell counting and FACS-analysis

Germ cells were counted in the right and left gonad in whole mount HH16-HH19 embryos (n=3 females; n=4 males) and sequential paraffin sections of HH25-HH30 embryos (n=3 females; n=3 males) immunostained for DDX4.

The left and right gonads were collected individually from HH35 females (n=3), HH35 males (n=2), HH42 females (n=3) and HH42 males (n=3) and isolated in DPBS0 (Life Technologies, UK). To obtain single-cells, gonads were first cut into small pieces

and then incubated in TRIPLE 5x (Life Technologies, UK) at 37°C for 30 minutes, with pipetting from time to time. The single cell suspension was washed in DPBS0 and cells were fixed in 4% PFA for 10 minutes on ice. Thereafter, cells were permeabilized using 0.1% Triton/DPBS0 for 30 minutes on ice, incubated with 1:1000 rabbit anti-DDX4 in blocking solution [1% bovine serum albumin (BSA, Life Technologies, UK) in 0.05% Tween/DPBS0] for 1 hour on ice, washed with DPBS0 and incubated with 1:500 Alexa fluor goat anti-rabbit 488 in blocking solution for 1 hour on ice. After a final wash in DPBS0 cells were resuspended in FACS buffer (1%BSA, 10mM EDTA in PBS) and FACS analysis was performed using BD FACSAriaTM III (BD BioSciences, the Netherlands). Results were processed using the software BD FACSDivaTM version 6.0 (BD BioScience, the Netherlands).

The percentage of PGCs in the left and right gonad was calculated in relation to the total number of germ cells counted per embryo. The Student's T-test (two-tailed distribution, two-sample unequal variance) was used to compare the percentage of DDX4 positive germ cells between the right and left gonads. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Quantitative reverse transcription-PCR (qPCR)

The left and right gonads were harvested individually from HH35 females (n=3), HH35 males (n=3), HH42 females (n=5) and HH42 males (n=5). RNA was isolated using RNeasy Micro Kit (Qiagen, Netherlands) and cDNA made using the iScript™ cDNA Synthesis Kit (Biorad, USA). QPCR was performed using iQ™ SYBR® Green Supermix (Biorad, USA) on a CFX96TM Real-time system, C1000TM Thermal Cycler (Biorad, USA) with the condition 1x (95°C, 3 minutes), 40x (95°C, 15 seconds; 60°C, 30 seconds; 72°C, 45 seconds) and 1x (95°C, 10 seconds; 65°C, 5 seconds; 95°C, 50 seconds). The primers used here for DDX4, DAZL and the housekeeping genes RPS17 and ACTB were described elsewhere [39,40]. All reactions were performed in triplicate. Data was normalized using the DDCt method. Data from HH35 and HH42 is relative to the right male gonads.

Immunofluorescence on cryosections

Paired gonads from HH35 to prior to hatching (n=19) were fixed in 4% PFA/PBS for 10 minutes at room temperature (RT), washed three times in PBS, cryoprotected in 30% sucrose in PBS o/n at 4˚C, included in optimal cutting temperature (OCT) compound (Tissue-Tek, The Netherlands) and frozen at -80˚C. The gonads were sectioned (10µm) using a CM3050S cryotome (Leica Instruments GmbH, Germany) and mounted on Superfrost plus slides (Thermo Scientific, Germany). Cryosections were washed with PBS, blocked 1 hour at RT in 1% BSA/ PBS. The rest of the procedure was as described

above for paraffin sections. Primary antibodies used were rabbit anti-SYCP3 (1:500, NB300-232, Novus Biologicals, USA), rabbit anti-FASLG (1:100, sc-6237, Santa Cruz Biotechnology, USA) and mouse anti-H2AFX (Ser139) (1:500, 05-636, Millipore, USA) and secondary antibodies used were as above.

Imaging

Slides were either analysed on a Leica DMRA fluorescence microscope (Leica, Germany) and pictures taken with a CoolSnap HQ2 camera (Photometrics, USA); or scanned using a Panoramic MIDI digital scanner (3DHISTECH, Hungary) and representative areas for images selected using the software program 'Panoramic viewer' (3DHISTECH, Hungary). Confocal images were made on a Leica TCS SP8 confocal microscope (Leica, Mannheim, Germany) operating under the Leica Application Suite Advanced Fluorescence software (Leica, Mannheim, Germany).

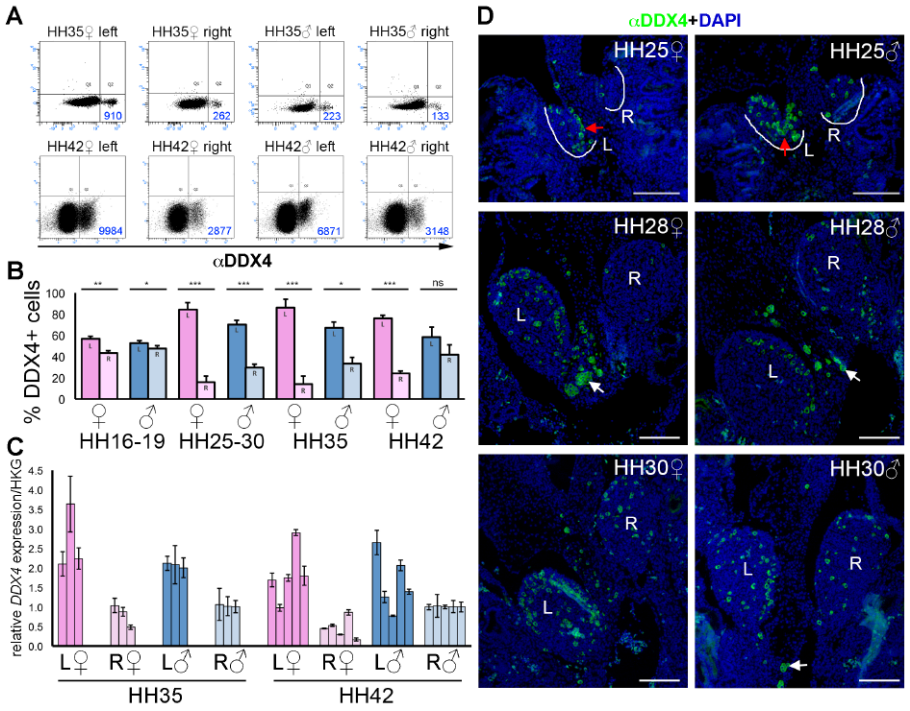

Figure 1. Difference in germ cells localization between the left and right gonads of female and male chicken at HH16-HH42. (A) Flowcytometric analysis showed less DDX4-positive cells in right gonad of each gonadal pair in both HH35 and HH42 females and males. (B) The percentage of DDX4-positive germ cells present in the in the left gonad (L) was significantly higher than in the right gonad (R) at HH16-HH19, HH25-HH30, HH35 and HH42 in both sexes, except for HH42 male gonads where this difference was not significant (ns). *, P<0.05; **, P<0.01; ***, P<0.001. (C) Relative DDX4 expression in left and right gonads of HH35 and HH42 embryos, both females and males, compared to the male right gonad. Each bar represents one gonad and data are expressed as the mean ± standard deviation of technical triplicates. Expression of DDX4 was normalized to the housekeeping genes (HKG) RPS17 and ACTB. (D) Immunostaining for DDX4 (green) showed more germ cells localized to the left gonad (L) than to the right gonad (R) in both females and males at HH25 (upper panels), HH28 (middle panels) and HH30 (lower panels). The white lines delimitate the place of the left and right gonads. Red arrows indicate germ cells accumulating just underneath the coelomic epithelium at the hinge between the gut mesentery and the developing left gonad. White arrows indicate ectopic PGC clusters. Scale bar (same for all images) is 100 µm.

RESULTS

Preferential asymmetric distribution and localization of germ cells between HH16-42

It has been reported that until HH35 both male and female chicken embryos contained a larger number of germ cells in their left gonads [5,7]. This led us to investigate the precise distribution of the germ cells in the developing gonadal ridges from the time of their arrival in the gonads at HH16 until HH42. Using immunostaining for the germ cell-marker DDX4 (also known as CVH), we either counted or FACS-analysed (DDX4)-positive germ cells in embryos from HH16 to HH42 and confirmed a consistently higher number in the left gonad independent of the gender until HH42, even though this differences were less pronounced in males (Figure 1A, 1B). At HH42, the left female gonad contains about 80% of the gonadal germ cells, whereas the male left gonad contains about 60%. This asymmetry was also observed by quantitative reverse transcriptase-PCR for DDX4 (Figure 1C) and similarly for DAZL, another germ cell marker using dissected whole gonads (Supplementary Figure S1). As expected [2,5,7], the number of germ cells in each gonad shows some variability. Between HH35-42, left-right differences in germ cell numbers persisted in the female gonads, but got less pronounced in the male gonads.

In terms of distribution, at HH25, germ cells strikingly accumulated underneath the coelomic epithelium at the hinge between the gut mesentery and the developing left gonad in both males and females (red arrows Figure 1D); this preferential localization just underneath the thick epithelial layer of the left gonad remained at HH28-30 (Figure 1D). Interestingly, outside the gonads, ectopic tight clusters of germ cells were observed both in females and males (white arrows in Figure 1D), where many remained localized in the gut mesentery. At HH35-42, the localization of germ cells to the “cortex” developing in the left female gonad became very pronounced, even though many germ cells were scattered in the “medulla” of the left and right female gonad (Figure 2A, 2B). Interestingly, between HH45 and just before hatching (BH), the germ cells in the medulla of both the right and left female gonad remained numerous (Figure 2C-2F). In the right medulla, germ cells also localized to the surface, however those did not become enclosed in germ cells cords as in the cortex of the left gonad (Figure 2C-2F).

Germ cells undergo a meiotic wave in the left cortex between HH38 and hatching

To explore further the developmental capacity of the asymmetrically distributed germ cells in the cortex and medulla of the left female gonad, we investigated

the timing of meiosis entry and progression until hatching. For this, we analysed the expression pattern of the meiotic-specific marker SYCP3 together with that of (phosphorylated) H2AFX, a marker of DNA double-strand breaks not only associated with DNA damage in apoptosis during mitosis [13], but also those DNA double-strand breaks that occur early during meiotic recombination [14,15].

At HH38, germ cells along the whole length of the left cortex as well as in the medulla of both the left and right female gonads expressed punctated H2AFX, but showed overall no/low levels of (nuclear) SYCP3 (Figure 3A, 3B; Supplementary Figure S2). However, in the cortex center of the left female gonad between HH38 and HH42, H2AFX is downregulated and at HH42, in the cortex center germ cells showed either only (punctated nuclear) SYCP3 or both SYCP3 and H2AFX (Figure 3C, 3Ci, 3Cii). In the extremities of the left cortex and in the medulla of both the left and right female gonads, germ cells maintained the characteristic pattern of H2AFX (Figure 3C, 3Ciii, 3Civ, 3D, 3Di) as observed at HH38. Combining our localization data using the intact gonads with previous data from chromosome spreads from dispersed germ cells [16-18], we conclude that the expression pattern of H2AFX and SYCP3 at HH38 corresponded to that of germ cells in pre-leptotene and, at HH42, germ cells located in the central and lateral part of the left cortex either in early leptotene (punctated SYCP3 and low/no H2AFX) or are in late leptotene/early zygotene (punctated SYCP3 and H2AFX), and those at the extremities of the left cortex and all medullary germ cells are still in pre-leptotene (no SYCP3 and high H2AFX). At HH45, the expression pattern of H2AFX and SYCP3 along the left cortex showed that germ cells in late leptotene/early zygotene (punctated SYCP3 and H2AFX) not only occupied the central part of the cortex, but now also occupied most of the lateral parts of the cortex, whereas germ cells in early leptotene (punctated SYCP3 and low/no H2AFX) became fewer and restricted to a narrow band, adjacent to the pre-leptotene germ cells (no SYCP3 and high H2AFX) in the cortex extremities (Figure 4A, 4Ai-4Aiii; representative higher magnifications in Supplementary Figure S3). This was less pronounced in the long axes of the cortex of the left gonad, but we could still find pockets of H2AFX-positive SYCP3-negative germ cells at the cortex extremities (Supplementary Figure S4).

Since several studies on chromosome spreads have described germ cells in pachytene around hatching [16-18], we extended our analysis to female gonads just before hatching (BH). We observed that the central part of the cortex indeed now contained many germ cells in late zygote/early pachytene (containing clear SYCP3-positive synaptonemal complexes but still expressing H2AFX) (Figure 4B, 4Bi; representative higher magnifications in Supplementary Figure S3). The lateral part of the left cortex still contained germ cells in late leptotene/early zygotene, flanked by germ cells in the cortex extremities in early leptotene and pre-leptotene (Figure 4B, 4Bii, 4Biii).

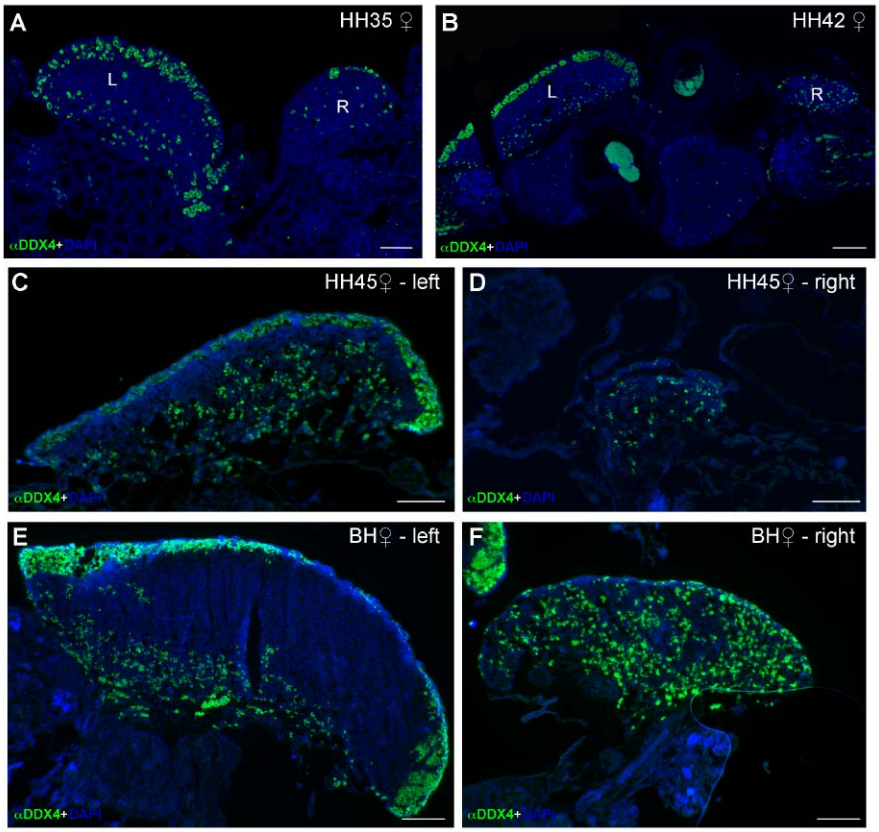

Figure 2. Distribution of (DDX4-positive) germ cells in left and right gonads in female chicken from HH35 to before hatching (BH). DDX4 (green) expression in transverse sections of female gonads showing the distribution of germ cells in left (L) and right (R) gonad at HH35 (A), HH42 (B), HH45 (C,D) and BH (E,F). Scale bars are 200 µm (A, C, D, E, F) and 500 µm (B).

Medullary germ cells in left and right female gonads are not apoptotic, but in pre-leptotene until hatching

Germ cells localized in the medulla of both left and right gonads at HH45 and before hatching (Figure 4C, 4D) showed similar nuclear features to pre-leptotene germ cells (no SYCP3 and high H2AFX) located in the extremities of the left cortex and are most probably in pre-leptotene. Interestingly, we noted that at HH45 and before hatching some small clusters of germ cells in the left and right gonadal medulla were either positive for SYCP3 (Figure 4Aiv, white arrow in Figure 4D) or expressed both SYCP3 and H2AFX (Figure 4Biv) suggesting that some medullary PGCs may in fact enter meiosis. As (phosphorylated) H2AFX is associated with DNA double-strand breaks both during apoptosis and meiosis entry, we wanted to discriminate between these two processes to understand the fate of left and right medullary germ cells. First, we investigated the expression of the proliferation cell nuclear antigen (PCNA), a marker for DNA replication in mitosis, but also involved in DNA replication during meiotic prophase [19]. At HH35-HH42, the great majority of germ cells expressed PCNA (Figure 5A; Supplementary Figure S5). The expression of both H2AFX and PCNA in medullary germ cells, suggest that these cells were in pre-leptotene, instead of apoptosis.

To further distinguish between apoptosis and meiosis, we then investigated the expression of the early apoptosis marker cleaved caspase 3 (CASP3) and assessed DNA fragmentation during late apoptosis with the TUNEL assay. In the HH35 and HH42 female gonads, we found only a few cleaved CASP3-positive cells (white arrowheads in Figure 5B) and a few TUNEL-positive germ cells and somatic cells (white arrowheads in Figure 6A) but no massive loss of germ cells in the medulla, which has been previously reported using electron microscopy [11,12]. Furthermore, the germ cells in the surface of the right gonad at HH35 and HH42 are clearly not in (early or late) apoptosis (white arrows in Figure 6A) and therefore, this cannot be the reason why a germ cell-rich cortex does not develop in the right female gonad. Until hatching, we only detected a very reduced number of either CASP-3-positive or TUNEL-positive cells in both gonads (data not shown).

We next examined the expression of FASLG (also known as FAS ligand), a marker involved in the FASLG-FAS system that plays a role in inducing apoptosis in mammalian spermatocytes undergoing meiosis [20,21]. Interestingly, HH42 germ cells in the extremities of the left cortex and some in the left and right medulla seem to upregulate FASLG (white arrowheads in Figure 6B); and at BH, many presumably germ cells in both the left and right medullas seem to become strongly positive for FASLG (white arrows in Figure 6C), suggesting that after hatching those may be eliminated by apoptosis via a FASLG-FAS dependent mechanism.

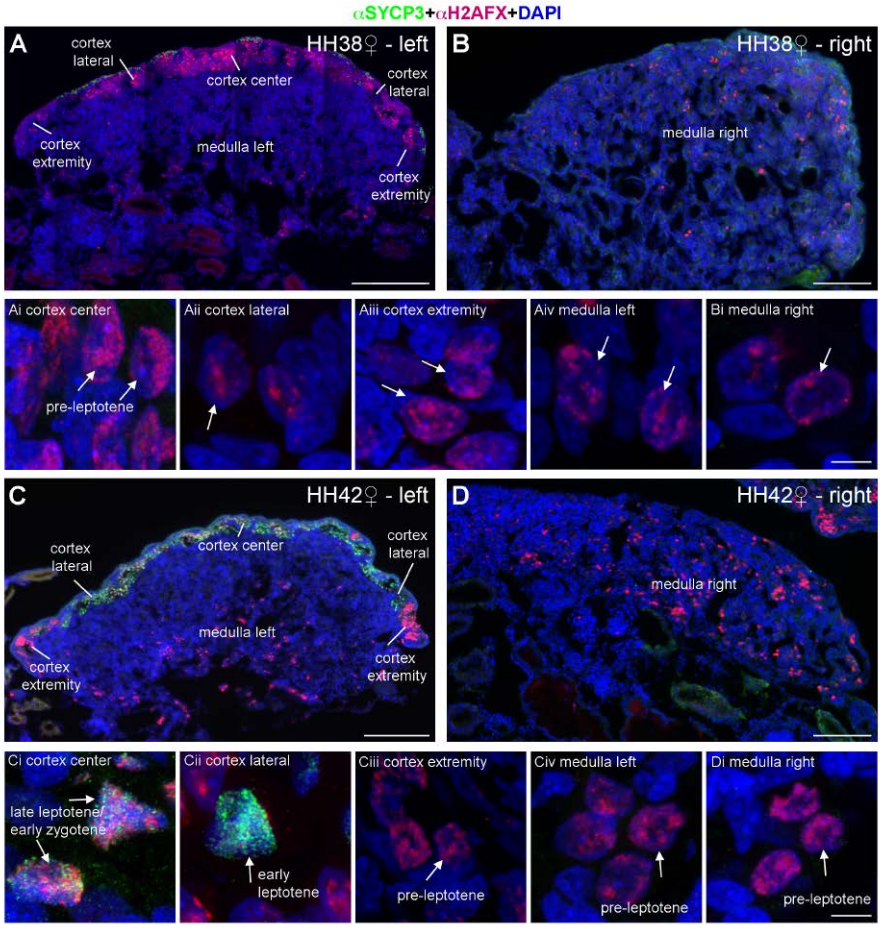

Figure 3. SYCP3 and H2AFX expression in chicken female gonads at HH38 and HH42. SYCP3 (green) and H2AFX (red) expression in HH38 female left (A) and right (B) gonads and HH42 female left (C) and right gonads (D). High magnifications are representative from three different regions of the left cortex (cortex center, cortex lateral, cortex extremity), the left medulla and the right medulla. White arrows point to germ cells illustrating specific meiotic stages (pre-leptotene, early leptotene, late leptotene/early zygotene). Scale bars are 100 μm (A, B, C, D) and 5 μm (Ai-Aiv, Bi, Ci-Civ, Di).

DISCUSSION

Sex-independent preferential migration of PGCs to the left side

Our results and those of others [5-7] show that chicken PGCs preferentially colonize the gonad on the left side of the body as they arrive at HH15, independent of their sex. Differentially expressed signaling cues could direct this asymmetric migration. Molecules that might play a role are BMP7, which is expressed in the left gonad around HH21 (3.5 days of incubation) in both sexes [22] and the transcription factor PITX2, which is expressed in the left gonad around HH18 (3 days of incubation) in both sexes [9,23-25]. Experiments by Naito and colleagues (2009) that transferred PGCs from the bloodstream of HH14-15 donor embryos to the bloodstream of age-matched recipient embryos and analysed them at 16.5 days of incubation (± HH42) suggested that female PGCs migrate preferentially to the left side of both female and male embryos but also that female embryos attracted more male or female PGCs to the left gonad [26]. It is interesting to note that also in humans, between 140-212 mm crown-rump length (about 22 weeks of gestation) the right gonad weighs more (and has a higher DNA and protein content) than the left gonad in both sexes [27], suggesting that an initial sex-independent left-right asymmetry may be conserved at least between humans and chicken.

No evidence for apoptosis in chicken medullary germ cells until before hatching

Left-right asymmetry of the chicken female gonads has been noticed and reported in morphological detail a long time ago [28]. However, since the right gonad of the chicken embryo has been considered as a degenerating structure containing germ cells in apoptosis [10,12], its study has been neglected. Ishimaru and colleagues (2008) analysed apoptosis levels at HH27 and HH29 and observed a very reduced number of TUNEL-positive germ cells in cortex and medulla in both sides [23], but we have extended this here and analysed apoptosis in both sexes between HH35 and hatching in the right and left gonad. We were unable to detect signs of robust apoptosis in the germ cells in the right gonad and our data suggest that medullary germ cells are not apoptotic, but presumable in pre-leptotene until hatching (and a few may even manage to enter meiosis). The reason for the discrepancy with previous literature is the fact that we presently use a combination of meiotic and apoptotic markers together with a robust germ cell marker, whereas previously only histological sections imaged by light microscopy of transmission electron microscopy were analyzed.

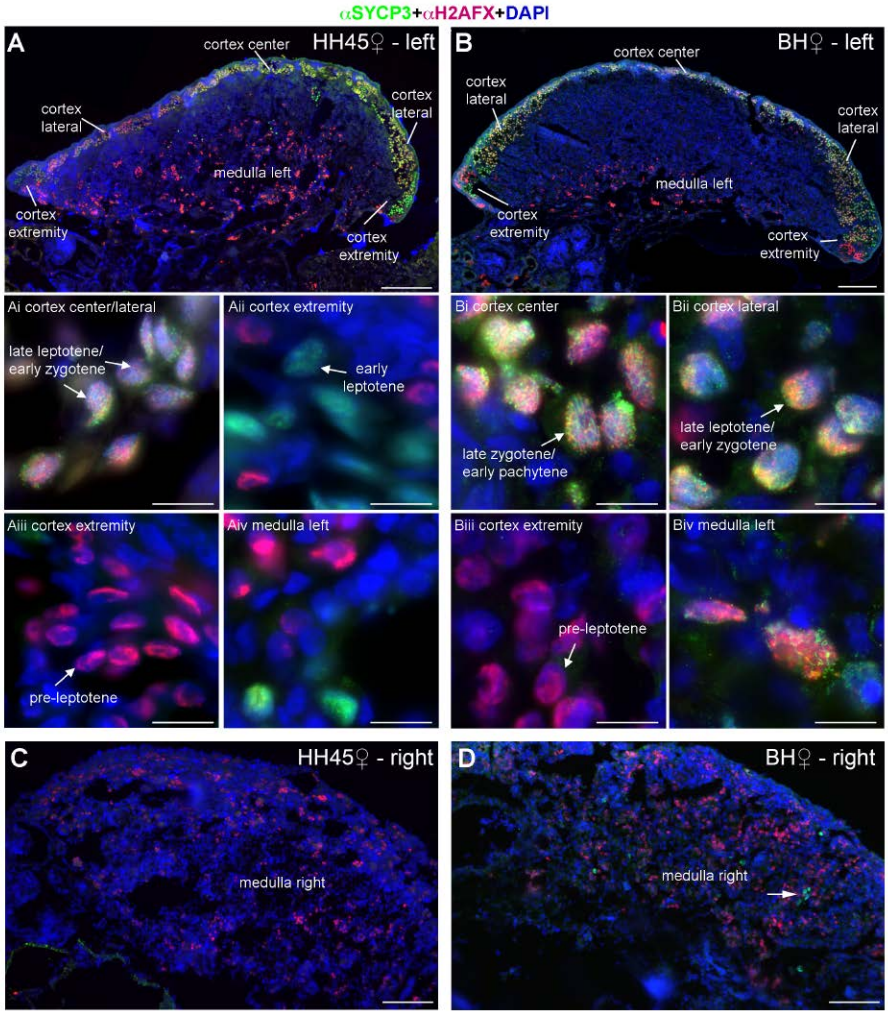

Figure 4. SYCP3 and H2AFX expression in chicken female left and right gonads at HH45 and before hatching (BH). SYCP3 (green) and H2AFX (red) expression in HH45 female left (A) and right (B) gonads and BH female left (C) and right gonads (D). High magnifications are from three different regions of the left cortex (cortex center, cortex lateral, cortex extremity), and the left medulla. White arrows point to germ cells illustrating specific meiotic stages (pre-leptotene, early leptotene, late leptotene/early zygotene, late zygotene/early pachytene). In D, the white arrow points to a cluster of germ cells that upregulated SYCP3. Scale bars are 100 µm (A, B, C, D) and 10 µm (Ai-Aiv, Bi-Biv).

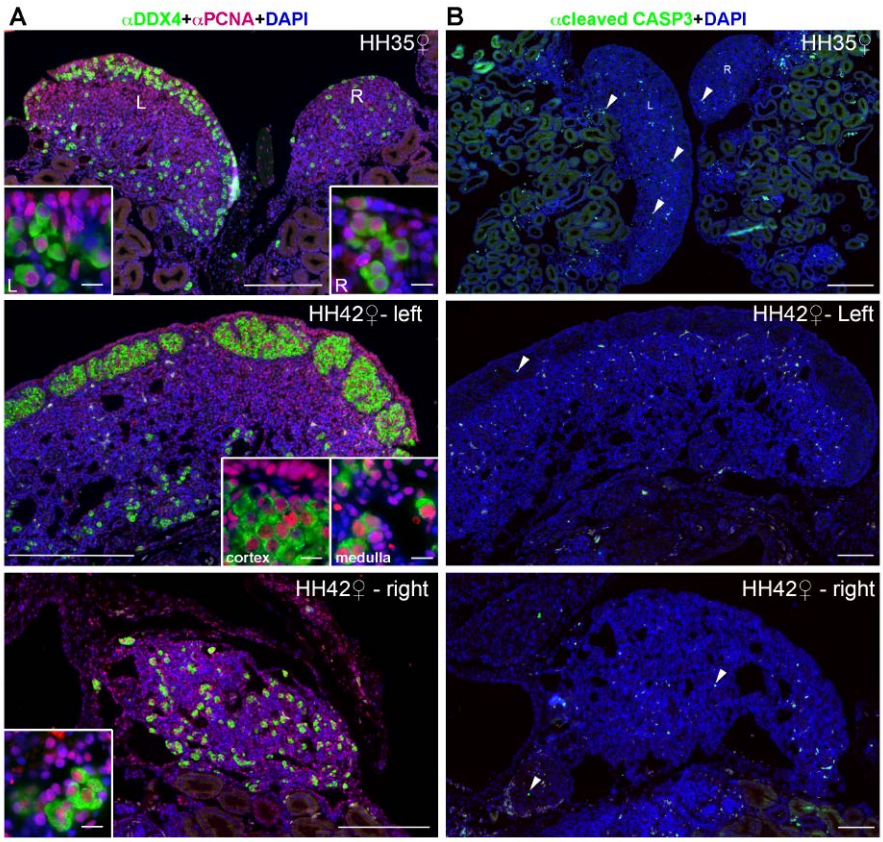

Figure 5. Expression of PCNA and cleaved CASP3 in chicken female gonads at HH35 and HH42. (A) PCNA (red) expression in female left (L) and right (R) gonads at HH35 and HH42. Most germ cells (DDX4-positive cells) at HH35 and HH42 are PCNA-positive. Inserts show a magnified detail. Ectopic PGC clusters, indicated by white arrows, also showed PCNA-positive nuclei. (B) Cleaved CASP3 expression (green) in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to cleaved CASP3-positive cells. Scale bars in (A) are 200 μm (HH35 and HH42 right gonad) and 500 μm (HH42 left gonad); in (B) are 200 μm (HH35) and 100 μm (HH42 left and right gonad); and in the inserts are 10 μm.

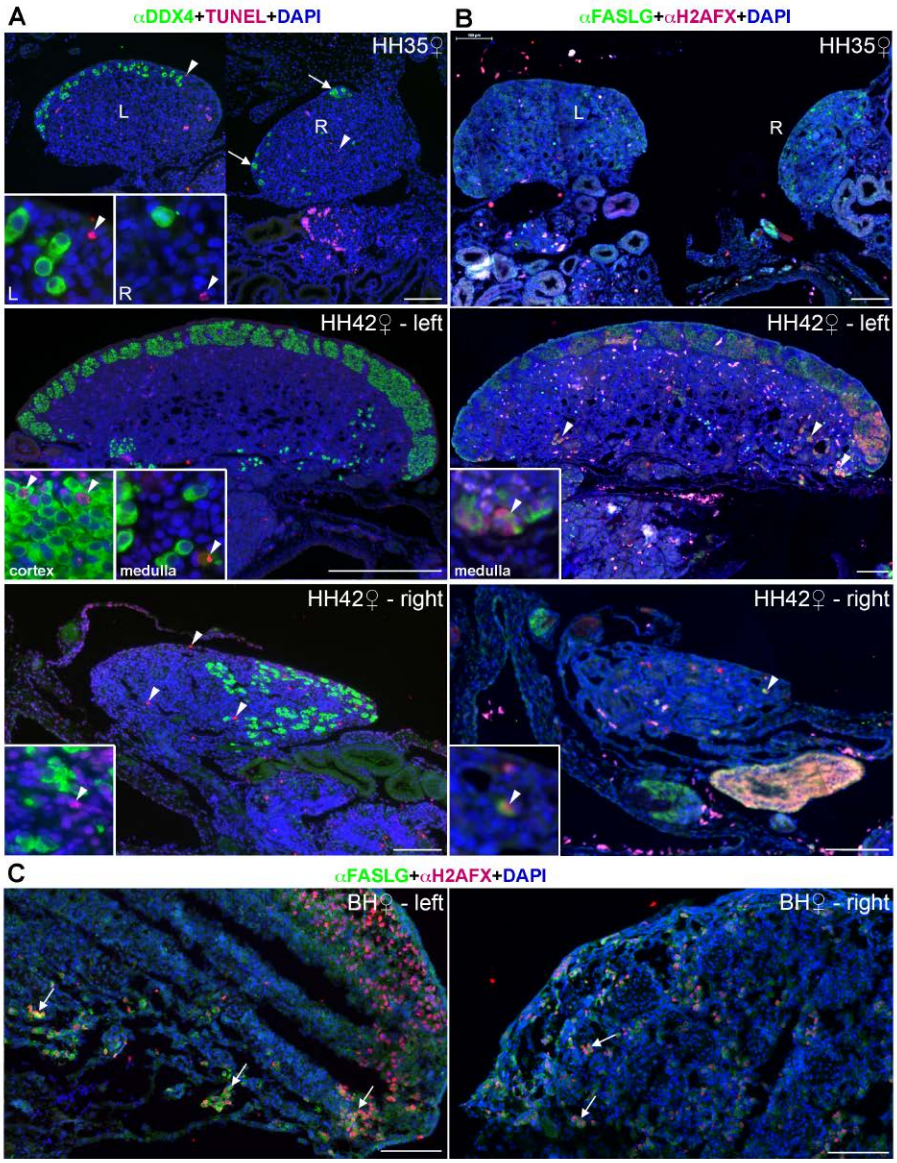

Figure 6. TUNEL and FASLG expression in chicken female gonads. (A) TUNEL assay (red) shows late apoptotic cells in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to apoptotic somatic cells and germ cells (green, DDX4-positive). Inserts show a magnified detail. White arrow point to TUNEL negative germ cells in the surface of the HH35 right gonad. (B) FASLG (green) and H2AXF (red) expression in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to double positive FASLG and H2AXF cells (C) FASLG (green) and H2AXF (red) expression in female left and right gonads before hatching (BH). White arrows point to double positive FASLG and H2AXF cells. Scale bars in A and B are 100 µm, in C are 200 µm and in the inserts are 10 µm.

In agreement, the overexpression of PITX2 *in ovo* in the right female gonad from HH8-10 has been demonstrated sufficient to induce a robust cortex formation in the right gonad similar to that of the left gonad containing H2AFX-positive germ cells at HH38-39 [9]. Those results indicate that germ cells in right female gonad have the capacity to develop into a normal cortex and enter meiosis if given the right environment. We suggest that until hatching, germ cells have equal potential to develop, as in males, in both left and right females gonads but they only receive the correct molecular signals, initiated by PITX2, to do so in the central and lateral part of the cortex of the left female gonad.

Interestingly, we detected expression of FASLG in both the H2AFX-positive germ cells in the extremities of the left cortex and medulla. FASLG-positive cells are not undergoing apoptosis, but are potential targets for destruction. The FASLG-FAS system plays a role in inducing apoptosis in mammalian spermatocytes undergoing meiosis [20,21]. Even though the role of the FASLG-FAS system early during oogenesis is less well studied, it is well known to play a role regulating atresia at different stages during folliculogenesis [29,30]. Whether the germ cells in the extremities of the left cortex and medulla are eliminated by apoptosis after birth, eventually via a FASLG-FAS dependent mechanism remains to be investigated.

Meiotic wave in the chicken left cortex from the center to the extremities

The development of the asymmetry between left and right in female gonads is primarily orchestrated by PITX2 expression [9,23,24]. Thereafter, meiosis entry in chicken seems to be directly related to the synthesis and breakdown of retinoic acid [31,32]. Several authors have described asynchrony in meiotic stages in dispersed chromosome spreads of chicken germ cells during late developmental stages and the first week post-hatching [16-18] or have reported immunostaining in intact gonads for either H2AFX or SYCP3 separately, but not in combination [9,31-34]. The existence of a meiotic wave in chicken has therefore remained elusive to date.

In his 1963 paper, Hughes did not refer to a meiotic wave in the left cortex although he noted that “germ cells in the central parts of the ovarian cortex are consistently more advanced in development than those at the extremities of the cortex” [28]. Our systematic study of the expression of H2AFX and SYCP3, from HH38 until hatching showed the existence of a meiotic wave in the left cortex (Figure 7).

A meiotic wave has been described in female mice from the anterior to the posterior part of the gonad during mid-gestation [35,36] and even in humans it seems to occur from the inside to the outside of the gonadal cortex during the second trimester of development (AMH and SMCSL, unpublished results), suggesting that a meiotic wave may be a conserved mechanism in animals.

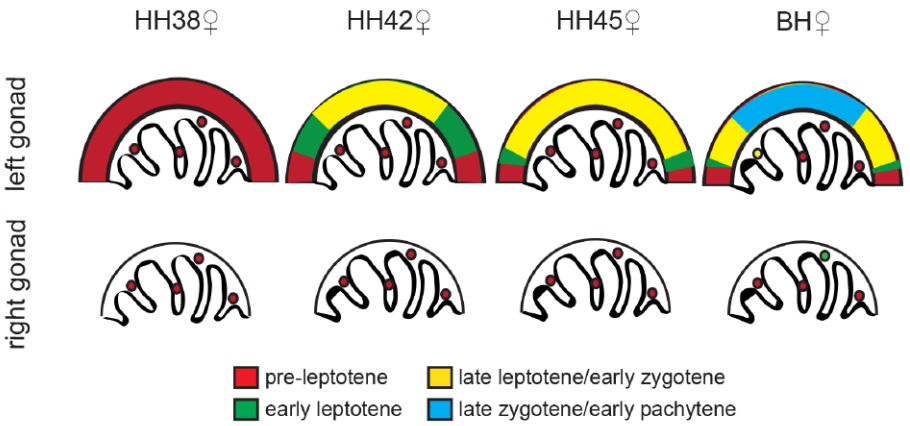

Figure 7. Proposed model of meiotic wave from HH38 until hatching in the female left and right gonads. At HH38, all germ cells independent of their localization express H2AFX and are in pre-leptotene. From HH42 until before hatching (BH), the germ cells in the left gonadal cortex exhibited a pronounced meiotic wave from the central part of the cortex to its extremities. Just BH, germ cells localized in the central and lateral part of the left cortex are in late zygote/early pachytene and late pachytene/early zygotene and germ cells in the extremities of the left gonad are in early leptotene or in pre-leptotene. The majority of the germ cells in the medulla of the left and right female gonads are in pre-leptotene

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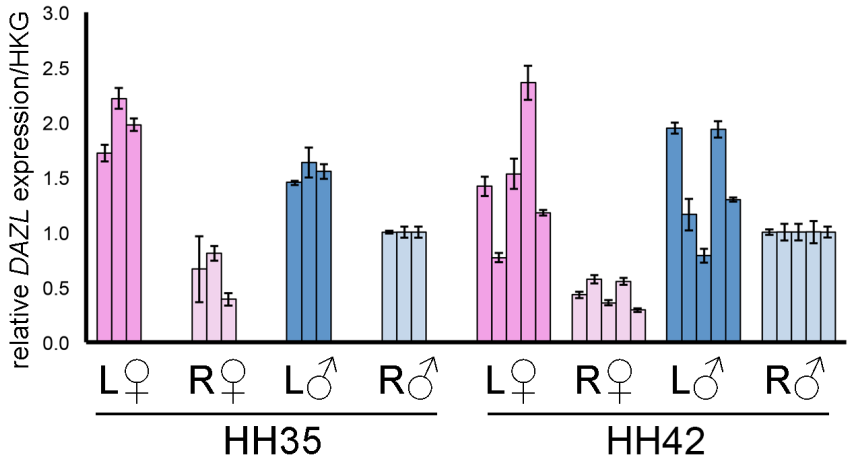
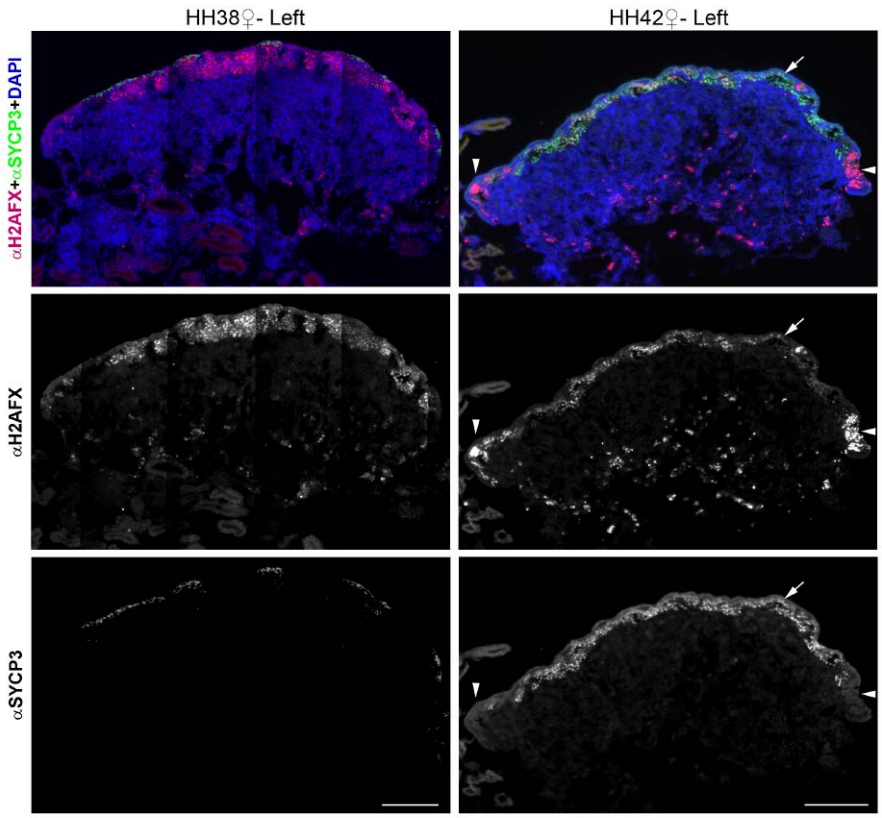
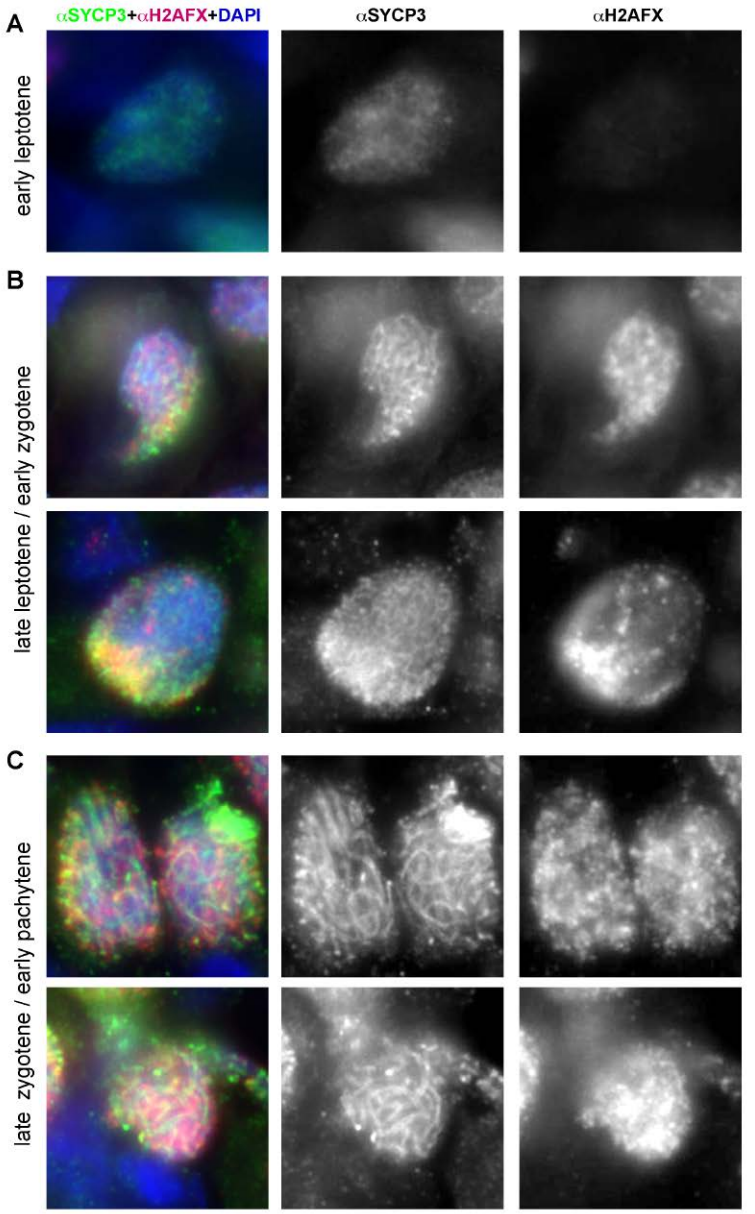
SUPPLEMENTARY FIGURES



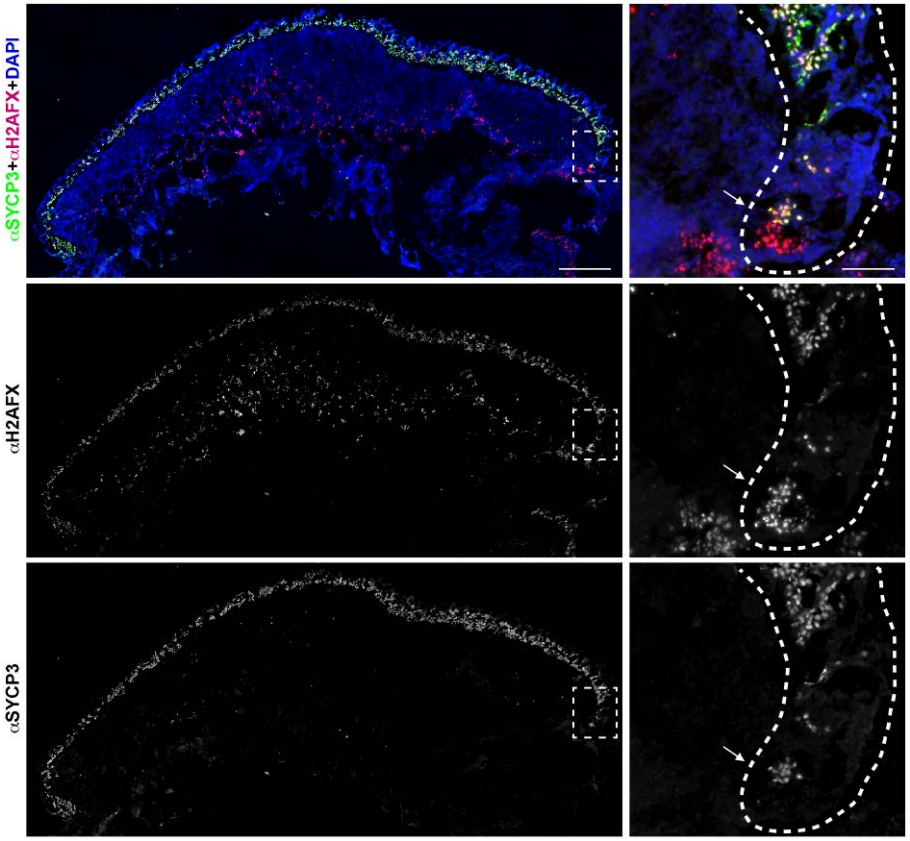
Supplementary Figure S1. DAZL expression in chicken gonads at HH35 and HH42. DAZL expression in left (L) and right (L) gonads of HH35 and HH42 chicken embryos, both females and males, compared to the male right gonad. Each bar represents one gonad and data are expressed as the mean ± standard deviation of technical triplicates. Expression of DAZL was normalized to the HKG RPS17 and ACTB.



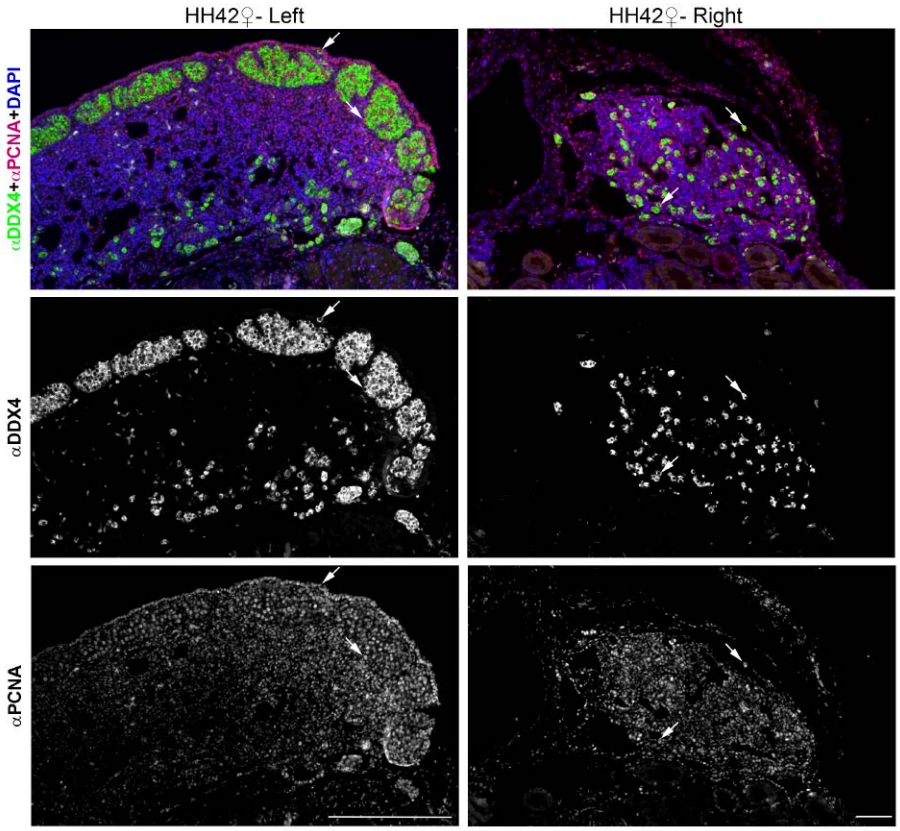
Supplementary Figure S2. Expression of H2AFX and SYCP3 in the chicken left gonad at HH38 and HH42. Sections of HH35 and HH42 female left gonads showing the merged and single channels for H2AFX (red) and SYCP3 (green). White arrows indicated germ cells with lower H2AFX and higher SYCP3 expression compared to the germ cells indicated with white arrowheads. Scale bar for HH38 is 100 μm and for HH42 is 200 μm.



Supplementary Figure S3. Different meiotic phases encountered at HH45 to before hatching. Magnified and single-channel images from Figure 4 showing different meiotic phases in female chicken gonads: (A) early leptotene stage marked by single expression of H2AFX, (B) late leptotene/early zygotene stage marked by the punctuated expression of both H2AFX and SYCP3, and (C) late zygotene/early pachytene stage marked by the expression of H2AFX and chromosomal synaptic expression of SYCP3. Scale bars are 5 μm.



Supplementary Figure S4. Expression of H2AFX and SYCP3 in the chicken left gonad at HH45. Sagittal section of HH45 female left gonad showing the merged and single channels for H2AFX (red) and SYCP3 (green). The dashed area is showed magnified in the right panel. In the left panel the dashed are depicts the cortex. White arrows indicate germ cells expressing H2AFX, but not SYCP3, in the extremity of the cortex. Scale bar is 500 μm in left panel and 75 μm in right panel.



Supplementary Figure S5. Expression of PCNA and DDX4 in chicken left and right gonad at HH42. Section of HH42 female left and right gonad showing the merged and single channels for DDX4 (green) and PCNA (red). White arrows indicate germ cells expressing PCNA. Scale bar are 500 μm for left gonad and 100 μm for right gonad.

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