Meiotic Wave Adds Extra Asymmetry to the Development of Female Chicken Gonads

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SUMMARY

Development of female gonads in the chicken is asymmetric. This asymmetry affects gene expression, morphology, and germ cell development; consequently only the left ovary develops into a functional organ, whereas the right ovary remains vestigial. In males, on the other hand, 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 migrated preferentially to the left gonad, accumulating in the left coelomic hinge between the gut mesentery and developing gonad in both males and females. Using the meiotic markers SYCP3 and phosphorylated H2AFX, we identified a previously undescribed, pronounced asymmetryc meiotic progression in the germ cells located in the central, lateral, and extreme cortical regions of the left female gonad from HH38 until hatching. Moreover, we observed that—in contrast to the current view—medullary germ cells are not apoptotic, but remain arrested in pre-leptotene until hatching. In addition to the systematic analysis of the asymmetric distribution of germ cells in female chicken gonads, we propose an updated model suggesting that the localization of germ cells—in the left or right gonad; in the cortex or medulla of the left gonad; and in the central part or the extremities of the left cortex—has direct consequences for their development and participation in adult reproduction.

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Abbreviations: DDX4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; CASP3, caspase 3; FASLG, Fas ligand; H2AFX, H2A histone family member X; HH, Hamburger and Hamilton stage; PCNA, proliferation cell nuclear antigen; PITX2, paired-like homeodomain 2; SYCP3, synaptonemal complex protein 3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

INTRODUCTION

Primordial germ cells, the progenitors of the gametes, are of extraembryonic origin in chicken, and are found in the developing blood islands in a region of the yolk sac anterior to the head at Hamburger and Hamilton stage (HH) 10−12 (Hamburger and Hamilton, 1992). From there, they migrate axially through the bloodstream, concentrating in the sinus terminalis, and enter the embryo mainly through the anterior vitelline veins (De Melo Bernardo et al., 2012). The primordial germ cells then travel through the embryonic vasculature to reach the gonadal ridges. After the primordial germ cells colonize both left and right gonadal ridges, this embryonic region undergoes morphological sex differentiation and develops into ovaries or testes according to their genetic inheritance. In males, both gonads develop into functional testes, but in females only the left gonad develops into a functional ovary; the right gonadal tissue remains rudimentary (Zaccanti et al., 1990; Smith and Sinclairm, 2004).

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 (Nakamura et al., 2007), HH22-26 (Van Limborgh, 1968), and HH35 (Intarapat and Stern, 2013). 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 (Carlon and Stahl, 1985; Guioli and Lovell-Badge, 2007).

Differences between the female left and right gonads are enhanced during sex differentiation (Smith and Sinclair, 2004). The left female gonad develops a strong spatial asymmetry by forming a germ cell-rich "cortex" and germ cell-poor "medulla" from HH32 onwards (Ukeshima and Fujimoto, 1991; Gonzalez-Moran, 2011), whereas the right female gonad does not develop a "cortex" and seems to consist of only germ cell-poor "medulla". The left "cortex" harbors the great majority of germ cells, which cluster into compact cords, whereas the lacunar medulla of both left and right female gonads contains single or small clusters of dispersed germ cells (Ukeshima and Fujimoto, 1991; Gonzalez-Moran, 2011).

Here, we examined the sequential steps of gonadogenesis that lead to the asymmetric development of the female chicken gonads from HH16 until hatching. 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 germ cells present in the germ cell-poor "medulla" in both left and right female gonads (Ukeshima, 1996). We were unable to confirm the massive wave of apoptosis in medullary germ cells reported previously in the left or right female gonad until hatching. Instead, using immunostaining for phosphorylated H2AFX (also known as γH2A.X), a marker of both apoptosis and meiosis, and the meiotic marker SYCP3, we detected a pronounced spatial wave of meiotic progression in the cortex of the left chicken gonad from the central part of the cortex to its extremities. This previously overlooked wave in meiotic progression adds a novel layer of asymmetric development to germ cell development in the female chicken.

RESULTS

Preferential Asymmetric Distribution and Localization of Germ Cells Between HH16-42

It was previously reported that until HH35, both male and female chicken embryos contained a larger number of germ cells in their left gonads (Nakamura et al., 2007; Intarapat and Stern, 2013). This led us to investigate the precise distribution of germ cells in the developing gonadal ridges from the time of their arrival at HH16 until HH42. Using immunostaining for the germ cell-marker DDX4 (DEAD [Asp-Glu-Ala-Asp]-box polypeptide 4; also known as chicken Vasa homolog, CVH), we either counted or sorted for DDX4-positive germ cells in embryos from HH16 to HH42. By both methods, we observed a consistently higher cell number in the left gonad of both genders until HH42, even though this difference was less pronounced in males (Fig. 1A,B). At HH42, the left female gonad contains about 80% of the gonadal germ cells, whereas the left male gonad contains about 60%. This asymmetry was also observed in dissected whole gonads by quantitative reverse-transcriptase PCR for the germ-cell markers *DDX4* (Fig. 1C) and *DAZL* (deleted in azoospermia-like) (Supplementary Figure S1). As expected (Nakamura et al., 2007; De Melo Bernardo et al., 2012; Intarapat and Stern, 2013), the number of germ cells in each gonad was somewhat variable. Between HH35-42, left-right differences in germ cell numbers persisted in female gonads, but became less pronounced in male gonads.

Germ cells strikingly accumulated beneath the coelomic epithelium, at the hinge between the gut mesentery and the developing left gonad, in both HH25 males and females (Fig. 1D, red arrows); this preferential localization just underneath the thick epithelial layer of the left gonad persisted at HH28-30 (Fig. 1D). Interestingly, ectopic tight clusters of germ cells were also observed outside the gonads in the gut mesentery both in females and males (Fig. 1D, white arrows), where many remained at later stages. At HH35-42, most germ cells localized to the developing "cortex" in the left female gonad, even though many germ cells were also scattered in the "medulla" of the left and right female gonad (Fig. 2A,B). Unexpectedly between HH45 and just before hatching, the number of germ cells in the medulla of both the right and left female gonad remained relatively high (Fig. 2C−F). In the right medulla, germ cells also resided at the surface, but did not become enclosed in germ-cell cords as occurred in the cortex of the left gonad (Fig. 2C−F).
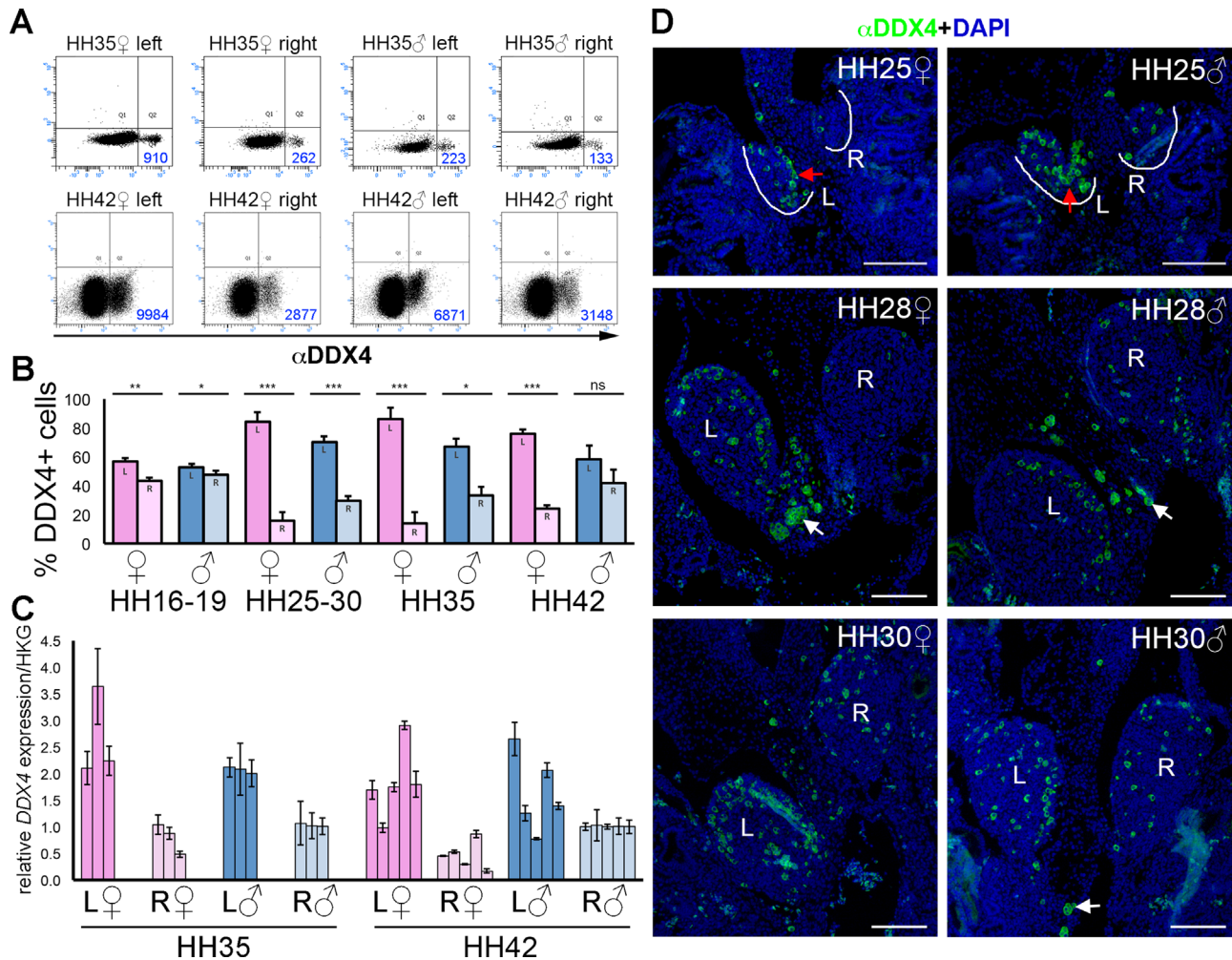
Figure 1. Differences in germ cell localization between the left and right gonads of female and male chicken at HH16-HH42. **A**: Flow-cytometric analysis showed fewer DDX4-positive cells in the right gonad of each gonad pair in both HH35 and HH42 females and males. **B**: The percentage of DDX4-positive germ cells present in the left gonad (L) was significantly higher than in the right gonad (R) at HH16-HH19, HH25-HH30, and HH35 in both sexes, as well as in HH42 females. The difference in HH42 male gonads was not significant (ns). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. **C**: Relative DDX4 expression in the left and right gonads of HH35 and HH42 embryos, relative to the male right gonad. Each bar represents one gonad. Data are expressed as the mean ± standard deviation of technical triplicates. Expression of DDX4 was normalized to the housekeeping genes RPS17 (ribosomal protein S17) and ACTB (beta actin). **D**: Immunostaining for DDX4 (green) revealed 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 delineate the boundaries 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 germ cell clusters. Scale bars, 100 μm.

Germ Cells Undergo a Meiotic Wave in the Left Cortex Between HH38 and Hatching

To further explore the developmental capacity of the asymmetrically distributed germ cells in the cortex and medulla of the left female gonad, we investigated the timing of meiotic entry and progression until hatching. We analysed the expression pattern of the meiosis-specific marker SYCP3 (synaptonemal complex protein 3) together with that of phosphorylated H2AFX (histone H2A, family member X), a marker of DNA double-strand breaks associated with both DNA damage in apoptosis (Rogakou et al., 1999) as well as DNA double-strand breaks introduced early

during meiotic recombination (Hunter et al., 2001; Ismail and Hendzel, 2008).

At HH38, germ cells along the entire length of the left cortex as well as in the medulla of both the left and right female gonads exhibited punctate phospho-H2AFX staining, but showed no-to-low nuclear SYCP3 staining (Fig. 3A,B; Supplementary Figure S2). In the cortex of the left female gonad, however, phospho-H2AFX is downregulated between HH38 and HH42, and germ cells in the cortex center showed either punctated nuclear SYCP3 or both SYCP3 and phospho-H2AFX staining at HH42 (Fig. 3C,Ci-ii). Germ cells maintained the
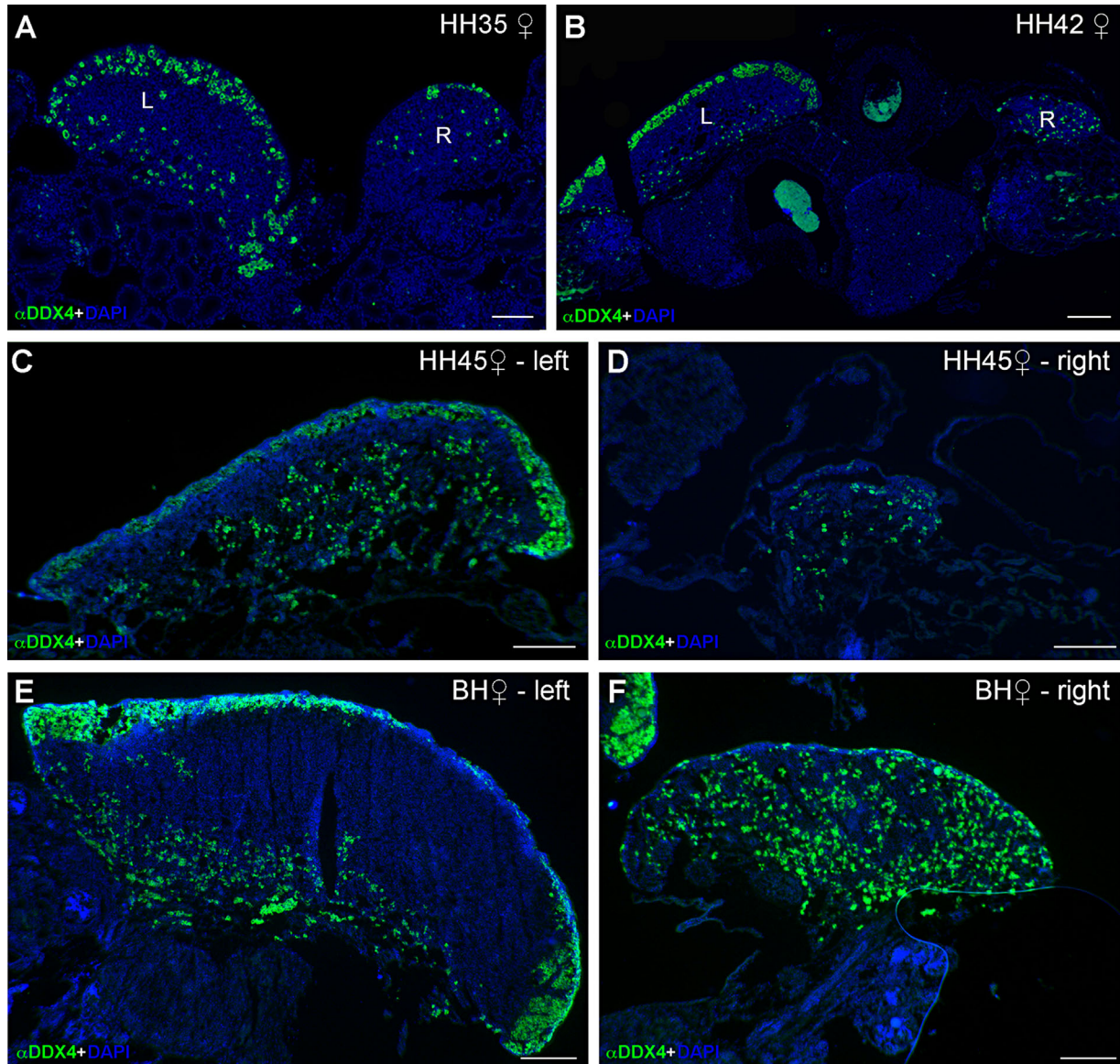
Figure 2. Distribution of DDX4-positive germ cells in left and right female gonads from HH35 to before hatching. DDX4 (green) expression in transverse sections of female gonads showing the distribution of germ cells in the left (L) and right (R) gonad at (**A**) HH35, (**B**) HH42, (**C**, **D**) HH45, and (**E**, **F**) before hatching (BH). Scale bars, 200 μm (A, C−F) and 500 μm (B).

characteristic phospho-H2AFX pattern in the extremities of the left cortex and in the medulla of both the left and right female gonads, as first observe at HH38 (Fig. 3C, Ciii-iv,D, Di). Combining our localization data using intact gonads with previous data from chromosome spreads from dispersed germ cells (Schoenmakers et al., 2009; del Priore and Pigozzi, 2012; Guioli et al., 2012), we concluded that the expression pattern of phospho-H2AFX and SYCP3 at HH38 corresponded to germ cells in pre-leptotene. Germ cells located in the central and lateral part of the left cortex at HH42 are either in early leptotene (punctated SYCP3 and no-to-low phospho-

H2AFX) or in late leptotene/early zygotene (punctated SYCP3 and phospho-H2AFX), while those at the extremities of the left cortex and all medullary germ cells are still in pre-leptotene (no SYCP3 and high phospho-H2AFX).

At HH45, phospho-H2AFX and SYCP3 staining along the left cortex showed that germ cells in late leptotene/early zygotene (punctated SYCP3 and phospho-H2AFX) occupied the central part of the cortex and were expanding to most of the lateral parts of the cortex, whereas the number of germ cells in early leptotene (punctated SYCP3 and no-to-low phospho-H2AFX) were fewer
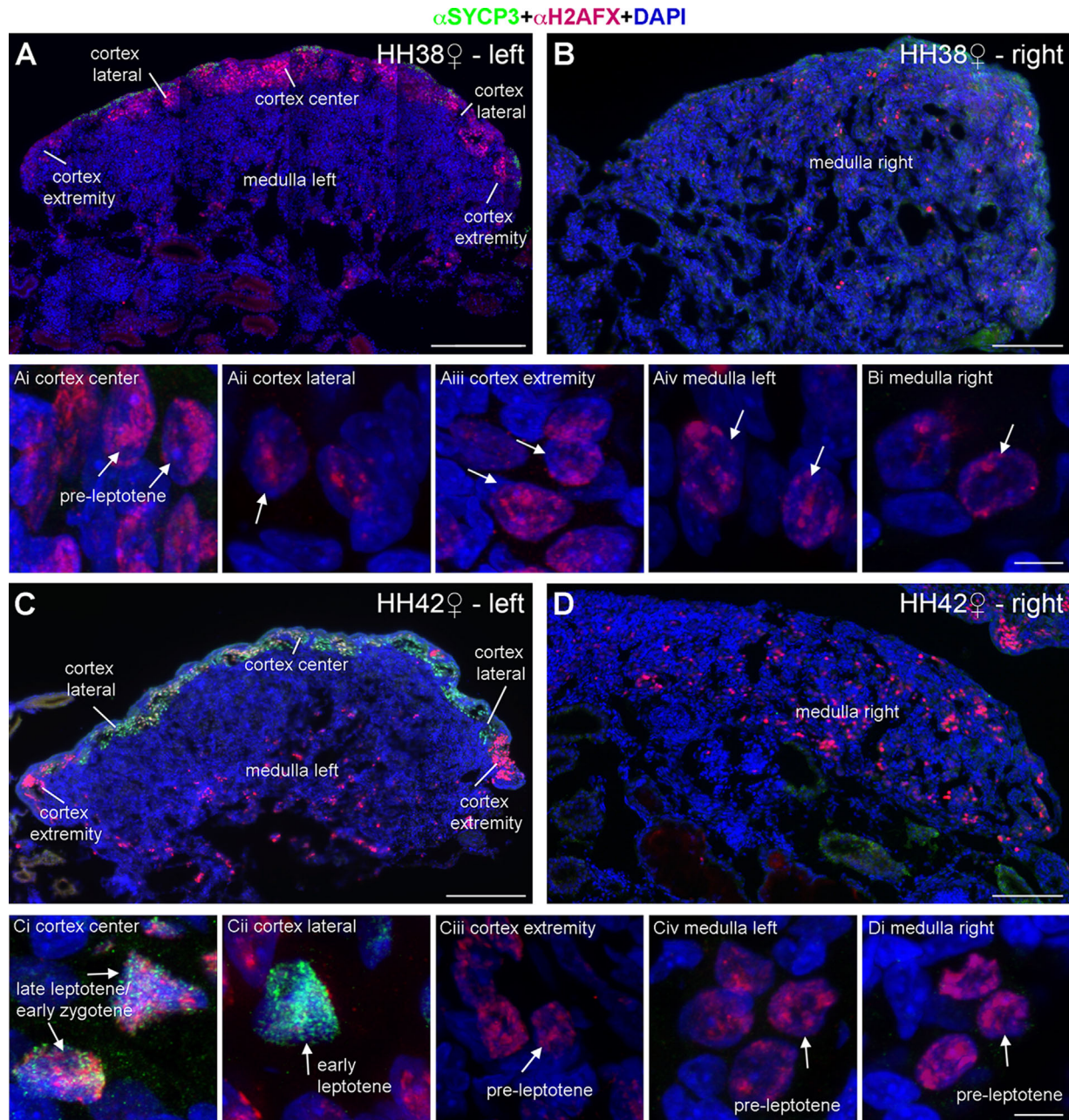
Figure 3. SYCP3 and phospho-H2AFX expression in female gonads at HH38 and HH42. SYCP3 (green) and phospho-H2AFX (red) expression in HH38 female (**A**) left and (**B**) right gonads, and HH42 female (**C**) left and (**D**) right gonads. High magnifications are representative from three different regions 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, 100 μm (**A-D**) and 5 μm (Ai-iv, Bi, Ci-iv, Di).

and restricted to a narrow band adjacent to the pre-leptotene germ cells (no SYCP3 and high phospho-H2AFX) in the cortical extremities (Fig. 4A,Ai-iii; representative higher magnifications in Supplementary Figure S3). This pattern was less pronounced along the long axes of the cortex of the left gonad, although we could still find pockets of phospho-H2AFX-positive, SYCP3-negative germ cells at the cortical extremities (Supplementary Figure S4).

Since several studies on chromosome spreads have described germ cells in pachytene around hatching (Schoenmakers et al., 2009; del Priore and Pigozzi, 2012; Guioli et al., 2012), we extended our analysis to female gonads just before hatching. At this stage, the
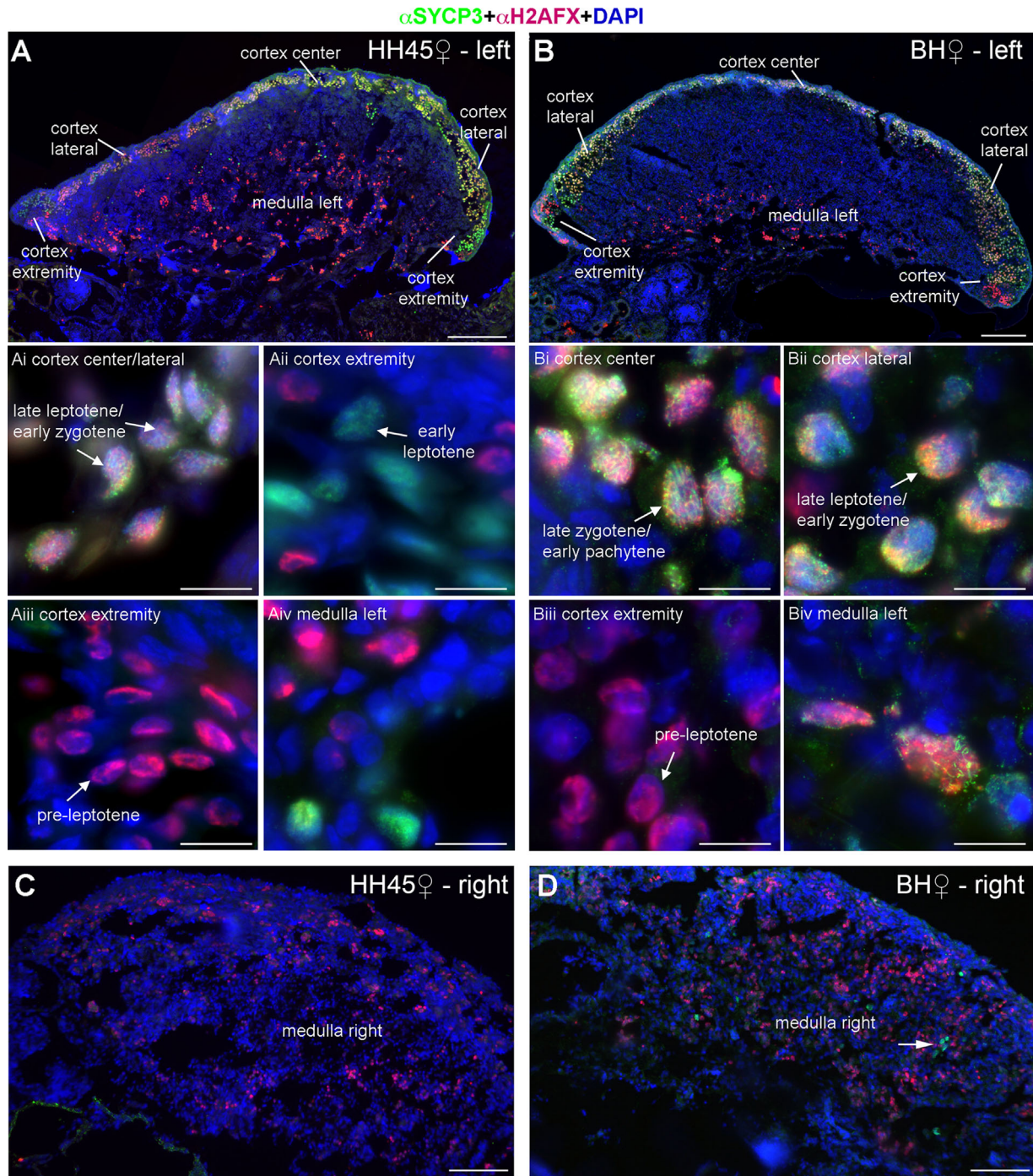
Figure 4. SYCP3 and phospho-H2AFX expression in female left and right gonads at HH45 and before hatching. SYCP3 (green) and phospho-H2AFX (red) expression in HH45 female (**A**) left and (**B**) right gonads and before-hatching female (**C**) left and (**D**) right gonads. High magnifications are from 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). **D**: The white arrow points to a cluster of germ cells that up-regulated SYCP3. Scale bars, 100 μm (**A-D**) and 10 μm (Ai-iv, Bi-iv).

central part of the cortex indeed contained many germ cells in late zygote/early pachytene (containing clear SYCP3-positive synaptonemal complexes, but still expressing phospho-H2AFX) (Fig. 4B,Bi; representative

higher magnifications in Fig S3). The lateral part of the left cortex still contained germ cells in late leptotene/early zygotene, flanked by germ cells in the cortical extremities in early leptotene and pre-leptotene (Fig. 4B,Bii-iii).
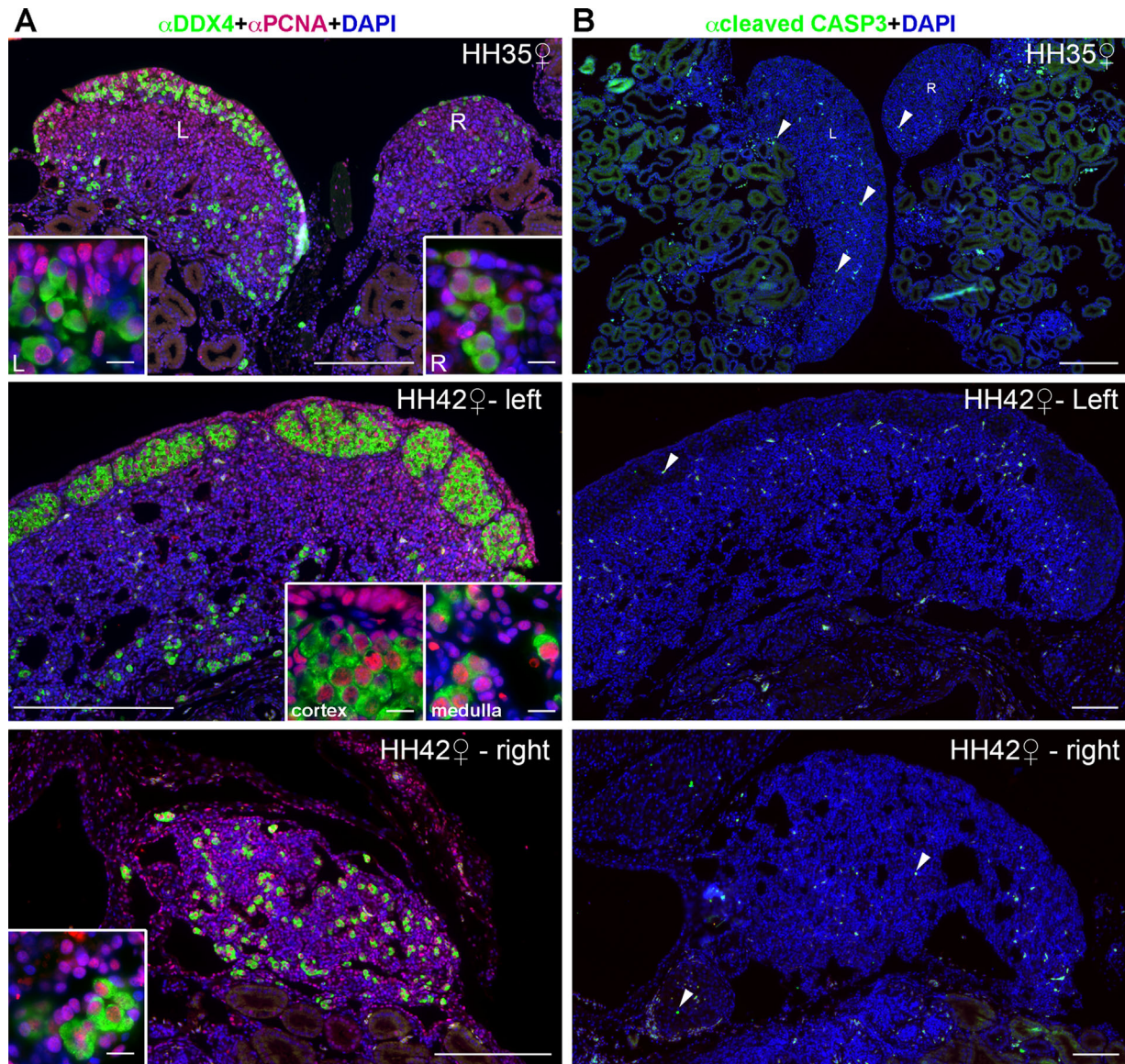
Figure 5. Expression of PCNA and cleaved CASP3 in chicken female gonads at HH35 and HH42. **A**: PCNA (red) expression in female left (L) and right (R) gonads at HH35 and HH42. Most germ cells (DDX4-positive cells) at HH35 and HH42 are PCNA-positive. Insets show a magnified detail. **B**: Cleaved CASP3 expression (green) in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to cleaved CASP3-positive cells. Scale bars, 200 μm (A, HH35 and HH42 right gonad) and 500 μm (A, HH42 left gonad); 200 μm (B, HH35) and 100 μm (B, HH42 left and right gonad); and 10 μm (insets).

Medullary Germ Cells in the Left and Right Female Gonads Are Not Apoptotic, but Remain in Pre-Leptotene Until Hatching

Germ cells localized in the medulla of both left and right gonads at HH45 and before hatching (Fig. 4C,D) showed nuclear features similar to the pre-leptotene germ cells (no SYCP3 and high phospho-H2AFX) located in the extremities of the left cortex, and are most probably in pre-leptotene. Interestingly, some small clusters of germ cells in the left and right gonadal medulla were either positive for SYCP3 (Fig. 4Aiv,D, white arrow) or expressed both

SYCP3 and phospho-H2AFX (Fig. 4Biv) from HH45 to before hatching, suggesting that some medullary germ cells may in fact be entering meiosis.

As phosphorylated H2AFX is associated with DNA double-strand breaks both during apoptosis and meiosis, we wanted to better discriminate between these two processes to understand the fate of left and right medullary germ cells. First, we investigated the expression of PCNA (proliferation cell nuclear antigen), a marker for DNA replication in mitosis that is also involved in DNA replication during meiotic prophase (Stone et al., 2008). At HH35 to HH42, the

majority of germ cells expressed PCNA (Fig. 5A and Supplementary Figure S5). The expression of both phospho-H2AFX and PCNA in medullary germ cells suggested that these cells were in pre-leptotene instead of undergoing apoptosis. To further distinguish between apoptosis and meiosis, we investigated the expression of the early apoptosis marker CASP3 (cleaved caspase 3) and assessed DNA fragmentation during late apoptosis with the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay. We found only a few cleaved CASP3-positive cells in HH35 and HH42 female gonads (Fig. 5B, white arrowheads) and a few TUNEL-positive germ cells and somatic cells (Fig. 6A, white arrowheads), but no massive loss of germ cells in the medulla, which has been previously reported using electron microscopy (Ukeshima and Fujimoto, 1991; Ukeshima, 1996). Furthermore, the germ cells at the surface of the right gonad at HH35 and HH42 are clearly not in early or late stages of apoptosis (Fig. 6A, white arrows). Thus, germ cell apoptosis is not the cause of the absence of a germ cell-rich cortex in the right female gonad. Until hatching, we detected few CASP3- or TUNEL-positive cells in both gonads (data not shown).

Finally, we examined the expression of FASLG (FAS ligand), a marker involved in the FASLG-FAS system that can induce apoptosis in mammalian spermatocytes undergoing meiosis (Kuerban et al., 2012; Aitken and Baker 2013). Interestingly, germ cells in the extremities of the left cortex and some in the left and right medulla at HH42 seem to upregulate FASLG (Fig. 6B, white arrowheads). Before hatching, many presumed germ cells in both the left and right medullas became strongly positive for FASLG (Fig. 6C, white arrows), suggesting that a FASLG-FAS-dependent apoptosis may occur in that female germ cell population after hatching.

DISCUSSION

Sex-Independent Preferential Migration of Primordial Germ Cells to the Left Side

Our results and those of others (Van Limborgh 1968; Nakamura et al., 2007; Intarapat and Stern, 2013) show that chicken primordial germ cells 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, such as *BMP7* (bone morphogenic protein 7), which is expressed in the left gonad around HH21 (3.5 days of incubation) in both sexes (Hoshino et al., 2005), and/or the transcription factor *PITX2* (paired-like homeodomain 2), which is expressed in the left gonad around HH18 (3 days of incubation) in both sexes (Smith et al., 2003; Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Rodriguez-Leon et al., 2008). Transplantation experiments performed by Naito et al. (2009) —involving the transfer of primordial germ cells from the bloodstream of HH14-15 donor embryos to the bloodstream of age-matched recipient embryos, followed by analysis at 16.5 days of incubation (about HH42)— suggested that female primordial germ cells preferentially migrate to the left side of both female and male embryos, although female embryos attracted more primordial germ cells from either sex to the left gonad.

It is interesting to note that 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 (Mittwoch and Mahadevaiah, 1980). Thus, an initial sex-independent left-right asymmetry may be conserved at least between humans and chicken.

No Evidence for Apoptosis in Chicken Medullary Germ Ccells Until Before Hatching

Left-right asymmetry of the chicken female gonads was first reported in morphological detail over 50 years ago (Hughes, 1963). Since the right gonad of the chicken embryo was considered a degenerating structure that contained apoptotic germ cells (Ukeshima, 1996; Gonzalez-Moran, 2011), its study has been neglected. Yet Ishimaru et al. (2008) analysed apoptosis levels at HH27 and HH29, and observed a reduced number of TUNEL-positive germ cells in the cortex and medulla on both sides; we extended this observation, and analysed apoptosis in both sexes between HH35 and hatching in the right and left gonad. Consistent with their observations, we were unable to detect signs of robust apoptosis in the germ cells in the right gonad. Instead, our data suggest that medullary germ cells are not apoptotic, but largely remain in pre-leptotene until hatching—although a few may manage to enter meiosis. The source of the discrepancy of our findings with previous literature may lie in the fact that we used a combination of meiotic and apoptotic markers together with a robust germ-cell marker, whereas only histological sections imaged by light microscopy of transmission electron microscopy were analyzed in previous reports. Further supporting our observation is the report that overexpression of *PITX2* in ovo in the right female gonad from HH8-10 is sufficient to induce robust cortex formation that is morphologically similar to that of the left gonad, including H2AFX-positive germ cells, at HH38-39 (Guioli and Lovell-Badge, 2007). This indicates that germ cells in the right female gonad have the capacity to develop into a normal cortex and enter meiosis if given the right environment. We therefore suggest that until hatching, germ cells in both gonads have an equal potential to develop. In males, the germ cells are equally likely to develop, whereas in females these cells only receive the correct molecular signals (via PITX2) to develop in the central and lateral part of the cortex of the left gonad.

Interestingly, we detected expression of FASLG in phospho-H2AFX-positive germ cells in the extremities of the left cortex and medulla. While FASLG-positive cells are not undergoing apoptosis, they are potential targets for destruction; indeed, the FASLG-FAS system has been shown to induce apoptosis in mammalian spermatocytes undergoing meiosis (Kuerban et al., 2012; Aitken and Baker, 2013). Even though the role of the FASLG-FAS system during early oogenesis is not well studied, it does
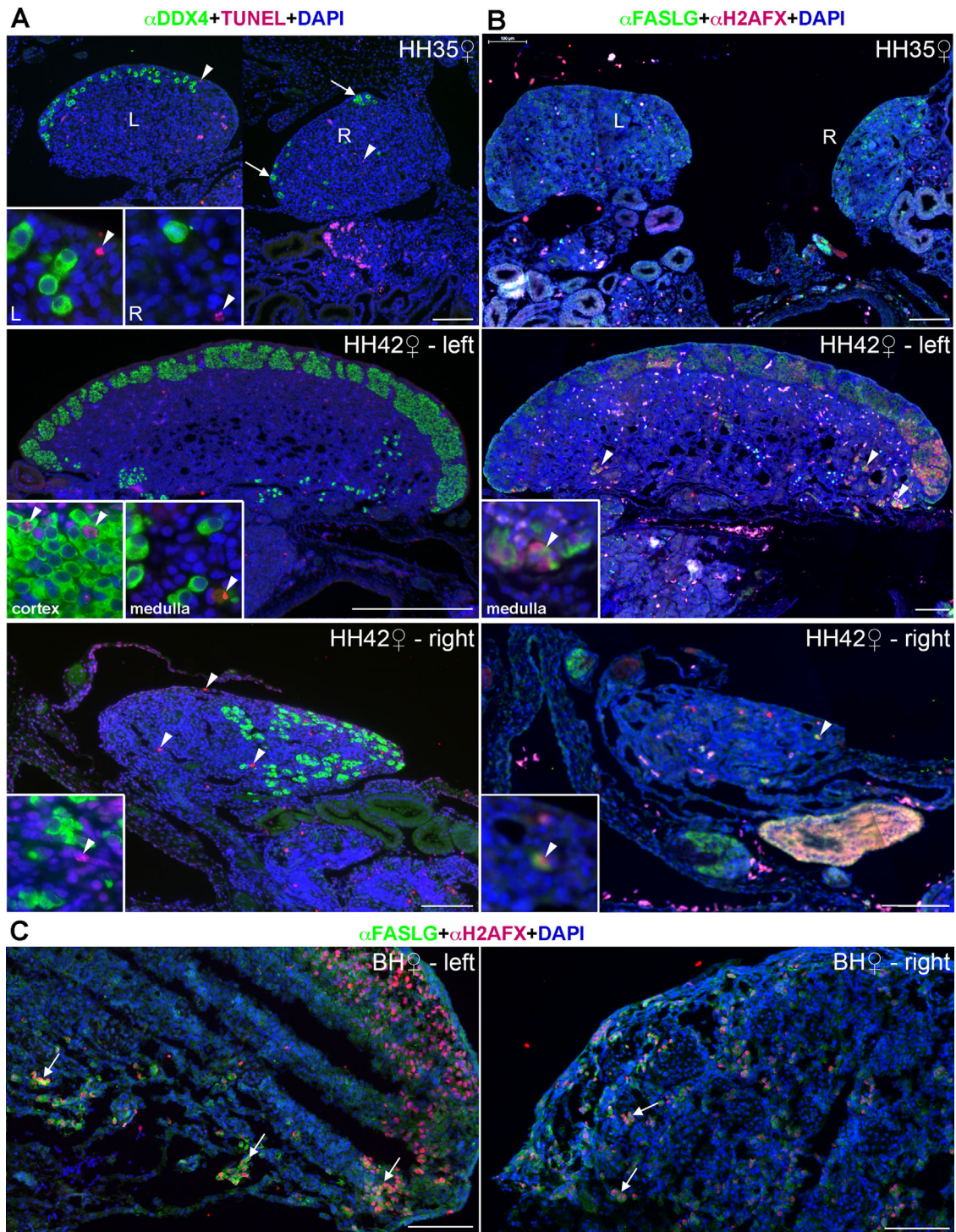
Figure 6. TUNEL and FASLG expression in female gonads. **A**: TUNEL labeling (red) shows late apoptotic cells in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to apoptotic somatic cells and germ cells (green, DDX4-positive). Insets show a magnified detail. White arrows point to TUNEL negative germ cells in the surface of the HH35 right gonad. **B**: FASLG (green) and phospho-H2AXF (red) expression in female left (L) and right (R) gonads at HH35 and HH42. White arrowheads point to double-positive FASLG and phospho-H2AXF cells. **C**: FASLG (green) and phospho-H2AXF (red) expression in female left and right gonads before hatching (BH). White arrows point to double-positive FASLG and phospho-H2AXF cells. Scale bars, 100 μm (**A-B**); 200 μm (**C**); or 10 μm (insets).

participate in the process of atresia during different stages of folliculogenesis (Guo et al., 1997; Mor et al., 2002). Whether or not germ cells in the extremities of the left cortex and medulla are eventually eliminated by apoptosis after hatching via a FASLG-FAS-dependent mechanism remains to be investigated.

Meiotic Wave in the Chicken Left Cortex From the Center to the Extremities

The asymmetry between left and right female gonads is primarily orchestrated by *PITX2* expression (Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Rodriguez-Leon et al., 2008). Thereafter, meiotic entry in the chicken seems to be directly related to the synthesis and breakdown of retinoic acid (Smith et al., 2008; Yu et al., 2013). Several authors have described asynchronous meiotic staging in chromosome spreads of chicken germ cells from late developmental stages through the first week post-hatching (Schoenmakers et al., 2009; del Priore and Pigozzi, 2012; Guioli et al., 2012). Others have reported immunostaining in intact gonads for either H2AFX or SYCP3 separately, but not in combination (Guioli and Lovell-Badge, 2007; Smith et al., 2008; Zheng et al., 2009; Yu et al., 2013; Guioli et al., 2014). Consequently, the existence of a meiotic wave in chicken has 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" (Hughes, 1963). Our systematic study of the expression of phospho-H2AFX and SYCP3, from HH38 until hatching, revealed the existence of a meiotic wave in the left cortex (Fig. 7). A meiotic wave has also been described in female mice from the anterior to the posterior part of the gonad

during mid-gestation (Menke et al., 2003; Yao et al., 2003), and even in humans it seems to occur from the inside to the outside of the gonadal cortex during the second trimester of development (AM Heeren and SM Chuva de Sousa Lopes, unpublished). Therefore, a meiotic wave in the developing female gonad may be a conserved event among vertebrates.

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 according to Hamburger and Hamilton (1992). The sex of HH35 embryos until prior to hatching was determined by eye, whereas the sex of HH16-30 embryos was determined by genomic PCR, as previously described (Griffiths et al., 1998). Embryos were isolated and used whole from HH16-HH30, whereas the paired gonads were further dissected out of the embryo from HH35 until prior to hatching.

Immunofluorescence on Whole-Mount Embryos and Paraffin Tissue Sections

Embryos and gonads were fixed overnight at 4°C in 4% paraformaldehyde (MERCK, Germany) in phosphate-buffered saline (PBS). Immunofluorescence on HH16-HH19 embryos (n = 7) was performed as whole mount, and on HH25-HH42 embryos (n = 14) using paraffin sections, essentially as previously described (De Melo Bernardo et al., 2012; Locher et al., 2013).

Primary antibodies used were rabbit anti-DDX4 (1:500 dilution; gift from T Noce); mouse anti-PCNA (1:500 dilution;
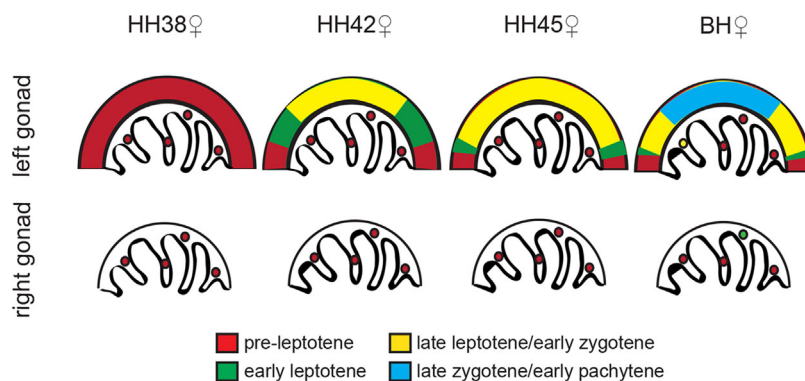

Figure 7. Proposed model of a meiotic wave occurring from HH38 until hatching in the female left and right gonads. At HH38, all germ cells—independent of their location—express H2AFX and are in preleptotene. From HH42 until before hatching (BH), the germ cells in the left gonadal cortex exhibited a pronounced meiotic wave from the central part of the cortex to its extremities. Just before hatching, germ cells localized in the central and lateral part of the left cortex are in late zygote/early pachytene and late pachytene/early zygotene while 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, even just before hatching.

sc-56, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-cleaved CASP3 (Asp 175) (1:300 dilution; 9661S; Cell Signaling Technology, Danvers, MA); mouse anti-H2AFX (phospho-Ser139) (1:500 dilution; 05-636, Millipore, Teme-cula, USA); and rabbit anti-FASLG (1:100 dilution; sc-6237, Santa Cruz Biotechnology). Secondary antibodies used were Alexa Fluor 568 goat anti-mouse (1:500 dilution; A-21124, Molecular Probes, Eugene, OR) and Alexa Fluor 488 goat anti-rabbit (1:500 dilution; A11008, Life Techno-logies, UK). DNA fragmentation was determined by TUNEL assay with a TMR red In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany), following manufacturer's instructions. The development reaction was performed for 1 hr at 37°C. All slides were counter-stained with a 1:1000 dilution of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies) in PBS for 1 min, and then mounted with ProLongGold antifade reagent (Life Technologies).

Immunofluorescence on Cryosections

Paired gonads from HH35 to before hatching (n = 19) were fixed for 10 min at room temperature in a 4% parafor-maldehyde-PBS solution, washed three times in PBS, cry-oprotected overnight at 4°C in 30% sucrose in PBS, transferred to optimal-cutting-temperature (OCT) com-pound (Tissue Tek, Zoeterwoude, The Netherlands), and frozen at −80°C. The gonads were sectioned (10 μm) using a CM3050S cryotome (Leica Instruments GmbH, Nussloch, Germany), and mounted on Superfrost-plus slides (Thermo Scientific, Karlsruhe, Germany). Cryosections were washed with PBS, and then blocked for 1 hr at room temperature in 1% bovine serum albumin (BSA)-PBS. The rest of the procedure was as described above for paraffin sections. Primary antibodies used were rabbit anti-SYCP3 (1:500 dilution; NB300-232, Novus Biologicals, Littleton, CO), rab-bit anti-FASLG (1:100 dilution; sc-6237, Santa Cruz Bio-technology), and mouse anti-H2AFX (phospho-Ser139) (1:500 dilution; 05-636, Millipore). The same secondary antibodies were used, as described above.

Imaging

Slides were either analysed on a Leica DMRA fluores-cence microscope (Leica) and pictures taken with a Cool-Snap HQ2 camera (Photometrics, Tucson, AZ) or scanned using a Panoramic MIDI digital scanner (3DHISTECH, Hungary). Representative areas for images were selected using the software program 'Panoramic viewer' (3DHIS-TECH, Budapest, Hungary). Optical sections were taken on a Leica TCS SP8 confocal microscope (Leica, Man-nheim, Germany) operating under the Leica Application Suite Advanced Fluorescence software (Leica).

Germ Cell Counting and Flow-Cytometry Analysis

Germ cells were counted in the right and left gonad in whole-mount HH16-HH19 embryos (n = 3 females; n = 4

males) and in sequential paraffin sections of HH25-HH30 embryos (n = 3 females; n = 3 males) immunostained for DDX4. The percentage of primordial germ cells 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 vari-ance) was used to compare the percentage of DDX4-positive germ cells between the right and left gonads. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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 then isolated in DPBS0 (Life Technologies). To obtain single-cells, gonads were first cut into small pieces and then incubated at 37°C for 30 min in TRIPLE 5x (Life Technologies), with occasion-al pipetting. The single-cell suspension was washed in DPBS0, and cells were fixed for 10 min on ice in 4% paraformaldehyde. Thereafter, cells were permeabilized for 30 min on ice using 0.1% Triton/DPBS0, incubated for 1 hr on ice with a 1:1000 dilution of rabbit anti-DDX4 (gift from T Noce) in blocking solution (1% BSA in 0.05% Tween/ DPBS0), washed with DPBS0, and incubated for 1 hr on ice with a 1:500 dilution of Alexa Fluor 488 goat anti-rabbit in blocking solution. After a final wash in DPBS0, cells were resuspended in flow-cytometry buffer (1% BSA, 10 mM EDTA in PBS). Fluorescence-activated cell sorting analy-sis was performed using a BD FACSAriaTM III (BD Bio-Sciences, Erembodegem, Belgium). Results were processed using the software BD FACSDivaTM version 6.0 (BD BioScience).

Quantitative Reverse-Transcriptase PCR

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 a RNeasy Micro Kit (Qiagen, Hilden, Germany), and cDNA was made using the iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA). Quantitative PCR was performed using iQ™ SYBR® Green Supermix (Biorad) on a CFX96™ Real-time system, C1000™ Thermal Cycler (Biorad) with the conditions: 95°C for 3 min; 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec; and a final melting curve at 95°C for 10 sec, 65°C for 5 sec, and 95°C for 50 sec. The primers for DDX4, DAZL (deleted in azoospermia-like), and the housekeeping genes RPS17 (ribosomal protein S17) and ACTB (beta actin) were described elsewhere (Lavial et al., 2009; Caetano et al., 2014). All reactions were performed in triplicate. Data were normalized using the ΔΔCt method, and are presented relative to the right male gonads.

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