© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 1354-1365 doi:10.1242/dev.104828

RESEARCH ARTICLE

The APC/C activator FZR1 is essential for meiotic prophase I in mice

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ABSTRACT

Fizzy-related 1 (FZR1) is an activator of the Anaphase promoting complex/cyclosome (APC/C) and an important regulator of the mitotic cell division cycle. Using a germ-cell-specific conditional knockout model we examined its role in entry into meiosis and early meiotic events in both sexes. Loss of APC/C^{FZR1} activity in the male germline led to both a mitotic and a meiotic testicular defect resulting in infertility due to the absence of mature spermatozoa. Spermatogonia in the prepubertal testes of such mice had abnormal proliferation and delayed entry into meiosis. Although early recombination events were initiated, male germ cells failed to progress beyond zygotene and underwent apoptosis. Loss of APC/C^{FZR1} activity was associated with raised cyclin B1 levels, suggesting that CDK1 may trigger apoptosis. By contrast, female FZR1Δ mice were subfertile, with premature onset of ovarian failure by 5 months of age. Germ cell loss occurred embryonically in the ovary, around the time of the zygotene-pachytene transition, similar to that observed in males. In addition, the transition of primordial follicles into the growing follicle pool in the neonatal ovary was abnormal, such that the primordial follicles were prematurely depleted. We conclude that APC/C^{FZR1} is an essential regulator of spermatogonial proliferation and early meiotic prophase I in both male and female germ cells and is therefore important in establishing the reproductive health of adult male and female mammals.

KEY WORDS: FZR1, Mouse, Meiosis, Spermatogenesis, Oogenesis, APC/C, CDH1

INTRODUCTION

Despite temporal differences in their meiotic programs, both sexes and indeed all eukaryotes, have a requirement for a prolonged prophase I to allow for homologous chromosome organization. In yeast, this is achieved through targeted proteolysis of M-phase promoting proteins by the Anaphase promoting complex/cyclosome (APC/C) complex, together with a meiosis specific coactivator protein, AMA1 (Cooper et al., 2000; Okaz et al., 2012). The mammalian meiotic program is punctuated by more complex 'stops' and 'starts' than those of lower eukaryotes, and the regulatory mechanisms behind these events – such as whether the meiotic role of the APC/C is conserved, has remained largely unexplored.

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Received 12 October 2013; Accepted 15 January 2014

In mammals, germ cell development encompasses a highly coordinated series of cell cycle changes. In the female, meiosis is initiated embryonically such that by birth oocytes are arrested at dictyate stage of prophase I within a primordial follicle (Adams and McLaren, 2002). Meiosis I is re-initiated at the time of ovulation but arrested once again at metaphase II until fertilization. By contrast, after a period of mitotic proliferation, after sex determination, embryonic male germ cells (gonocytes) enter quiescence. Postnatally they migrate to the basement membrane, re-enter the cell cycle and develop into undifferentiated spermatogonial cells. Some gonocytes establish the self-renewing stem cell population whereas others become differentiated spermatogonia that complete transit amplifying mitotic divisions and then enter meiosis, completing the two nuclear divisions in succession before undergoing terminal differentiation to form spermatids and finally, post-spermiation, spermatozoa (Hilscher et al., 1974; Oatley and Brinster, 2012).

Recognized as essential for mitotic progression, the APC/C, an E3 ubiquitin ligase, has an established role in the cell cycle promoting the metaphase-anaphase transition (Peters, 2006). In mammals, substrate specificity is determined by the binding of one of two coactivators, cell division-cycle protein 20 (CDC20) or FZR1 through discrete binding motifs (Pfleger and Kirschner, 2000; Zur and Brandeis, 2002; Reis et al., 2006b). APC/C^{CDC20} activity appears to be restricted to metaphase, where it has an essential function in promoting chromosome segregation by mediating cyclin B1 and securin degradation. Although CDC20 is the preferred APC/C coactivator in metaphase when cyclin-dependent kinase 1 (CDK1) activity is high, FZR1 is more promiscuous. APC/C^{FZR1} is found when CDK1 activity is low (Peters, 2006; Thornton and Toczyski, 2006) and is thought to regulate a wide range of cell cycle events, including G1/G0 maintenance, initiation of DNA replication, cell differentiation, signal transduction and the DNA damage response (García-Higuera et al., 2008; Qiao et al., 2010; Cotto-Rios et al., 2011; Tudzarova et al., 2011; Wan et al., 2011; Takahashi et al., 2012). More than 30 proteins have been identified as APC/C^{FZR1} substrates, including mitotic cyclins, cell cycle kinases and phosphatases, DNA replication proteins and metabolic proteins (reviewed by Qiao et al., 2010; Wäsch et al., 2010).

A meiotic role for the APC/C has been established in budding yeast, which relies upon the coactivator AMA1 (Okaz et al., 2012). However, although mammals appear to lack an AMA1 homolog, a role for APC/C^{FZR1} activity in mammalian meiosis is emerging. We, and others, have identified that FZR1 is important for maintaining the dictyate-stage arrest of fully-grown mammalian germinal vesicle (GV) oocytes, as well as the timing and fidelity of the first meiotic division (Reis et al., 2006a; Marangos et al., 2007; Reis et al., 2007; Yamamuro et al., 2008; Homer et al., 2009; Schindler and Schultz, 2009; Holt et al., 2011; Holt et al., 2012). Using a germ-cell-specific Cre-loxP model of FZR1 loss, we now report that APC/C^{FZR1}

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activity plays an essential role in male fertility and in maintaining the normal reproductive lifespan of the female.

RESULTS

FZR1 protein is expressed in male and female germ cells

To examine the expression pattern of FZR1 in the testes, we performed immunohistochemical detection on adult testis sections. FZR1 protein was ubiquitously expressed, with prominent immunostaining of germ cells in tubules of different stages and therefore representing the majority of germ cell stages (Fig. 1A). Immunoblotting of isolated populations of male germ cells confirmed the presence of FZR1 in meiotic pachytene spermatocytes, as well as postmeiotic round spermatids and the mitotic spermatogonial population (Fig. 1B). FZR1 protein was also present in the primordial follicle oocytes and mitotic cells of the PND7 ovary (supplementary material Fig. S1). Therefore, this APC/C activator in males is present in germ cells from the spermatogonial stage onwards and in females is also present in quiescent oocytes not examined previously.

Generation of a germ cell-specific knockout of FZR1

Detection of FZR1 protein in male and immature female germ cells suggested APC/C^{FZR1} may have an important role in spermatogenesis and oogenesis. Consistent with this hypothesis, preliminary examination of FZR1-deficient Sox2-Cre/FZR1$^{\Delta/-}$ mice (see supplementary material Table S2) revealed abnormalities in the gonads of both sexes. In the Sox2-Cre model, Cre is expressed in most cells of the developing embryo except those of the placenta, which prevents the embryonic lethality associated with complete embryonic FZR1 deficiency (García-Higuera et al., 2008). FZR1-deficient males and females showed gonadal atrophy, which was particularly severe in males (supplementary material Fig. S2). Additionally 8-week-old male or female FZR1-deficient mice mated with wild-type animals over a period of 4 months produced no litters. To examine in more detail the contribution of FZR1 to the meiotic program, we went on to develop a mouse model in which FZR1 deletion was restricted to germ cells, as mutant somatic cells of Sox2-Cre/FZR1$^{\Delta/-}$ may have contributed to the reproductive phenotype of these animals.

Mice possessing floxed *Fzr1* alleles (*Fzr1*$^{loxP/loxP}$) (García-Higuera et al., 2008) were mated with those expressing Cre under the control of the DEAD (Asp-Glu-Ala-Asp) box protein 4 (DDX4, also known as mouse vasa homolog, MVH promoter) (Fig. 1C). Expression of Cre recombinase in Ddx4-Cre mice begins at embryonic day 15.5 (E15.5) in both males and females (Soriano, 1999; Gallardo et al., 2007; John et al., 2008). In the male, this corresponds to the period of germ cell mitotic arrest, before meiotic entry. In the female almost all germ cells have entered meiosis by E15.5 to become oogonia at the zygotene or pachytene stages (Fig. 1C) (Kolas et al., 2005).

All mice from *Fzr1*$^{loxP/loxP}$ × Ddx4-Cre matings were born at expected Mendelian frequencies (supplementary material Table S1) and pups were viable and overtly phenotypically normal. As anticipated, FZR1 protein levels in nonreproductive tissues were equivalent to wild-type animals (supplementary material Fig. S3), but significantly decreased in testicular tissue of young FZR1Δ males (Fig. 1D) and we assume that the residual FZR1 is from Leydig and interstitial cells (Fig. 1Ai). No immunoreactivity was observed in GV oocytes isolated from the ovaries of 24-day-old females, confirming successful deletion of FZR1 in these animals (Fig. 1D).

Loss of FZR1 results in complete male infertility

To determine if premeiotic FZR1 loss had any impact on male fertility, we mated 6- to 8-week-old males FZR1Δ, FZR1+/Δ, and their control littermates with wild-type (C57Bl6) females, over a period of 4 months. Control and FZR1+/Δ males all produced at least five litters over this period whereas FZR1Δ males appeared infertile (Fig. 2A). Examination of juvenile and adult male testes

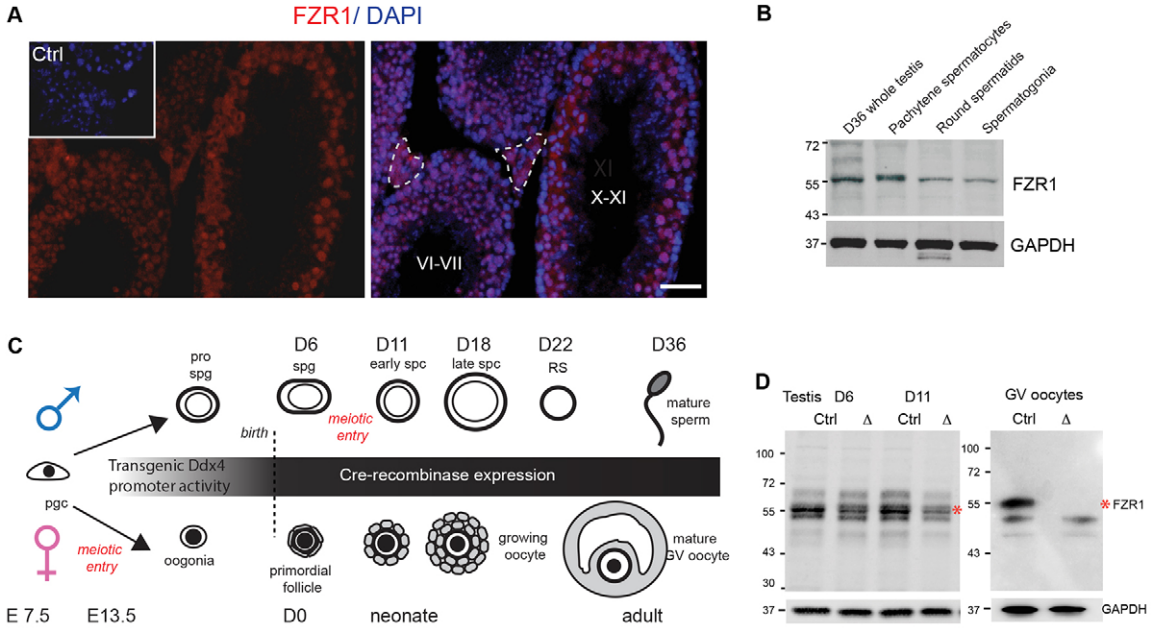

Fig. 1. FZR1 protein expression and conditional gene knockout by Cre-loxP recombination in mouse germ cells. (A) FZR1 immunostaining of adult mouse testes showing ubiquitous expression of the protein. The interstitial tissue is outlined with a dashed line. (B) Representative immunoblot for FZR1 in whole juvenile testis and isolated germ cells; GADPH used as a control for protein loading. (C) Schematic model for *Ddx4*-driven Cre recombinase-induced loss of FZR1 in male and female germ cells. (D) FZR1 immunoblotting in FZR1Δ whole neonatal testis and isolated oocytes from D24 females. Scale bar: 50 µm. E, embryonic day; D, postnatal day; pgc, primordial germ cell; RS, round spermatid; spc, spermatocyte; spg, spermatogonia.

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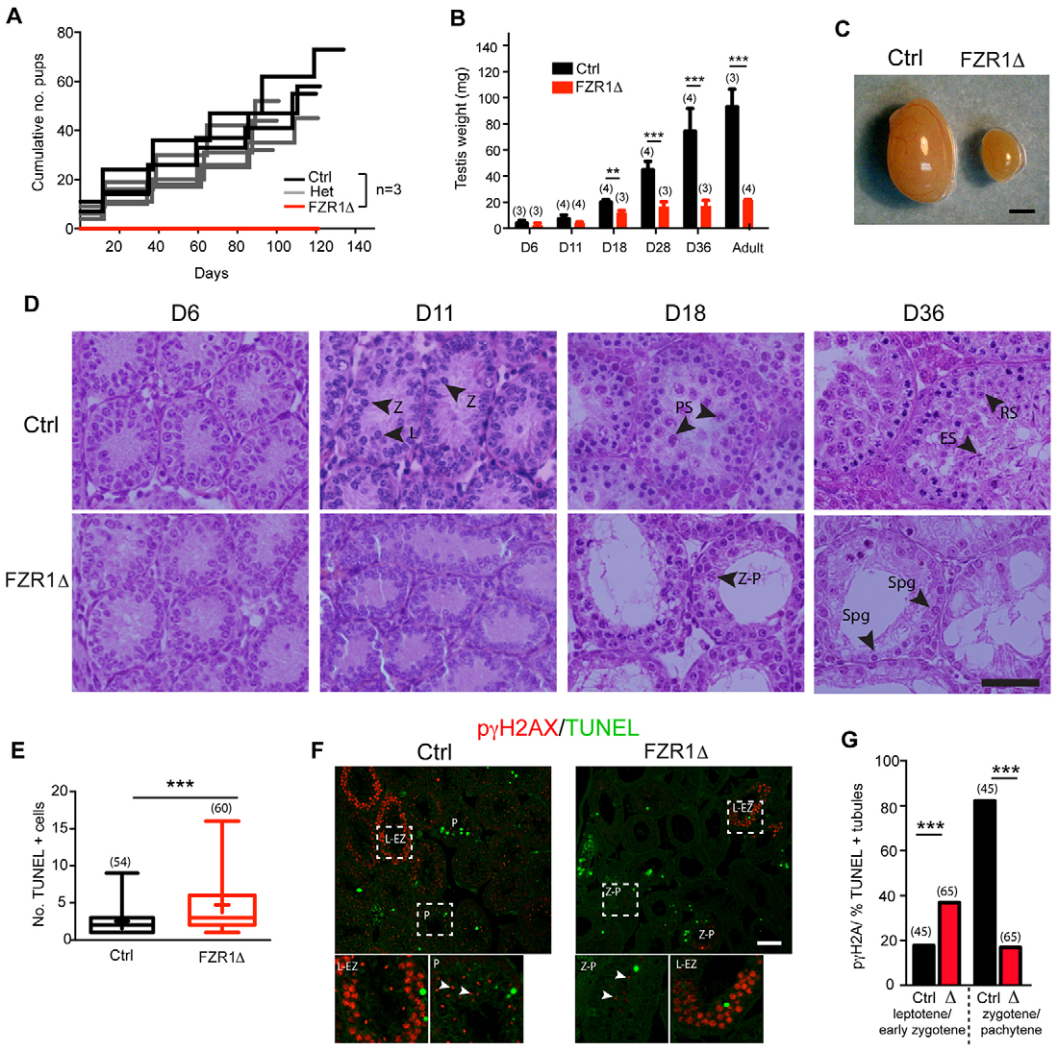

Fig. 2. Infertility in FZR1Δ males is a consequence of germ cell depletion before adulthood. (A) Cumulative number of pups born to mated FZR1Δ (Ddx4-Cre/Fzr1$^{\Delta/\Delta}$), heterozygote (Ddx4-Cre/Fzr1$^{\Delta/wt}$) or control Fzr1$^{loxP/loxP}$) males, where Day 0, equals day of first litter. (B) Mean testis weight for control and FZR1Δ mice between D6 and adulthood (**P<0.01, ***P<0.001, Student's t-test; error bars, s.d.; n=3-5 animals/genotype). (C) Control and FZR1Δ adult testes imaged immediately following recovery. (D) Histological cross sections of control and FZR1Δ testes. (E) Number of TUNEL-positive cells per tubule, from tubules possessing at least one TUNEL-positive cell. ***P<0.0001, Mann-Whitney U-test; n=3 animals/genotype. Mean shown as +; box outline, 25th/75th percentiles; centerline, median; whiskers, min./max. (F) Representative images of dual TUNEL-processed/pγH2AX immunostained D18 testis sections. (G) Percentages of TUNEL-positive tubules displaying L-EZ or Z-P type pγH2AX labelling for control and FZR1Δ D18 testes (***P<0.0001, Chi-squared test; n=3 animals/genotype). Parentheses, n animals/tubules examined. Scale bars: 500 µm in C; 50 µm in D. ES, elongating spermatid; L, leptotene; L-EZ, leptotene/early zygotene; PS, pachytene spermatocyte; RS, round spermatid; Spg, spermatogonia; Z, zygotene; Z-P, zygotene/pachytene like spermatocyte.

revealed no difference in fresh tissue weights at day 6 (D6) and D11; however, testes from FZR1Δ males at D18 or older were significantly smaller, such that by adulthood (7-8 weeks of age) mean testes weights were less than 25% of controls (Fig. 2B,C).

Histological examination revealed that D6 FZR1Δ testes possessed cells of germ-cell-like morphology located on the seminiferous tubule membrane similar to controls (Fig. 2D). At D11 leptotene and zygotene stage cells were clearly present in controls, identified by punctate intense chromatin staining in the nucleus, but few were observed in FZR1Δ testes. By D18 when large pachytene spermatocytes were evident in controls, FZR1Δ testes showed few cells of pachytene-like morphology, and instead tubules appeared depleted of the majority of germ cells (Fig. 2D). Additionally, transmission electron microscopy imaging of testis sections failed to identify any mature spermatocytes, but instead found evidence of degenerating and sloughed germ cells in these testes (supplementary material Fig. S4). Consistent with these cells being lost through apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) identified significantly more positively labeled cells predicted to be germ cells, per tubule by D18 in FZR1Δ testis (Fig. 2E). Additionally, immunolabelling with meiotic marker phosphorylated γ-histone 2AX (pγH2AX) was used to correlate meiotic stage with apoptosis. Tubules with leptotene/early zygotene cells were identified as having strong positive staining dispersed throughout the nuclei consistent with unrepaired recombination double-strand break (DSB), in contrast to zygotene/pachytene stages, which showed punctate regions of staining as pγ-H2AX became restricted to the asynapsed sex chromosomes in the XY body. More TUNEL-positive FZR1Δ tubules were found to possess germ cells that were predominantly at leptotene to early zygotene stage compared with controls (Fig. 2F,G; 38% versus 18%). By contrast, the majority of TUNEL-positive tubules of control animals

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had mid-pachytene-like pγH2AX labeling (82%), which is a distinguishing feature of the normal first spermatogenic wave (Jahnukainen et al., 2004).

The significant germ cell loss and developmental arrest observed in neonates was even more severe in the juvenile testes (D36), with most commonly only a single row of spermatogonial-like germ cells remaining in the basement compartment of the tubules of FZR1Δ testes (Fig. 2D). In FZR1Δ males aged to 10 months, no germ cells remained and tubules contained only Sertoli cells (supplementary material Fig. S5). Together these data indicate that in the absence of FZR1, there is both a progressive loss of spermatogonia with age, together with a defect in meiotic progression during the first spermatogenic wave.

APC/C^{FZR1} is required for normal spermatogonial proliferation

Loss of FZR1 has previously been associated with defective mitotic cells cycles such as those of *Fzr1*$^{-/-}$ mouse embryonic fibroblasts (MEFs), which spontaneously enter a quiescent state (García-Higuera et al., 2008; Li et al., 2008; Sigl et al., 2009). As spermatogonia must undergo several rounds of mitotic division before entry into meiosis, we hypothesized that FZR1Δ spermatogonia may have had a proliferation defect (Spradling et al., 2011), leading to the catastrophic loss of germ cells in the adult. We therefore immunostained for a marker of proliferating gonocytes and spermatogonia, cyclin D1, which is present at highest levels at the G1 and G2 phases of the cell cycle (Beumer et al., 2000; Yang et al., 2006). Intriguingly, no cyclin D1 was detected in tubules at D6, although immunosignal was detectable in a small number of spermatogonia by D11 (Fig. 3A). Loss of this marker suggested cell quiescence might be occurring in the absence of APC^{FZR1} activity. To further confirm this hypothesis, we examined two additional markers: KI67 (MKI67 – Mouse Genome Informatics), which is expressed at all dividing cell cycle stages; and phospho-histone H3(Ser10), which is present at G2/M transition and M phase (Gerdes et al., 1984; Hendzel et al., 1997). Similar to cyclin D1, these markers were absent or present at very low levels in the neonatal testis of FZR1Δ animals (supplementary material Fig. S6).

We next sought to determine whether expression of an important regulator of the spermatogonial stem cell pool, promyelocytic leukaemia zinc finger (PLZF; ZBTB16 – Mouse Genome Informatics) was altered in the absence of FZR1, which could account for the defect in spermatogonial function. A marker of the undifferentiated stem cell pool, transcriptional repressor PLZF is normally expressed from E17.5, peaking in the neonatal testis as spermatogonia begin to proliferate and becoming restricted to spermatogonial stem cells in juvenile and adult testis (Costoya et al., 2004). Similar to cyclin D1, PLZF was detectable by immunohistochemistry in D6 and D11 control testis, but not in the D6 FZR1Δ testis (Fig. 3B). Some FZR1Δ tubules displayed PLZF signal by D11 and 18 (Fig. 3B). This data suggests that the establishment of the spermatogonial pool is affected by the loss of APC/C^{FZR1} such that unexpected germ cell cycle quiescence occurs and there is a delay in the timely transition of gonocytes into proliferating spermatogonia and ultimately mitotic exit into meiosis.

Key known APC/C^{FZR1} protein substrate levels are unaltered in FZR1Δ neonatal testis

To explain this spermatogonial proliferation defect we next investigated whether protein levels of known APC/C^{FZR1} substrates that also have reported roles in spermatogonial maintenance were altered in neonatal FZR1Δ testes, including DNA replication protein,

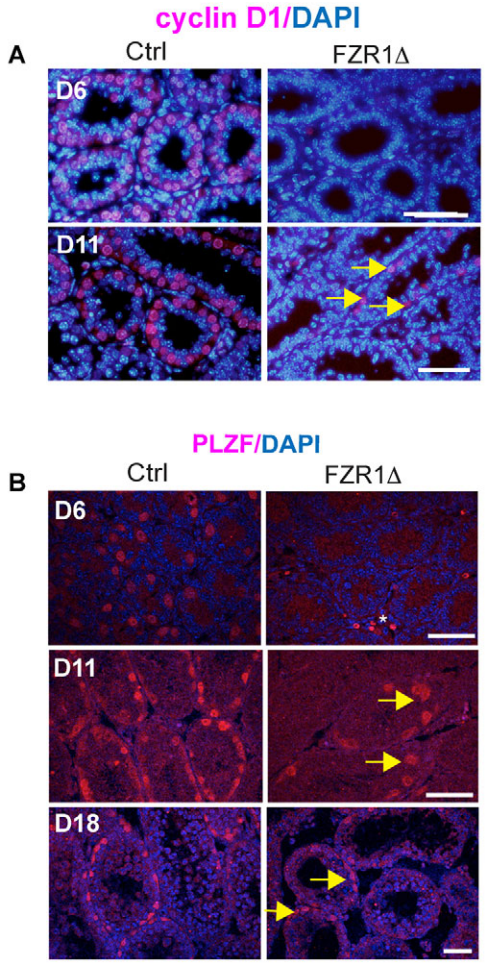

Fig. 3. Delayed and reduced expression of spermatogonia proliferation markers in FZR1Δ testes. (A) Cyclin D1 immunostaining in neonatal testes. (B) PLZF immunostaining in neonatal and juvenile testes. Arrows show positively labeled cells. Asterisk marks nonspecific interstitial staining. Scale bars: 50 µm.

geminin and transcription factor ETS2 (Barry et al., 2012; Shreeram et al., 2002; Wohlschlegel et al., 2000). We also analysed target cell senescence associated p16^{INK4a} protein, which accumulates in serum-starved FZR1Δ MEFs, in addition to other APC/C^{FZR1} substrates such as cyclin A2, cyclin E2, EHMT1 (GLP) and EHMT2 (G9a). Intriguingly, we did not detect any change in these proteins in D6 testes compared with controls (supplementary material Fig. S7) and therefore conclude that they are not APC/C^{FZR1} substrates in spermatogonia.

Meiotic entry is delayed in male germ cells lacking FZR1

Despite abnormal and delayed spermatogonial proliferation, histology and immunohistochemistry analysis (Figs 2, 3) suggested that a proportion of spermatogonia were entering meiosis, but dying around the time of transition into pachytene spermatocytes. We consequently examined markers of early meiotic events to characterize more fully the germ cell defect in these mice, including STRA8, which is associated with premeiotic S-phase entry once gonocytes have differentiated into spermatogonia (Mark et al., 2008). In control mice, STRA8 was detected in tubules at D6 but was often absent in D6 FZR1Δ testes (Fig. 4A). Notably, low levels of expression were apparent by D11 in FZR1Δ testis, which was

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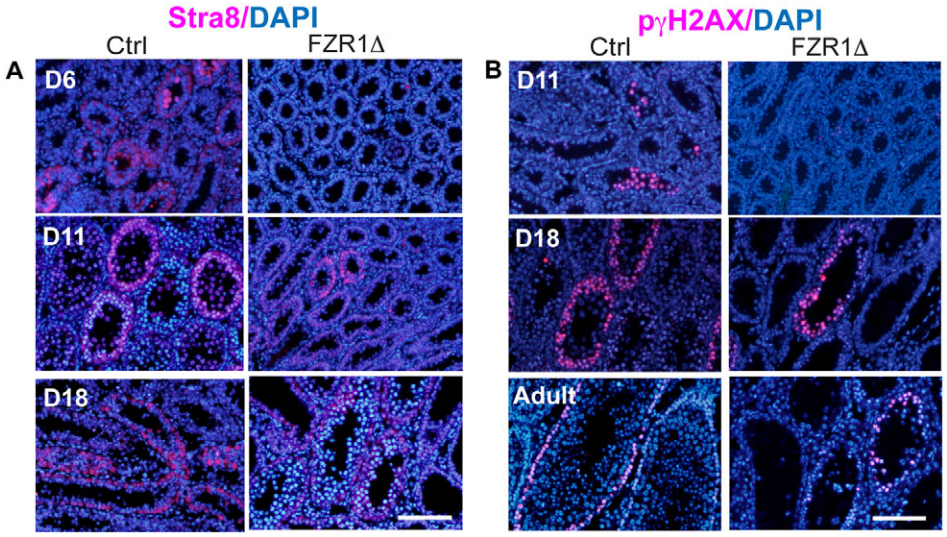

Fig. 4. Delayed and reduced expression of meiotic markers in FZR1Δ testes. (A) STRA8 immunostaining of neonatal and juvenile testes. (B) Phosphorylated γH2AX immunostaining in juvenile and adult testes. Scale bars: 100 µm.

verified by immunoblotting (supplementary material Fig. S7). Similarly, pγH2AX, a marker of SPO11-induced recombination-associated DSBs at leptotene (Keeney et al., 1997; Mahadevaiah et al., 2001; Kinner et al., 2008; Lukas et al., 2011) was observed in control testes at D11, but not apparent until D18 in FZR1Δ mice (Fig. 4B). The late onset of these early meiotic markers confirms that at least some spermatogonia lacking APC/C^{FZR1} activity are competent to enter meiosis during the first wave of spermatogenesis, albeit with some delay.

Evidence for initiation of synapsis and DSB detection in the absence of FZR1

To examine more closely the fidelity of early meiosis in these late-entry FZR1Δ spermatocytes, we performed cell spreads and assessed the progression of recombination by visualizing synaptonemal complex (SC) assembly. The SC scaffold begins to assemble after DSB formation (leptotene stage), and can be visualized by the gradual deposition of the axial/lateral element synaptonemal complex protein 3 (SYCP3) along the length of the chromosome, followed by accumulation of central element protein, synaptonemal complex protein 1 (SYCP1) which then links the homologs together (pachytene stage) (de Vries et al., 2005, Dobson et al., 1994; Yuan et al., 2000). Meiotic spreads from D18 control testes displayed SYCP3 and SYCP1 staining patterns characteristic of leptotene/zygotene/pachytene meiotic stages (Fig. 5A,B). By contrast, only leptotene or early zygotene-like staining patterns were evident in FZR1Δ spreads, with no cells displaying the complete lateral or central element pattern that is associated with pachytene spermatocytes, an observation also noted for spreads from Sox2-Cre/FZR1$^{\Delta/-}$ males (Fig. 5A; supplementary material Fig. S8). These observations suggest that synapsis is not completed in FZR1Δ germ cells.

Similar to control leptotene/zygotene cells, pγH2AX signals persisted on the chromatin in FZR1Δ cells consistent with the presence of unrepaired meiotic DSBs at this stage of prophase I (Fig. 5A). To assess whether DSB repair was initiated in the absence of FZR1, we next examined RAD51, an important homologous recombination repair protein, which normally forms foci in zygotene cells and gradually disappears during pachytene as DSBs are repaired (Baumann et al., 1996; Shinohara et al., 1992; Moens et al., 2002; Wang and Höög, 2006). RAD51 foci were present in FZR1Δ germ cells, suggesting that initiation of RAD51-dependent DNA repair does occur in mutant animals, and persistence of these foci was consistent with failure of these germ cells to progress beyond zygotene stage (Fig. 5C). Taken together, our data confirm that FZR1Δ spermatocytes undergo DSB formation, initiation of DSB repair and loading of synaptonemal complex proteins; however, recombination is not completed in these germ cells.

Cyclin B1 is upregulated in germ cells lacking FZR1

We previously observed that cyclin B1 protein levels were found to be regulated by APC/C^{FZR1} in fully grown oocytes (Holt et al., 2010; Holt et al., 2011). To determine whether cyclin B1 levels may also become critically regulated by APC/C^{FZR1} activity in male germ cells we performed immunohistochemistry on D18 testis. In agreement with previous reports we observed strong immunoreactivity to cyclin B1 in the cytoplasm of spermatocytes from control mice (Fig. 6A) (Brandeis et al., 1998; Chapman and Wolgemuth, 1994; Gromoll et al., 1997; Illert et al., 2012; Liu et al., 2000; Zhu et al., 1997). By contrast, germ cells from FZR1Δ testis possessed significantly enhanced signal for cyclin B1 in their nuclei, which was confirmed by analysis of confocal sections prepared and imaged in tandem (Fig. 6B). To confirm further that the nucleus was a site for FZR1 and cyclin B1 interaction in normal spermatocytes, we carried out an *in situ* proximity ligation assay (Duolink), which allows visualization of protein-protein interaction at single-molecule resolution (Söderberg et al., 2006; Redgrove et al., 2011; Redgrove et al., 2013). Positive signal was obtained for this interaction in the vicinity of spermatocyte chromatin (Fig. 6C). Taken together, our results suggest that APC/C^{FZR1} interacts with, and mediates the degradation of cyclin B1 in the nuclei of early prophase I male germ cells.

In addition to cyclin B1 we also questioned whether Polo kinase (PLK1), may have been inappropriately regulated in FZR1Δ germ cells. In *Saccharomyces cerevisiae*, APC/C^{AMA1} targets both cyclin B1 and the yeast PLK1 homolog Polo (Cdc5) to maintain appropriate prophase I timing (Okaz et al., 2012). Consistent with a previous recent study (Jordan et al., 2012) immunohistochemistry revealed chromatin-associated localization of PLK1 in control spermatocytes, which was similar in the few detectable FZR1Δ spermatocytes. Additionally, immunoblotting of D11 testis suggested that PLK1 protein was not prematurely increased in FZR1Δ animals (supplementary material Fig. S9). Therefore, this substrate is not affected by loss of APC/C^{FZR1} activity during male germ cell development.

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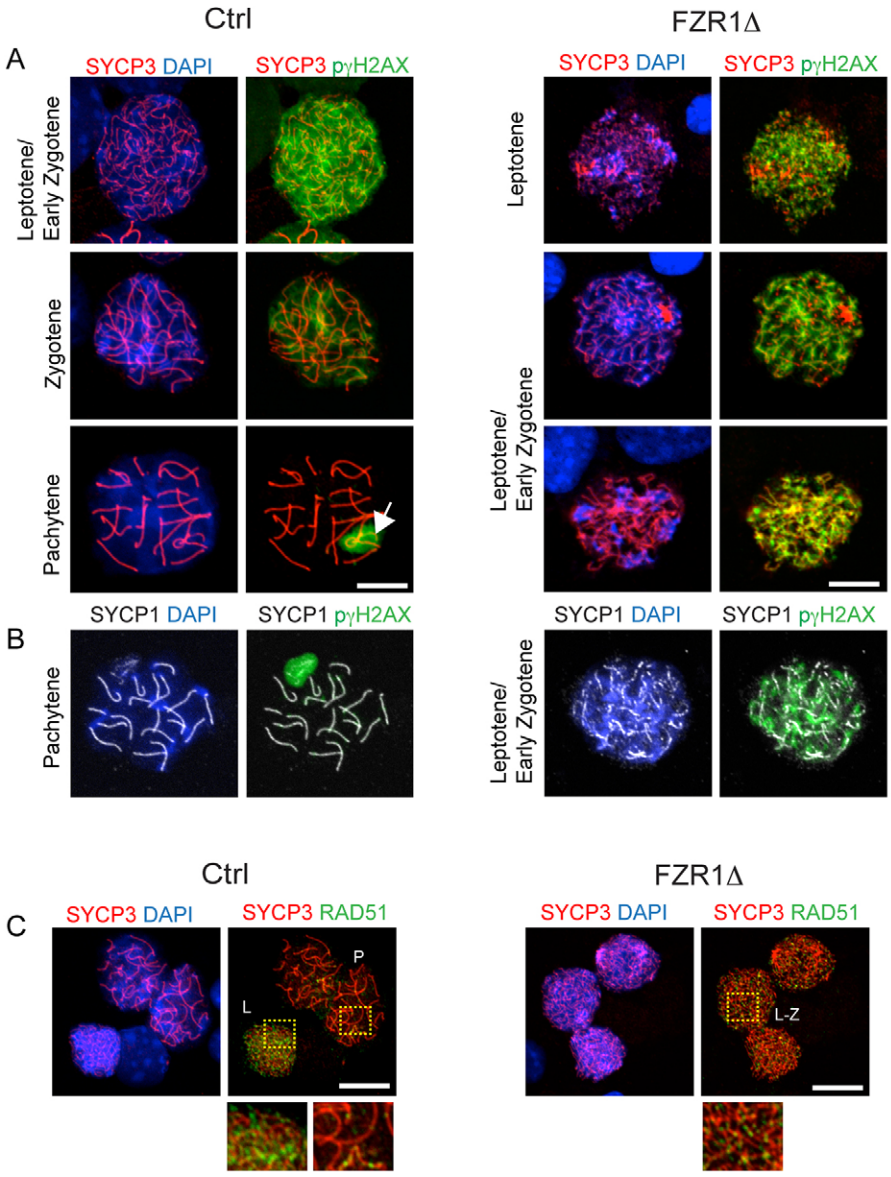

Fig. 5. Failure of spermatocytes to progress beyond zygotene stage. (A) Immunostaining of SYCP3 and pγH2AX, examined in control and FZR1Δ D16 cell spreads (representative of spermatocytes). Arrow shows XY body restricted pγH2AX labeling in control pachytene spermatocytes. (B) Immunostaining for SYCP1 and pγH2AX in pachytene cells from control animals and the most advanced FZR1Δ germ cells. (C) RAD51 immunostaining of D16 cell spreads showing RAD51 foci in leptotene and pachytene control cells and the most advanced FZR1Δ spermatocytes. Scale bars: 5 μm in A; 10 μm in C. L, leptotene; L-Z, leptotene/zygotene; P, pachytene.

Loss of FZR1 results in poor fertility and premature reproductive senescence in the female

To examine whether loss of FZR1 also had an impact on the early events of female germ cell development, we next assessed the fertility of FZR1Δ females. Importantly, however, in this model we were examining the effect of FZR1 loss midway through the meiotic program, as *Ddx4*-driven Cre recombinase activity begins at E15.5, when the majority of female germ cells are at the zygotene/pachytene stages (Gallardo et al., 2007). Reproductively mature control females (4-6 weeks old) consistently produced litters; however, low fertility was observed for age-matched FZR1Δ females, with three out of four females failing to produce more than one litter (Fig. 7A; supplementary material Fig. S10). FZR1Δ females also displayed significantly smaller litter sizes and no litters were born after 130 days (~4-5 months of age) (Fig. 7A; supplementary material Fig. S10).

In order to understand the subfertility of female mutant mice, and their associated infertility with age, we went on to examine ovaries from juvenile females. We assessed the ability of 4- to 6-week-old females to produce preovulatory cumulus-oocyte complexes (COCs), and found that FZR1Δ mice produced ~66% fewer COCs than controls (Fig. 7B). A reduction in COCs was consistent with our previous observations using the ZP3CreFZR1-loxP model that revealed FZR1 loss in the mature oocyte leads to premature GV breakdown and follicular degeneration (Holt et al., 2011).

As young adult FZR1Δ females were capable of producing mature GV oocytes, we next sought to histologically evaluate the follicle pool of D24 mice. We used granulosa cell morphology to differentiate between the nongrowing and growing follicle populations: primordial follicles were identified as those with squamous (flattened) granulosa cells, small growing follicles (preantral follicles), those with one or more layers of cuboidal granulosa cells, and early antral follicles, those with emerging cavities between granulosa cells (Fig. 7C). FZR1Δ ovaries possessed 66% fewer follicles than controls (Fig. 7D), and although we noted no difference between numbers of preantral and early antral follicles present between groups, very few primordial follicles were observed in FZR1Δ D24 ovaries (Fig. 7E). By 10-12 weeks of age (Ddx4-Cre) FZR1Δ and Sox2-Cre/FZR1$^{\Delta/-}$ mutants had much smaller ovaries and lacked follicles of any stage (Fig. 7F; supplementary

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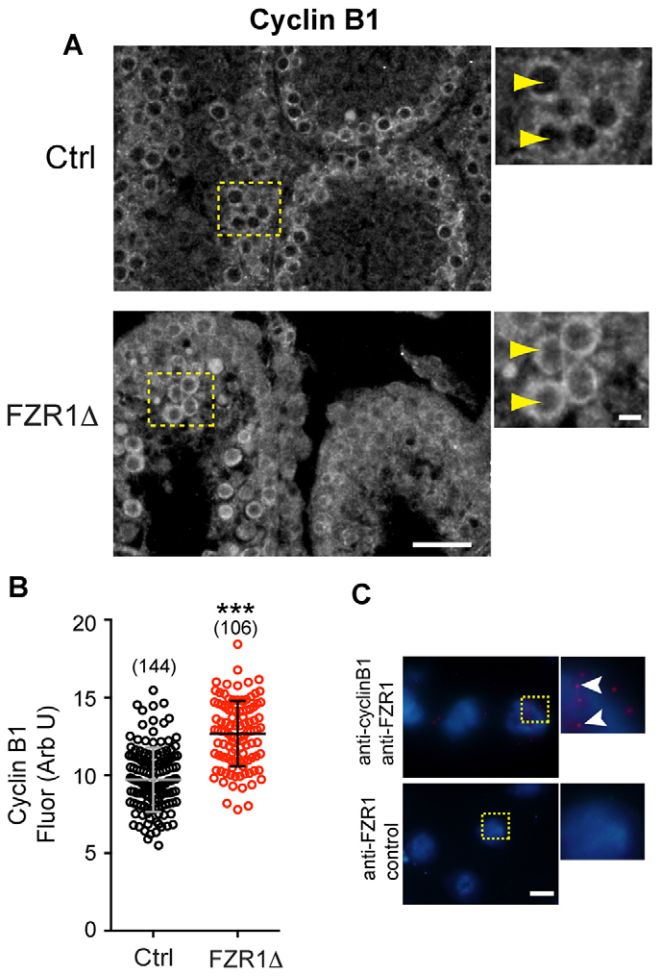

Fig. 6. Increased nuclear cyclin B1 in FZR1Δ male germ cells. (A) Confocal images of cyclin B1 immunostaining in FZR1Δ and control testis at D18. Inset arrowheads highlight spermatocyte nuclei. (B) Nuclear cyclin B1 levels quantitated from germ cells imaged in A (mean, ***P<0.001, Student's t-test; error bars, s.d.). (C) FZR1 and cyclin B1 interaction detected by DUOLINK in spermatocytes isolated from control D18 testes. Chromatin is DAPI stained. Insets show magnified region with positive foci indicative of protein-protein interaction (arrowheads). Scale bars: 50 µm in A; 5 µm in insets and C.

material Figs S10, S11). As the primordial follicle population represents the entire pool from which all fully grown oocytes will develop, our observations suggested that reduced numbers of primordial follicles in young adult FZR1Δ females was a key underlying cause for their premature reproductive senescence.

FZR1 loss leads to a small endowment of primordial follicles

The primordial follicle pool in the mouse is established from a germ cell pool that develops in the embryonic ovary. Shortly after birth, ovarian somatic cells invade germ cell cysts and enclose the oocytes to form individual primordial follicles (Pepling and Spradling, 2001; McLaughlin and McIver, 2009). To determine whether the embryonic germ cell pool, and thus the size of the primordial follicle pool, was being affected by FZR1 loss, we next examined embryonic gonads from females around the time of Cre-recombinase-mediated FZR1 deletion. Using an MVH (DDX4) antibody as a germ cell marker, we performed whole-mount immunostaining of embryonic ovaries at E14.5 and E16.5. As expected, at E14.5 the germ cell complement of control and FZR1Δ ovaries were similar, because at this time point, Ddx4-Cre recombinase activity, and therefore FZR1 loss, has not yet begun (Fig. 8B; supplementary material Fig. S12). However, by E16.5 we noted that germ cell density was significantly decreased in FZR1Δ ovaries by ~50% (Fig. 8A,B; P<0.001, Mann-Whitney U-test). In addition, high numbers of pyknotic nuclei and TUNEL-positive cells were observed at this time point, as well as less pγH2AX-positive cells (supplementary material Fig. S12), suggesting that similar to the scenario in the male, germ cells lacking APC/C^{FZR1} activity undergo apoptosis at the zygotene/pachytene stage. Consistent with these observations, on the day after birth, FZR1Δ ovaries were smaller in size than controls and contained ~60% fewer germ cells than controls. Here germ cells were defined as oogonia, which lack squamous granulosa cells, plus the primordial follicle population (Fig. 8C,D; supplementary material Fig. S12). Therefore, the premature reproductive senescence of FZR1Δ females is due, at least in part, to germ loss during embryonic life, which results in a decreased ovarian reserve of primordial follicles and hence earlier depletion of the germ cell pool in the adult.

FZR1 loss influences primordial follicle survival and subsequent growth

A smaller initial pool of primordial follicles in FZR1Δ ovaries could be the exclusive reason for the premature ovarian failure seen in these mice. However, the ability of those remaining primordial follicles to survive and enter the growing follicle pool normally could also influence fertility. So for example, overactivation of the neonatal ovarian pool has been associated with premature ovarian failure in other mouse models (Castrillon et al., 2003; Rajareddy et al., 2007; John et al., 2008; Reddy et al., 2008). We therefore assessed ovaries at D5, a time point at which many primordial follicles start to enter the growing follicle pool, and used the presence of cuboidal granulosa cells to identify follicles that were no longer quiescent (Hirshfield, 1991; McGee and Hsueh, 2000; McLaughlin and McIver, 2009). As predicted from germ cell counts at earlier time points, D5 FZR1Δ ovaries contained fewer total follicles than control ovaries, with significantly fewer primordial follicles than controls, but similar numbers of follicles with cuboidal granulosa cells (Fig. 9A). Intriguingly, examination of those FZR1Δ follicles possessing cuboidal granulosa revealed that they had oocytes that were significantly smaller in diameter (Fig. 9B). To determine whether these granulosa cells had begun to mitotically divide, we immunostained with proliferating cell nuclear antigen (PCNA) (Fig. 9C,D) (Oktay et al., 1995; Wandji et al., 1997; Tomic et al., 2002). In contrast to controls, the vast majority of FZR1Δ follicles failed to show PCNA immunoreactivity. Taken together, these data are consistent with abnormal entry of follicles into the growing pool in the absence of APC/C^{FZR1} activity, which leads to the appearance of some aspects of follicle growth, such as granulosa cell morphology changes, but not others, such as granulosa cell proliferation and an increase in oocyte size.

As mature oocytes can be produced by young adult FZR1Δ mice (Fig. 7), we conclude that despite abnormal features displayed by immature FZR1Δ follicles, at least some primordial oocytes lacking APC/C^{FZR1} activity are able to progress to the antral follicle stage. However, the pronounced loss of primordial follicles by D24, without a corresponding increase in preantral follicle numbers, suggests that many FZR1Δ follicles are lost at the earliest stages of growth. We failed to detect positive immunosignal for activated caspase 3 or TUNEL in D5 ovaries (data not shown); however, this is in agreement with reports that confirm that *in vivo* mouse and human immature follicles do not display classic markers of

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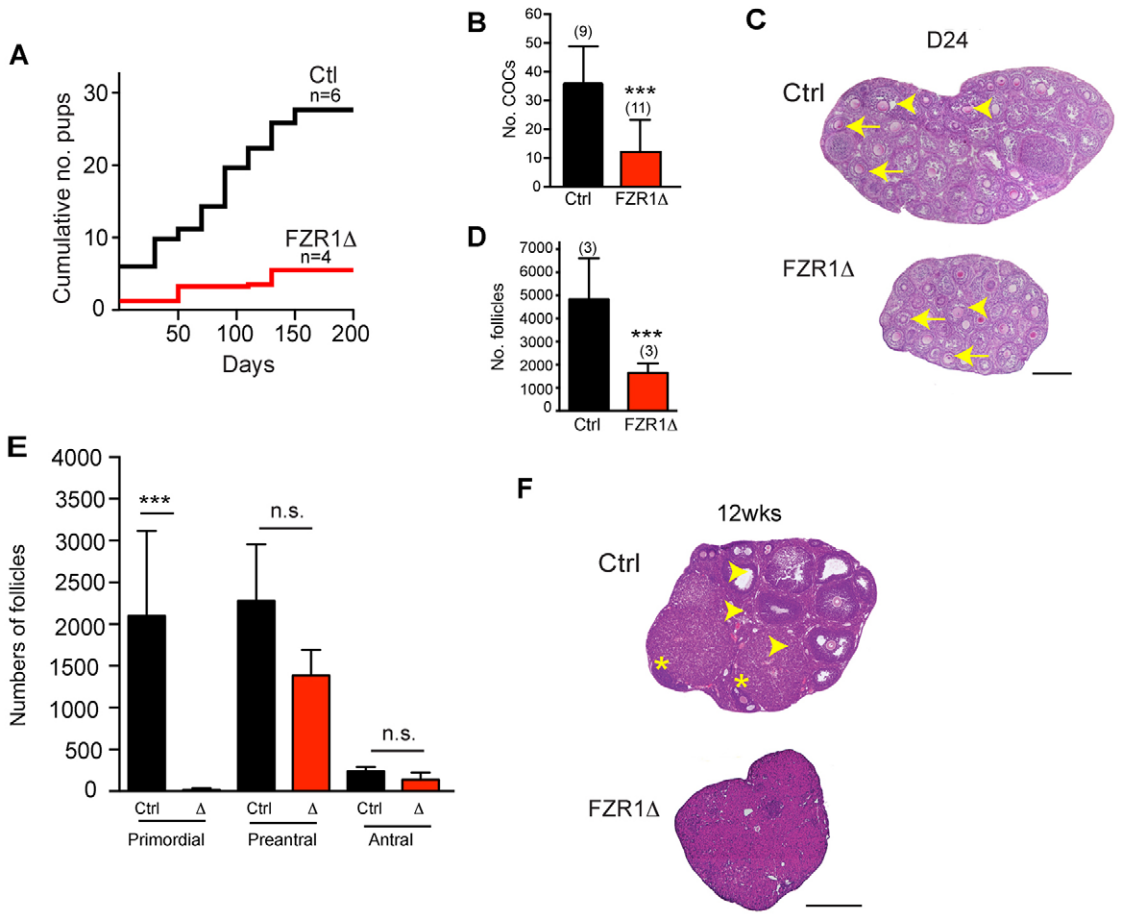

Fig. 7. Subfertility in FZR1Δ females is associated with premature depletion of the follicle pool. (A) Cumulative number of pups born to mated FZR1Δ and control females. (B) Mean numbers of cumulus-oocyte complexes (COCs) isolated from 4- to 6-week-old control and FZR1Δ females (***P=0.005, Student's t-test). (C) Representative sections of D24 ovaries. (D) Total number of follicles per D24 ovary (***P=0.005, Student's t-test). (E) Numbers of follicles per developmental stage in D24 ovaries (***P<0.0001, ANOVA with Tukey's test; n=3 animals/genotype). (F) Representative ovarian sections of 12-week-old ovaries. C,D: Arrows, preantral follicles; arrowheads, antral follicles; asterisks, corpora lutea. Parentheses, number of animals examined. Scale bars: 200 µm in C; 500 µm in F.

apoptosis (Tingen et al., 2009). It remains possible that abnormal immature FZR1Δ follicles could be lost to autophagy, which has recently been implicated in perinatal mouse ovary germ cell loss (Escobar et al., 2008; Gawriluk et al., 2011) and so together with an initial small primordial follicle pool, this further loss of immature follicles could contribute to premature the reproductive senescence of FZR1Δ females.

DISCUSSION

Using conditional knockout of FZR1, and so APC/C^{FZR1} activity, we have shown here that the premeiotic period and early prophase I are severely affected by loss of this protein in both sexes. In male FZR1Δ mice, we observed multiple defects during the first wave of spermatogenesis involving: (1) reduced mitotic proliferation of spermatogonia; (2) a delay of several days in the onset of meiotic entry; and (3) increased apoptosis at the around the time germ cells normally enter the zygotene/pachytene stage.

Potentially relevant to our observations regarding abnormal spermatogonial proliferation, cultured MEFs lacking APC/C^{FZR1} proliferate more slowly, and this is associated with premature S-phase onset, DNA replication stress leading to instability, and early quiescence (García-Higuera et al., 2008; Li et al., 2008; Sigl et al., 2009). The reduction in cell cycle marker expression in neonatal mutant testis suggests that many spermatogonia may enter a quiescent state during the first wave of spermatogenesis. We predict that some of these cells later re-enter the cell cycle, and undergo the required number of mitotic divisions before meiotic entry (Hess and Renato de Franca, 2008). Therefore, the observed late meiotic entry of FZR1Δ spermatogonia is likely to be a direct consequence of their reduced proliferation in neonatal life. Together, our observations suggest that, similar to mitotic cells, premeiotic germ cells also require APC/C^{FZR1} activity for the fidelity of their cell cycles. The effect becomes more pronounced with age, such that ultimately spermatogonia cease proliferation altogether and no meiotic cells are observed in aged testes. Interestingly, however, in contrast to previous studies using MEFs, we did not observe the accumulation of known APC/C^{FZR1} substrates in neonatal FZR1Δ testes. Therefore, the cohort of APC/C^{FZR1} substrates in spermatogonia that affect growth are not the same as those that influence MEFs, and the underlying mechanism for altered premeiotic germ cell proliferation in these mice remains to be elucidated. The fact that known APC/C^{FZR1} substrates were present, but not altered, in FZR1Δ testis, suggests that substrate-specific regulation of the APC may be particularly important in germ cells. Post-translational modifications such as phosphorylation, acetylation and protein-protein interactions have previously been shown to influence the degradation of substrates by the APC (Crane et al., 2004; Choi et al., 2009; Song and Rape, 2010).

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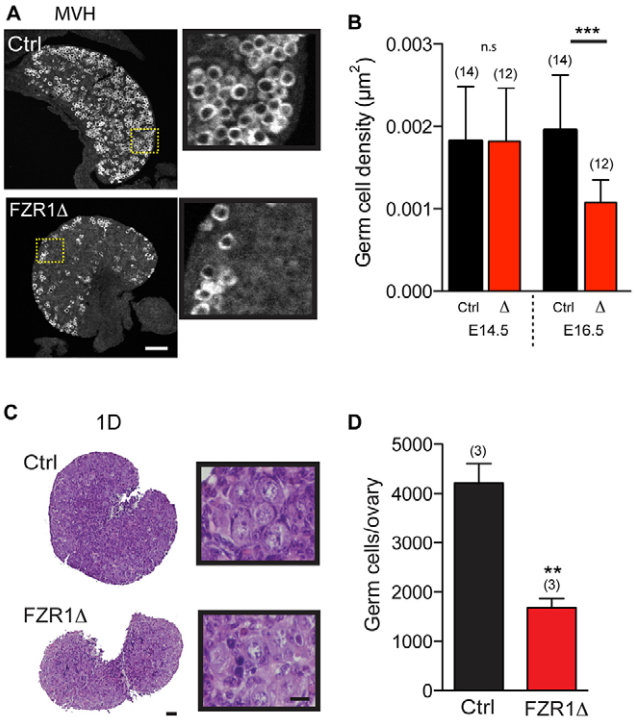

Fig. 8. Loss of FZR1 depletes embryonic ovaries of germ cells. (A) DDX4/MVH immunostaining at E16.5 in control and FZR1Δ ovaries. (B) Mean germ cell density per ovary from embryonic FZR1Δ ovaries immunostained in A and supplementary material Fig. S12. ***P<0.001, Mann-Whitney U-test. (C) Representative ovarian sections of 1D FZR1Δ and control ovaries. Insets show primordial follicles. (D) Germ cell number per 1-day-old ovary. Germ cells were defined as oogonia (lacking clearly defined pre-granulosa cells) or primordial follicles (oocytes with squamous granulosa cells) (**P<0.01, Mann-Whitney U-test). Error bars in B,D show s.d.; parentheses, n number sections examined, 3 animals/genotype. Scale bars: 50 μm in A,B; 10 μm in insets.

Role of APC/C^{FZR1} during meiotic prophase I

Following delayed entry into meiosis, male germ cells from FZR1Δ juveniles underwent apoptosis around the time of the zygotene/pachytene transition. This timing of germ cell loss matches that of many mouse knockout models for proteins now identified as playing a role in recombination (Pittman et al., 1998; de Vries et al., 1999; Kneitz et al., 2000; Romanienko and Camerini-Otero, 2000; Yuan et al., 2000; Hunt and Hassold, 2002). This phenomenon has been referred to as the 'pachytene checkpoint'. It is thought to be the result of multiple checkpoint mechanisms converging to cause apoptosis, such as a failure in sex chromosome silencing, or Sertoli cell-derived development signals or checkpoints at this stage of the spermatogenic cycle (Fernandez-Capetillo et al., 2003; Barchi et al., 2005; Royo et al., 2010). We suggest that the loss of germ cells lacking APC/C^{FZR1} activity at around the time of the pachytene checkpoint is due, at least in part, to elevated cyclin B1. A cyclin B1-driven rise in CDK1 activity in early prophase I may affect the completion of meiotic recombination. Recombination is normally associated with a period of low CDK1 activity, with an increase occurring only in late pachytene/diplotene, which drives the final stages of chromosome condensation and synaptonemal protein relocation (Chapman and Wolgemuth, 1994; Wiltshire et al., 1995; Sun and Handel, 2008). A premature rise in CDK1, which inappropriately brings forward these events, may subsequently cause the demise of germ cells. Interestingly, neurons also undergo apoptosis in response to a rise in cyclin B1 when APC/C^{FZR1} is inactivated, although an analogy to meiotic germ cells is limited because these are postmitotic cells in G0 (Almeida et al., 2005; Maestre et al., 2008).

Consistent with a model in which cyclin B1 is low in early meiotic prophase I, downregulation of cyclin B1 transcripts have been observed around the time of meiotic entry in the female germ cell (Miles et al., 2010). Furthermore, infertility defects similar to those of our model have been reported when CDK1 activity is indirectly affected by other means, such as via loss of chaperone HSPA2, or cyclin A1, which plays a role in the CDK1 activity amplification cycle (Zhu et al., 1997; Liu et al., 1998). Another model closely resembling the FZR1Δ phenotype is that of the nuclear interacting partner of ALK (NIPA/ZC3HC1) mutant nonconditional knockout. NIPA, the substrate recognition subunit of SKP1-CULL-F-Box E3 ligase (SCF), has also been implicated in cyclin B1 regulation in germ cells. NIPA-knockout males are infertile with complete germ cell arrest (Illert et al., 2012).

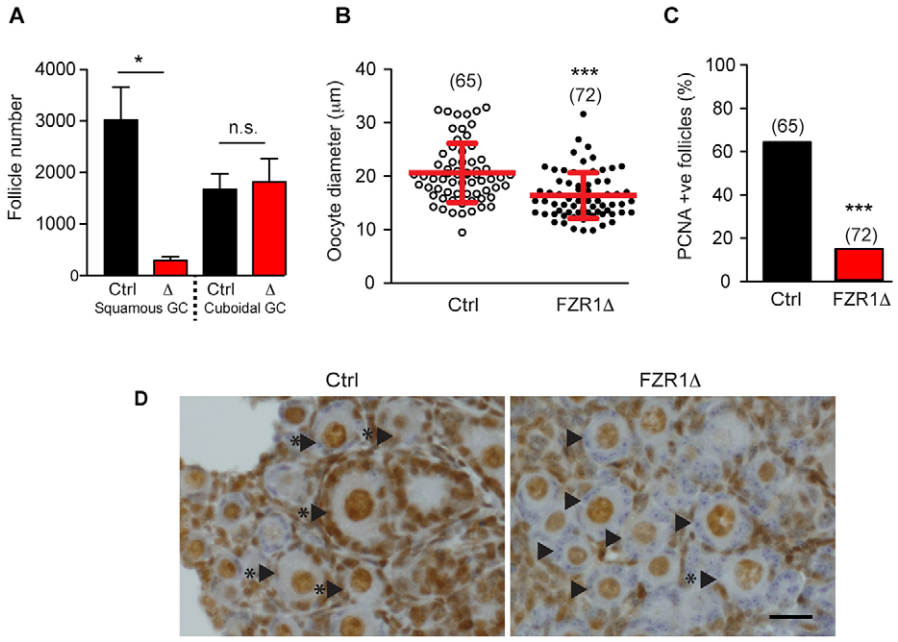

Fig. 9. Abnormal follicle activation following loss of FZR1. (A) Numbers of follicles classified as having squamous or cuboidal granulosa cells at postnatal day 5 (D5) (*P<0.013, Student's t-test). (B) Plot of oocyte diameters in follicles possessing cuboidal granulosa cells (***P<0.0001, Student's t-test). (C) Percentages of follicles with PCNA-positive cuboidal granulosa cells in FZR1Δ and control ovaries (***P<0.0001, Chi-squared test). (D) Representative D5 FZR1Δ and control ovarian sections showing PCNA immunostaining. Arrowheads, primary follicles; asterisks, follicles with PCNA-positive granulosa cells. Parentheses, n of follicles/oocytes examined from three ovaries per genotype. Error bars, s.d. Scale bar: 20 μm. GC, granulosa cell.

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Interestingly in these mice, meiotic arrest occurs later, with some cells reaching pachytene. Therefore, we predict that the requirement for APC/C^{FZR1}-mediated cyclin B1 degradation precedes that of NIPA. Taken together, both these studies support the argument for a model in which cyclin B1 must be kept in check during early prophase I to allow the completion of recombination events.

Sexually dimorphic response to loss of APC/C^{FZR1} activity

The observed apoptosis of FZR1Δ male germ cells resulted in the complete absence of haploid germ cells in the testes, and so male infertility. However in the female, loss of FZR1Δ germ cells was incomplete. Such sexual dimorphism in response to loss of meiotic proteins has been observed for a number of other knockout models genes (SPO11, DMC1, MSH4/5, SYCP3, MEI1), and has been hypothesized to arise from the more rapid and synchronous transit through prophase in the female (~4 days versus ~7-8 days in the male) or alternatively through the process of oogenesis being more 'robust' (Hunt and Hassold, 2002; Kolas et al., 2005). Although the sex difference observed in the current study could reflect these phenomena, a key issue to note is the timing of FZR1 loss in our conditional knockout females. *Ddx4*-driven Cre recombinase expression begins at around D15.5, when some female germ cells have already reached the pachytene stage. Data from the male model suggests that the zygotene-to-pachytene transition is particularly sensitive to loss of APC/C^{FZR1} activity. Therefore, it is conceivable that in the female at E15.5, some cells have already reached pachytene stage before Cre-recombinase activity and may be unaffected by FZR1 deletion. By contrast, those cells still at the zygotene stage when Cre activity begins are the population that succumb to the effects of loss of APC/C^{FZR1} activity and undergo apoptosis. Despite this potentially confounding factor, our observations nevertheless suggest a common model in which at least one sensitive window to loss of APC/C^{FZR1} activity in both sexes is the zygotene-to-pachytene transition, when crucial events recombination events are taking place.

We attribute the premature ovarian failure (POF) of FZR1Δ females primarily to the reduced number of primordial follicles present in their ovaries at birth as a result of this embryonic loss of germ cells. However, it is important to note that similar timing of POF onset has been observed in other mouse models, including oocyte-specific knockouts of PTEN and FOXO3A and nonconditional knockout of P27KIP1 (CDKN1B – Mouse Genome Informatics), all proteins now identified as regulators of primordial follicle quiescence (Rajareddy et al., 2007; John et al., 2008; Reddy et al., 2008; Reddy et al., 2010). The unifying phenotype for these models was an enhanced rate of primordial follicle activation with subsequent death of a high proportion of these follicles leading to POF. As the number of remaining primordial follicles dictates the reproductive lifespan of the female, we predict that the significant depletion of germ cells in embryonic life was the major cause of fertility loss in the adult FZR1Δ female. However, our observation that primordial follicles themselves displayed abnormal characteristics in the absence of APC/C^{FZR1} suggests that fertility loss in these mice might also be related to a premature loss of those primordial follicles that we observed in the early postnatal ovary, which subsequently were absent in gonads from female animals after weaning (D24).

In conclusion, we have described an unexpected role for APC/C^{FZR1} activity in completion of mammalian meiosis in both sexes. In the male, the impact of FZR1 loss appears to be complex such that both spermatogonial proliferation and spermatocyte progression are catastrophically affected. In the female, FZR1 loss during early prophase I causes significant depletion of the embryonic germ cell pool, and abnormal primordial follicle characteristics, which together contribute to premature ovarian failure in the adult. These findings show that APC/C^{FZR1} activity plays an essential role in maintaining germ cell number in the mammalian adult, as well as being needed to transition the cell cycle from mitosis to meiosis.

MATERIALS AND METHODS

Reagents

All reagents were obtained from Sigma-Aldrich unless otherwise specified.

Generation of FZR1Δ mice

Use of animals for this study was approved by the University of Newcastle Animal Care and Ethics Committee. Fzr1$^{loxP/loxP}$ mice were created by García-Higuera et al. (García-Higuera et al., 2008) and maintained on a C57Bl6 background. Transgenic Ddx4-Cre mice [FVB-Tg(Ddx4-Cre)1Dcas/J] were obtained from Jackson Laboratory (ME, USA). Ddx4-Cre/Fzr1$^{loxP/wt}$ males were used as studs with Fzr1$^{loxP/loxP}$ females to produce, Ddx4-Cre/Fzr1$^{\Delta/\Delta}$ (FZR1Δ) or heterozygotes (Ddx4-Cre/Fzr1$^{\Delta/wt}$). Fzr1$^{loxP/loxP}$ were used as controls for all experiments. Genotyping primers were as described previously [Cre (Holt et al., 2011); Fzr1 allele (García-Higuera et al., 2008)].

Tissue collection and histology

Reproductive organs were fixed in Bouin's solution, paraffin-embedded and sectioned at 5-8 µm before immunohistological processing. Follicle counts were performed blind on serial sections by scoring oocytes with nuclei in every fifth section across whole ovaries.

Germ cell isolation

GV oocytes were collected in M2 media with 4% bovine serum albumin (BSA) from ovaries of 4-week-old mice 44-52 hours after intraperitoneal injection of 10 IU of pregnant mare's serum gonadotrophin (PMSG). Germ cells were isolated from testes by loading dissociated tissue onto a 2-4% continuous BSA gradient, as previously described (Baleato et al., 2005).

Immunoblotting

Protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer with ProteCEASE protease inhibitor (G-Biosciences, MO, USA) and quantitated using the Pierce BCA Protein Assay Kit (Thermo Scientific). Proteins were separated on a NuPage or Tris-glycine 10% gel (Life Technologies) blotted using a XCell Blot Module (Life Technologies). Primary antibodies used are listed in supplementary material Table S2. Anti-mouse/rabbit IgG horseradish peroxidase (HRP) secondary antibodies were used (DAKO) and detection performed using ECL Reagents (GE Healthcare).

Meiotic spread preparation

D16 whole testes were dissected into phosphate-buffered saline (PBS), tubules separated with forceps and incubated in hypotonic buffer [30 mM Tris, 50 mM sucrose, 17 mM trisodium citrate dehydrate, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), ProteCEASE protease inhibitor] before transfer to 0.1 M sucrose. Cell suspensions were fixed with 1% paraformaldehyde, pH 9.2, with 0.15% Triton X-100 and slides air-dried before washing in 0.4% Photo-Flo 200 solution (Kodak). Slides were rinsed and blocked with 10% goat serum/3% BSA /0.015% Triton X-100 PBS before immunostaining. Counterstaining was performed with 0.2 µg/ml 4′-6-diamidino-2-phenylindole (DAPI).

Immunohistology

Primary antibodies used are listed in supplementary material Table S2. Secondary antibodies conjugated to Alexa Fluor 488, 594, 633 (Life Technologies) were used. Counterstaining was performed with 0.2 µg/ml DAPI. Images were acquired using an Olympus FV1000 and data analysis was performed using FluoView Software FV10-ASW2.0 (Olympus) and ImageJ (National Institutes of Health, MD, USA).

TUNEL

TUNEL staining was performed as described previously (Sobinoff et al., 2012) using the ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Millipore).

Duolink proximity ligation assay

A Duolink proximity ligation assay was performed as previously described (Redgrove et al., 2011; Redgrove et al., 2013) using the Duolink *in situ* kit (OLINK Biosciences, Sweden).

Electron microscopy

Testis were fixed in 2.5% (v/v) glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated through an acetone series and embedded in Spurr's resin. Tissue was sectioned at 70-100 nm and stained with 1% uranyl acetate in 50% (v/v) methanol followed by lead citrate. Micrographs were taken with a JEOL-100CX electron microscope operating at 80 kV.

Statistical analysis

All statistical analysis was performed using GraphPad Prism software. Two-way unpaired Student's *t*-tests, Mann-Whitney U or chi-squared tests were used.

Acknowledgements

We thank R. Hobbs and M. Carrington for the kind gifts of anti-PLZF and anti-Cyclin A2 antibodies, respectively.

Competing interests

The authors declare no competing financial interests.

Author contributions

J.E.H. and E.A.M. designed the work, with input from other authors. I.G.-H. and S.M. made the floxed Fzr1 and SoxCre-Fzr1lox mutants, with other experiments conducted by J.E.H., V.P., E.B., R.R. and J.L.S. Writing of the manuscript was by J.E.H., K.T.J. and E.A.M., with input from all other authors.

Funding

This work was supported by project grants from the Australian Research Council (ARC) to K.T.J., S.M. and E.A.M.; an ARC Early Career Researcher Award to J.E.H.; and Fundación Científica de la Asociación Española contra el Cáncer to I.G.-H.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.104828/-/DC1

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