GENETIC DISSECTION OF CHECKPOINT SIGNALING IN MOUSE PRIMORDIAL GERM CELLS

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Abstract

Although mutations are the force of evolution, most mutations are deleterious. Faithful inheritance of genetic information is the key to evolutionary success. The only cell lineage that is capable of transferring genetic information is the germ line. Therefore, refined control of mutation frequency in germ cells is of great importance. The control mechanism mainly involves the DNA damage response (DDR), since DNA damage is the major source of mutations. In mammals, most studies on germ line mutation and DDR focus on meiosis. In contrast, little is known about these processes in primordial germ cells (PGCs). In particular, checkpoint signaling has not been characterized in PGCs. Multiple analysis showed that the mutation rate in germ cells is consistently lower than that in somatic cells. Therefore, it has been proposed that there is a fundamental difference in DDR between germ cells and somatic cells. Indeed, a few DDR mutants have revealed a hypersensitivity of PGCs to DNA damage. In this dissertation, I characterized two such genes in mouse model: $Fancm^{C4/C4}$ and $Mcm9^{XG/XG}$. Deficiency of either *Fancm* or *Mcm9* causes genome instability as well as PGC depletion. PGC depletion in both mutants is largely due to reduced PGC proliferation between E11.5 and E13.5. In order to understand the correlation between genome instability and reduced PGC proliferation, I

conducted genetic studies using 5 checkpoint mutants, including *Hus1*, *Atm*, *Chk2*, *p53* and *p21*. My studies indicated that ATM-p53-p21 signaling is partially responsible for the germ cell deficiency in $Fancm^{C4/C4}$ males. None of the 5 genes is required for the germ cell depletion in $Fancm^{C4/C4}$ females or either sex of $Mcm9^{XG/XG}$. Additionally, there is an additive germ cell depletion in $Fancm^{C4/C4}$ and $Mcm9^{XG/XG}$ compound mutants, suggesting $Fancm^{C4}$ and $Mcm9^{XG}$ alleles have independent impacts on DDR. Together, my results imply that there are at least 3 checkpoint pathways responsible for DDR in PGCs.

BIOGRAPHICAL SKETCH

Yunhai Luo was born in Beijing, China. In 2004, he was accepted to Tsinghua University and majored in biological sciences and biotechnology. During four years of undergraduate study, he not only studied basic knowledge in natural science but also was fortune enough to joined Dr. Zihe Rao's structural biology lab, to experience some real scientific research. In two years of lab-life, he was guided two talented graduate students, Hui Wang and Xiuna Yang. Together with them, he studied the purification of BTG2 and CNOT6L in the CCR4-NOT transcription complex. Those studies opened his mind and extended his knowledge beyond textbooks. More importantly, the two-year studies helped him finally make up his mind to further pursue biological researches. In 2008, he graduated from Tsinghua University and accepted Cornell's offer into the field of Genetics and Development for graduate study. With great honor, he joined the Dr. John Schimenti's lab in 2009 and began his graduate project in studying DNA damage response in primordial germ cells.

This dissertation is dedicated to my family for their lifelong support, to scientists in every field of every generation, who have dedicated their lives to extend our understanding of the world, and to all supports that make scientific researches possible in the modern society.

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CHAPTER 1

Introduction

Heredity and variation are two important aspects of modern evolution theory. Although mutations are important source of variation, most mutations are deleterious [1]. Therefore, faithful inheritance of most genetic material from the previous generation together with a few beneficial mutations in the context of natural selection is the key to evolutionary success. One major source of mutation is DNA damage that occurs in germ cells. In mammals, studies focusing on checkpoint mechanisms during meiosis have been started [2,3]. This dissertation, however, focuses on genome maintenance in germ cells prior to meiosis, especially primordial germ cells (PGCs) in mammalian embryos.

1.1 Germ cell development in the mouse

Unlike *C. elegans* and *D. melanogaster*, the germline in mouse is not specified in early cleavage-stage embryos. The very first germ cell precursors could be labeled by tissue nonspecific alkaline phosphatase at the base of allantois during embryonic day E6.0 to 6.5, and approximately 45 cells will commit to the germ cell lineage by E7.5 [4]. Started from E8.0, PGCs migrate into the hindgut and move along the gut epithelium [5]. The movement is unidirectional toward the urogenital ridge, under the guidance of chemo-attractants [5]. After 1 day, PGCs move first ventrally into the dorsal midline between E9.0 and E9.5, before moving laterally to the genital ridges between E10 and E10.5 [6]. Upon the arrival in the primitive gonad, PGCs receive signals from surrounding somatic cells and start their sexual differential development. PGCs in male will exit the cell cycle and enter a prolonged phase of G0 arrest until approximately

postnatal day 3 (3dpp) [7]. PGCs in female will enter meiosis, progress through leptotene, zygotene, and pachytene stages before arresting after birth (2dpp) in diplotene [8].

PGCs actively proliferate during embryogenesis, increasing from ~45 to ~25000 cells by E13.5 [9]. Based on cell counting and on BrdU incorporation, the PGC doubling time is about 11 to 15 hours from E10.5 to E13.5 and 32 hours during E9.5-10.5 [10,11]. It has been noticed that PGCs become transcriptionally silent, undergoing a transient G2 phase cell cycle arrest [12]. This arrest coincides with an extensive epigenetic reprogramming and the arrest be gradually reversed between E9.5 to E10.5 [12]. In comparison, the proliferation rate of PGCs are generally slower than cells in early mouse development (<E7.5) [13], comparable to cultured mouse embryonic fibroblasts and embryonic stem cells[14,15], but faster than cells in late embryos and adults [16], and faster than some tumor cells [17,18].

1.2 DNA damage and mutation

DNA replication is one of the most vulnerable stages during cell proliferation. Not only is the DNA itself, especially the exposed single strand DNA, sensitive to DNA damage, but also unrepaired DNA damage causes replication stress, which aggravates the damage. Incorrectly repaired DNA damage is repaired and inherited as mutation. In this regard, rapid or large scale cell proliferation becomes a challenge for genome maintenance, since it predisposes to mutations in the cells, and expands the mutant cell population. In response to this challenge, DNA damage response (DDR) signaling coordinates multiple cellular activities to limit the expansion of mutations. As a result, the observed mutation rate, which is different in different cell types, is a combination of DNA damage level, DDR and proliferation rate in each specific cell population. Mutation

rate has been traditionally estimated by measuring the mean mutation frequency of a given locus in the genome, including specific genes and genetic markers such as microsatellites [19,20]. With the development of sequencing technology, genome wide mutation rates have also been estimated [21]. Although the estimates vary a lot between different studies, it is clear that mutation rates are significantly higher in somatic cells than in germ cells [20,22]. This difference could result from hypothetical differences in DNA damage levels, DDR, and/or proliferation rate between somatic cells and germ-line cells. Although the number of DNA lesions per PGC is not known, there seems to be no evidence to suggest the DNA damage level is significantly lower in germ cells than somatic cells [23]. Since PGC proliferation is not faster than somatic cells during embryogenesis [13] and many somatic cells in adult tissues are terminally differentiated, a difference in proliferation rate may not be enough to explain the low mutation rate in the germ-line. Therefore, it is reasonable to hypothesize that the DDR in the germ-line is more stringent in eliminating mutations. In line with this hypothesis, PGCs are more sensitive to low dose irradiation (<0.5Gy), a dose that has little effect on somatic cells [24]. This hypothesis has been further extended to the study of testicular germ cell tumors (TGCTs). It is believed that the high degree of curability among TGCTs, namely an enhanced responsiveness to p53-mediated apoptosis induced by DNA damage and a reduced ability of TGCTs to repair cisplatin-induced DNA damage, is related to the origin of these tumor cells from PGCs [25,26].

1.3 DNA damage response in general

The DNA damage response has been studied for over 30 years [27]. In general, it senses DNA damage and replication stress and coordinates multiple cellular activities to

repair the damage and relieve the stress. The binding of damage sensor proteins would trigger both DNA repair and checkpoint signaling, which are two main aspects of DDR.

So far, several DNA repair mechanisms have identified. These include direct DNA damage reversal, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), single strand break (SSB) repair, interstrand cross-link (ICL) repair, and double strand break (DSB) repair through homologous recombination (HR) or non-homologous end joining (NHEJ) (recently reviewed in [28-34] respectively). It is worth mentioning that these mechanisms are not mutually exclusive but rather interconnected along with the processing of DNA damage [35]. The relationship between DNA repair and checkpoint signaling remains to be fully understood. It is believed that rapid recruitment of repair factors and the formation of a multi-protein repair complex are the first and immediate response of DNA damage. Indeed, the binding of repair factors and/or intermediates during DNA repair have been shown to be required by checkpoint signaling [36-40]. On the other hand, checkpoint signaling is known to regulate or even be required by NER, ICL repair, HR and NHEJ [41,42]. Furthermore, although simple repair reactions can happen without checkpoint activation, an optimal repairing environment, such as nucleotide synthesis, gene transcription, chromatin remodeling, can only be established through checkpoint signaling [43].

Checkpoint signaling is primarily mediated by proteins of the phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family — DNA-PK, ATM, and ATR (Figure 1-1, reviewed in [42]). The three proteins mediate three relatively independent yet highly interactive checkpoint signaling pathways. DNA-PK responds to DSBs via the binding of its subunit, Ku heterodimer (Ku70-Ku80), which then recruits its

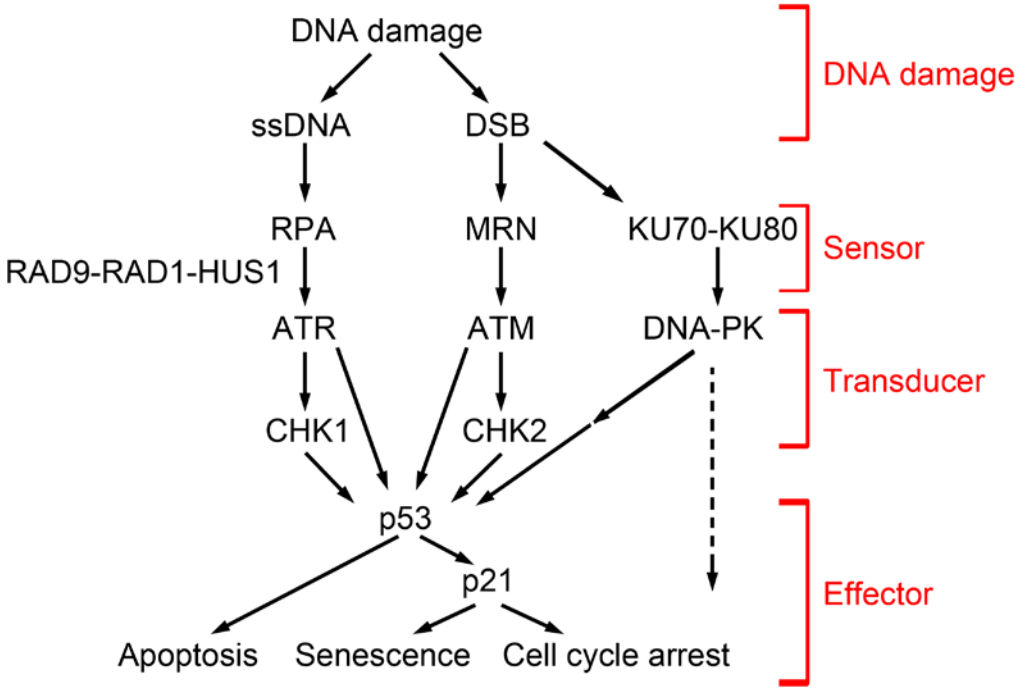

Figure 1-1. Summarization of key components in checkpoint signaling.
Checkpoint signaling can be divided into three layers: sensor, transducer and effector. DNA damage can be processed by DNA repair machinery into ssDNA or DSB. ssDNA will be coated by RPA proteins, and DSB can be recognized by MRN complex or KU70-KU80 heterodimer. These sensors then recruit and/or stimulate protein kinase ATR, ATM and DNA-PK to initiate the signaling cascade. Transcription factor p53 can integrate signals and cause cell cycle arrest, cell senescence and apoptosis by activating transcription of different genes. For example, p53 regulates the expression of cyclin-dependent kinase inhibitor, p21, which can regulate cell cycle progression and can mediate cellular senescence.

catalytic partner, DNA-PKcs, to the DSB site. The binding of DNA–PKcs/Ku complex activates its kinase activity. Unlike ATM or ATR, substrates of DNA-PK known to date are relatively limited, and are involved in DNA repair (e.g., Artemis), transcription (e.g., RNA Pol I and II), and downstream checkpoint signaling (e.g., p53) (reviewed partially in [44]).

Most previous studies and much of the current understanding of checkpoint signaling focus on ATM and ATR. ATM is recruited to DNA double strand breaks (DSBs) by a damage sensor, the MRN (MRE11, RAD50 and NBS1) complex. The activation of ATM is not yet fully understood, although it is believed to be related in part to autophosphorylation [45,46]. ATR is recruited by RPA-coated single stranded DNA (ssDNA), which arises during certain DNA repair (e.g., NER) and replication stress. The RAD9-RAD1-HUS1 ("9-1-1") checkpoint clamp stimulates TopBP1, an activator of ATR, although the biochemistry of this activation is not yet fully understood [47]. Activated ATM and ATR then initiate a signaling cascade by phosphorylating proteins including CHK2 and CHK1, which can stimulate a secondary wave of phosphorylation. A transcriptional response is one final target of this signaling cascade. The most extensively studied component of this response is p53, which can integrate signals directly from ATM, ATR, CHK1, CHK2, and DNA-PK [48]. p53 regulates multiple cellular and developmental processes [49]. In the context of PGC development, some best known targets of p53 are members of cell cycle arrest (e.g., p21), apoptosis (e.g., BAX) and senescence (e.g., $p16^{INK4a}$) pathways [50].

Not only is p53 a master regulator of the checkpoints, it has also recently become clear that the canonical Atm-Chk2-p53 and ATR-Chk1-p53 pathways were over-

simplified. Over 700 substrates of ATM and ATR has been extensively identified in a large scale unbiased screen [51]. 35 substrates of CHK2 and CHK1 were identified in similar studies [52,53]. Emerging evidences suggest that ATM and ATR share common substrates. For example, ATM may activate CHK1 under ionizing radiation [54,55], and ATR may activate CHK2 under replication stress [56-58]. Physiological consequences of the cross-talk between ATM and ATR have been studied in mice [59]. It might be worth to consider that the checkpoint signaling is a protein network with different modules regulating different biological processes [51].

1.4 DNA damage response in the germ-line

DNA damage response in the mammalian germ-line is poorly characterized. In mouse testis, recent studies suggest that RAD9A and HUS1 are essential for DSB repair in meiotic prophase I, whereas ATR and BRCA1 play roles in meiotic sex chromosome inactivation [60-63]. In mouse ovary, it has been shown that ATR-CHK2-p53/p63 is the potential pathway eliminating damaged oocytes [2]. Considering the fundamental differences between mitosis and meiosis, however, the DDR in mitotic germ cells need to be clarified.

After birth, the only mitotic germ cells besides PGCs in mammals are spermatogonial stem cells (SSCs). Rübe and colleagues have tried to dissect the DDR mechanisms in SSCs [64]. They irradiated the whole mouse and studied the response of SSC to DSBs specifically. Although the checkpoint signaling in SSCs is not clarified in their report, it is found to be independent of γH2AX/MDC1 [64]. Despite such a lack of knowledge, we could probably get some insights from the *C. elegans* germ line. In adult *C. elegans*, germ cells undergo mitotic and meiotic cell divisions, whereas somatic

tissues are entirely post-mitotic. Consequently, DNA damage checkpoints function specifically and therefore studied in the germ line. In summary, the counterpart of "9-1-1"-ATR-CHK1 pathway in worms is the major signaling transducer which regulates cell cycle arrest and apoptosis [65]. CEP-1, the worm p53 homolog, is a major effector of checkpoint signaling in the germ line. It has an indispensable role in germ cell apoptosis, but has a controversial role in cell cycle arrest [65]. Besides, a few p53 independent checkpoint effector were found to have an effect on DNA damage-induced apoptosis [65].

In general, PGC development can be divided into three aspects: specification, migration, and survival. Many PGC specific genes have been identified as inductive signals, stemness factors and extracellular factors [5,66]. Only a few of those play a role in genome maintenance. These include the Fanconi anemia gene family, *Pin1*, *Espl1*, *Rev7*, *Helq* and *Rad54* [67-77]. However, checkpoint signaling has not been identified for mutants of any of these genes in PGCs. In this dissertation, two such mouse models, $Fancm^{C4}$ and $Mcm9^{XG}$, will be introduced. Genetic approaches will be applied on both models to address the following questions:

1. What are the checkpoint signaling pathways in PGCs?
2. Is the checkpoint signaling the same in male PGCs as in female PGCs?
3. Do different mutants, which potentially have different DNA damage profiles, share the same checkpoint response?

1.5 Fanconi anemia gene family and *Fancm*

Fanconi anemia is a disorder named for Swiss paediatrician Guido Fanconi, who first described the disease in 1927 [78]. Typical symptoms of Fanconi anemia include bone marrow failure, predisposition to cancer, congenital abnormalities and infertility

[79]. It is now clear that Fanconi anemia is a genetic disease involving at least 15 genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM*, *FANCN*, *FANCO*, *FANCP*) [80]. Products of these genes function coordinately in the Fanconi anemia (FA) pathway to repair DNA interstrand crosslinks (ICL) and maintain genome integrity during DNA replication. FANCM-FAAP24 heterodimers recognizes ICLs at stalled replication forks and recruit the other eight proteins of FA core complex (FANCA, B, C, E, F, G, L) to the damage site [81]. The FA core monoubiquitinates FANCD2-FANCI (ID) complex, which is thought to be an adaptor for DNA repair proteins. The ID complex can regulate ICL repair by coordinating nucleolytic incision, translesion DNA synthesis (TLS), and HR [81].

Impaired fertility is common in FA patients. Although pregnancy has been reported in some female patients, about half of all female patients are infertile [79]. Most male patients are infertile with extremely few exceptions [82]. Semen analyses show low or absent sperm counts and abnormal spermatogenesis [79]. Hypogonadism has been reported in most FA mouse models (*Fanca*, *Fancc*, *Fancd2*, *Fancf*, *Fancg*, *Fancl*, *Fancm*, *Fancp*) except for *Fancd1* [67-71]. In *Fanca*, *Fancc*, *Fancd2*, *Fancl* mice, it has been suggested that PGC deficiency might contribute to the hypogonadism [67,83-85].

Fancm was identified as a FA gene based on lack of gene expression and the presence of biallelic mutations in patient EUFA867. Unlike other FA genes, the FANCM phenotype is unclear because EUFA867 was later found to also carry biallelic mutations in *Fanca* [86]. *In vitro* study shows that *Fancm* is not required for, but rather facilitates FANCD2 monoubiquitination [87]. In *Fancm* knockout human colon cancer cells (HCT-116), ICL induced ATR activation is not affected [87]. Apart from ICL repair, the DNA

translocase activity of FANCM could protect stalled replication forks by promoting replication traverse of ICLs [88]. In yeast and *Arabidopsis*, FANCM promotes non-crossover and limits crossover during meiosis [89,90].

1.6 MCM gene family and *Mcm9*

MCM gene family has 8 members, *Mcm2*, *Mcm3*, *Mcm4*, *Mcm5*, *Mcm6*, *Mcm7*, *Mcm8* and *Mcm9*. MCM2-7 form a heterohexamer and act as the eukaryotic replicative helicase [91]. *Mcm8* and *Mcm9* were later identified as novel MCM gene family members by their sequence homology [92-95]. Human and mouse *Mcm8* contains the conserved AAA^+ core domain of the MCM gene family, including Walker A motif, Walker B motif and Arginine finger motif [96]. Human *Mcm9* has three isoforms, $Mcm9^S$, $Mcm9^M$ and $Mcm9^L$, with $Mcm9^M$ and $Mcm9^L$ containing the full AAA^+ core domain and $Mcm9^S$ containing only the Walker A motif [97]. Mouse *Mcm9* has two isoforms, $Mcm9^S$ and $Mcm9^L$, with $Mcm9^L$ containing the full AAA^+ core domain and $Mcm9^S$ containing only the Walker A motif [98]. Despite the indispensable role of MCM2-7 in DNA replication, neither *Mcm8* nor *Mcm9* is essential for DNA replication [99-101]. Emerging evidences suggest that MCM8 and MCM9 form a complex and function in ICL induced HR repair by recruiting RAD51 [100-102].

Mcm9 knockout females are sterile, while *Mcm9* knockout males are fertile [101]. Histological analysis showed that adult $Mcm9^{-/-}$ ovaries had no follicles at all [101]. Adult $Mcm9^{-/-}$ testes had both atrophied and normal seminiferous tubules, which had been correlated to a proliferation defect of spermatogonia at around 16 days after birth [101]. However, potential defects in newborn mice and/or in PGCs were not investigated.

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CHAPTER 2

Hypersensitivity of primordial germ cells to compromised replication-associated DNA repair involves ATM-p53-p21 signaling

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YL's contributions, specifically, include writing the manuscript, conceiving and designing the experiments, performing the experiments and analyzing the data for figures 2-5, 2-6, 2-7, 2-8, S2-2 and S2-3.

2.1 Abstract

Genome maintenance in germ cells is critical for fertility and the stable propagation of species. While mechanisms of meiotic DNA repair and chromosome behavior are well-characterized, the same is not true for primordial germ cells (PGCs), which arise and propagate during very early stages of mammalian development. Fanconi anemia (FA), a genomic instability syndrome that includes hypogonadism and testicular failure phenotypes, is caused by mutations in genes encoding a complex of proteins involved in repair of DNA lesions associated with DNA replication. The signaling mechanisms underlying hypogonadism and testicular failure in FA patients or mouse models are unknown. We conducted genetic studies to show that hypogonadism of *Fancm* mutant mice is a result of reduced proliferation, but not apoptosis, of PGCs, resulting in reduced germ cells in neonates of both sexes. Progressive loss of germ cells in adult males also occurs, overlaid with an elevated level of meiotic DNA damage. Genetic studies indicated that ATM-p53-p21 signaling is partially responsible for the germ cell deficiency.

2.2 Author Summary

The precursors to sperm and eggs begin as a group of <100 cells in the embryo, called primordial germ cells (PGCs). They migrate in the primitive embryo to the location of the future gonads, then undergo a rapid proliferation over the next few days to a population of many thousands. Because these cells contain the precious genetic information for our offspring, and the DNA replication associated with rapid PGC proliferation is subject to spontaneous errors, mechanisms exist to avoid propagation of mutations. A manifestation of this is the high sensitivity of PGCs to genetic perturbations

affecting DNA repair. We studied mice defective for a gene called Fanconi anemia M (*Fancm*) that is important for repair of DNA damage that occurs during replication. Although it is expressed in all tissues, only the PGCs are affected in mutants, and are reduced in number. We find that PGCs lacking *Fancm* respond by slowing cell division, and identified the genetic pathway responsible for this protective response.

2.3 Introduction

Fanconi anemia (FA) is a genomic instability (GIN) syndrome characterized by developmental abnormalities affecting the renal, gastrointestinal and reproductive systems, the skeleton, skin pigmentation, and heart. It also causes progressive bone marrow failure and increased incidence of cancer [1,2]. It can be caused by germline mutations in any of at least 16 genes (*FANCA, FANCB, FANCC, FANCD1(BRCA2), FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN (PALB2), FANCO(RAD51C), FANCP(SLX4), FANCQ(ERCC4)*) [3,4]). The products of these genes coordinately function in the repair of DNA interstrand crosslinks (ICL) during DNA replication [5]. A key event in FA pathway activation is the monoubiquitination of FANCI-FANCD2 (ID) heterodimers by the FA "core complex" (FANCA/B/C/E/F/G/L/M) [6-8]. The monoubiquitinated ID complex is recruited to DNA ICLs, and coordinates ICL repair together with downstream FA proteins (D1/J/N/O/P) and other (BRCA1, ATR) DNA repair proteins [1,9,10]. FANCM complexed with FAAP24 initiates FA pathway activity by recognizing DNA damage and loading the FA core complex. FAAP24 is particularly important in activating ATR in response to ICLs [11]. FANCM also has translocase activity that promotes branch migration of Holliday junctions and replication forks independent of FAAP24 [12].

FA deficient cells are hypersensitive to agents that induce ICLs, such as mitomycin C [MMC] or cisplatin. Most FA patients manifest anemia and bone marrow failure during childhood and are predisposed to cancer. Reduced fertility, hypogonadism and testicular failure, which is a consequence of impaired gametogenesis, are also common [13,14], and this is reflected in most mouse models for FA, including knockouts

for *Fanca*, *Fancc*, *Fancd2*, *Fancf*, *Fancg*, *Fancl*, *Fancm*, and *Fancp*, though *Fancd1* is an exception [15-22]. While the severity varies amongst mutants, males generally present a partial Sertoli Cell Only-like phenotype whereby a subset of seminiferous tubule sections are depleted of germ cells. In mutant females, the number of ovarian follicles is typically reduced. Although most of these mutants have been characterized only as adults, the germ cell defects in three have been investigated perinatally or earlier. Germ cell depletion in *Fancd2*$^{-/-}$ is evident in newborn mice [22], and defects in the proliferation of PGCs were reported in *Fancc* and *Fancl* mutants [15,23]. While defects in DNA repair presumably underlie these germ cell phenotypes, the downstream DNA damage signaling pathway(s) that respond to these defects, ultimately leading to germ cell depletion, have not been identified.

The FA pathway appears to function in all cell types, including germ cells. However, experimental difficulties in studying the mammalian germline – particularly those stages occurring during embryonic development – have limited investigations into the roles of the FA and other DNA damage response (DDR) pathways in these cells. Importantly, the germline mutation rate is significant lower than that in somatic cells [24,25], indicating a fundamental difference in genome maintenance that appears to reflect the biological importance of minimizing the germline mutation rate. While specific DDRs in the *C. elegans* germline have been identified [26], the DDRs operative in mammalian PGCs have not.

Here we investigate a *Fancm* mouse model (*Fancm*^{Chaos4}) that was recovered in a forward genetic screen for GIN mutants. Mutant mice exhibit GIN and PGC depletion during embryogenesis. Using a genetic approach, we found that the ATM-p53-p21 axis

contributes to the PGC depletion in this model, underscoring the critical importance of genome maintenance in these cells that undergo rapid cellular proliferation during a short period of time during development.

2.4 Results

Isolation of a new Fancm allele, Fancm^{Chaos4}, from a forward genetic screen for GIN mutations in mice.

We previously conducted an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen in mice for mutants showing chromosome instability, as assessed by micronucleus levels in erythrocytes [27]. *Chaos4* (chromosome aberrations occurring spontaneously 4) was one mutation identified in this screen. Homozygous mutants show a mildly elevated (3 fold) frequency of erythrocytes with micronuclei (Figure 2-1A). Using combined SNP- [28] and microsatellite-based mapping, *Chaos4* was genetically localized to a 9-Mb region between *RS13481482* and *D12Mit71* containing 9 RefSeq genes, including *Fancm* (Figure 2-1B). Sequencing of *Fancm* cDNA from mutants and controls identified a *de novo* T to C transition at nucleotide 524 of the coding region (Figure 2-1C). This point mutation changes a highly conserved cysteine residue to arginine (C142A) that is located within the DEXDc domain of this DEAD-like helicase superfamily region of FANCM (Figure 2-1D).

To confirm that the point mutation in *Chaos4* underlies the GIN phenotype, we performed complementation analysis with a *Fancm* gene-trap allele, *Fancm*$^{Gt(XH297)Byg}$, abbreviated hereafter as *Fancm*^{XH}. The gene-trap vector resides in exon 14, between the helicase and endonuclease domains (Figure 2-1D). *Fancm*^{XH} homozygotes also had elevated erythrocyte micronuclei (Figure 2-1A) as did *Fancm*$^{C4/XH}$ mice, providing strong

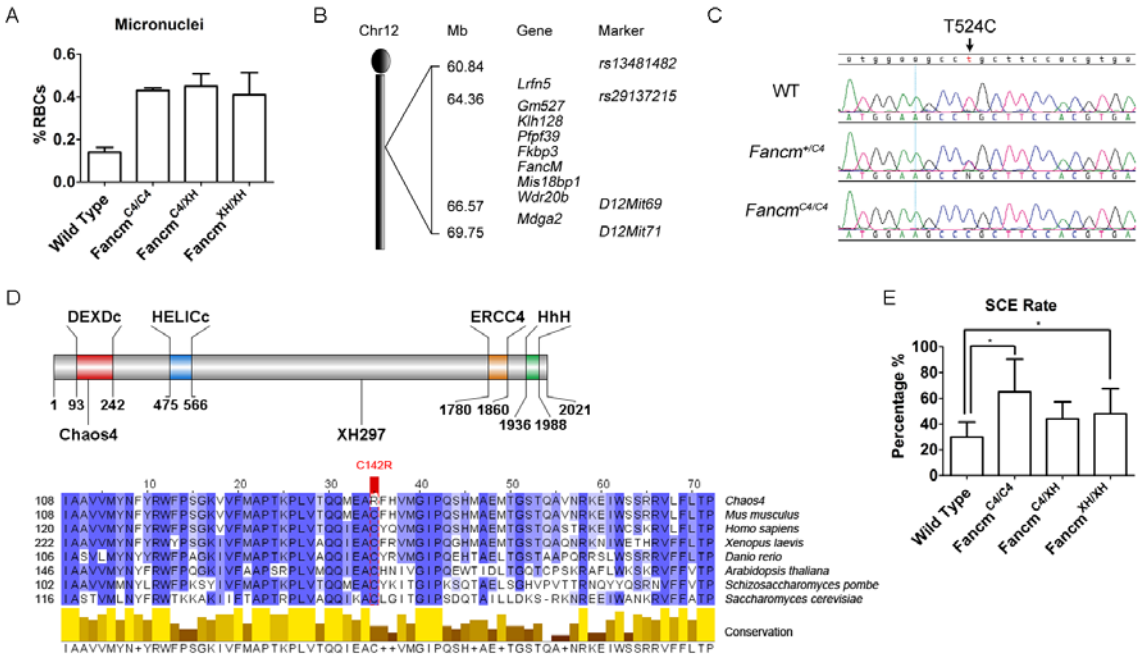

Figure 2-1. The *Chaos4* allele is a point mutation in *Fancm*.
(A) Flow cytometric analysis of erythrocytes to quantify red blood cells (RBC) with micronuclei. (B) Genetic mapping of *Chaos4* to a 9-Mb region of chromosome 12 containing *Fancm* between *rs13481482* and *D12Mit71*. (C). Sequence traces showing the T524C transversion (arrows) identified in the *Chaos4* allele of *Fancm*. (D) The *Chaos4* point mutation is in the first exon, and the *XH297* gene-trap is in the 14th exon. DEXDc, DEAD-like helicase domain; HELICc, Helicase superfamily c-terminal domain; ERCC4, ERCC4 endonuclease domain; HhH, Helix-hairpin-helix domain which interacts with FAAP24. Sequence alignment surrounding C142 is highly conserved from human to budding yeast. (E) SCE rates are significantly increased in *Fancm*$^{C4/C4}$ MEFs ($p<0.05$).

evidence that the *Fancm*^{Chaos4} allele (hereafter abbreviated *Fancm*^{C4}) is responsible for the GIN phenotype. We further assessed the chromosomal instability phenotype of our alleles via the sister chromatid exchange (SCE) assay. Consistent with results from a *Fancm*$^{\Delta 2}$ knockout mouse model [18], untreated *Fancm*$^{C4/C4}$ and *Fancm*$^{XH/XH}$ MEFs both had elevated DNA breaks and radial chromosomes (Figure 2-1E; Figure S2-1), further confirming that the Chaos4 phenotype is attributable to the mutation in *Fancm*. Both *Fancm*$^{C4/C4}$ and *Fancm*$^{XH/XH}$ mice were born at a Mendelian ratios, indicating that the mutations do not compromise embryonic viability (Table S2-1).

Fancm$^{C4/C4}$ *primary MEFs undergo premature immortalization and mutant mice are cancer prone.*

The proliferation of untreated *Fancm*$^{C4/C4}$ primary MEFs during early passages was diminished compared to wild-type (Figure 2-2A, B). However, they recovered from senescent crisis and became immortalized much earlier (by passage 7) than wild-type (passage 10 or later) (Figure 2-2A, B).

Cancer predisposition is a defining feature of Fanconi Anemia. To determine if the early immortalization was an indicator of cancer susceptibility, *Fancm*^{C4} mutants were aged for up to 1.5 years. *Fancm*$^{+/C4}$ and *Fancm*$^{C4/C4}$ females congenic in the C3HeB/FeJ background had significantly elevated cancer/neoplasia susceptibility (Table S2-2), developing multiple tumor types (Table S2-3). Thirty-three percent (33%) of heterozygotes (9/27) and 58% of homozygous females (15/26) developed tumors by ~1 year of age, compared to none of the 28 WT controls ($p=0.004$ and $p=0.0002$, respectively). The most common tumor types were ovarian, mammary and uterine. Heterozygous and homozygous *Fancm*^{C4} males also were significantly tumor prone

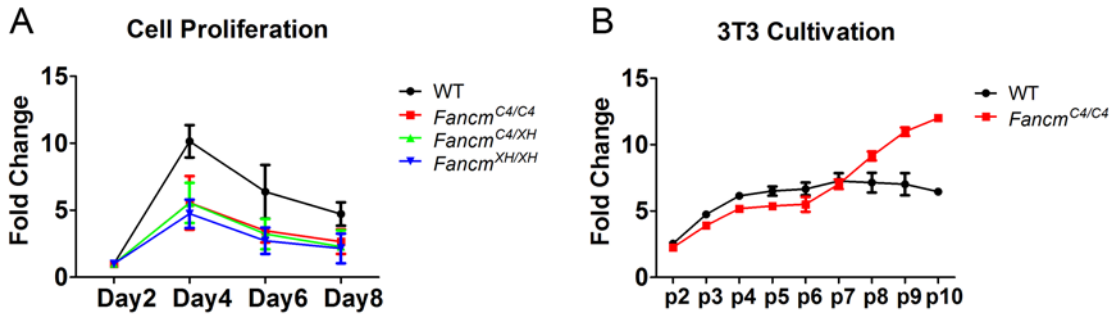

Figure 2-2. *Fancm*$^{C4/C4}$ MEFs undergo premature senescence.
(A) MEF growth assays. (B) Immortalization timeline of primary MEFs using a 3T3 growth protocol. Cultures were passaged every 3 days.

(42%, p=0.001 and 47%, p=0.002, respectively, vs. 9% of WT males; Tables S2-2, S2-3). *Fancm* null mice were reported to have a similar degree of tumor susceptibility [18].

FANCM deficiency compromises primordial germ cell proliferation and causes meiotic defects

In a limited gross and histological study, adult *Fancm* null mice were reported to have smaller gonads, germ cell loss in a subset of seminiferous tubule sections, and a reduced number of ovarian follicles [18]. Similar to those findings, we found that although $Fancm^{C4/C4}$ males appear grossly normal and were fertile, they had markedly smaller testes and about 60% the amount of sperm as wild-type littermates at 12 weeks of age (Figure 2-3A,B). Testis histology of young mice (≤ 16 weeks of age) revealed subtle seminiferous tubule abnormalities, namely the presence of occasional giant multinucleated cells that are not present in WT (Figure 2-3C, D). Prior to inbreeding onto strain C3HeB/FeJ, young $Fancm^{C4/C4}$ also exhibited germ-cell depleted individual tubules (not shown). Spermatogenesis defects in $Fancm^{C4/C4}$ mice (but not WT controls) became more severe over time, such that most seminiferous tubules in mice over 1 year of age were highly disrupted (Figure 2-3E, F). Gonadal defects in $Fancm^{C4/C4}$ mutants were sex independent; females manifested a significant depletion of primordial follicles compared to WT animals (Figure 2-3G).

The presence of multinucleate cells in younger animals was suggestive of abnormal meiotic or premeiotic cell divisions. To investigate potential meiosis defects, we immunolabeled meiotic chromosomes from 12-week $Fancm^{C4/C4}$ males with markers of DSB signaling (γH2AX, the phosphorylated form of H2AX), DSB repair (RAD51), and meiotic chromosome structure (SYCP3, which detects axial elements of the

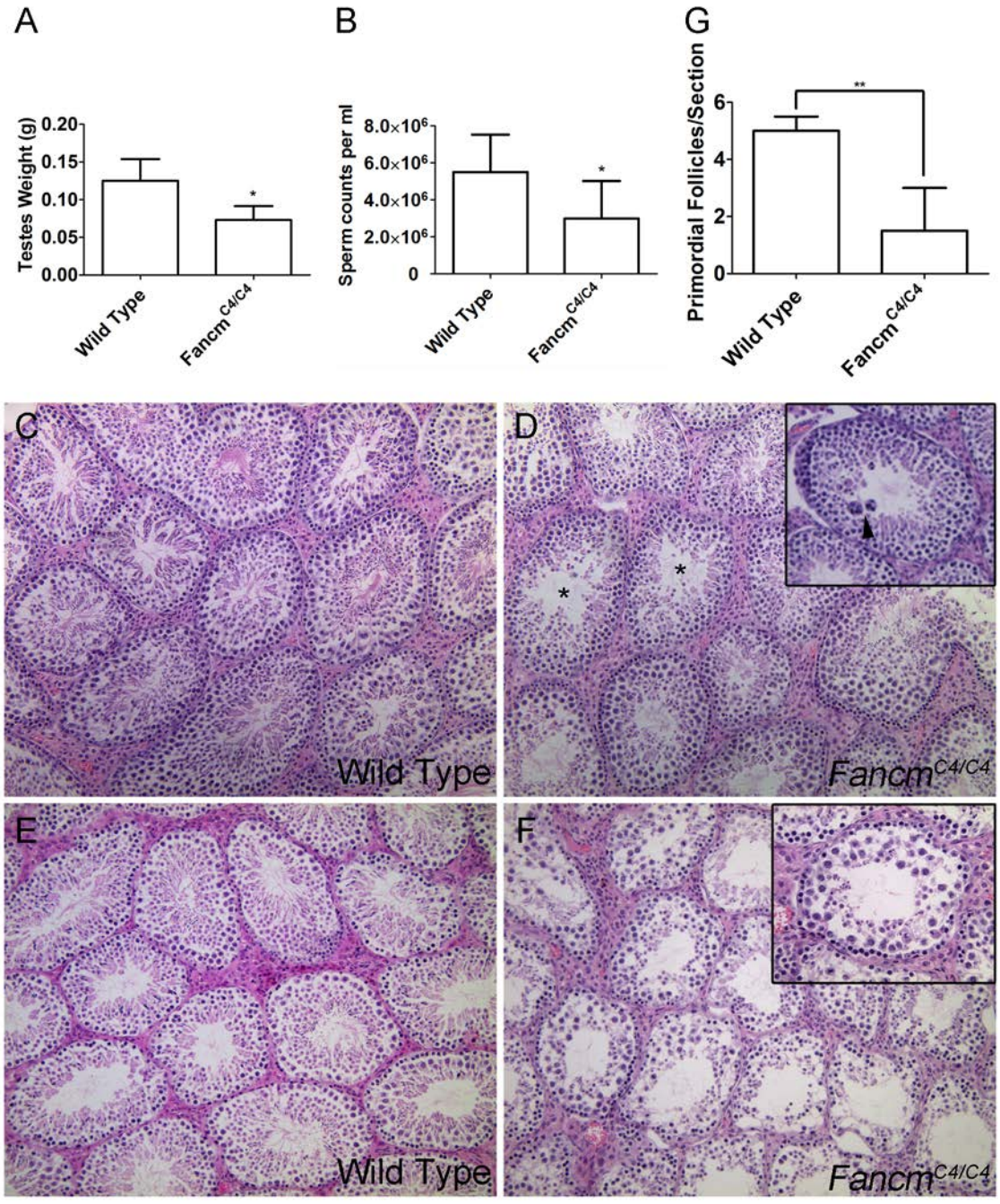

Figure 2-3. Hypogonadism and spermatogenesis defects in *Fancm* mutant males. (A,B) *Fancm*$^{C4/C4}$ mice (12 weeks old) have smaller testes and lower epididymal sperm counts. (C-F) H&E-staining of testis sections of the indicated genotypes. Samples in panels C-D are from 16 week old males. Arrowhead points to a giant multinucleated cell. (*) indicates an example of a seminiferous tubule with abnormal spermatogenesis. Panels E and F are from 80 week old males. Most tubules in 80 week *Fancm*$^{C4/C4}$ testes have only Sertoli cells. (G) Primordial follicles quantification in mutants. * $p<0.05$, ** $p<0.01$ $n>10$ for each genotype.

synaptonemal complex). H2AX phosphorylation is also a marker of, and is involved in, transcriptional Meiotic Silencing of Unsynapsed Chromatin (MSUC) during meiosis [29]. As in WT (Figure 2-4A, E), most mutant pachytene spermatocytes had a normal XY body (marked by an intense γH2AX domain) and no RAD51 foci or autosomal γH2AX staining (Figure 2-4B, F), indicative of proper chromosome synapsis and recombinational repair of programmed (SPO11-induced) meiotic DSBs. However, 42% of the pachytene nuclei showed abnormal γH2AX staining, either spreading as a cloud into autosomes (Figure 2-4C) or as punctate foci on chromosome axes (Figure 2-4D), reflective of unsynapsed chromosomes and unrepaired DSBs, respectively. Consistent with the γH2AX results, twenty-seven percent of the spreads showed persistent RAD51 foci (Figure 2-4G, H). The data suggest that *Fancm*$^{C4/C4}$ spermatocytes have a defect in meiotic DSB repair, which in turn may affect synapsis of chromosomes in a subset of spermatocytes.

The incomplete, sex-independent germ cell depletion in young adults, characterized by primordial follicle reduction, reduced testis size, and germ cell losses in some seminiferous tubules was suggestive of premeiotic germ cell defects. To explore this, newborn gonads were serially sectioned and probed with the germ cell-specific marker MVH (mouse vasa homolog) to quantify the number of germ cells at birth. In *Fancm*$^{C4/C4}$ males and females, there were markedly fewer germ cells (55% and 30%, respectively) compared to wild-type littermates (Figure 2-5). This indicates that the germ cell depletion is initiated during embryogenesis.

To identify the stage at which germ cell depletion starts, we examined the PGC population at various times of gestation. PGCs are first specified extra-embryonically at

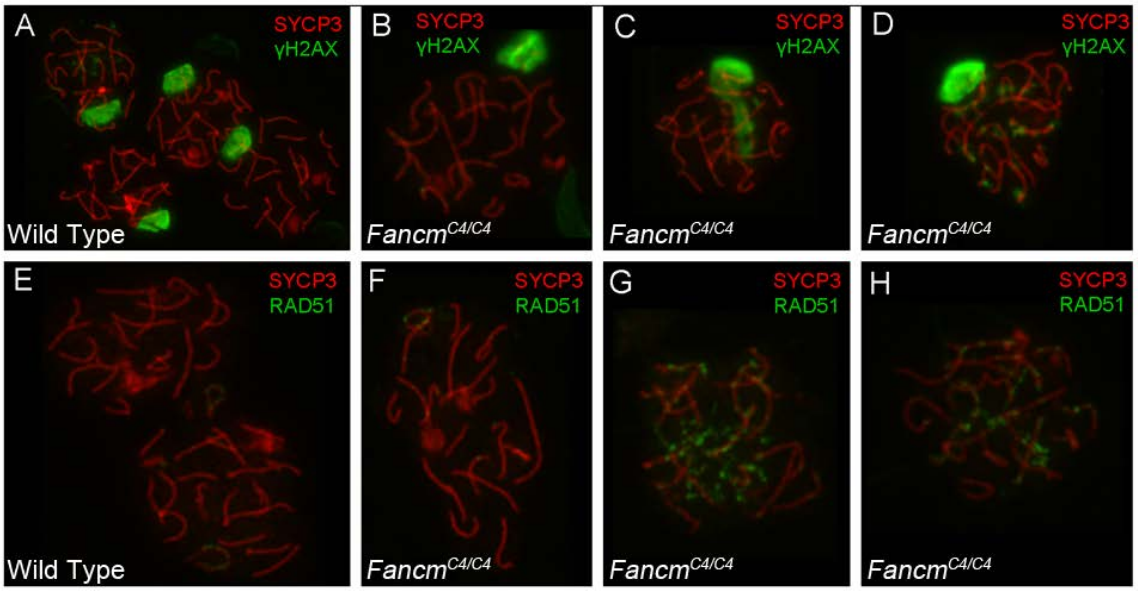

Figure 2-4. Meiotic defects in *Chaos4* mutant spermatocytes.
(A-H) Shown are surface spread meiotic chromosomes from the indicated genotypes of males. Antibodies used for immunolabeling are as indicated with color coding. SYCP3 is a synaptonemal complex protein marking chromosome cores. The large domains of γH2AX staining in A,B and D correspond to the XY body. In panel C, there is an extended region of XY body-like staining over autosomes, a pattern typically called a "pseudo sex body" and usually marks asynapsed autosomes. All nuclei are in the pachytene stage of meiosis.

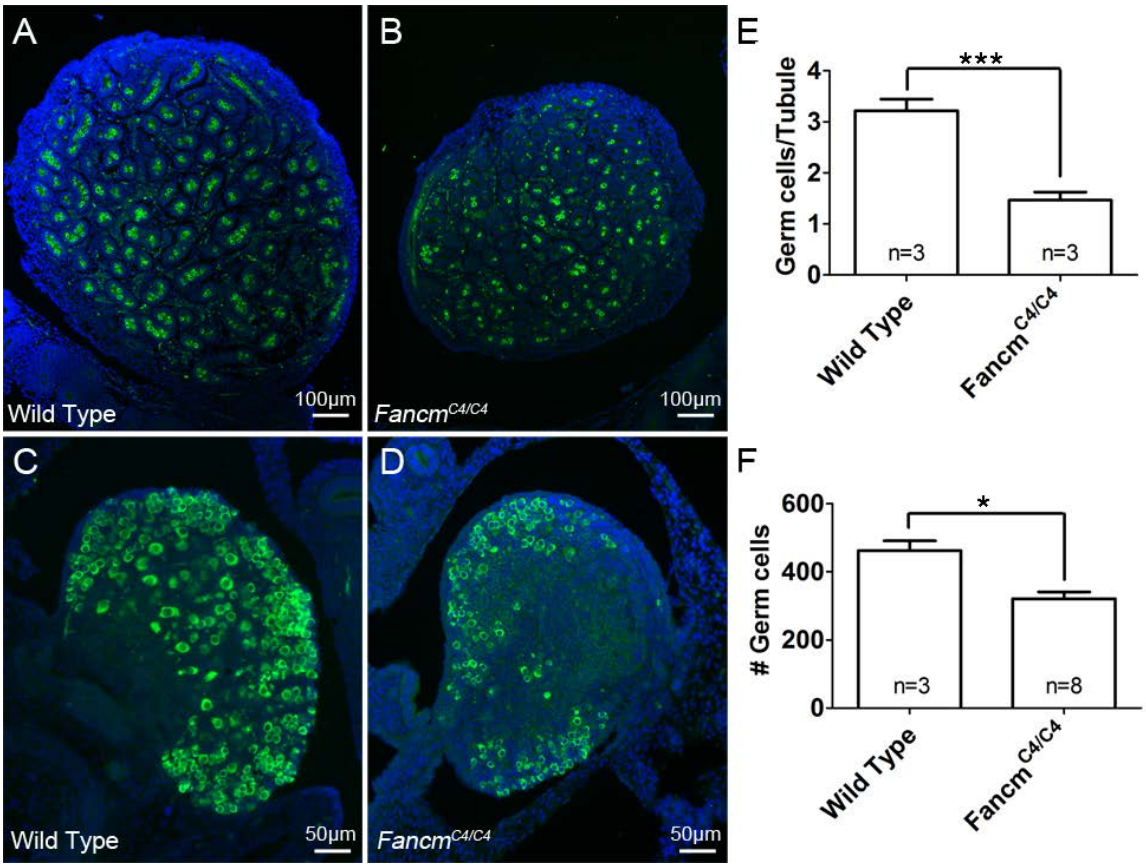

Figure 2-5. Germ cell depletion in *Fancm*$^{C4/C4}$ occurs before birth.
Immunofluorescence of 1 dpp testes (A and B) and ovaries (C and D) of the indicated genotypes. *MVH* (green) stains germ cells and DAPI stains nuclei (blue). (E and F) Germ-cell counts at 1 dpp. Germ cell number is averaged on a per seminiferous tubule cross-section basis for males. Female counts correspond to the total from three medial sections. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; Error bars indicate SD.

embryonic day 7.5 (E7.5). Between E8.5 and E10.5, this pool of alkaline phosphatase-positive PGCs then migrates along the epithelia of the hindgut towards the urogenital ridge, undergoing a modest degree of proliferation along the way. From there, they traverse the dorsal mesentery and populate the primitive gonad. They then undergo a dramatic proliferation after which male PGCs enter mitotic arrest until 3-4 dpp, while female PGCs enter meiosis at ~E13.5 and arrest in meiotic prophase I until puberty (reviewed in [30]). We quantified PGCs at E11.5, E12.5 and E13.5. The numbers were not significantly decreased in either male or female *Fancm*$^{C4/C4}$ embryos at E11.5 (Figure 2-6). However, a significant reduction was evident by E12.5 and E13.5 (Figure 2-6).

These combined data suggest that FANCM deficiency does not significantly impair PGC specification or migration, but rather that mutant PGCs either proliferate more slowly or undergo elevated apoptosis. To distinguish between these possibilities, we assessed PGC proliferation and apoptosis using BrdU incorporation and TUNEL assays, respectively. The BrdU incorporation assays indicated that PGC proliferation is reduced in both male and female *Fancm*$^{C4/C4}$ gonads at E12.5 and E13.5 (Figure 2-6B; Figure A2-2). Furthermore, apoptosis was not evident in either wild type or *Fancm*$^{C4/C4}$ gonads at E12.5 (Figure A2-3).

Previous studies estimated the number and the doubling time of PGCs between E11.5 and E13.5 [31,32]. The doubling time of wild type PGCs is 15.8h in males, and 16.1h in females (see Methods). Based on our PGC quantification, the doubling time of *Fancm*$^{C4/C4}$ PGC increased to 17.9±0.2h in males, and 18.9±0.3h in females.

DNA damage response pathways involved in PGC depletion

Although hypogonadism and testicular failure is characteristic of FA, a possible

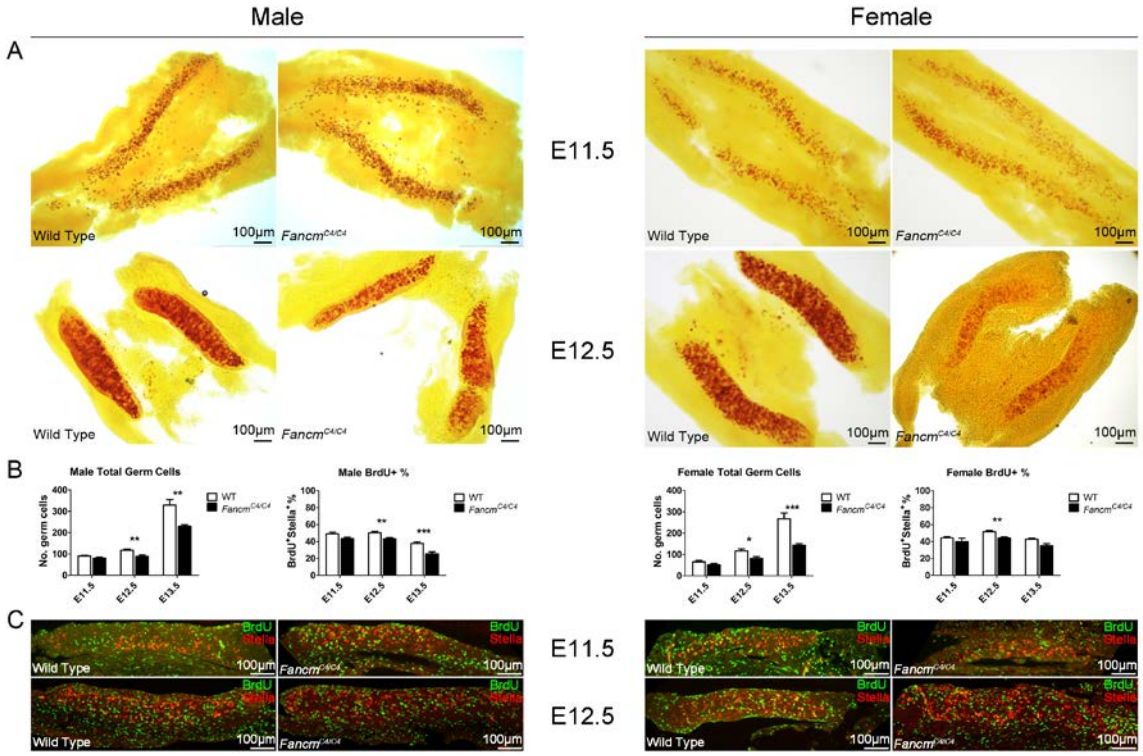

Figure 2-6. PGC depletion in *Fancm*$^{C4/C4}$ mice is associated with reduced proliferation, not apoptosis.

(A) Male and female embryonic gonads from E11.5 and E12.5 stained for alkaline phosphatase activity. A decrease in PGCs is becomes evident only at the latter time point. The graphs of germ cells in (B) represent quantification of germ cells by immunolabelling fetal gonads with either Stella (E11.5 and E12.5) or MVH (E13.5). Representative images for E13.5 gonads are shown in Figure A2-2. The graphs of BrdU+ cells represent data from BrdU incorporation assays shown in (C and Figure A2-2). BrdU and Stella double-positive cells, which represent PGCs in S phase, were quantified as percentage of total Stella-positive PGCs.

link between this and FA-related GIN has not been established. We hypothesized that if activation of a particular DDR pathway triggers PGC growth arrest or attenuation, then genetic disruption of that pathway would relieve the PGC depletion. Accordingly, we crossed *Fancm*$^{C4/C4}$ with various checkpoint mutants, including alleles of *Atm*, *Chk2* (*Chek2*), *p53* (*Trp53*), *p21* (*Cdkn1a*), and *Hus1* to obtain double mutants. All mutations were congenic or near congenic (at least 7 backcross generations) on the C3H strain background. The numbers of MVH-positive germ cells in newborn gonads were then quantified.

We first analyzed the role of p53 and its downstream effector p21 [33,34]. Deletion of one or both p53 alleles partially but significantly rescued germ cell loss in *Fancm*$^{C4/C4}$ male newborns (Figure 2-7A). This partial rescue implies that some but not all germ cell depletion is due to p53 activation. Similar partial rescue was observed in *Fancm*$^{C4/C4}$ *p21*$^{-/-}$ males (Figure 2-7B). The involvement of p21, a CDK inhibitor and downstream effector of p53 [35,36], is consistent with our previous finding that PGC depletion in *Fancm*$^{C4/C4}$ is a result of reduced proliferation. Surprisingly, the partial rescue was sexually dimorphic; neither *p53* nor *p21* knockout ameliorated the germ cell deficiency in newborn *Fancm*$^{C4/C4}$ females.

Next, we focused on the upstream kinases of two major DDR pathways, ATM and ATR [37]. These two proteins primarily respond to DSBs and sites of replication errors (RPA-coated ssDNA), respectively. Intercrosses of *Fancm*$^{C4/C4}$ *Atm*$^{+/-}$ mice produced 49 pups, none of which were homozygous for both mutations ($p<0.001$; expected = 12.25). Whereas doubly deficient mice were not born, *Fancm*$^{C4/C4}$ mice heterozygous for *Atm* were viable, and the genetic reduction of ATM partially rescued the

germ cell loss in males but not females (Figure 2-7C). Therefore, *Atm* may respond to increased DNA damage in *Fancm*$^{C4/C4}$ PGCs, ultimately activating p53-p21 signaling to protect the fidelity of genetic information in the PGC pool. In contrast, a hypomorphic viable allele (*Hus1*^{neo}) of the ATR-pathway gene *Hus1* [38] had no apparent impact on the depletion of *Fancm*$^{C4/C4}$ PGCs (Figure 2-7D).

Given the partial phenotypic rescue of *Fancm*$^{C4/C4}$ PGCs by *Atm* haploinsufficiency and *p53* nullizygosity, we hypothesized that the ATM target CHK2 served as the intermediate transducer kinase. However, *Chk2* deficiency did not rescue germ cells loss in *Fancm*$^{C4/C4}$ males, but significantly rescued the germ cell population in *Fancm*$^{C4/C4}$ females (Figure 2-7E). Interestingly, *Chk2*$^{-/-}$ newborn females had more germ cells than WT controls (Figure 2-7E). Therefore, the rescue effect of *Chk2* mutation is probably independent of *Fancm*^{C4} mutation. As previously reported [39], we observed that *Chk2*$^{-/-}$ adults had histologically normal gonads. *Chk2*$^{-/-}$ males did not have more gonocytes at birth than WT siblings (Figure 2-7E). Since female but not male PGCs enter meiosis before birth, and *Chk2* was recently found to play a crucial DNA damage checkpoint role in female meiosis [40], this may account for the elevated number of oocytes in double mutants.

2.5 Discussion

FANCM is a key component of the FA signaling pathway. Numerous *in vitro* studies have suggested that FANCM is a sensor of DNA damage at replication forks and helps anchor the FA core complex to chromatin [8,41-44]. *Fancm* was also reported to have the non-canonical function of regulating meiotic crossovers in *Arabidopsis thaliana* and *Saccharomyces pombe*, specifically by catalyzing interference-independent

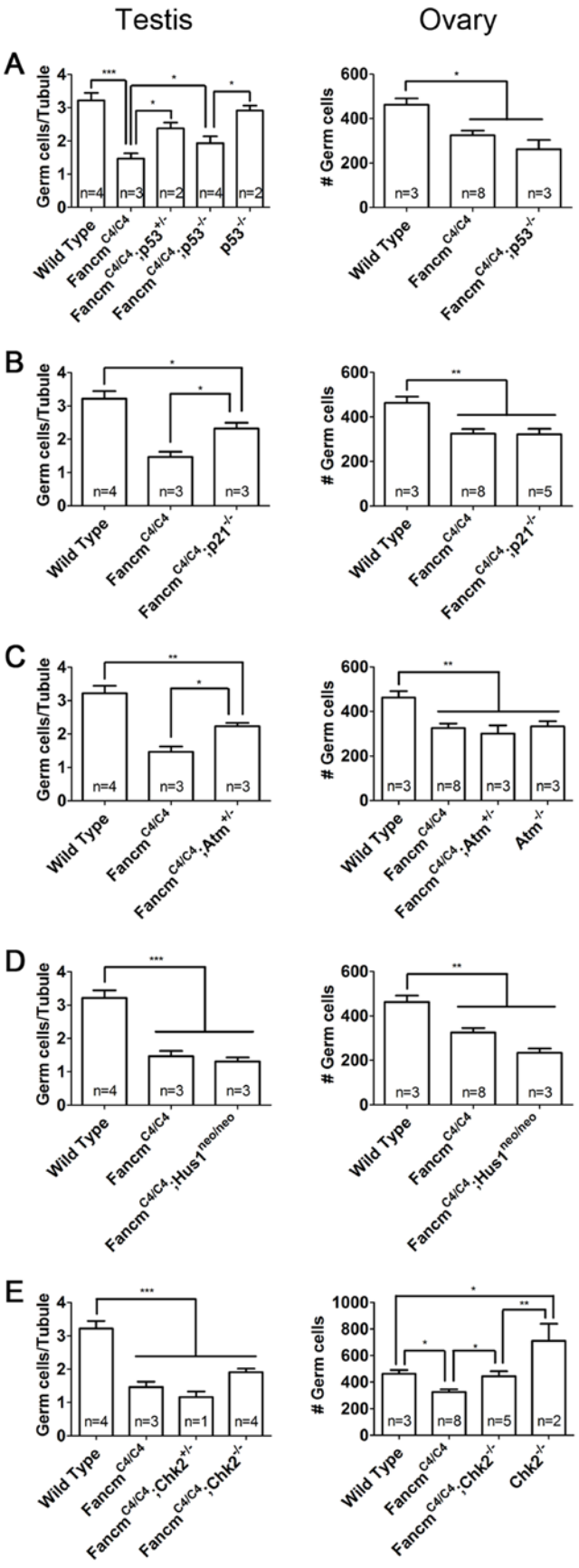
Testis
Ovary
A
Germ cells/Tubule
# Germ cells
n=4
n=3
n=2
n=4
n=2
n=3
n=8
n=3
Wild Type
Fancm C4/C4
Fancm C4/C4;p53+/-
Fancm C4/C4;p53-/-
p53-/-
Fancm C4/C4;p53-/-
B
n=4
n=3
n=3
n=3
n=8
n=5
Wild Type
Fancm C4/C4
Fancm C4/C4;p21-/-
C
n=4
n=3
n=3
n=3
n=8
n=3
n=3
Wild Type
Fancm C4/C4
Fancm C4/C4;Atm+/-
Atm-/-
D
n=4
n=3
n=3
n=3
n=8
n=3
Wild Type
Fancm C4/C4
Fancm C4/C4;Hus1neo/neo
E
n=4
n=3
n=1
n=4
n=3
n=8
n=5
n=2
Fancm C4/C4;Chk2+/-
Fancm C4/C4;Chk2-/-
Chk2-/-

Figure 2-7. Genetic analysis of checkpoint signaling in *Fancm*$^{C4/C4}$ germ cells.
(A-E) Compound mutant gonads with indicated genotypes were collected at 1 dpp. Germ-cell counts were performed following MVH labeling. Male germ cell number was averaged on a per tubule cross-section basis for males. Values for females equal the total of germ cells detected in three medial sections. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; Error bars indicate SD.

recombination intermediates to undergo noncrossover rather than crossover resolution [45-47]. It was recently shown that FANCM, via its translocase activity, interacts with MHF to allow replication to "traverse" ICLs without repair, and that this activity is independent of other FA members [48]. Despite the substantial biochemical and mechanistic information on *Fancm* function, the physiological roles of *Fancm* in vertebrates are incompletely characterized.

A previous study found that *Fancm* null mice not only phenocopied other FA mouse models in causing hypogonadism and hypersensitivity to cross-linking agents (in MEFs), but also had decreased longevity and tumor-free survival [18]. As with the null mutant, $Fancm^{C4/C4}$ mice had elevated SCE and tumor susceptibility, and $Fancm^{C4/C4}$ MEFs underwent senescence prematurely. The general similarity in phenotypes between the null and $Fancm^{C4}$ alleles indicates that the single amino acid change in the DEAH helicase domain disrupts the crucial function of this protein in mice. This domain has no detectable helicase activity, but does encode the translocase activity of FANCM that is important for promoting the recovery of stalled replication forks [49,50]. Given that mutating the translocase function of FANCM alone disrupts replication traverse of ICLs in the same manner as null cells [48], we speculate that the $Fancm^{C4}$ mutation disrupts translocase function to yield phenotypes that are essentially indistinguishable from nulls. Future studies to test this and other possibilities, such as protein stability, would be of interest.

We traced the cause of germ cell depletion in newborn FANCM-deficient mice to defects in PGC proliferation, which was not reported for the knockout, but which has been noted for knockouts of other FA genes (discussed earlier). Specifically, we found

that the ATM-p53-p21 DDR pathway is operative in regulating PGC proliferation in males. Mutations of each partially restored germ cell numbers in newborns. However, the results with compound *Atm* mutants suggest a complex relationship with FANCM in PGCs. It has been reported that FANCM is actually regulated in part by ATR and ATM in response to damaged DNA in a *Xenopus* extract system [51], but the synthetic lethality between *Fancm* and complete ATM deficiency ($Atm^{-/-}$) suggests that ATM and FANCM also have parallel, non-epistatic roles in DDRs during development. The $Fancg^{-/-}$ $Atm^{-/-}$ genotype also causes embryonic lethality [52], and inhibition of the FA pathway selectively kills ATM-deficient cells [53,54], supporting the idea that the DNA damage to which the ATM and the FA pathway responds overlap. The viability of, and partial rescue of PGC loss in, $Fancm^{C4/C4}$ $Atm^{+/-}$ males suggests that the parallel DNA repair role of reduced ATM is sufficient to overcome the lack of functional FA pathway repair, but compromises checkpoint-mediated cell cycle delay in PGCs, presumably via reduced signaling to p53.

p53 is a key transcription factor that regulates several signaling pathways involved in the response to cellular stress, DNA damage, oncogene activation and other physiological signals [55]. Genetic experiments in mice have shown that p53 plays a role in FA signaling. p53 deficiency partially rescues the embryonic lethality in *Fancn* (*Palb2*) and *Fanco* (*Rad51c*) mutants [56,57] and bone marrow failure in *Fancd2* mutants [58]. Our studies provide the first evidence that p53 is involved in genome surveillance of PGCs during their expansion phase in development, at least in males. In the context of *Fancm* deficiency and the presumed increase of DNA lesions this causes, p53 appears to slow cell cycle progression rather than causing apoptosis (see model in Figure 2-8).

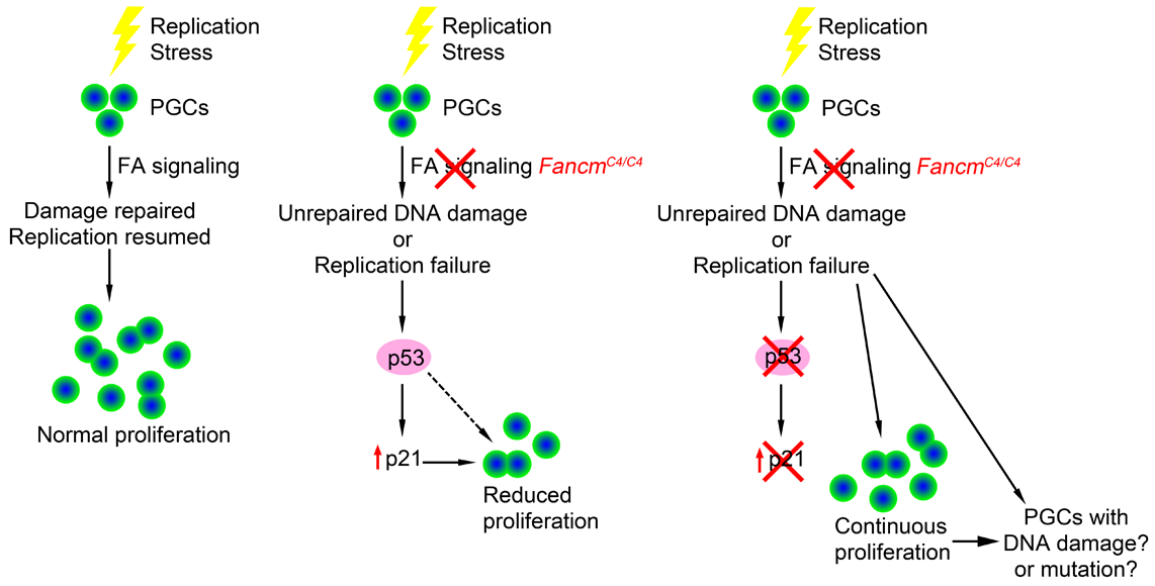

Figure 2-8. Model of checkpoint responses to replication stress in primordial germ cells.

Mutations in *Fancl* and *Fancc* also cause germ cell reduction traced to reduced PGC proliferation and not apoptosis [15,23], suggesting that the level of endogenous DNA damage induced by FA pathway defects is not sufficient to stimulate p53-mediated apoptotic signaling. In contrast, p53 was reported to mediate germ cell apoptosis in Zebrafish *fancl* mutants [59], implying either that germ cells in this organism are more sensitive to DNA replication defects, the p53 pathway is more active in zebrafish germ cells, and/or zebrafish lack a redundant repair pathway(s).

The activity of p53 alone doesn't fully account for germ cell depletion in *Fancm* mutants. Aside from only partial rescue in $Fancm^{C4/C4}$ males by *p53* deletion, which suggests that an additional or parallel DDR pathway might still be operative such as one involving paralogs p63 and p73, p53 deficiency did not rescue loss of oocytes in newborn females. One possible explanation for this sexual dimorphism may relate to the direct entry of female PGCs into meiosis at ~E13.5, unlike the mitotic arrest that male PGCs undergo. Since quantification of germ cell number in compound mutants was conducted in newborns, the number of oocytes at birth reflects events that occur both during PGC proliferation and during meiotic prophase I. Considering that male $Fancm^{C4/C4}$ meiocytes had substantially elevated DSBs, and mouse oocytes have a stringent meiotic DNA damage checkpoint that causes apoptotic elimination perinatally [60], it is possible that any rescue of PGC proliferation in $Fancm^{C4/C4}$ $p53^{-/-}$ females was counteracted by subsequent meiotic losses of those oocytes derived from damage-bearing “rescued” PGCs. Importantly, the oocyte DNA damage checkpoint involves signaling of CHK2 to both p53 and TAp63, and that in the absence of p53, DSB-bearing oocytes are still efficiently eliminated by CHK2-activated TAp63 [40]. As mentioned earlier, our observation that

perinatal *Fancm*$^{C4/C4}$ germ cell numbers were rescued in CHK2-deficient females but not males likely reflects this oocyte-specific meiotic DNA damage pathway, not a PGC DDR.

Few DNA repair gene mutations are known to impact PGC growth or maintenance. Beyond FA mutants, *Pin1*, *Mcm9, Rev7* and *Helq* are four other genes that have been correlated with both a function in genome maintenance and a PGC depletion phenotype [61-66]. *Pin1* is a prolyl isomerase which directly regulates cell cycle genes. *Pin1* deletion depletes PGCs by delaying their proliferation [64]. *Mcm9* and *Helq* appear to be involved in homologous recombination repair (HRR) of ICLs. HELQ interacts with the RAD51 paralog complex, but appears to function in a pathway in parallel to FA [61,62,67-70]. MCM9 is required for normal homologous recombination, promoting recruitment of RAD51 to DNA damage sites and repair of ICLs [68-70] It also appears to act downstream of the FA pathway [70]. Interestingly, FANCM was reported to be required for HR-independent ICL repair [11]. Despite these indications of multiple pathways for DNA repair in PGCs, that these cells remain highly sensitive to perturbations of any of them.

Fancm$^{C4/C4}$ males also exhibited progressive germ cell depletion with age. The reason for this is unclear, since histological analysis revealed only subtle seminiferous tubule abnormalities in young mice. The progression to a near Sertoli Cell Only-like phenotype in many tubules suggests a defect in spermatogonial proliferation or renewal. The lack of more dramatic testicular pathology in young mice is also curious in light of evidence for DNA repair and XY-body defects in a substantial fraction of spermatocytes. Aside from the occasional appearance of abnormal multinucleated cells near the lumen of seminiferous tubules, coordinated arrest of pachytene stage spermatocytes was not

observed as is typical for mutants that are recombination-defective and which disrupt XY silencing, an event proposed to underlie meiotic arrest [71]. One possible explanation is that the level of defects is below the threshold that would trigger a checkpoint, or that the unrepaired DNA damage is eventually repaired before checkpoint-mediated elimination. It may be relevant in this regard that we have not noticed visual abnormalities in offspring of *Fancm* mutants. Another possibility is that the DNA damage in $Fancm^{C4/C4}$ spermatocytes, inferred as such by the presence of γH2AX and RAD51 foci, may be of a nature that does not trigger elimination. For example, it is possible that these foci correspond to sites of damage incurred during premeiotic DNA replication, as opposed to SPO11-induced DSBs. Another example of apparently tolerated meiotic damage is the case of $Rad54^{-/-}$ spermatocytes, which are not eliminated despite bearing extensive RAD51 foci in late pachynema [72]. Finally, it is possible that *Fancm* has a hitherto unknown role in meiotic checkpoint activation in addition to DNA repair.

This study contributes to an emerging picture that the FA pathway is particularly important in stem cell biology [2]. Reprogramming of fibroblasts into induced pluripotent stem cells requires FA pathway function [73,74]. Furthermore, not only is bone marrow failure a hallmark of FA, but this failure depends upon p53/p21 signaling [58]. The involvement of p53/p21 activation in both hematopoietic and germline stem cells bearing FA mutations, and the particular sensitivity of these lineage, emphasizes the importance of expanding studies of the FA pathway into diverse cell types including additional stem cell lineages.

2.6 Materials and methods

Micronucleus Assays

These were performed as described [75].

Positional cloning

The *Chaos4* mutation was ENU-induced on the C57BL/6J ("B6") background [27]. To identify the causative mutation, the mutation was outcrossed to strain C3HeB/FeJ ("C3H"), then intercrossed to produce potential homozygotes. F2 offspring were screened for micronucleus levels and a genome scan with a collection of microsatellite markers polymorphic between C3H and the parental strain B6 was performed [28]. This localized *Chaos4* to a 44-Mb interval on chromosome 12, between *D12Mit285* and *D12Mit71*. Subsequently, we conducted an inter-subspecific mapping cross with *Mus castaneus* (CAST/Ei). The F1s were either intercrossed or backcrossed to CAST/Ei and scored for micronuclei. A total of 956 informative meioses were examined, defining a 9-Mb critical region (Figure 2-1B).

Embryonic stem cell culture and microinjection for chimera production

The XH297 ES cell line (derived from the 129/Ola strain; BayGenomics) [76] bearing a gene trap insertion of *Fancm* (abbreviated $Fancm^{XH}$) were cultured in DMEM (Gibco) supplemented with 15% FBS (HyClone), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, penicillin-streptomycin (100 units/ml), 100 µM beta-mercaptoethanol (Sigma) and recombinant leukemia inhibitory factor (produced in-house). Cells were microinjected into C57BL/6J blastocysts by standard methods. $Fancm^{+/XH}$ mice were then backcrossed into the C3HeB/FeJ background.

Mice and genotyping

Genotyping of $Fancm^{C4}$ mice was performed by PCR amplification of a 240bp mutated segment with two primers: Chaos4L (CTTCTGGCAAGGTGGTTTTC) and

Chaos4R (TTTGCTACCCACAGACGATG). PCR products were then digested by restriction enzyme *Aci*I, which is present in the *Chaos4* allele only. The *Chaos4* allele is cut into 180bp and 60bp fragments. Genotyping of $Fancm^{XH}$ mice was performed indirectly using microsatellite markers *D12Mit69* and *D12Mit71* that flank *Fancm*, and which are polymorphic between strain C3H and B6 (B6 alleles at *D12Mit69* and *D12Mit71* are indicative of the *Chaos4* allele). The use of mice in this study was approved by Cornell's Institutional Animal Care and Use Committee. Mice bearing alleles of other mutations were: *Atm* (Atm^{tm1Led}, abbreviated as Atm^{-}), *Chk2* ($Chek2^{tm1Mak}$, abbreviated as $Chk2^{-}$), *p53* ($Trp53^{tm1Tyj}$, abbreviated as $p53^{-}$), *p21* ($Cdkn1a^{tm1Tyj}$, abbreviated as $p21^{-}$), and *Hus1* ($Hus1^{tm2Rsw}$, abbreviated as $Hus1^{neo}$) [39,77-80]. The stocks of mice bearing the *p53*, *p21* and *Hus1* alleles were all congenic in the C3H background (N10 or greater). The *Atm*, *Chk2* stocks were at the N7 backcross generation. Euthanasia was performed by CO_2 administration.

Mouse embryonic fibroblast (MEF) growth analyses

MEFs were generated from 12.5- to 14.5-dpc embryos. Cells were cultured in DMEM supplemented with 15% FBS (fetal bovine serum), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, penicillin-streptomycin (100 units/ml), and beta-mercaptoethanol. For cell proliferation assays, 0.5×10^6 cells were seeded per 100-mm plate and then cultured and harvested to count cell numbers at various time points. For the cell senescence assay, 0.5×10^6 cells were seeded per 100-mm plate and then cultured and passaged every 3 days until they became immortalized. MEF metaphase spreads and the sister chromatid exchange assay were performed as previously described [18,81].

Histology and Immunohistochemistry

For basic histology, tissues were fixed in 4% paraformaldehyde (PFA) overnight, paraffin-embedded, sectioned at 5μm, and stained with H&E (hematoxylin and eosin). Statistical differences in tumor types were assessed via Fisher's exact test. For germ-cell counts on embryonic or newborn gonads, 5μm sections were immunostained as previously described [82]. Germ cells in postnatal gonads were counted in three sections from the midportion of each gonad and averaged. Antibodies: Rabbit anti-DDX4/MVH (Abcam ab13840; 1:250); rabbit anti-Stella (Abcam ab19878; 1:250); goat anti-mouse Alexa 594 conjugate (Molecular Probes A11005; 1:1,000); goat anti-rabbit Alexa 488 conjugate (Molecular Probes A11008; 1:1,000). The data were analyzed using one-way ANOVA with Bonferroni correction (Prism software package). The resulting P values were used to determine significance ($P < 0.05$).

BrdU incorporation assay

Pregnant females received a single BrdU intraperitoneal injection (50 mg/kg) at 11, 12, or 13 days after vaginal plug detection (their corresponding embryos were E11.5, E12.5 and E13.5). Injected mice were sacrificed two hours later, and embryos were collected. Embryonic gonads together with mesonephric tubules (for E12.5 and E13.5 embryos) or the dorsal part of the trunk without other internal organs (for E11.5 embryos) were fixed in 4% PFA. Tissues were embedded in paraffin and sectioned. BrdU was detected by the Invitrogen BrdU Staining Kit (Cat. No. 93-3944), and PGCs were detected with rabbit anti-Stella (Abcam ab19878; 1:250). At least three sagittal sections across the central part of the gonads were used for PGC quantification and BrdU scoring.

Since no cell apoptosis was obvious and no cell migration occurs between E11.5 and E13.5, PGC doubling time was calculated based on an exponential growth model:

$$T_{Doubling} = \frac{48}{\log_2 \frac{N_{E13.5}}{N_{E11.5}}}$$

$N_{E13.5}$ and $N_{E11.5}$ are the absolute number of PGCs in the whole gonad, which was estimated based on the previous studies and the relative ratio between wild type and mutants.

Alkaline phosphatase staining

Embryonic gonads were stained as described [83]. Briefly, fixed gonads were washed with dH_2O and stained with freshly made staining solution (0.1 mg/ml Sodium α-naphthyl phosphate, 5 mg/ml Borax, 0.6 mg/ml $MgCl_2$, and 0.5 mg/ml Fast Red TR salt) for 15-30 min. Tissues were then washed in dH_2O and cleared with 70% glycerol.

TUNEL staining

Five μm paraffin sections of embryonic gonads were TUNEL stained using the In Situ Cell Death Detection Kit (Roche 11684817910). *Atm*$^{-/-}$ adult testes were used as a positive control [84].

Meiotic chromosome analysis

This was performed as described [75]. Primary antibodies used in this study: rabbit anti-SYCP3 (1:500, Abcam); mouse anti-γH2AX (1:500, JBW301 Upstate Biotechnology); rabbit anti-RAD51 (1:250, this polyclonal antibody recognizes both RAD51 and DMC1; Oncogene Research Products).

Ethics statement regarding vertebrate animal use

The use of mice in this study was approved by Cornell's Institutional Animal Care and Use Committee, under the approved protocol of JCS (2004-0038). Euthanasia was performed by CO_2 administration.

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2.8 Supporting Information

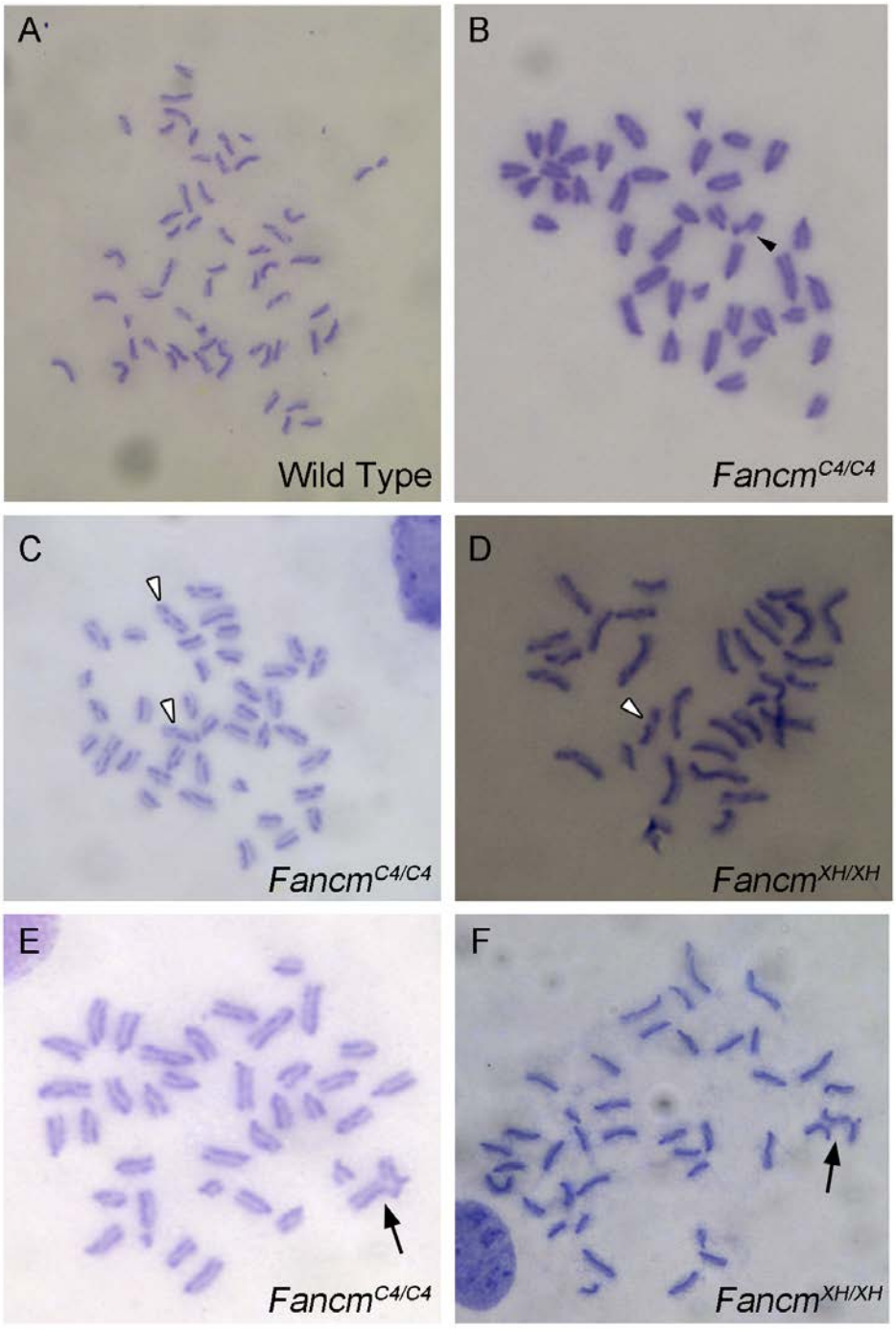

Figure S2-1. Chromosomal instability in *Fancm* mutant MEFs.
(A-F) Metaphase chromosomes from the indicated genotypes of MEFs. Chromosomal breaks (black arrowhead in B), sister chromatid exchanges (white arrowheads in C and D), and radial chromosomes (arrow in E and F) are observed in *Fancm* mutant MEFs, but not wild type MEFs (A).

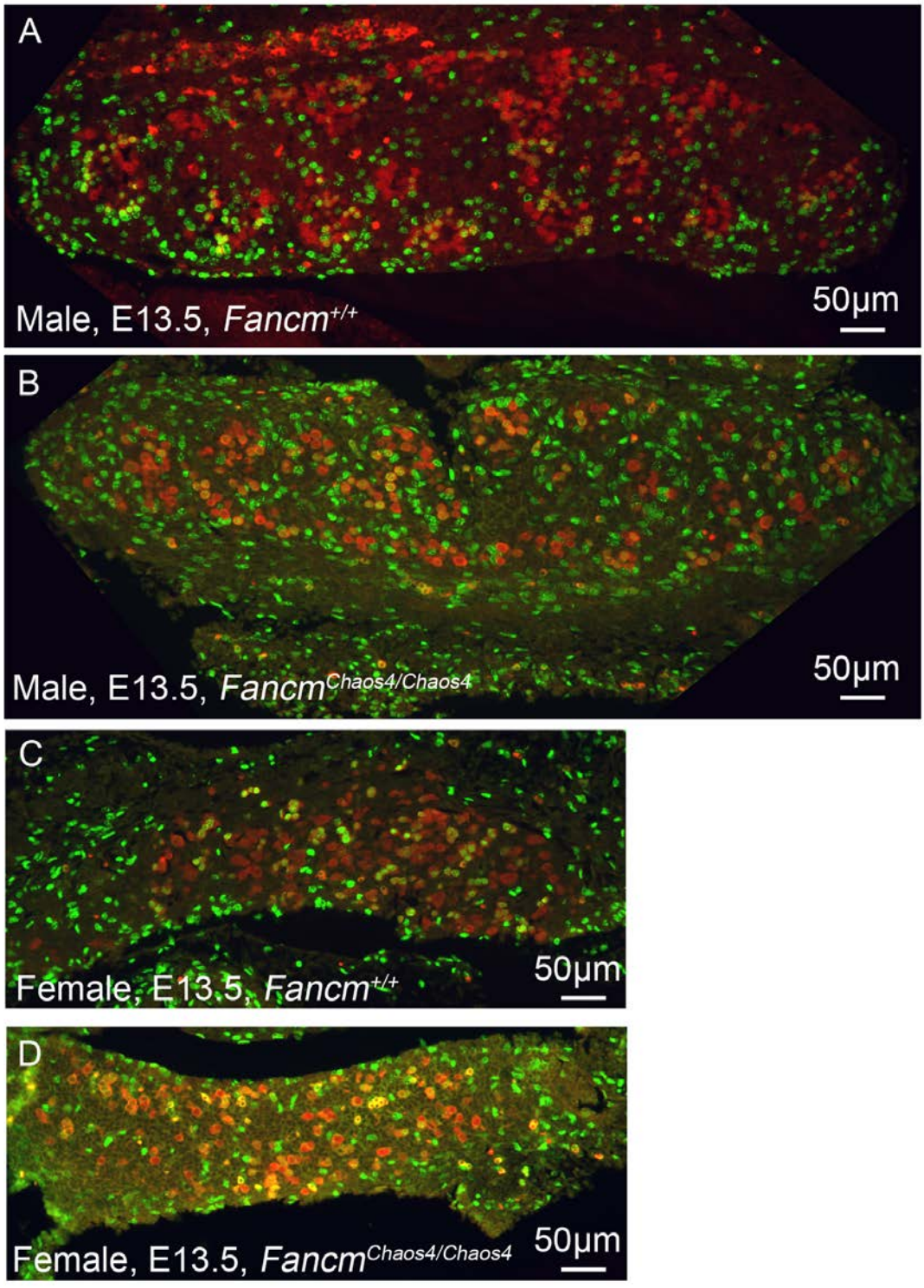

Figure S2-2. Representative images for PGC quantification and proliferation in E13.5 embryonic gonads.
Wild type (A, C) and *Fancm*$^{C4/C4}$ (B, D) male (A, B) and female (C, D) gonads are immunolabeled for Stella, a PGC marker, in red and BrdU in green.

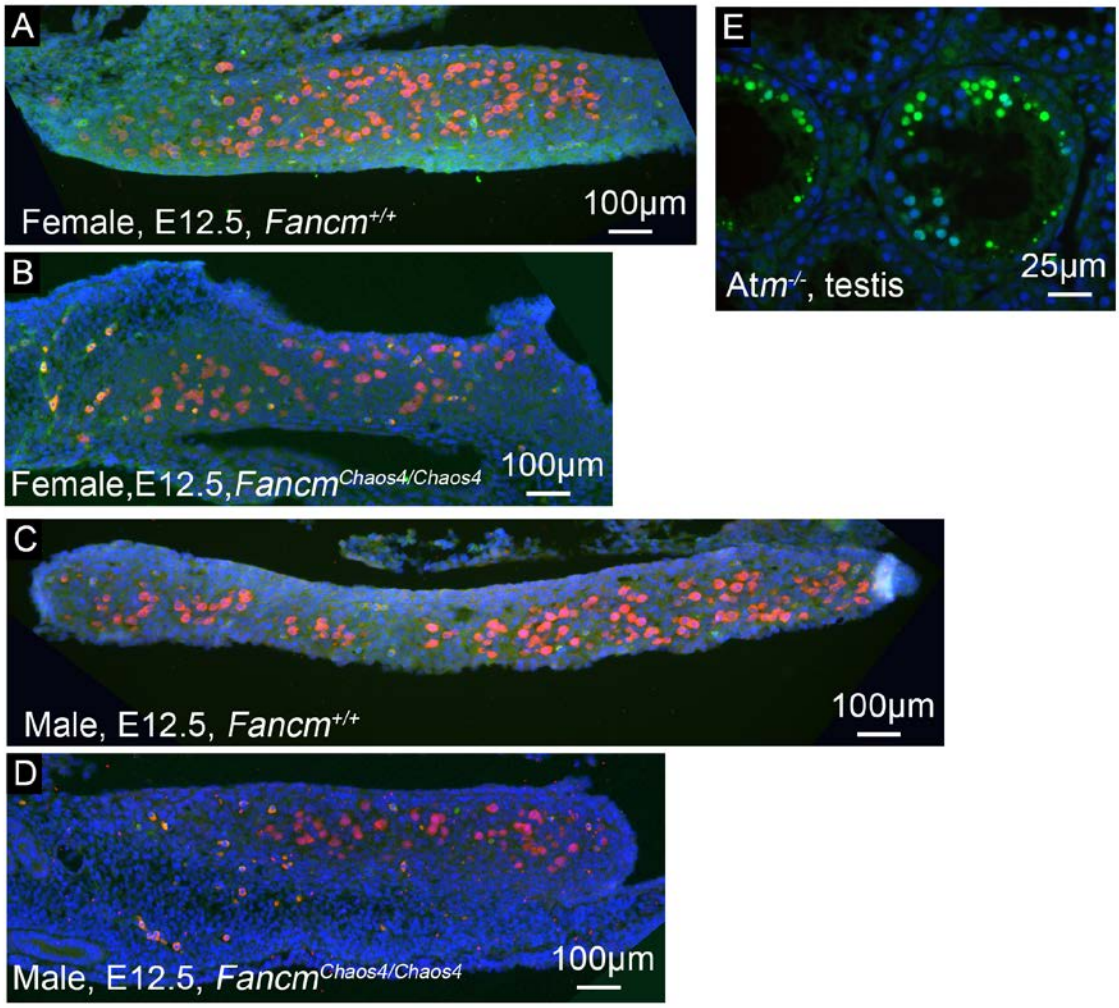

Figure S2-3. TUNEL assay of PGCs in E12.5 gonads.
Wild type (A, C) and *Fancm*$^{C4/C4}$ (B, D) female (A, B) and male (C, D) gonads are immunolabeled for Stella, a PGC marker, in red and TUNEL in green. (E) *Atm*$^{-/-}$ testis was used as a positive control for TUNEL signal (green).

Table S2-1. Viability of *Fancm* mutant mice.

Fancm$^{+/C4}$× *Fancm*$^{+/C4}$						
	Male		Female		Total	
	Observed	Expected	Observed	Expected	Observed	Expected
+/+	49	44	44	44	93	88
Fancm$^{+/C4}$	81	88	84	88	165	175
Fancm$^{C4/C4}$	40	44	52	44	92	88
χ^2 test	p=0.48		p=0.43		p=0.56	

Fancm$^{+/XH}$× *Fancm*$^{+/XH}$						
	Male		Female		Total	
	Observed	Expected	Observed	Expected	Observed	Expected
+/+	12	8	11	8	21	16
Fancm$^{+/XH}$	10	16	15	16	25	33
Fancm$^{XH/XH}$	12	8	5	8	17	16
χ^2 test	p=0.05		p=0.31		p=0.10	

Fancm$^{+/C4}$× *Fancm*$^{+/XH}$						
	Male		Female		Total	
	Observed	Expected	Observed	Expected	Observed	Expected
+/+	4	3	4	3	6	6
Fancm$^{+/C4}$	5	3	2	3	3	6
Fancm$^{+/XH}$	1	3	3	3	8	6
Fancm$^{C4/XH}$	2	3	4	3	8	6
χ^2 test	p=0.36		p=0.83		p=0.44	

Table S2-2. Tumor Frequency of *Fancm* mutants.

Tumor Frequency-Females		
Wild type	0/28 (44-80wks)	0.00%
Fancm$^{+/C4}$	9/27 (44-80wks)	33.33%
Fancm$^{C4/C4}$	15/26 (44-80wks)	57.69%
Fancm$^{XH/XH}$	0/5 (28-52wks)	0.00%

Tumor Frequency-Males		
Wild type	4/45 (50-85wks)	9.00%
Fancm$^{+/C4}$	15/36 (60-90wks)	41.67%
Fancm$^{C4/C4}$	8/17 (70-80wks)	47.06%
Fancm$^{XH/XH}$	0 out of 8 (12-78wks)	0.00%

Table S2-3. Histopathology of *Fancm* mutant mice.

Animal	Age (wks)	Genotype	Sex	Histopathology	Tumor
17251	54	WT	F	Ovary - follicular cyst; spleen - red pulp mildly expanded by extramedullary heperplasia (EMH) and abundant hemosiderin laden macrophages, white pulp also mildly hyperplastic; uterus - mild cystic endometrial hyperplasia and dilation of lumen	
13116	45	WT	F	Follicular cyst	
11125	81	WT	F	ovary - 2 small follicular cysts	
16096	80	WT	F	Follicular cyst and hemorrhagic cyst	
2611	106	C4/+	F	Epithelial Tumor	√
13894	81	C4/+	F	one fluid filled cyst on each ovary	
13896	81	C4/+	F	Follicular cysts	√
21225	66	C4/+	F	Ovarian Teratoma	√
21226	66	C4/+	F	uterine edema, adrenal gland tumor?, small spleen, small black tumors on lung	
404	46	C4/+	F	uterus - some proteinaceous fluid and neuts in lumen	
11123	81	C4/+	F	uterus - unilateral hydro/mucometra; cystic endometrial hyperplasia, potential adenomyosis	
13118	45	C4/+	F	ovary - multifocal mineralized follicles unilateral;	√
14389	75	C4/+	F	Liver Tumor	
14391	75	C4/+	F	1 fluid cyst on each, fluid filled uterus, but not too enlarged, there ma have been a small mammary tumor.	
14652	83	C4/+	F	Follicular cyst	
14964	81	C4/+	F	Hemorrhagic ovarian cyst	√
15364	80	C4/+	F	ovary - large follicular cyst; uterus - multifocal perivascular lymphoid aggregates; liver - multifocal perivascular lymphoid aggregates; cervical lymph node - paracortex expanded by sheets of lymphocytes, small cell lymphoma	√
15868	50	C4/+	F	ovary - unilateral follicular cyst; uterus - abundant nondegenerate neutrophils in glands, endometrium, lumen; liver - mild multifocal emh	
16095	80	C4/+	F	liver - hepatocellular carcinoma and hematoma;	√

#154	102	C4/+	F	adrenal - mild subcapsular cell hyperplasia;liver - mild multifocal EMH and necrotizing hepatitis; mammary gland - mild lymphoid infiltrates; spleen - moderate hemosiderin in white and red pulp	
11108	95	C4/+	F	Ovary - tubulostroma hyperplasia; liver - large biliary cyst with peripheral inflammatory infiltrates	
11110	95	C4/+	F	Liver, spleen - histiocytic sarcoma; kidney - mineral in distal collecting ducts, mild multifocal tubular ectasia and proteinosis; adrenal gland - locally extensive moderate subscapsular cell hyperplasia; ovary and uterus - some neoplastic cells in peripheral adipose;brain - multifocal mineralized blood vessels in brainstem and thalamus	√
11118	81	C4/+	F	ovary - paraovarian cyst; mineralized follicles; mammary gland adenocarcinoma	√
11119	81	C4/+	F	ovary - follicular cyst;	
17247	54	XH/+	F	Uterus - mild hydrometra	
2708	58	C4/C4	F	Basisquamous carcinoma	√
17817	24	C4/C4	F	Uterine Tumor	√
21223	66	C4/C4	F	cystic ovary, edema, start of a liver tumor, cyst on kidney, brown hair stripes, intestinal tumors, small lump on tail	√
402	46	C4/C4	F	endometrial hyperplasia-adenoma based on size(UT), Ov-cavernous sinuses that may have lead to the corpora hemorrhagia	√
3368	85	C4/C4	F	Mammary or epithelial tumor	√
11124	81	C4/C4	F	ovary - multiple cysts compressing and distorting ovarian architecture, Liver and Lung adenoma	√
13117	45	C4/C4	F	Skin Tumor, hemorrhagic ovarian cyst	√
14390	75	C4/C4	F	Uterine Tumor, skin tumor, follicular cysts	√
14650	83	C4/C4	F	Mammary Tumor	√
14965	81	C4/C4	F	Mammary Tumor, follicular cysts	√
14967	81	C4/C4	F	Liver Tumor	√
16097	80	C4/C4	F	Uterine Tumor, follicular cysts	√
19169	24	C4/C4	F	Follicular cyst	
#155	102	C4/C4	F	Brain and epithelial tumors	√
11113	99	C4/C4	F	Multiple small liver tumors, mammary tumor	√
11116	81	C4/C4	F	caverous hemoraghioma cysts, and paraovarium cyst in second ovary. Tumor-	√

				secratory mammary adenocarcinoma	
11117	81	C4/C4	F	tubularstromal adenoma/carcinoma low grade, bloody cystic oavary- another potentian tubular stromal carcinoma, UT- polypoid adenoma, endometric hypersplasia, Lung- 2 early adenomas	√
17248	54	C4/XH	F	Spleen - mild lymphoid hyperplasia; ovary - large (9 mm) follicular cyst with mineralization	
20397	31	C4/XH	F	Spleen - mild lymphoid hyperplasia	
20692	26	C4/XH	F	Uterus - some hemosiderin laden macrophages in wall; spleen - mild lymphoid hyperplasia, many hemosiderin laden macrophages in white pul	
20761	21	C4/XH	F	Spleen - lymphoid hyperplasia, mild increase in EMH	
21003	21	C4/XH	F	Bone tumor on Jaw 7X11mm	√
16802	52	WT	M	Liver Adenoma	√
17877	55	WT	M	Islet cell tumor	√
13115	85	WT	M	Liver Tumor	√
13113	85	WT	M	Liver Tumor	√
13889	72	C4/+	M	Liver Tumor	√
14199	80	C4/+	M	Liver Tumor	√
11307	89	C4/+	M	liver - focal adenoma	√
11311	89	C4/+	M	preputial gland - keratin filled cyst, focal suppurative and granulomatous inflammation; liver - multifocal areas of mineralization surrounded by fibrous connective tissue	
14388	69	C4/+	M	Liver Tumor	√
14648	83	C4/+	M	liver - hepatocellular carcinoma and hematoma	√
14960	81	C4/+	M	liver - hepatocellular carcinoma	√
14646	83	C4/+	M	liver - hepatocellular carcinoma	√
14200	80	C4/C4	M	Lung Tumor	√
11310	89	C4/C4	M	liver - hepatocellular carcinoma;	√
13984	80	C4/C4	M	kidney - mild multifocal tubular mineralization; liver - multiple adenomas	√
13985	80	C4/C4	M	lung - multifocal peribronchiolar and perivascular lymphoid aggregates	
14647	83	C4/C4	M	liver - hepatocellular carcinoma	√
15359	80	C4/C4	M	testis - mineralized and sclerotic	

16092	80	C4/C4	M	haired skin - focal pyogranulomatous inflammation and sebaceous gland hyperplasia (is this near the ear?; lymph nodes, moderately reactive; liver - adenoma and focus of cellular alteration	√
16094	73	C4/C4	M	Testis - rare multinucleate cells, <10% of tubules lined by single layer vacuolated Sertoli cells; Spleen - marked myeloid hyperplasia and splenomegaly; haired skin - infundibular keratinizing acanthoma; locally extensive hepatic necrosis; peritonitis and pancreatitis;	
11104	106	C4/C4	M	Liver - hepatocellular carcinoma; testis - focal hemangioma with fibrin thrombin, mild degeneration	√
11105	106	C4/C4	M	Testis - severe degeneration, most tubules lined by single layer of vacuolated sertoli cells, focal cavernous hemangioma	
20345	34	C4/XH	M	expanded T cell areas in spleen, lymph nodes, thymus	
20346	34	C4/XH	M	spleen - mild lymphoid hyperplasia	
20389	29	C4/XH	M	Lymph nodes, thymus - lymphoma	√
20393	31	C4/XH	M	Subcutis - follicular cyst; lymphoid hyperplasia in spleen	
20689	30	C4/XH	M	Spleen - red pulp mildy expanded by extramedullary hyperplasia(EMH)	
20690	26	C4/XH	M	liver - multifocal telangiectasia; lymph node - mild expansion of paracortex	
20691	26	C4/XH	M	spleen - mild expansion of red pulp (EMH) and white pulp (lymphoid hyperplasia)	
20998	45	C4/XH	M	Lung - adenoma; lymph nodes - many hemosiderin laden macrophages in sinuses	√
20999	45	C4/XH	M	Liver - adenoma	√
21001	48	C4/XH	M	lymph nodes - increased hemosiderin laden macrophages in one node	
20329	33	XH/XH	M	Liver - centrilobular hepatocytes large and vacuolated, multiple foci of necrotizing hepatitis; spleen - lymphoid hyperplasia and increased EMH	

CHAPTER 3

Primordial germ cell loss in MCM9 deficient mice is independent of ATM-p21 signaling

Yunhai Luo[1] and John C. Schimenti

[1] This chapter forms the basis of a manuscript that will eventually submitted to a journal of publication. A part of my work related to this chapter, but not included is summarized in Appendix.

3.1 Abstract

Genome stability is important for the health of organisms. However, quite a few genome maintenance genes are not essential for life. Most mutant phenotypes of those genes mainly involve cancer predisposition. Mutations in a few of the genome maintenance genes cause phenotypes other than tumorigenesis. These non-tumor phenotypes can reveal hypersensitivity of specific tissues to genome instability and indicate a possible uniqueness in their genome maintenance mechanism. *Mcm9* is a non-essential gene in the Mcm gene family. It is known to play a role in DNA homologous recombination repair. A hypomorphic mutation of *Mcm9*, the $Mcm9^{XG}$ allele, causes germ cell depletion in newborn mice. Here, I showed that the germ cell depletion is a result of reduced primordial germ cell (PGC) proliferation. In order to dissect the DNA damage response in $Mcm9^{XG}$ PGCs, I conducted genetic studies and discovered that the reduced cell proliferation is independent of ATM-p53-p21 signaling or HUS1 signals. Moreover, the mechanism of PGC depletion in $Mcm9^{XG/XG}$ mutant is different from that in the $Fancm^{C4/C4}$ mutant which also displays PGC depletion in response to genome instability, revealing diversified mechanisms of genome maintenance in PGCs.

3.2 Introduction

Mcm9 is a member of the Mcm gene family (*Mcm2-9*). Proteins in this family have a MCM helicase domain containing Walker A and Walker B motifs, which are essential for ATP binding and hydrolysis respectively [1]. MCM2-7 proteins from this family form a heterohexamer which functions as the eukaryotic replicative helicase [2]. Each of the MCM2-7 proteins is essential [3]. Dysfunction of, or decreased levels of MCMs can cause genome instability and cancer in mice [3-6]. *Mcm8* and *Mcm9* have significant homology with *Mcm2-7* [7,8]. Comparative genomics and phylogenetics studies suggested that *Mcm8* and *Mcm9* were present in the last common ancestor of the eukaryotes [9]. *Mcm8* and *Mcm9* are conserved in many eukaryotic species but seem to have been lost together in yeast and nematode lineages [9]. Drosophila species have only *Mcm8* [9]. A previous study from our lab has shown that two MCM9 isoforms are ubiquitously expressed in mice, a short isoform ($MCM9^{S}$) containing a partial MCM domain and a long isoform ($MCM9^{L}$) containing the whole MCM domain [10].

Because of their homology with *Mcm2-7*, *Mcm8* and *Mcm9* were initially proposed to play an essential role in DNA replication [11]. However, experimental results using Xenopus egg extracts were controversial with two groups giving discrepant results [11,12]. Furthermore, studies from several groups including our own suggested that *Mcm8* or *Mcm9* are not essential in mice or chicken DT40 cells [10,13,14]. Viable knockout mice and chicken DT40 cells lacking *Mcm8* or *Mcm9* were successfully generated [10,13,14]. Although neither *Mcm8* nor *Mcm9* is required for DNA replication, independent results from knockout mice, DT40 cells, human cells and Xenopus egg extracts indicated that *Mcm8* and *Mcm9* are required for resistance to DNA damage, such

as DNA interstrand crosslinks (ICLs) [10,12-15]. The exact roles and mechanisms of MCM8 and MCM9 in response to DNA damage remain to be clarified. A few independent studies agreed that MCM8 and MCM9 form a complex and function in homologous recombination repair by recruiting RAD51 [13-15]. However, the recruitment of MCM8 and MCM9 themselves remains to be studied and seems to depend on the type of damage. MCM8 and MCM9 deficient MEFs and chicken DT40 cells are hypersensitive to replication-related or ICL-related DNA damage but not to ionizing radiation (IR) [13,14].

At least two independent MCM9 deficient mouse lines have been characterized [10,13]. They share similar but slightly different phenotypes. Mutant mice have a normal mortality rate but display genome instability and are predisposed to cancer, including hepatocellular carcinoma and ovarian tumors [10,13]. MCM9 deficiency impacts fertility of mice. *Mcm9*^{XG}, a hypomorphic allele generated in our lab, causes germ cell loss, although males and females are fertile [10]. In contrast, *Mcm9* knockout females are sterile [13]. Although *Mcm9* knockout males are fertile, there is a severe depletion of germ cells and few postmeiotic cells in *Mcm9*$^{-/-}$ testis [13]. In our previous report, I proposed based on the quantification of germ cells in newborn mice that the germ cell loss occurred during gestation [10]. However, the exact stage and cause of primordial germ cell (PGC) loss was not determined. I also showed that germ cell loss is independent of p53-p21 signaling [10]. In a study of another PGC depletion mouse model, *Fancm*^{C4}, I found that the PGC depletion depends on ATM-p53-p21 checkpoint signaling in males but not in females [16]. There are three major checkpoint signaling pathways in response to DNA damage. ATM and DNA-PK kinases transduce signals from DNA

double strand breaks, and ATR kinase transduces signals from exposed single stranded DNA (ssDNA) [17]. It is not clear whether ATM signals or other checkpoint signaling pathways play a role in causing the phenotype of *Mcm9*$^{XG/XG}$ mutants. PGC depletion in *Fancm*$^{C4/C4}$ and *Mcm9*$^{XG/XG}$ could be triggered by either independent pathways or by a same signaling. Also, there might be a yet to be determined sexual dimorphic DNA damage response in *Mcm9*$^{XG/XG}$ PGCs.

Here, I did an immunohistochemical analysis of *Mcm9*$^{XG/XG}$ embryonic gonads, showing that there is a reduced proliferation in mutant PGCs. I also showed through a genetic approach that the DNA damage response in PGCs is independent of ATM signals and is different from the response in *Fancm*$^{C4/C4}$ PGCs. On the other hand, ATR signaling might be required in *Mcm9*$^{XG/XG}$ PGCs to prevent them from further depletion and thus causing infertility.

3.3 Results

Mcm9 deficiency compromises primordial germ cell proliferation

In our previous study of *Mcm9*^{XG} allele, I reported that *Mcm9*$^{XG/XG}$ mice showed a 72% decrease of germ cells in newborn testis and 82% decrease of germ cells in newborn ovary [10]. This result suggests that the germ cell depletion started during embryogenesis. To identify the stage at which germ cell depletion starts, I examined the PGC population at various times of gestation. About 45 PGCs are first specified extra-embryonically at embryonic day 7.5 (E7.5) [18]. Between E8.5 and E10.5, this pool of PGCs then migrates along the epithelium of the hindgut toward the urogenital ridge, undergoing a modest degree of proliferation along the way. From there, the PGCs traverse the dorsal mesentery and populate the primitive gonad between E10.5 and E11.5. They then

undergo a dramatic proliferation until E13.5, after which male PGCs enter mitotic arrest until 3-4 dpp, while female PGCs enter meiosis and arrest in meiotic prophase I until puberty (reviewed in [19]). I quantified PGCs at E11.5, E12.5 and E13.5. There is a significant decrease of PGCs at all three stages in both male and female mutants (Figure 3-1).

Previous studies have estimated the number and the doubling time of PGCs between E11.5 and E13.5 [20-22]. The doubling time of wild type PGCs is 15.8h in males, and 16.1h in females [16]. Based on our PGC quantification, the doubling time of *Mcm9*$^{XG/XG}$ PGC increased to 37.8±1.2h in males, and 32.7±4.5h in females. To verify whether there is a dramatic decrease in PGC proliferation, I did a BrdU incorporation assay. Indeed, the percentage of S-phase PGCs is reduced in both male and female *Mcm9*$^{XG/XG}$ gonads, ranging from E11.5 to E13.5 (Figure 3-1).

DDR pathways involved in PGC depletion

In our previous study, I tried to identify DDR pathways involved in PGC depletion through genetic studies. I found that PGC depletion in *Mcm9*$^{XG/XG}$ mutants is not triggered through *p53* signaling [10]. Rather, introducing a *p53* deficiency into *Mcm9*$^{XG/XG}$ mice could further decrease the numbers of germ cells in *Mcm9*$^{XG/XG}$ newborn males but not newborn females [10]. In order to identify the relevant signaling, I extended this study to other known DDR signaling components, including *p21*, *Atm*, *Chk2* and *Hus1*. Double mutants of *Mcm9*^{XG} and DDR deficiency were all congenic or near congenic (at least 7 backcross generations) on the C3H strain background. The numbers of germ cells were then quantified in newborn gonads.

Because there is a correlation between PGC proliferation slow down and PGC

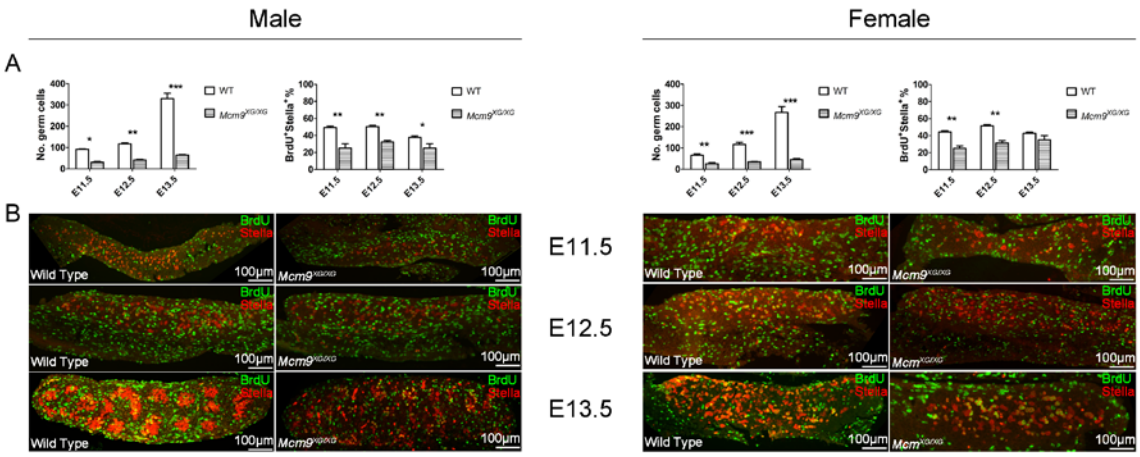

Figure 3-1. PGC depletion in *Mcm9*$^{XG/XG}$ mice is associated with reduced proliferation before E11.5.

(A) Quantification of PGC is done by immunolabelling of fetal gonads with either Stella (E11.5 and E12.5) or MVH (E13.5) in red. Representative images are shown in (B). BrdU^{+} cells immunolabelled in green in BrdU incorporation assays are those that replicated their DNA during 2-hour BrdU pulse. BrdU and Stella double-positive cells, which represent PGCs in S phase, were quantified as percentage of total Stella-positive PGCs.

depletion, I first analyzed the role of *p21*, which is the cyclin-dependent kinase inhibitor 1 (*Cdkn1a*) and also a downstream effector of *p53*. Results from congenic double mutants verified our previous reports that *p53* deficiency would not rescue the germ cell depletion in 1dpp $Mcm9^{XG/XG}$ testes or ovaries [10], and further showed that *p21* deficiency failed to rescue the germ cell depletion in 1dpp $Mcm9^{XG/XG}$ ovaries (Figure 3-2A). Moreover, with an increased number of samples, I couldn't prove a significant further decrease of germ cells in $Mcm9^{XG/XG};p21^{-/-}$ testis (Figure 3-2A). Collectively, these results suggest that *p21* may not be directly involved in the germ cell depletion caused by $Mcm9^{XG}$ or compound deficiency of *Mcm9* and *p53*.

Next, I focused on kinases of one major DDR pathways upstream to P53, ATM and CHK2. These two proteins primarily respond to double strand breaks (DSBs) [17]. Compound mutants between $Mcm9^{XG}$ and *Atm* showed a similar level of germ cell loss in both 1dpp testis and 1dpp ovaries (Figure 3-2B). Consistent with this result, the CHK2 kinase, which is downstream to ATM and upstream to P53, also failed to rescue the germ cell loss in $Mcm9^{XG/XG}$ testis (Figure 3-2C). I observed a partial rescue by *Chk2* mutation in the germ cell loss in $Mcm9^{XG/XG}$ females (Figure 3-2C). However, similar to what I have seen in $Fancm^{C4/C4}$ females, this rescue effect of *Chk2* mutation is probably independent of the $Mcm9^{XG}$ mutation since $Chk2^{-/-}$ newborn females had more germ cells than WT controls [16]. Collectively, these genetic studies indicate that ATM-p53-p21 signaling is not directly involved in the PGC depletion in $Mcm9^{XG/XG}$ gonads.

I then continued my study of the other major DDR pathway upstream of P53: ATR signaling, which primarily responds to sites of replication errors (RPA-coated ssDNA). Homozygosity for a hypomorphic viable allele ($Hus1^{neo}$) of the ATR-pathway

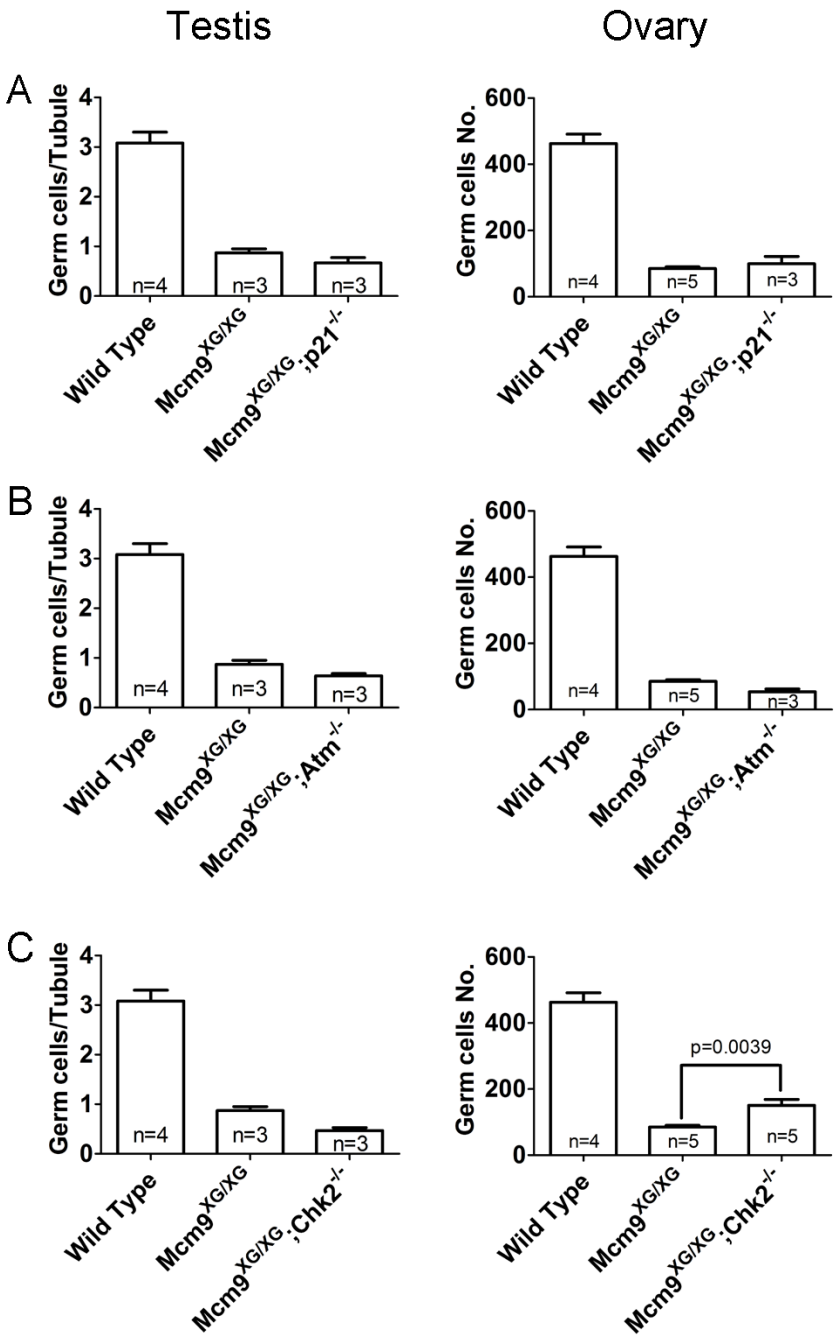

Figure 3-2. Germ cell depletion in *Mcm9*$^{XG/XG}$ is independent of ATM-CHK2-p21 signaling.

Compound mutant gonads with indicated genotypes were collected at 1 dpp. Germ-cell counts were performed following anti-MVH immunostaining. Male germ cell numbers were averaged on a per tubule cross-section basis. Female germ cell number equals the total of germ cells detected in three medial sections. No significant difference between single and double mutants was evident except for *Mcm9*$^{XG/XG}$;*Chk2*$^{-/-}$ with p value indicated. Error bars indicate SD.

gene *Hus1* [23] failed to rescue the depletion of *Mcm9*$^{XG/XG}$ PGCs (Figure 3-3A, B). Although *Hus1*^{neo} didn't significant aggravate the PGC depletion in newborn *Mcm9*$^{XG/XG}$ males and *Mcm9*$^{XG/XG}$*;Hus1*$^{neo/neo}$ males are fertile (Table 3-1), there seems to be a dramatic depletion of germ cells in the testes of 6-month-old males of this genotype (Figure 3-3C). It is not clear whether double mutant males have a reduction in fertility or not. *Mcm9*$^{XG/XG}$*;Hus1*$^{neo/neo}$ females are sterile (Table 3-1) even though newborn double mutant females do not have an elevated depletion of germ cells (Figure 3-3B), suggesting there is a defect in meiosis in *Mcm9*$^{XG/XG}$*;Hus1*$^{neo/neo}$ females.

DDR pathways in Mcm9$^{XG/XG}$ *and Fancm*$^{C4/C4}$ *PGCs are different*

I have shown previously that ATM-p53-p21 signaling is partially responsible for the PGC depletion in *Fancm*$^{C4/C4}$ males and that there might be another novel pathway responsible for the PGC depletion in *Fancm*$^{C4/C4}$ females [16]. To test whether PGC depletions in *Mcm9*$^{XG/XG}$ and *Fancm*$^{C4/C4}$ mice share the same pathway, I did an epistasis analysis. Both male and female *Mcm9*$^{XG/XG}$*;Fancm*$^{C4/C4}$ mice are infertile (Table 3-2). Germ cells are rarely seen in 1dpp double mutant males and are significantly reduced compared to single mutants (Figure 3-4). This result implies that *Mcm9*^{XG} *and Fancm*^{C4} cause DNA damage probably through different mechanisms and/or trigger PGC depletion via different signaling. Therefore, it supports the finding that ATM-p53-p21 signaling is not responsible in *Mcm9*$^{XG/XG}$ PGCs.

3.4 Discussion

Although *Mcm9* is a member of the Mcm gene family, multiple independent studies have proven that MCM9 does not play a role in DNA replication and is not an essential gene in mouse, chicken DT40 cells and human cells. Mutations in *Mcm9* have

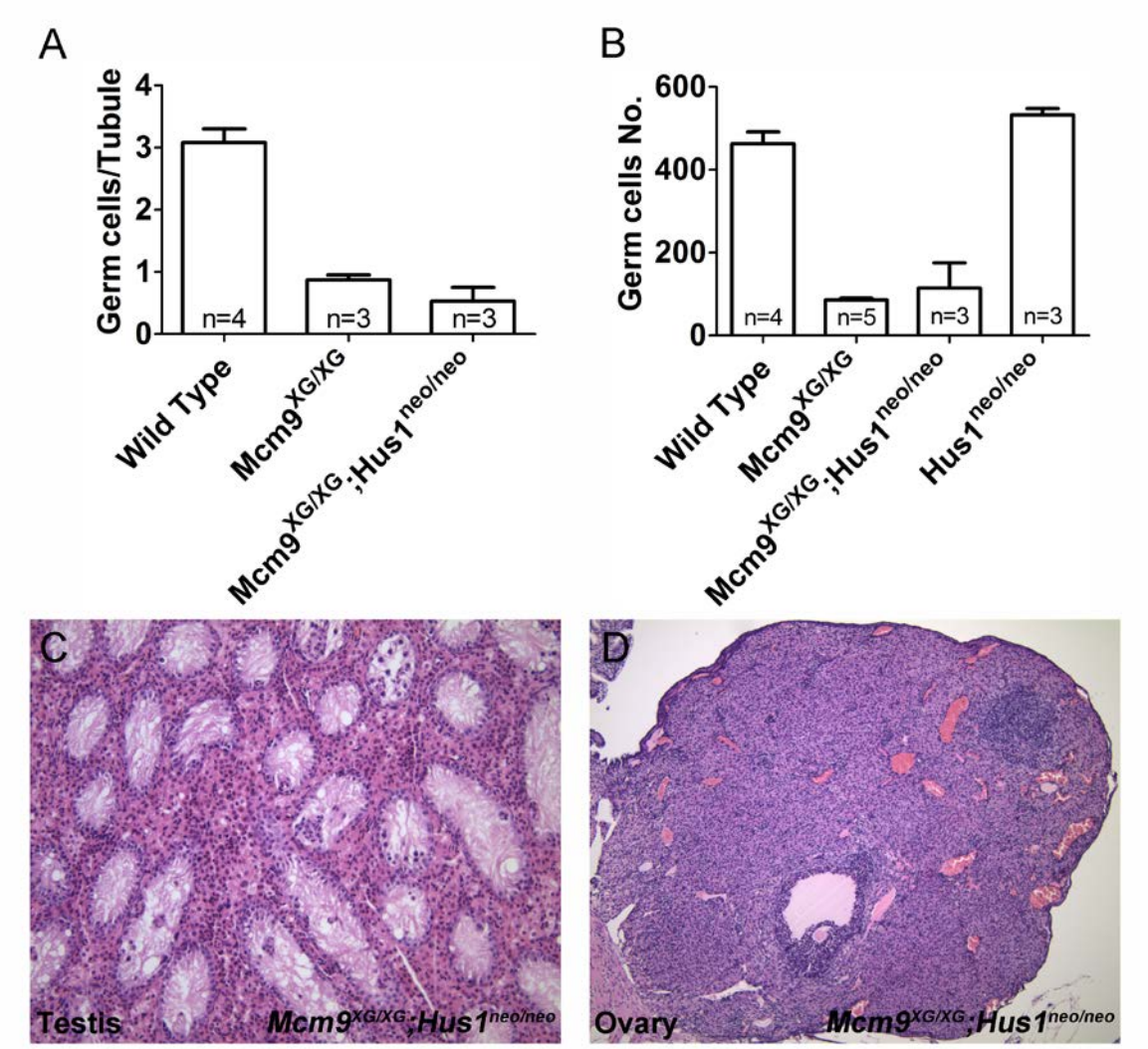

Figure 3-3. *Hus1* interruption in *Mcm9*$^{XG/XG}$ does not affect PGCs but may further disrupt meiosis.

Newborn male and female compound mutants were analysis similarly in (A) and (B), respectively. No significant difference in the number of germ cells between single and double mutant was evident ($p>0.05$). However, elevated spermatocytes and follicle depletion was observed in 6-month testis (C) and 5-month ovary (D).

Table 3-1. Fertility of *Mcm9*$^{XG/XG}$;*Hus1*$^{neo/neo}$ mice

	Animal No.	No. copulatory plugs	No. pups born
Males	24250	6	Litter #1: 1
	24705	3	0
	25412	2	Litter #1: 2 Litter #2: 5
Females	24249	3	0
	25410	6	0
	25411	6	0
	31094	1	0
	31830	2	0

Table 3-2. Fertility of *Mcm9*$^{XG/XG}$;*Fancm*$^{C4/C4}$ mice

	Animal No.	No. copulatory plugs	No. pups born
Males	22504	3	0
	25729	6	0
	27662	6	0
	27908	6	0
Females	22503	3	0
	25304	3	0
	25305	1	0
	25725	3	0
	25727	3	0
	27907	3	0

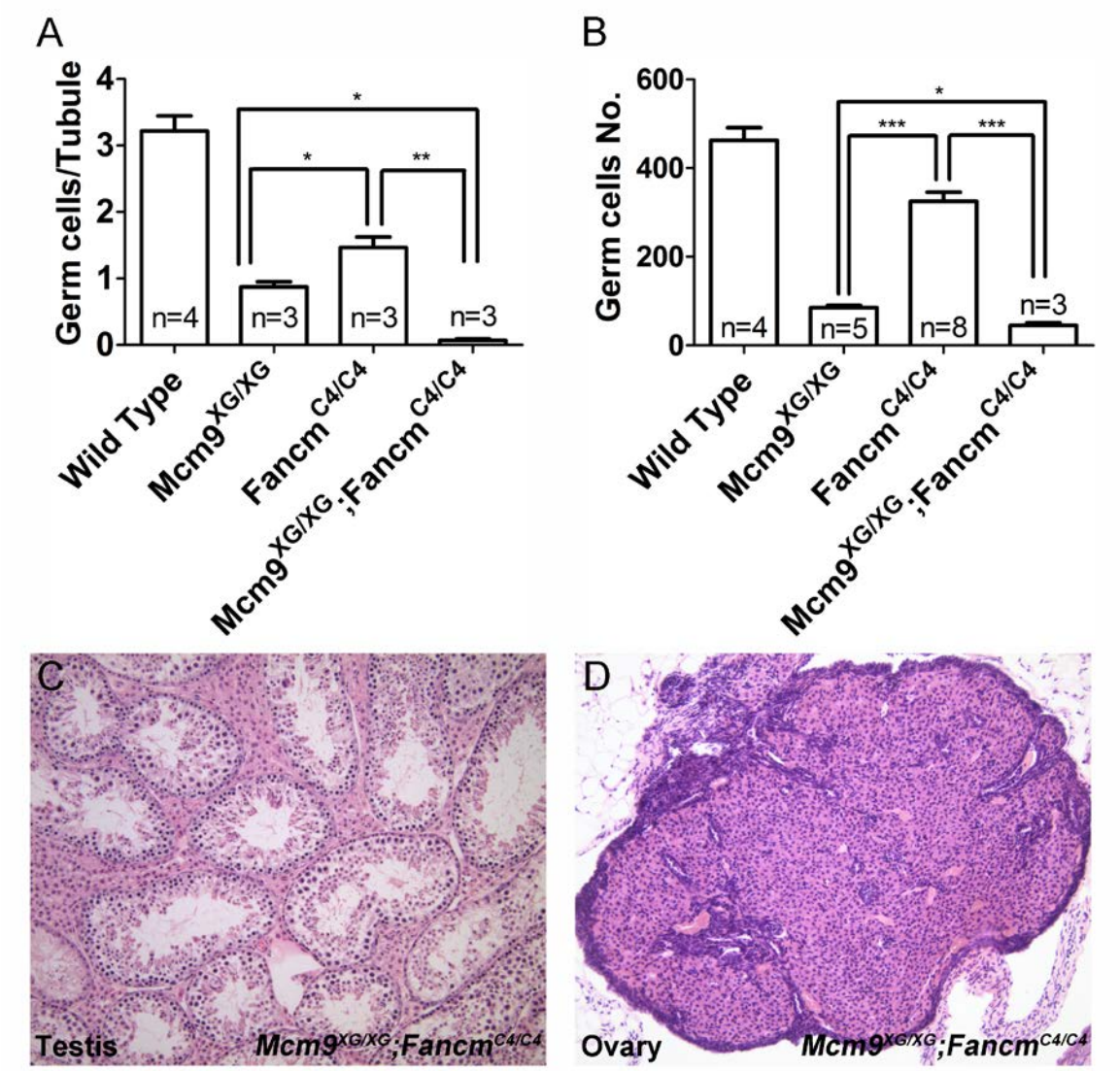

Figure 3-4. Additive PGC depletion in *Mcm9*$^{XG/XG}$;*Fancm*$^{C4/C4}$ mutant.
Significantly increased germ cell depletion was evident in newborn males (A) and females (B). Hypogonadism was observed in 4-month testis (C) and 4-month ovary (D).

relatively limited effects on mice, especially on young mice. However, different mutant alleles of *Mcm9* all cause a deficiency in mouse reproduction. This seemingly specific phenotype reveals a hypersensivity of germ cells to DNA damage. Consistent with this hypersensivity, the germline mutation rate in general is significant lower than that in somatic cells [24,25], indicating a fundamental difference in genome maintenance that appears to reflect the biological importance of minimizing the germline mutation rate. A hypersensitivity of PGCs to DNA damage has been reported in some other mutants, including most Fanconi anemia (FA) mouse models, *Pin1* and *Helq*[16,26-33]. The hypogonadism in a few of these mutants (*Fanca*, *Fancc*, *Fancd2*, *Fancl*, *Fancm*, *Pin1* and *Helq*) has been correlated with PGC deficiency [16,26,32-36]. In *Fancc*, *Fancm*, *Pin1* mutants, direct evidence showed that PGC proliferation has been slowed down [16,32,34]. The mechanism underlying this hypersensivity of mammalian PGCs to DNA damage is far from clear. Our previous study suggested that ATM-p53-p21 signaling is only partially responsible for the PGC depletion in *Fancm* mutant males, while an unknown pathway plays a role in the females [16]. The results in this report suggest that traditional DDR signaling might not be enough to explain the PGC depletion in *Mcm9* mutants either.

The fact that I observed a significant PGC depletion in E10.5 embryonic gonads implies that $Mcm9^{XG}$ mutation might be different from other mutants described above. PGC depletion in $Mcm9^{XG/XG}$ mutants started before they reached urogenital ridge. It is possible that the $Mcm9^{XG}$ mutation also disrupts the specification and migration of PGCs. However, considering the dramatic slowdown in PGC proliferation and the fact that PGCs have a modest level of proliferation during migration, defects in proliferation may

still make a major contribution to the PGC depletion. Further quantification and localization of earlier stage PGC are required to resolve this issue.

Among all the checkpoint components I have tested, *Hus1* seems to be an essential gene in *Mcm9*$^{XG/XG}$ female germ line. Given the study that *Hus1* plays a role in meiotic chromosome maintenance in males [37], it is highly possible that the infertility of double mutant females is an additive effect. However, other possibilities remain. *Hus1*^{neo} is a hypomorphic allele of the *Hus1* gene, which is different from the conditional knockout generated in the study mentioned above [23,37]. Each *Hus1*^{neo} allele expressed roughly 40% of the wild-type level of *Hus1*, and *Hus1*$^{neo/neo}$ MEFs showed an intermediate level of chromosomal instability [23]. On the other hand, proliferation of *Hus1*$^{neo/neo}$ MEFs is normal, and *Hus1*$^{neo/neo}$ mice are normal and are born at the expected frequency [23]. Moreover, it is not known yet *Hus1*$^{neo/neo}$ mice have any fertility problem. I found that the number of germ cells in 1dpp *Hus1*$^{neo/neo}$ females was normal (Figure 3-3B). In considering the hypomorphic nature of *Hus1*^{neo} allele, the infertility in double mutant females could possibly be a synergistic effect. Indeed, it has been shown that *Hus1*$^{neo/neo}$ MEFs are capable of normal growth but is sublimiting for the response to extrinsic genotoxic stresses [23]. Since *Mcm9*^{XG} deficiency causes genome instability [10], this endogenous stress on genome maintenance could trigger a further failure in *Hus1*$^{neo/neo}$ oocytes.

Current studies of *Mcm9* propose that it functions in facilitating homologous recombination repair. Moreover, its function and the related repairing mechanism seem to depend on the type of damage. At least two independent groups suggested that MCM8 and MCM9 deficient cells are hypersensitive to replication related or ICL related DNA

damage but not to ionizing radiation (IR) [13,14]. Nevertheless, multiple groups using different models showed that *Mcm8* and *Mcm9* are required for resistance to DNA ICLs [10,12-15]. FA signaling is the major pathway in response to ICLs, most of which would be processed by homologous recombination repair [38]. Such relationship seems to put *Mcm8*/*Mcm9* complex downstream to FA signals. Therefore, it will be of great interest to check the interaction between *Mcm8*/*Mcm9* and FA pathway. The additive effect of *Mcm9* and *Fancm* observed in our epistasis analysis indicates that the increased DNA damage in $Mcm9^{XG/XG}$ and $Fancm^{C4/C4}$ PGC most likely act by different mechanisms and/or the specific pathways slowing down PGC proliferation are independent.

3.5 Materials and Methods

Mice and genotyping

Genotyping of $Mcm9^{XG/XG}$ mice was performed as described [10]. In general, Mcm^{XG} allele was identified using flanking markers *D10Mit20* and *D10Mit194* that are polymorphic between 129 and C3H. The use of mice in this study was approved by Cornell's Institutional Animal Care and Use Committee. Mice bearing alleles of other mutations were: *Atm* (Atm^{tm1Led}, abbreviated as Atm^{-}), *Chk2* ($Chek2^{tm1Mak}$, abbreviated as $Chk2^{-}$), *p21* ($Cdkn1a^{tm1Tyj}$, abbreviated as $p21^{-}$), and *Hus1* ($Hus1^{tm2Rsw}$, abbreviated as $Hus1^{neo}$) [23,39-41]. The stocks of mice bearing the *p21* and *Hus1* alleles were congenic in the C3H background (N10 backcross generation or greater). The *Atm* and *Chk2* stocks were at the N7 backcross generation.

Histology and Immunohistochemistry

Basic histology and immunohistochemistry were done as previously described [16]. In general, tissues were fixed in 4% paraformaldehyde (PFA) overnight, embedded

in paraffin and sectioned at 5μm. Sections were stained with H&E (hematoxylin and eosin) or immunostained with the following antibodies: rabbit anti-DDX4/MVH (Abcam ab13840; 1:250); rabbit anti-Stella (Abcam ab19878; 1:250); goat anti-mouse Alexa 594 conjugate (Molecular Probes A11005; 1:1,000); goat anti-rabbit Alexa 488 conjugate (Molecular Probes A11008; 1:1,000). Germ cells were quantified as previously described [16]. Prism software package was used for statistical analysis and the resulting P values were used to determine significance ($P < 0.05$).

BrdU incorporation assay

BrdU incorporation assay on PGCs was performed as previously described [16]. In general, pregnant females received a single BrdU intraperitoneal injection (50 mg/kg) at corresponding gestation stages. Embryonic gonads were collected after 2 hours and analyzed by immunohistochemistry as described above. BrdU was immunostained by the Invitrogen BrdU Staining Kit (Cat. No. 93-3944), and PGCs were immunorstained with rabbit anti-Stella (Abcam ab19878; 1:250). PGC doubling time between E11.5 and E13.5 was calculated based on an exponential growth model as described previously [16].

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CHAPTER 4

Summary & Discussion

Over 30 years of DNA damage response studies have established a huge signaling network for the checkpoint signaling. However, the complexity of DDR is not limited to the scale of this network. In the past 10 years, it has become more and more clear that DDR is both species-specific and cell-type specific [1,2]. Differential DDR in different species and in different cells has been further correlated with longevity, cancer susceptibility and cancer prognosis [3]. Unlike traditional studies, researches on specialized DDR face a few challenges. For example, they require *in vivo* studies because *in vitro* system may not fully recapitulate the environment for specialized DDR. In order to reveal the specificity *in vivo*, a comparison of measurable phenotypes with reliable controls is necessary. However, a lot of checkpoint mutants in mice are either non-viable or grossly normal except for increased cancer predisposition in aged adults. The lack of differential phenotypical responses makes it difficult to dissect mechanistic differences of DDR in different tissues. Differential expressions of DDR genes have been observed during embryonic development, although functional analysis is largely limited [4].

Here, I used two mouse models, $Fancm^{C4}$ and $Mcm9^{XG}$, which showed a hypersensitivity of PGCs to corresponding mutations. Using these models, I established a correlation between PGC hypersensitivity and endogenous DNA damage. Further analysis of the hypersensitivity showed that DDR in PGCs is multilevel, sexually dimorphic and genetic-background specific.

4.1 Endogenous DNA damage in PGCs

In both of my mouse models, mice were never treated with any DNA damaging agents. In other words, the genome instability caused by *Fancm*^{C4} or *Mcm9*^{XG} mutation was endogenous and spontaneous. So far, it is impossible to define specific types of DNA damage accumulated in PGCs, because no systematic research has characterized the types of DNA damage in *Fancm* or *Mcm9* deficient cells. We can only hypothesize based on the functions of these genes. Except for its role in regulating meiotic crossing-over, *Fancm* is known to deal with DNA ICLs [5], while *Mcm9* is involved in repairing DSBs [6]. Therefore, it is reasonable to hypothesize that ICLs accumulate in *Fancm*$^{C4/C4}$ PGCs, and DSBs accumulate in *Mcm9*$^{XG/XG}$ PGCs. Endogenous ICLs has never been proven to occur in mammalian cells [7]. Sources of endogenous ICL, which are products of normal metabolism, have been identified (Figure 4-1) [7]. DSBs can occur from actions of endogenous metabolites; they can also be byproducts during the repair of other endogenous DNA damage [8]. However, two groups noticed that DSB repair by *Mcm9* might be specific to the source of damage. *Mcm9*^{KO} chicken DT40 cells were not sensitive to IR [9], and RAD51 foci formation in response to IR is not disrupted in *Mcm9*$^{-/-}$ MEFs [10]. I, therefore, hypothesize that *Mcm9* functions downstream to FA signals, and responds specifically to ICL induced DSB (Figure 4-1).

Although it is difficult to quantify DNA damage directly in wild type cells, levels of spontaneous ICLs and DSBs have been estimated based on studies in DNA repair deficient cells. In cultured human cells, the ICL level can reach one per 10^7 bp [11], and DSB level is about one per 10^8 bp [8]. This damage level is comparable to levels of other types of DNA damage which could range from 0.1 to 500 damage per 10^7 bp [12].

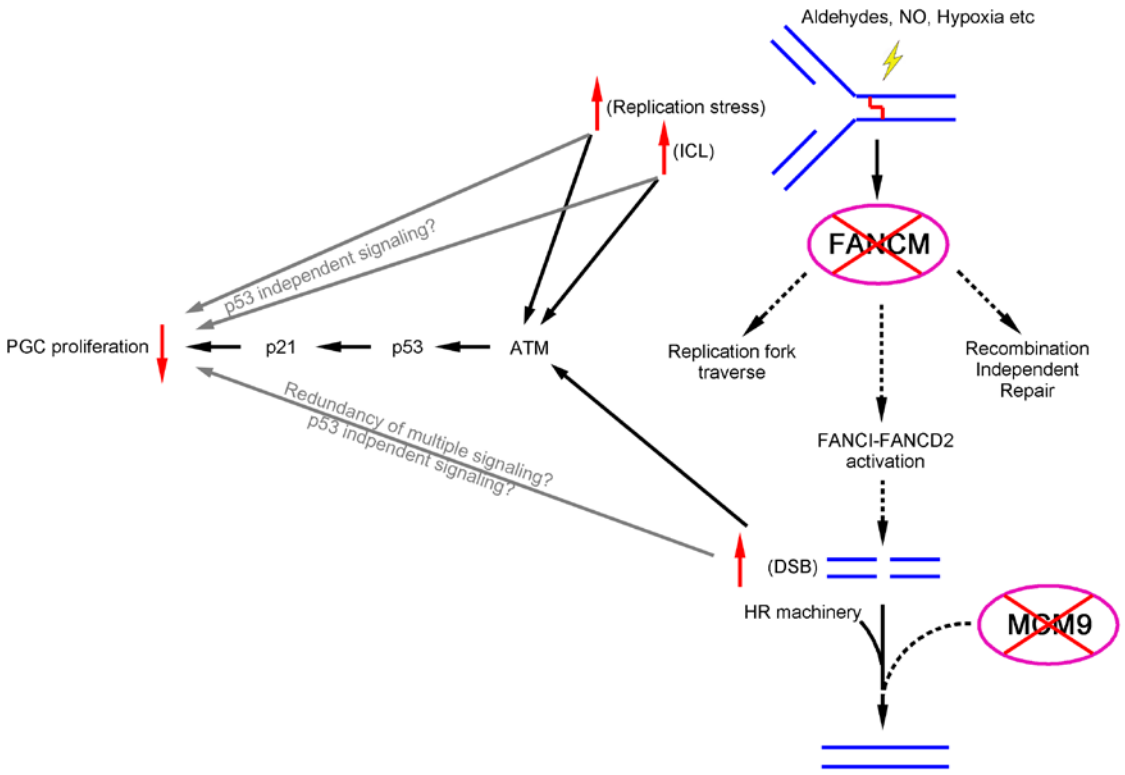

Figure 4-1. Working model for DDR in *Fancm*$^{C4/C4}$ and *Mcm9*$^{XG/XG}$ PGCs.
Fancm^{C4} mutation compromises replication traverse, FA signaling and recombination independent ICL repair. *Mcm9*^{XG} mutation decreases the replication efficiency of ICL related HR repair. As a consequence, there will be increases in replication stress, ICL and DSB. The resulting genome instability then triggers checkpoints, including but not limited to ATM-p53-p21 signaling, leading to reduced PGC proliferation.

Although the level of DNA damage varies from one cell type to another [12], spontaneous ICL and DSB level in PGCs are likely to fall in the same range.

4.2 DNA damage response in PGCs

My study indicates that there are at least 3 independent checkpoint pathways in PGCs. ATM-p53-p21 is only partially responsible for the PGC depletion in *Fancm*$^{C4/C4}$ males. Another p53-p21 independent pathway might play a role in both *Fancm*$^{C4/C4}$ males and in *Mcm9*$^{XG/XG}$ males. A third p53 independent pathway might be present in *Fancm*$^{C4/C4}$ and *Mcm9*$^{XG/XG}$ females. More specifically, analysis of *Fancm*$^{C4/C4}$ and *Mcm9*$^{XG/XG}$ embryos suggests that the depletion results from reduced proliferation by PGCs. Therefore, the unknown p53-p21 independent pathway should cause cell cycle arrest or senescence. p53 is a master regulator of checkpoint signaling, regulating most biological processes of the DDR. Current knowledge about p53-independent cell cycle regulation, rather than apoptosis, focuses on CDC25A and CDC25C which regulate G1/S and G2/M arrest, respectively [13,14]. Although the response through CDC25A/C was proposed to be an immediate arrest whereas p53 mediates a sustained cell cycle arrest [15], CDC25A and CDC25C might be enough to confer a depletion phenotype in continuously proliferating PGCs. Both CDC25 homologs could be phosphorylated and inactivated by CHK1, thus leading to cell cycle arrest. The ATM-CHK2-p53-p21 independent nature of the CDC25A/C signaling fits our genetic data. However, CHK1 activation depends on ATR which requires “9-1-1” clamp loader [16]. Therefore, why *Hus1* is dispensable for PGC depletion needs to be addressed. Moreover, there are two recent reports discussing SMG-1 phosphorylation of CDC25A and TGF-β induced senescence, both of which might be independent of ATM and p53 signaling [17,18]. Both

reports provided potential candidates for p53-p21 independent signaling in our model. It remains unclear whether ATR signaling is involved in those responses. Overall, it is worth checking the cell cycle profile of mutant PGCs to test the stage of cell cycle arrest and the possibility of senescence. In order to dissect the p53 independent checkpoint, it is also critical to examine the activity of CDC25A and CDC25C in PGCs since they seem to be major players in a p53 independent response.

PGC depletion in females has another layer of complexity. As introduced in Chapter 1, female PGCs enter meiosis at E13.5. Although it is clear that PGC depletion in $Fancm^{C4/C4}$ or $Mcm9^{XG/XG}$ females happens before meiosis, the interpretation of the genetic studies is not straightforward. The lack of rescue observed in 1dpp double mutant mice could be a combinatorial result of a rescue of pre-meiotic PGC depletion by E13.5 and a secondary germ cell depletion induced by the compound mutation during female meiosis between E13.5 and birth. To test this explanation, a PGC analysis prior to E13.5 is required in double mutants. Besides, it will be worthwhile to mention that a recent report on the oocyte meiotic checkpoint sheds light on another potential p53 independent, female specific checkpoint signaling, as was discussed in depth in Chapter 2 [19].

4.3 PGCs vs somatic cells

As introduced in Chapter 1, the DDR in PGCs is more stringent in eliminating mutations than, apparently, in the soma. This dissertation made the first step as to determine what the DDR is in PGCs. Although results here are not enough to explain differences in DDR between PGCs and somatic cells, we might be able to get clues from our data and other studies.

First, the expression of genome maintenance genes has been compared between germ-line and somatic cells. There are sporadic reports from single gene studies suggesting that the expression of DDR related genes, such as PARP1, is higher in PGC than in surrounding somatic cells [20]. There are also large-scale or genome-wide analyses of DDR genes in human testes or Drosophila PGCs that showed an enhanced expression compared to somatic cells [21,22]. Specifically, ATM is highly expressed in human testes and ATM and p53 are highly enriched in *Drosophila* PGCs [21,22]. At least one study has correlated this differential expression with cell cycle regulation. Spermatogonia in *Atm*-deficient mice are progressively depleted and lose their self-renewal potential in a p21-dependent manner [23]. Interestingly, our preliminary data also suggested that spermatogonial stem-cell renewal is defective in $Mcm9^{XG/XG}$ mice [24]. Mechanisms revealed in *Atm*-deficient mice might also be involved in $Mcm9^{XG/XG}$ mice, though *p21* is not required in $Mcm9^{XG/XG}$ PGC depletion. Collectively, a potential explanation of the stringent DDR in PGCs is the differential expression of checkpoint genes compared to somatic cells.

Second, the evolutionary pressure on selecting a stringent DDR in PGCs relies on its exclusive role in transferring genetic information from one generation to the next. Stem cells (SCs) face a similar challenge in maintaining genome integrity. SCs have a lifelong potential of proliferation. Mutations in SCs would potentially lead to either tumorigenesis or aging [25]. Studies in the past 10 years indeed revealed specialized responses to DNA damage in different stem cell systems (reviewed in [23]). In general, the basic mechanism is differential expression of checkpoint genes as discussed above. Cellular responses of SCs to DNA damage are diverse, involving apoptosis, cell cycle

arrest, or terminal differentiation [23]. Among those responses, cell cycle arrest is the most relevant one since it is also the primary response of our mutant PGCs here. Enhanced p53-p21 signaling occurs in hematopoietic SCs, intestinal SCs and muscle SCs [26-28]. In contrast, cell cycle arrest has been proposed as not being a major response in melanocyte SCs [29]. Therefore, to reveal the potential difference in DDR between PGCs and somatic cells, it will be interesting to know the expression level of p53 and p21 in PGCs. It is also interesting to examine what happens if the expression level of p53 and p21 in PGCs is experimentally changed.

4.4 Relationship between *Fancm* and *Mcm9*

Fancm and *Mcm9* might act in a common pathway. Multiple groups have found in different model organisms that *Mcm9* is required for DNA ICL response [6,9,10,30]. It was proposed that *Mcm9* is involved in HR repair of ICLs, thus placing its action downstream of *Fancm* signals. Moreover, it was suggested by some groups that MCM9 deficient cells are hypersensitive to replication related or ICL related DNA damage but not to ionizing radiation (IR) [9,10]. Fanconi anemia signaling, on the other hand, responds specifically to replication stress and ICL [31]. Therefore, it seems likely that *Fancm* and *Mcm9* fall into the same pathway. This notion, though, is not consistent with the aggravated PGC depletion that I saw in $Fancm^{C4/C4}$ and $Mcm9^{XG/XG}$ double mutants. Based on the known function of *Mcm9*, it was expected that *Mcm9* deficient cells have increased DSBs processed by *Fancm* coming from replication stress and/or ICLs. If a *Fancm* mutation abolishes DSB generation, the DNA damage level, checkpoint activation and thus the cell depletion in double mutants would be expected to be the same as in *Fancm* mutants.

Fancm is unique in the FA core complex, in not being essential for successful monoubiquitination of FANCI-FANCD2 [31]. In *Fancm*$^{-/-}$ cells, FANCD2 ubiquitination is compromised but not abolished [5]. It is expected that there will be an increase in ICLs due to reduced repair efficiency, while HR repair of ICLs is not completely disrupted. Moreover, *Fancm* mutant cells may also have increased replication stalls because replication traverse of an ICL is greatly reduced by *Fancm* inactivation [32]. Collectively, *Fancm* deficient cells have elevated ICLs and stalled replication forks, and might also show a reduction in DSB generation (Figure 4-1). As a result, *Fancm*$^{C4/C4}$ and *Mcm9*$^{XG/XG}$ double mutants potentially have an overall increase in DNA damage level which may lead to the aggravated PGC depletion.

Different types of DNA damage will trigger different DDR [33]. It is known that FA responds to ICLs and stalled replication forks activate the ATR checkpoint, while DSBs primarily activate the ATM checkpoint [33,34]. Therefore, the assumption that the same pathways function in both *Fancm*$^{C4/C4}$ and *Mcm9*$^{XG/XG}$ mice might be limited. Besides the overall increase in DNA damage level, the aggravated PGC depletion in *Fancm*$^{C4/C4}$ and *Mcm9*$^{XG/XG}$ double mutants may also reflect an additive activation of multiple checkpoints. Although the overall increase in DNA damage level and the combinatorial activation of different checkpoints could possibly have a synergistic effect, our data don't have enough power to test this. To examine the potential synergistic effect, quantitative analysis of checkpoint activity and cell cycle profile in PGCs is required.

4.5 Reference

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Appendix

Published and supplemental data for PGC depletion in *Mcm9*$^{XG/XG}$ mice

This part of my work was published in a separate paper, and was adapted with permission here:

> "Hartford SA, Luo Y, Southard TL, Min IM, Lis JT, et al. (2011) Minichromosome maintenance helicase paralog MCM9 is dispensible for DNA replication but functions in germ-line stem cells and tumor suppression. Proc Natl Acad Sci U S A 108: 17702-17707."

Works from Hartford SA showed that there is a hypogonadism in *Mcm9*$^{XG/XG}$ adult mice. Therefore, I decided to check the germ-line in mutant pups to find out when the defect starts. I collected gonads from newborn wild type and mutant pups, serially sectioned the gonad and probed it with the germ cell specific marker MVH (mouse vasa homolog). I observed a 72% decrease of gonocytes in testes (Figure A3-2A), and 82% decrease of gonocytes in ovaries (Figure A1D). I also observed a slightly decreased in the ovary size (Figure A1E), while I failed to see any significant difference in testis size between wild type and *Mcm9*$^{XG/XG}$ (Figure A1B,C). The hypogonadism in *Mcm9*$^{XG/XG}$ is qualitatively different from the phenotype in an *Mcm9* knockout mouse model (Lutzmann M, Grey C, Traver S, Ganier O, Maya-Mendoza A, et al. (2012) MCM8- and MCM9-deficient mice reveal gametogenesis defects and genome instability due to impaired homologous recombination. *Mol Cell* **47**: 523-534.). While *Mcm9*$^{XG/XG}$ females are fertile, *Mcm9* knockout females are sterile. I believe that this is at least partialy due to the hypomorphic feature of *Mcm9*^{XG} allele. I have previously established another mouse model in the lab, *Mcm9*$^{AWO/AWO}$, which has a null allele of *Mcm9*. In newborn ovaries of *Mcm9*$^{AWO/AWO}$, I observed a significantly higher decrease in gonocytes comparing with *Mcm9*$^{XG/XG}$ ovaries

(Figure A2B). I also found a more severe germ cell depletion in newborn testis (Figure A2A).

In trying to dissect the checkpoint signaling responsible for PGC depletion, I first tested the role of p53. A disruption of p53 signals failed to rescue the germ cell depletion in *Mcm9*$^{XG/XG}$ (Figure A3). Rather, introducing *p53* deficiency decreased the numbers of germ cells in *Mcm9*$^{XG/XG}$ newborn males but not females (Figure A3).

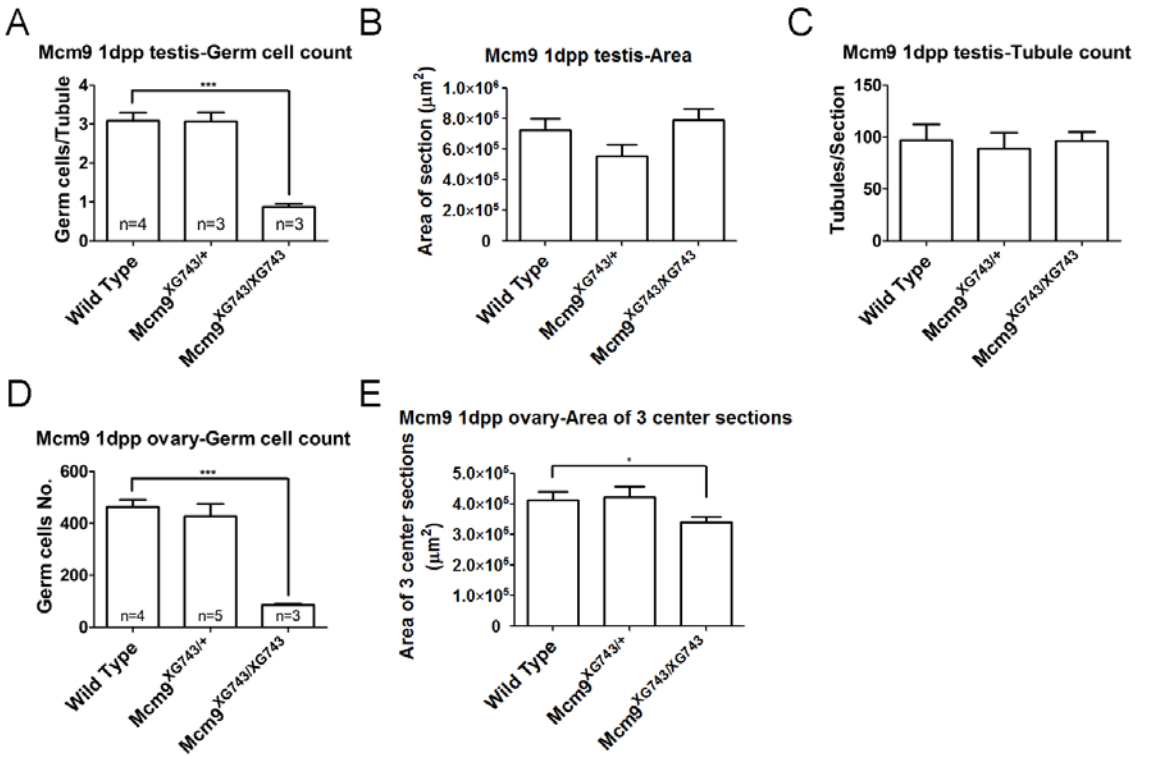

Figure A1. Germ cell depletion in *Mcm9*$^{XG/XG}$ occurs before birth.
Germ cells in 1dpp testes (A) and ovaries (D) of the indicated genotypes were immunelabeled then quantified by MVH. Male germ cell number was averaged on a per tubule cross-section basis for males. Female germ cell number equals to the total of germ cells detected in three medial sections. (B, E) Area of gonad sections were measured by using Image J. No significant difference was evident in 1dpp testes ($p>0.05$). However, 1dpp mutant ovary is smaller than the wild type ovary (C) Number of tubules in testis sections was shown and quantified by DAPI, revealing the histological structure. No significant difference was evident ($p>0.05$). *, $p<0.05$; ***, $p<0.001$; Error bars indicate ±SD. At least 3 animals were analyze for each genotype.

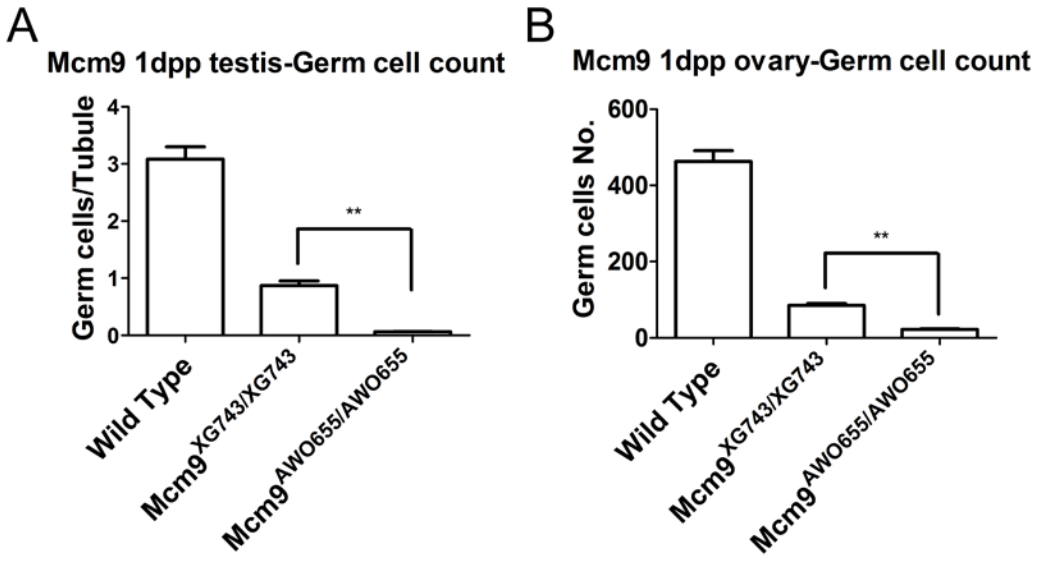

Figure A2. Germ cell depletion in *Mcm9*$^{AWO/AWO}$ occurs before birth.
Germ cells in 1dpp testes (A) and ovaries (B) of the indicated genotypes were quantified as in figure A1. Elevated germ cell depletion were observed, relative to that in *Mcm9*$^{XG/XG}$. **, p<0.01; Error bars indicate ±SD.

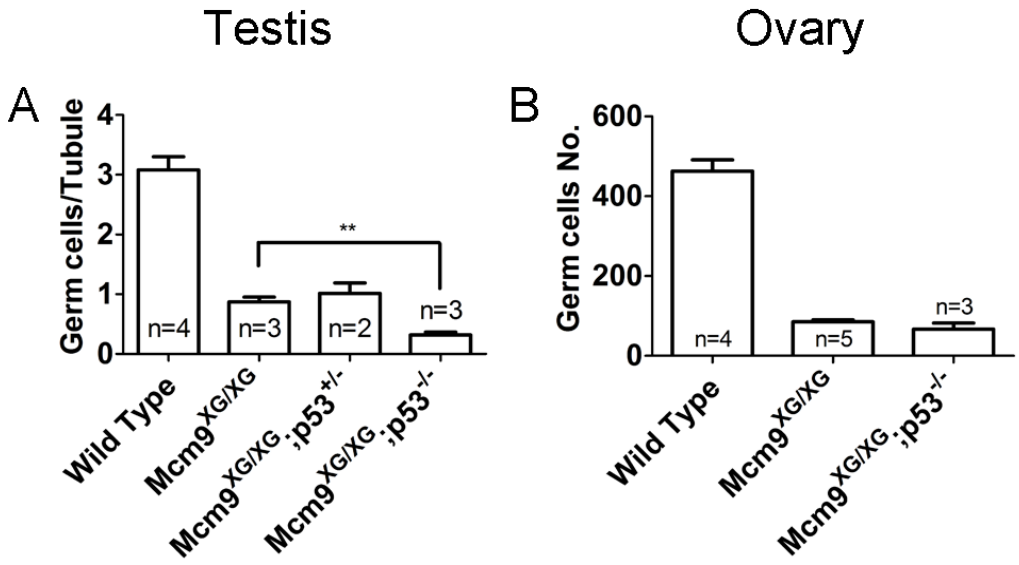

Figure A3. Germ cell depletion in *Mcm9*$^{XG/XG}$ is independent of ATM-CHK2-p21 signaling.
Double mutant gonads with indicated genotypes were collected at 1 dpp. Germ cells in 1dpp testes (A) and ovaries (B) of the indicated genotypes were quantified as in figure A1. *p53* deficiency will further deplete germ cells in 1dpp *Mcm9*$^{XG/XG}$ testes but neither aggravate nor rescue germ cell depletion in 1dpp *Mcm9*$^{XG/XG}$ ovaries. **, p<0.01 Error bars indicate ±SD.