Fancd2 is required for the repression of germline transposable elements

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Abstract

The Fanconi anemia (FA) DNA damage response (DDR) pathway regulate important cellular processes such as DNA replication, cell cycle control and DNA damage repair. Here we show that FANCD2, a key member of the FA DDR pathway, interacts with several important components of the germ-cell-specific Prmt5/piRNA pathways that orchestrate the repression of transposable elements (TEs). By using the *Pou5f1*-eGFP reporter mice, which marks pure populations of primordial germ cells (PGCs), we demonstrate that FA deficiency results in de-repression of TEs, depletion of PGCs, and defective spermatogenesis and oogenesis. *Fancd2–KO* PGCs exhibited excessive DNA damage and exacerbated apoptosis. Mechanistically, we observed a significant reduction of PRMT5-catalyzed H2A/H4R3me2s marks on the LINE1 TEs in E10.5 PGCs of *Fancd2-KO*, *Pou5f1*-eGFP and *Fanca-KO;Pou5f1*-eGFP embryos. Furthermore, we utilized the *Fancd2-KI* model to show that Fancd2 and Prmt5 co-occupied the promoter of LINE1 in WT PGCs, and that this co-occupancy was lost in FA-deficient (*Fanca-KO*) PGCs. These results suggest that the FA pathway takes part in TE repression in early PGCs, likely through a mechanism involving Fancd2-facilitated, Prmt5-catalyzed repressive H2A/H4R3me2s marks on TEs.

Keywords

DNA damage response (DDR); Fanconi anemia (FA); primordial germ cells (PGCs); Prmt5/ piRNA pathways; transposable elements (TEs)

Introduction

Fanconi anemia (FA) is a genetic disease associated with bone marrow failure, increased cancer, and severe germline defects and the FA pathway is known to play a major role in the DNA damage response (DDR) network (Bagby 2003, Tischkowitz & Hodgson 2003, Bagby 2018). FA is genetically heterogeneous, with at least 22 complementation groups (*FANCA-FANCW*) identified thus far (Dong *et al.* 2015, Sawyer *et al.* 2015, Knies *et al.* 2017). Eight of the FA proteins (FANCA, B, C, E, F, G, L, and M) form the FA core complex that functions as an ubiquitin ligase. In response to DNA damage or DNA replication stress, the FA core complex monoubiquitinates two downstream FA proteins, FANCD2 and FANCI, which then recruit the downstream FA proteins and other DNA repair factors, to nuclear loci containing damaged DNA and consequently influence important cellular processes such as DNA replication, cell-cycle control, and DNA damage response and repair (Kottemann & Smogorzewska, 2013; Deans & West, 2011).

DDR comprises an elaborate network of signaling pathways that enable cells to execute biological responses to genotoxic stress (Jackson & Bartek 2009). Defects in DDR pathways have been shown to be associated with infertility, gamete aneuploidy and diseased risk in offspring (Leduc *et al.* 2008). As a major DDR pathway, FA has been subjected to extensive study for its role in germline genomic maintenance. In addition to genome instability and cancer susceptibility, FA deficiency in mice has been shown to compromise male and female fertility. Specifically, deletion of the genes for the murine FA proteins *Fanca*, *Fancb*, *Fancc, Fancl, Fancm* and *Fancp* causes primordial germ cell (PGC) depletion, meiotic defects, and ultimately hypogonadism and infertility (Nadler & Braun 2000, Agoulnik *et al.* 2002, Wong *et al.* 2003, Crossan *et al.* 2011, Kato *et al.* 2015, Alavattam *et al.* 2016). More recently, it was shown that hypogonadism of *Fancm* mutant mice was a result of reduced proliferation of PGCs, likely resulting from elevated DNA damage (Luo *et al.* 2014). These studies highlight the importance of the FA DDR pathway in germ cell development and function. A recent study demonstrated that the FA pathway is critical for DNA crosslinking repair to safeguard genomic stability of PGCs (Hill RJ & Crossan GP 2019). However, the mechanism by which the FA pathway functions in genome maintenance in the germline, particularly in PGCs, remains largely undefined.

Transposable elements (TEs) induce DNA double-strand breaks (DSBs), insertional mutations, and chromosome rearrangements, leading to genomic instability (Goodier & Kazazian 2008). This genetic consequence is particularly dangerous in germ cells, as genome changes induced by TEs will be transmitted to the next generation. Thus, TE silencing is considered an essential mechanism for defending germline integrity and fertility. There are two key mechanisms for the repression of germline TEs: the H2A/H4R3me2s modification catalyzed by the protein arginine methyltransferase 5 (PRMT5) in early (E8.5-

E11.5) PGCs, and the *de novo* DNA methylation orchestrated by the Piwi-interacting small RNA (piRNA) pathway initiating at approximately E14.5 (Aravin *et al.* 2008, Kim *et al.* 2014). Consequently, mice deficient in genes encoding Prmt5 or factors in the piRNA pathway exhibit overexpression of TEs, loss of germ cells and ultimately male and/or female sterility (Saito & Siomi 2010, Siomi *et al.* 2011, Kim *et al.* 2014, Weick & Miska 2014, Berrens & Reik 2015). Despite extensive investigation, the mechanisms by which germ cells control TE activity are largely not known and, in particular, the DDR pathways functioning in this critical process have not been identified.

In the current study, we show that Fancd2, a key member of the FA DDR pathway, interacts with several important components of the Prmt5/piRNA pathways essential for TE silencing in primordial germ cells (PGCs). Loss of FANCD2 results in massive de-repression of TEs, depletion of PGCs, defective spermatogenesis and oogenesis, and ultimately infertility. Early FA-deficient PGCs show reduced H2A/H4R3me2s marks on LINE1 transposons. Further, we reveal the co-occupancy of Fancd2 and Prmt5 at the promoter of LINE1 TEs in early PGCs.

Results

Fancd2 interacts with germline-specific Prmt5/piRNA factors

We recently reported that by using a *Fancd2* knock-in mouse model and proteomic approach, we unveiled a FANCD2 in vivo interaction network (Zhang *et al.* 2017). Significantly, analysis of Fancd2-containing complexes from four different tissues (ES cells, E11.5 embryos, testes and spleen) revealed distinct patterns of Fancd2-associated proteins (Fig. 1A), suggesting the presence of developmental and tissue-specific differences in the repertoire of Fancd2 interactors. Specifically in testes, we observed several proteins of the germ-cell-specific Prmt5/piRNA pathways, which orchestrate the repression of transposable elements (TEs) (Saito & Siomi 2010, Siomi *et al.* 2011, Kim *et al.* 2014, Weick & Miska 2014, Berrens & Reik 2015). We validated the interaction between the FLAG-tagged Fancd2 and these Prmt5/piRNA factors in *Fancd2-KI* mice (Fig 1B). We also addressed whether the loss of Fancd2 affected these Prmt5/piRNA factors. Western analysis for Mael and Tdrd8 showed that these proteins were still present in the *Fancd2-KO* testes (Fig 1C). However, the levels of Prmt5, Mili, Tdrd1 and Tdrd6 were reduced in the *Fancd2-KO* testes compared to those in WT and *Fancd2-KI* mice (Fig 1C). These results indicate Fancd2 interacts with germline-specific Prmt5/piRNA factors and suggest that Fancd2 may play a role in the regulation of germline TEs.

Loss of Fancd2 leads to defective spermatogenesis and oogenesis

The observation that Fancd2 interacts with Prmt5/piRNA factors prompted us to determine whether Fancd2 plays a role in germline development. Consistent with previous report (Houghtaling *et al.* 2003), we found that *Fancd2-KO* mice were smaller than WT or *Fancd2-KI* mice at the embryonic stage (d.p.c 13.5) (Fig. 2A). Second, the size of testes and ovary in 8-week-old-adult *Fancd2-KO* mice were significantly reduced in comparison to age-matched WT or and *Fancd2-KI* mice (Fig. 2B). Third, H&E stained testis and ovary of 8-week-old WT, *Fancd2-KO* and *Fancd2-KI* mice show severely defective spermatogenesis and

oogenesis in *Fancd2-KO* mice, as compared to WT and *Fancd2-KI* mice (Fig. 2C). Consequently, loss of *Fancd2* results in complete sterility in both males and females (Zhang *et al.* 2017 and data not shown). Thus, these data confirm the past finding and suggest that *Fancd2* is required for germline maintenance.

Loss of *Fancd2* results in depletion of PGCs during embryonic development

It has been reported that deletion of the mouse FA genes *Fanca, Fancb, Fancc, Fancl, Fancm* and *Fancp* causes primordial germ cell (PGC) depletion, hypogonadism and ultimately infertility (Nadler & Braun 2000, Agoulnik *et al.* 2002, Wong *et al.* 2003, Crossan *et al.* 2011, Kato *et al.* 2015). In addition, it has been shown that hypogonadism in *Fancm* mutant mice is a result of reduced proliferation of PGCs (Luo *et al.* 2014). Therefore, we asked whether *Fancd2* deficiency would affect PGC maintenance during embryonic development. To facilitate the purification and analysis of PGCs by flow cytometry, we crossed $Fancd2^{+/-}$ mice to *Pou5f1* (*Oct4*)-eGFP (*Pou5f1*-eGFP hereafter) reporter mice, which have been used to mark and isolate pure populations of PGCs by FACS (Szabo *et al.* 2002). Using another PGC marker, SSEA1 (Hayashi *et al.* 2011), we observed a significant reduction of GFP$^+$ SSEA1$^+$ PGCs in *Fancd2-KO* mice compared to WT controls at both E11.5 (WT 384 ± 76 vs *Fancd2-KO* 147 ± 49, n =5) and E13.5 (WT 634 ± 61 vs *Fancd2-KO* 283 ± 44, n =6) (Fig. 3A, 3B). These results indicate that loss of *Fancd2* leads to PGC depletion.

Reduced proliferation and increased apoptosis in *Fancd2-KO* PGCs

Since we observed a significant reduction of PGCs in *Fancd2-KO* embryos, we sought to determine the effect of *Fancd2* deficiency on the proliferation and survival of mouse PGCs. To this end, GFP$^+$ PGCs from E11.5 embryos were sorted by FACS and cultured on STO feeder cells for 15 days (Fig. 4A). We found that *Fancd2-KO* PGCs proliferated significantly slower than WT PGCs in stroma-supported culture (Fig. 4B), suggesting that *Fancd2* is essential for proliferation of PGCs. Consistent with these observations, the number of BrdU-positive cells was reduced in *Fancd2-KO* PGCs (16.2 ± 4.1%, n =5) compared to WT controls (27.8 ± 6.4%, n =5) (Fig. 4C), indicating that Fancd2 is required for proliferation of mouse PGCs. To determine whether *Fancd2* is directly involved in the cell survival of mouse PGCs, we analyzed apoptosis using Annexin V staining followed by flow cytometry. Notably, Annexin V-positive cells were up-regulated in *Fancd2-KO* PGCs (5.40 ± 0.38%, n =5) compared to WT controls (1.73± 0.62%, n =5) (Fig. 4D). These data suggest that *Fancd2* plays a role in regulating proliferation and survival of mouse PGCs.

FA deficiency leads to de-repression of germline TEs

Our proteomic analyses suggested that the FA pathway may play a TE silencing function *in vivo* during germ cell development. For this reason, we decided to further study the link between the FA pathway and germ cell-specific TE silencing machinery. To determine if the loss of *Fancd2* results in TE de-repression in PGCs, we isolated GFP$^+$ SSEA1$^+$ PGCs from WT;*Pou5f1*-eGFP and *Fancd2-KO*;*Pou5f1*-eGFP mice, and determined the expression of two TEs, LINE1 (long interspersed nuclear elements) and IAP (intracisternal A particle), whose unrestrained expression has been linked to infertility (Hayashi *et al.* 2011), by quantitative qPCR. We observed a 4- to 7-fold increase in LINE1 and a 3- to 5-fold increase in IAP expression in E11.5 *Fancd2-KO* PGCs compared to WT controls (Fig. 5A). Thus,

Fancd2 is essential for transcriptional or posttranscriptional silencing of two TEs known to cause infertility in mice.

To determine whether the depression of LINE1 and IAP TEs in PGCs required the FA core complex, we generated WT; *Pou5f1*-eGFP and *Fanca⁻ᐟ⁻*;*Pou5f1*-eGFP mice, and analyzed the levels of LINE1 and IAP TEs in E11.5 PGCs. Consistent with the observation in *Fancd2-KO* PGCs, *Fanca* deficiency also resulted in de-repression of LINE1 and IAP TEs in PGCs (Fig. 5B). These results indicate that the Fancd2/FA pathway is essential for TE repression during early germline development.

Prmt5-catalyzed repressive H2A/H4R3me2s is essential for TE silencing in PGCs between E8.5–E11.5 (Kim *et al.* 2014), when these actively dividing PGCs are undergoing global DNA demethylation (Hajkova *et al.* 2002, Seisenberger *et al.* 2012). To investigate the mechanistic link between the Fancd2/FA pathway and Prmt5-directed TE silencing in early PGCs, we generated three mouse models: 1) *Fancd2-KO*,*Pou5f1*-eGFP for the effect of *Fancd2* deficiency on Prmt5-mediated H2A/H4R3me2s repressive chromatin modifications on LINE1 TEs; 2) *Fanca-KO;Pou5f1*-eGFP for the requirement of the FA core complex for these Prmt5-mediated H2A/H4R3me2s marks on LINE1; and 3) *Fancd2-KI*,*Pou5f1*-eGFP in a WT or *Fanca-KO* background for interaction between Fancd2 and Prmt5 on sites of H2A/H4R3me2s marks in LINE1 chromatin. We chose E10.5 PGCs to investigate the FANCD2-PRMT5 interaction in TE silencing because Prmt5-mediated H2A/H4R3me2s marks on LINE1 are enriched at E10.5-E11.5, which are responsible for TE repression during this specific stage of development when PGCs undergo global demethylation of DNA (Seisenberger *et al.* 2012, Hajkova *et al.* 2002). Since LINE1 TEs are silenced via 5′-UTR promoter methylation (Smith *et al.* 2012), we compared repressive H2A/H4R3me2s marks on the LINE1 5′-UTR promoter in E10.5 PGCs from WT;*Pou5f1*-eGFP, *Fancd2-KO*,*Pou5f1*-eGFP and *Fanca-KO; Pou5f1*-eGFP embryos by micro-chromatin immunoprecipitation (µChIP) assays (Dahl & Collas 2008). We observed that at E10.5, the LINE1 promoter was strongly enriched with H2A/H4R3me2s marks, indicative of a Prmt5-repressed chromatin state, in WT; *Pou5f1*-eGFP PGCs (Fig 5C). In contrast, these Prmt5-mediated H2A/H4R3me2s marks were significantly decreased in *Fancd2-KO*; *Pou5f1*-eGFP and *Fanca-KO;Pou5f1*-eGFP PGCs (Fig 5C), consistent with LINE1 transcriptional activation.

Next, we examined the recruitment of Fancd2 and Prmt5 to the promoter of LINE1 in E10.5 PGCs from WT (*Fanca+/+;Fancd2-KI*) and FA-deficient (*Fanca−/−;Fancd2-KI*) embryos by µChIP assays using agarose-conjugated antibodies against FLAG (tagged Fancd2) and PRMT5 coupled with protein A magnetic beads. We found that Fancd2 and Prmt5 co-occupied the promoter of LINE1 in WT PGCs, and that this co-occupancy was lost in *Fanca−/−;Fancd2-KI* PGCs (Fig 5D). Notably, *Fanca-KO* caused not only complete loss of FLAG-Fancd2 occupancy but also significant reduction of Prmt5 recruitment to the LINE1 promoter, indicating a pattern of Fancd2-dependent Prmt5 occupancy. Together, these results suggest that Fancd2 recruits Prmt5 to the LINE1 promoter to support TE repression.

The requirement of the FA core component Fanca for repression of LINE1 and IAP TEs, the establishment of the Prmt5-mediated H2A/H4R3me2s marks and the recruitment of Fancd2

and Prmt5 to the LINE1 promoter in PGCs prompted us to analyze the levels of Prmt5 and Fancd2 proteins in *in-vitro* culture-expanded PGCs from WT (WT;*Fancd2-KI*) and *Fanca*-deficient (*Fanca−/−;Fancd2-KI*) mice. We did not observe significant alteration in the protein levels of either Prmt5 or Fancd2 in *Fanca−/−;Fancd2-KI* mice compared to the WT;*Fancd2-KI* controls (Fig 5E). These results indicate that, while Fanca is not required for the stability of the Fancd2 and Prmt5 proteins, a functional FA core complex is essential for Fancd2-dependent and Prmt5-mediated germline TE repression.

We also investigated whether the observed increase in the expression of TEs could lead to an increase in the accumulation of DNA damage in *Fancd2*-deficient PGCs. To this end, we performed the Comet assay and revealed that the *Fancd2−/−* PGCs exhibited significantly more DNA damage (% comet tail DNA) (Figure 5F) than their wild-type counterparts. This result demonstrates excessive DNA damage in the *Fancd2−/−* PGCs, and suggests that de-repression of germline TEs could cause apoptosis of germ cells resulting from DNA damage.

Discussion

The FA DDR pathway plays critical roles in the maintenance of germline integrity; however, the underlying mechanisms are largely unknown. In this study, we show that (1) Fancd2 interacts with several important Prmt5/piRNA factors essential for germline TE silencing. (2) Loss of Fancd2 results in de-repression of TEs, depletion of PGCs, defective spermatogenesis and oogenesis, and infertility. (3) FA deficiency (*Fancd2-KO* and *Fanca-KO*) causes a significant reduction of Prmt5-catalyzed H2A/H4R3me2s marks on LINE1 TEs in early PGCs. And (4) Fancd2 and Prmt5 co-occupy the promoter of LINE1 in PGCs, which is lost in FA-deficient (*Fanca-KO*) PGCs. These results indicate a mechanistic link between the FA DDR pathway and germline maintenance, likely through regulation of TE expression in early PGCs.

De-regulated TE expression causes germ cell loss leading to infertility and induces germline genomic instability compromising the viability and health of offspring. Despite intensive investigation, the mechanisms by which germ cells control TE activity are largely not known and the DDR and repair pathway(s) functioning in this critical process have not been identified. The conventional model suggests that as a major DDR pathway involved in germline genomic maintenance, the FA pathway primarily engages in the processing and enzymatic repair of DNA damage during meiotic recombination or replication (Nadler & Braun 2000, Agoulnik *et al.* 2002, Wong *et al.* 2003, Crossan *et al.* 2011, Luo *et al.* 2014, Kato *et al.* 2015). Our results show that Fancd2 is required for Prmt5-catalyzed H2A/H4R3me2s marks, and loss of Fancd2 causes TE de-repression in early PGCs. These studies suggest that Fancd2/FA pathway protects germline genome from TE-induced DNA damage through regulating Prmt5-catalyzed H2A/H4R3me2s marks in TE repression. Our results are also consistent with a recent elegant study, in which the authors employed CRISPR-Cas9 screening strategies to identify genes in the control of the LINE-1 TE and showed that several FA proteins including FANCB, FANCI and FANCL, play a role in LINE-1 repression (Liu *et al.* 2018).

One novel finding of the current study is the link between the FA pathway and Prmt5-mediatedH2A/H4R3me2s repressive chromatin modification in PGC TE silencing. Prmt5-catalyzed repressive H2A/H4R3me2s chromatin modification is essential for TE silencing in PGCs between E8.5–E11.5, when actively dividing PGCs are undergoing global DNA demethylation (Hajkova *et al.* 2002, Hajkova *et al.* 2008, Seisenberger *et al.* 2012). We proposed that the FA pathway play a crucial role in TE repression through a mechanism that involves Prmt5-mediated repressive H2A/H4R3me2s marks on TEs. Indeed, we observed a significant reduction of H2A/H4R3me2s marks on LINE1 transposons in E10.5 PGCs of *Fancd2-KO; Pou5f1*-eGFP and *Fanca-KO; Pou5f1*-eGFP embryos compared to WT PGCs (Fig 5C). Our current study shows that the FA DDR pathway is required for establishing the repressive H2A/H4R3me2s marks catalyzed by Prmt5. These findings are consistent with previous reports that the repressive H2A/H4R3me2s marks is vital for TE silencing for early PGCs at a critical time when these cells reprogram their epigenome via a nearly complete erasure of DNA methylation (Reik *et al.* 2001, Hajkova *et al.* 2002, Lees-Murdock *et al.* 2003, Gulbert *et al.* 2012). Furthermore, we utilized our newly developed *Fancd2-KI* mouse model to show that Fancd2 and Prmt5 co-occupied the promoter of LINE1 in WT PGCs, and that this co-occupancy was lost in *Fanca–/–;Fancd2-KI* PGCs (Fig 5D). These studies established a potential mechanistic link between the Fancd2-Prmt5 interaction and Prmt5-catalyzed repressive H2A/H4R3me2s chromatin modification.

It is possible that the PRMT5 pathway is not the only Fancd2-dependent mechanism in germline TE repression. Another germline TE silencing machinery is the piRNA pathway (Weick & Miska 2014), which functions in a very strict development-stage- and cell-compartment-specific manner in the context of TE repression (Aravin *et al.* 2008). How these critical processes are regulated is unknown. Using our *Fancd2-KI* mouse model, we established biochemical interaction between Fancd2 and several components (Mili, Tdrd1, Tdrd6, Tdrd8, Mael) in the piRNA pathways (Fig. 1). In addition, Fancd2 is localized in both cytoplasm and the nucleus (Zhang *et al.* 2017). Loss of Fancd2 leads to TE overexpression at E11.5 and PGC depletion at E13.5 (Fig. 4–5). These data argue a functional link between Fancd2 and the piRNA pathway in the germline TE repression.

Genome maintenance in the germline is crucial for fertility and health of the next generation. Although DDR and DNA repair have been well characterized in meiocytes, the DDR pathway(s) that function in PGCs, which are the embryonic precursors to sperm and eggs, are less well defined. The current study unveils a novel interaction between a major DDR (FA) pathway and germ cell-specific TE silencing machinery in safeguarding the germline genome. From a clinical standpoint, TE silencing in the germline has a vital role in both parental fertility and offspring health. Therefore, defining the molecular collaboration between the FA DDR pathway and Prmt5/piRNA pathways in the context of germline TE repression will open up a new avenue of research designed to target these interacting pathways for developing innovative therapeutic strategies for reproductive diseases such as infertility and birth defects.

Materials and Methods

Mice

Fancd2-KI mice were generated in our laboratory (Zhang & Pang, et al., 2017). *Fancd2+/−* and *Fanca+/−* mice were provided by Dr. Markus Grompe (Oregon Health & Sciences University) (Houghtaling *et al.* 2003) and Dr. Madeleine Carreau (Laval University) (Wong *et al.* 2003), respectively. The *Fancd2-KI;Pou5f1*-eGFP, *Fanca-KO;Pou5f1*-eGFP or *Fancd2-KO; Pou5f1*-eGFP mice were generated by crossing the *Fancd2-KI*, *Fanca-KO*, or *Fancd2-KO* mice to the *Pou5f1*-eGFP reporter mice (B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J; JAX Stock #:004654), respectively. Mice were maintained on C57BL/6J background in the animal barrier facility at Cincinnati Children's Hospital Medical Center. Animals were kept in accordance with the protocol approved by the CCHMC Institutional Animal Care and Use Committee.

Isolation and culture of primordial germ cells (PGCs)

For isolation of PGCs, genital ridges of E10.5, E11.5 or E13.5 embryos from timed crosses of FA mice and *Pou5f1*-eGFP reporter mice were dissected, and single PGCs from individual genotyped embryos sorted by GFP expression or by SSEA1-APC (R&D Systems; Cat.# FAB2155A) positive cells using a MoFlo XDP high speed flow sorter (Beckman Coulter). For PGC culture, STO feeder cells (American Type Culture Collection (ATCC), Cat.# CRL-1503) were seeded in a 96 well plate coated with 1% gelatin (Sigma-Aldrich, Cat.# G1393) and cultured in DMEM (Gibco, Cat. # 11-960-044) supplemented with 10% FBS and 5% P/S. Upon confluency, STO cells were incubated with mitomycin C (Sigma-Aldrich, Cat.# M4287) at the concentration of 10 μg/ml for 4 hours. Then the cells were washed 3 times with 10 ml 1x PBS. The sorted PGCs were plated onto the STO feeder layer in PGC culture medium [DMEM supplemented with 1% P/S, 10% FBS, 100 ng/ml SCF (Peprotech, Cat.# 250–03), 25 nM bFGF (Peprotech, Cat.# 100–18B) and 25 nM LiF (Peprotech, Cat.# 250–02)].

Proliferation assay

A cell suspension containing GFP-positive PGCs obtained from E10.5 embryos of WT and *Fancd2−/−* females expressing the *Pou5f1*-eGFP reporter gene was prepared as described above. PGCs were subjected to pulse-labeling with Bromodeoxyuridine (BrdU; final concentration 10 μM) for 60 min. We used the reagents from the APC-BrdU Flow Kit (BD Biosciences,Cat. #552598), according to the manufacturer's instructions.

Analysis of apoptosis

Cell apoptosis was measured by Annexin V (BD Pharmingen, Cat. # 550474) and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; BD Pharmingen, Cat. # 564907) staining using flow cytometry according to the manufacturer's instructions.

Quantitative PCR analysis of TE expression

Total RNA from FACS-sorted (GFP+ SSEA1+) PGCs isolated from E11.5 embryos of WT, *Fanca−/−* and *Fancd2−/−* females expressing the *Pou5f1*-eGFP reporter gene was prepared

using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Reverse transcription was performed with random hexamers and Superscript II RT (Invitrogen, Grand Island, NY) and was carried out at 42 °C for 60 min and stopped at 95 °C for 5 min. First-strand cDNA was used for qPCR using the following primers: *L15UTR-F 5'-GGCGAAAGGCAAACGTAAGA-3', L15UTR-R 5'-GGAGTGCTGCGTTCTGATGA-3'; L1ORF2-F 5'-GGAGGGACATTTCATTCTCATCA-3', L1ORF2-R 5'-GCTGCTCTTGTATTTGGAGCATAGA-3'; IAP3LTR-F 5'-GCACATGCG CAGATTATTTGTT-3', IAP3LTR-R 5'-CCACATTCGCCGTTACAAGAT-3'; IAPGag-F 5'-AACCAATGCTAATTTCACCTTGGT-3', IAPGag-R 5'-GCCAATCAGCAGGCGTTAGT-3'.* Samples were normalized to the level of WT controls, and relative expression levels were determined by the standard curve method.

Preparation of protein extracts and immunoblotting

To prepare protein lysates, testes from 8–10 week old mice or in-vitro expanded PGCs isolated from E13.5 embryos were lysed using a homogenizer on ice in 3 volumes of ice-chilled Lysis buffer with Roche Complete proteinase inhibitor (Roche, Cat. # 04693116001). Lysates were cleared by centrifugation (40,000g, 20 min, at 4°C). Protein concentration was quantified by using Bio-Rad reagent and resolved on SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were then incubated with primary antibodies specific for Mili (Cell Signaling, Cat. #2071), Prmt5 (Sigma-Aldrich, Cat. # 07–405), Mael (Santa Cruz, Cat. # sc-398925), Tdrd1 (R&D Systems, Cat. # AF320), Tdrd6 (Sigma-Aldrich, Cat. # ABE231), Tdrd8/STK31 (Abcam, Cat. # ab155172), FLAG (Sigma-Aldrich, Cat. # F3165), β-actin (Sigma-Aldrich, Cat. # A5441).

µChIP analysis

Small-scale ChIP (µChIP) experiments were conducted as described previously (Dahl & Collas, 2008). Briefly, FACS-sorted (GFP+ SSEA1+) PGCs isolated from E10.5 embryos of WT, *Fanca-KO* or *Fancd2-KO* females expressing both *Fancd2-KI* transgene and the *Pou5f1*-eGFP reporter were crosslinked with 0.5% paraformaldehyde, lysed in radio immunoprecipitation assay (RIPA) buffer containing protease inhibitors, and sonicated to achieve a mean DNA fragment size of around 200–400 base pairs. After centrifugation, the chromatin extracts were then precleared with Dynal Magnetic Beads (Invitrogen) (4°C, 1 hr) followed by centrifugation (2,000 rpm, 30 min). Supernatant (precleared chromatin) was immunoprecipitated overnight with Dynal Magnetic Beads coupled with anti-H4R3me2s (1 µg per ChIP, ab5823, Abcam), anti-FLAG (Sigma-Aldrich, Cat. # F3165), anti-Prmt5 (Sigma-Aldrich, Cat. # 07–405), or normal rabbit serum. After reverse crosslinking (with protease K at 42°C for 2 hr and 68°C for 6 hr), DNA was purified (phenol-chloroform extraction) and used for qPCR analysis. Primers used were as follows: *LINE1 5UTR-Forward 5'-GGCGAAAGGCAAACGTAAGA-3', LINE1 5UTR-Reverse 5'-GGAGTGCTGCGTTCTGATGA-3'.*

Comet assay

Comet assay was performed using the Trevigen's Comet Assay Kit, following the manufacturer's instructions. In brief, the PGCs suspension was mixed with molten LMAgarose at a ratio of 1:10 (v/v), and transferred onto slides. After incubation in the lysis

buffer and alkaline treatment in electrophoresis buffer, electrophoresis (20 min, 25 V and approximately 300 mA) and neutralization were performed. Subsequently, slides were dehydrated with ethanol. For evaluation, the slides were stained with 100 μL of diluted SYBR Gold (1:10 000 in TE buffer, Invitrogen), and the comets were evaluated by image analysis using the software comet IV version 4.2 (Perceptive Instruments, Haverhill, UK). The percentage of tail DNA (% tail DNA) for each comet was calculated. The experiments were performed three times (duplicates, 50 cells per technical replicate).

Statistical analysis

Student's *t-test* was performed using GraphPad Prism v6 (GrapPad software). Comparison of more than 2 groups was analyzed by one-way ANOVA test. Values of $p<0.05$ were considered statistically significant. Results are presented as mean ± SD. * indicates $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Acknowledgements

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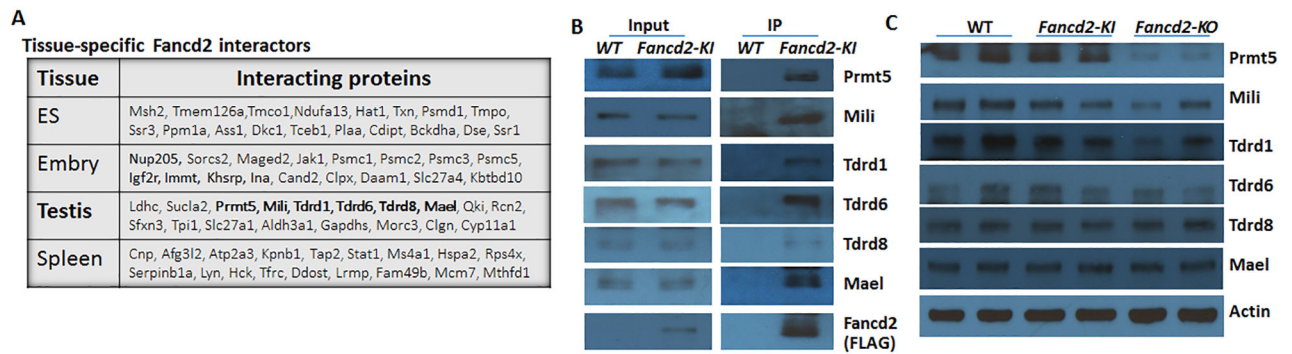
Figure 1. Fancd2 interacts with germline-specific Prmt5/piRNA factors.
(A) List of tissue specific Fancd2-associated proteins. The testis-specific Prmt5/piRNA factors are marked by bold. (B) Validation of the testis-specific Prmt5/piRNA factors by anti-FLAG immunoprecipitation (IP) from testis lysates and detected by antibodies against the indicated proteins. (C) Western analysis of the indicated Prmt5/piRNA factors in the testes of WT, *Fancd2-KI* and *Fancd2-KO* mice.
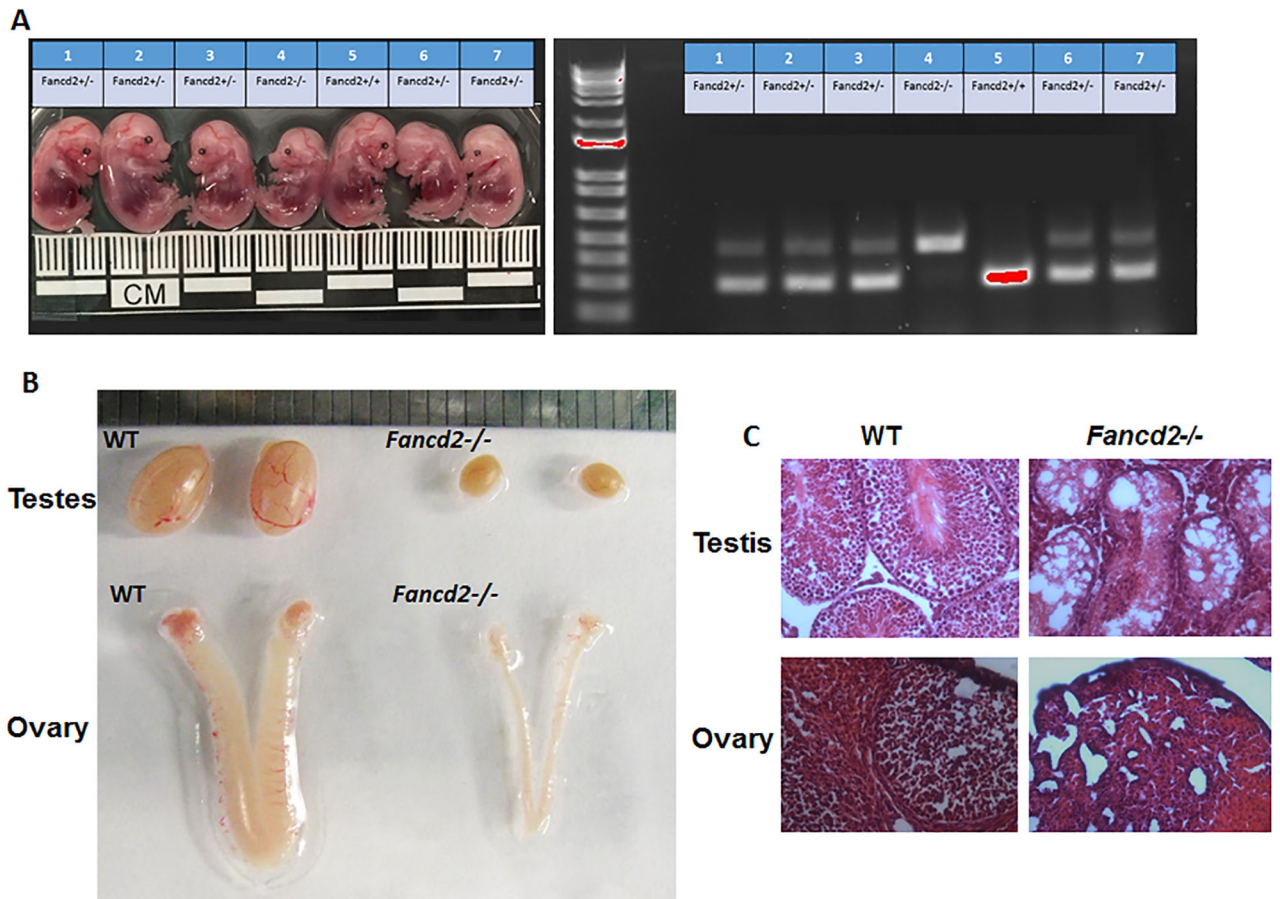
Figure 2. Loss of Fancd2 leads to hypogonadism.
(A) Images of embryos (Left) of the indicated genotypes at E13.5. Genotypes (Right) of the embryos were determined by PCR. (B) Representative photo of the testes and ovary of WT and *Fancd2-KO* mice. (C) Gonadal defects in *Fancd2–/–* mice. Shown are H&E stained sections of testes and ovaries from 8-week-old WT and *Fancd2–/–* mice (200× magnification).
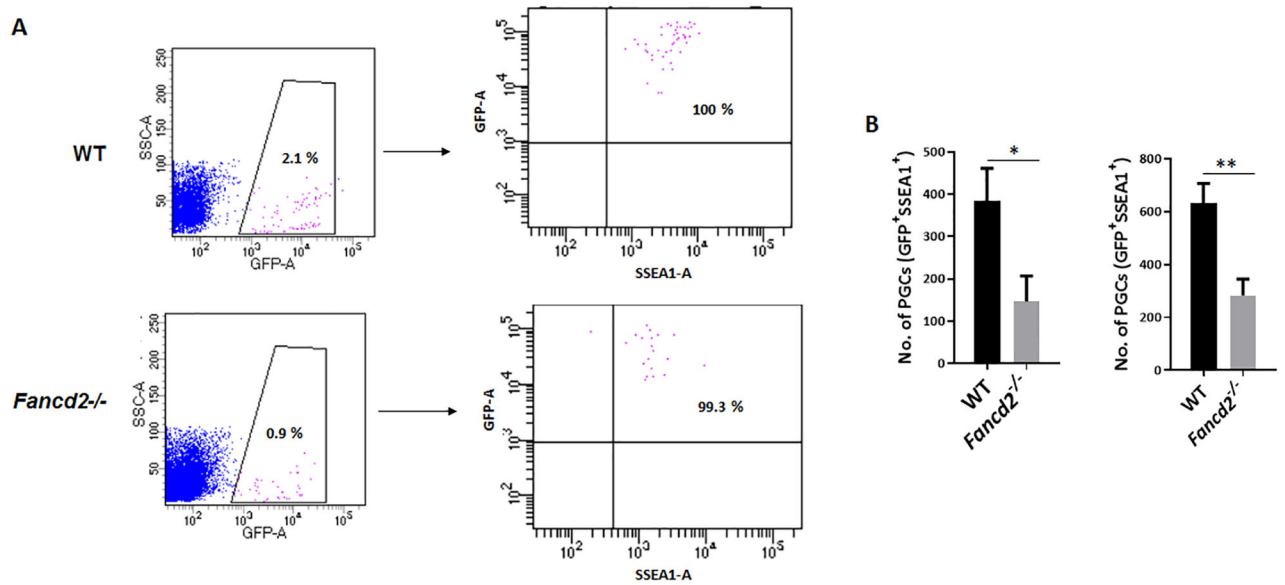
Figure 3. Loss of Fancd2 leads to PGC depletion.
(A) Representative dot plots of flow cytometric analysis of GFP+ SSEA1+ PGCs from
E11.5 embryos of WT and *Fancd2–/–* females expressing the *Pou5f1*-eGFP reporter gene.
(B) The number of PGCs at E11.5 and E13.5 embryos of WT and *Fancd2–/–* females
expressing the *Pou5f1*-eGFP reporter gene was determined by flow cytometry for GFP+
SSEA1+ PGCs. Results are presented as mean ± SD of three independent experiments. *p <
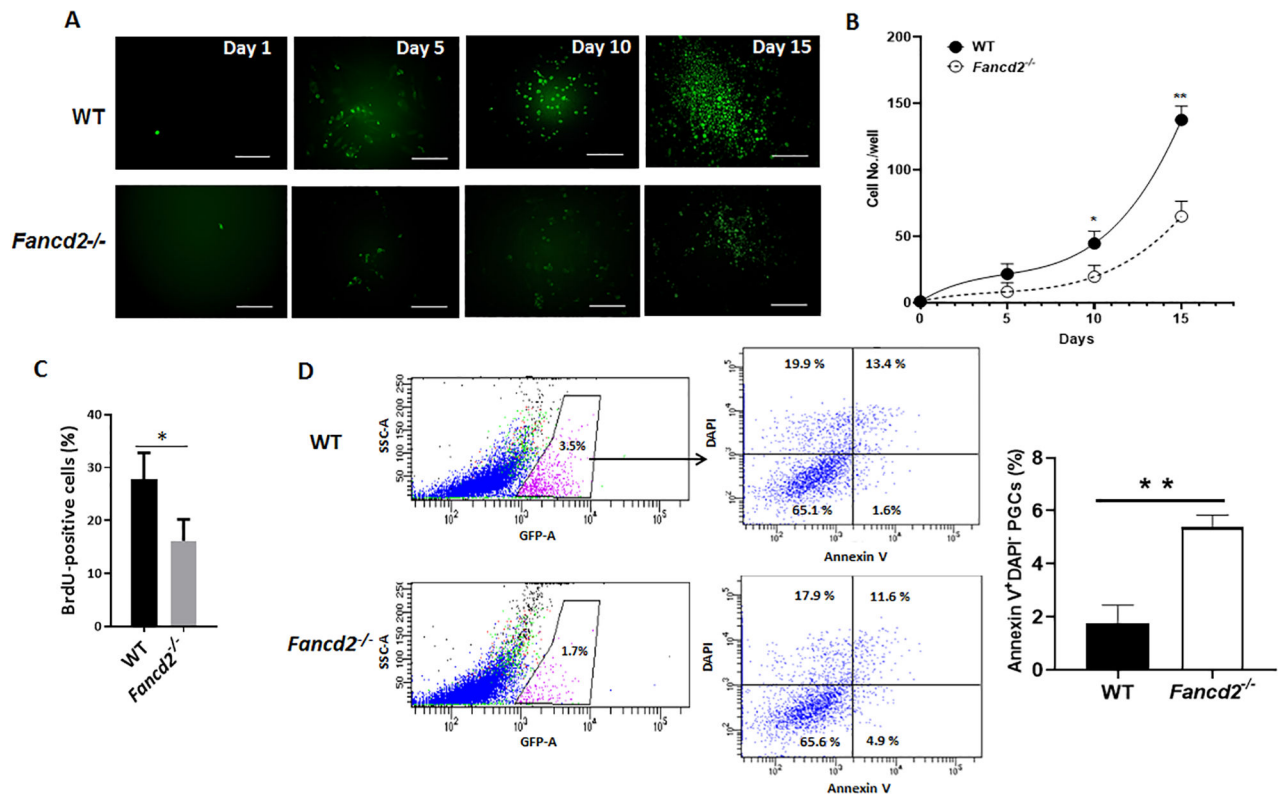0.05, **p < 0.01

Figure 4. Reduced proliferation and increased apoptosis in *Fancd2-KO* PGCs.

(A) Reduced proliferation in *Fancd2-KO* PGCs. GFP⁺ PGCs from E11.5 embryos of WT and *Fancd2–/–* females expressing the *Pou5f1*-eGFP reporter gene were sorted by FACS and cultured on STO feeder cells for 15 days. Images were taken at the indicated time points. (B) Cumulative growth curve of PGCs during PGC culture initiation. GFP⁺ PGCs from E11.5 embryos of WT and *Fancd2–/–* females expressing the *Pou5f1*-eGFP reporter gene were sorted by FACS and cultured on STO feeder cells for the indicated days. Values indicate mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$. (C) Diminished BrdU incorporation in *Fancd2-KO* PGCs. GFP⁺ PGCs from E11.5 embryos of WT and *Fancd2–/–* females expressing the *Pou5f1*-eGFP reporter gene were sorted by FACS and subjected to pulse-labeling with BrdU followed by flow cytometric analysis for BrdU-positive cells. Values indicate mean ± SD of three independent experiments. $*p < 0.05$. (D) Increased apoptosis in *Fancd2-KO* PGCs. GFP⁺ PGCs from E11.5 embryos of WT and *Fancd2–/–* females expressing the *Pou5f1*-eGFP reporter gene were subjected to flow cytometric analysis for Annexin V-positive, DAPI-negative apoptotic cells. Results are presented as mean ± SD of three independent experiments. $**p < 0.01$
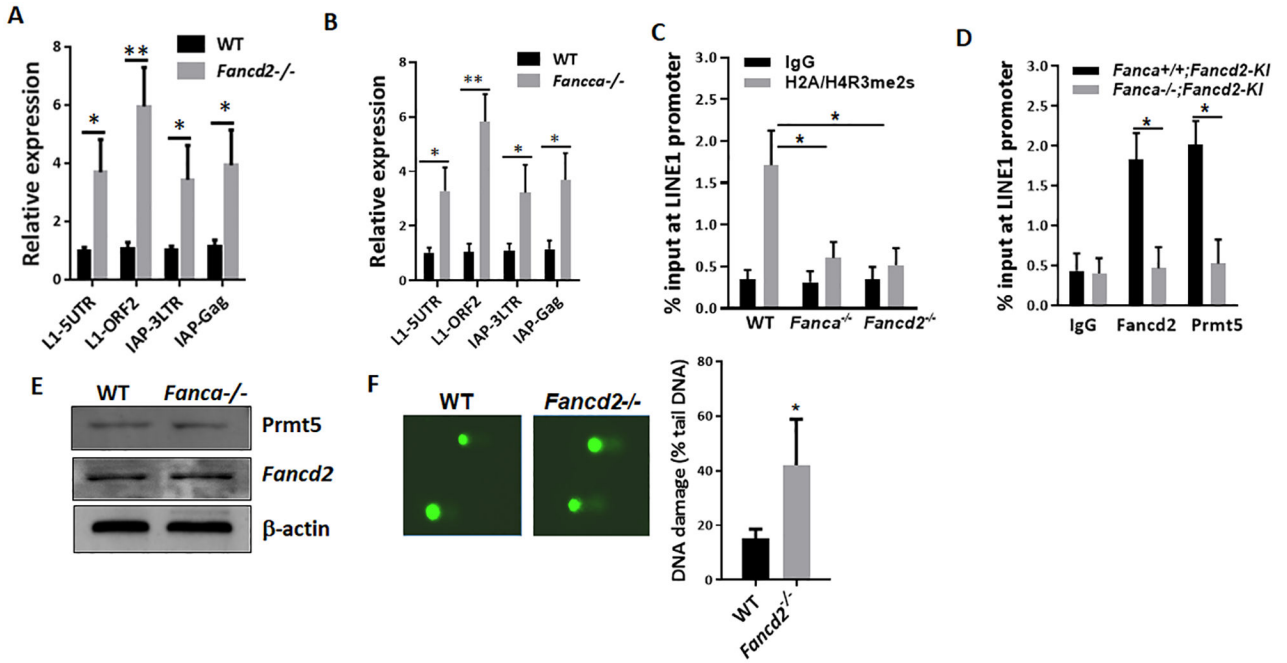
Figure 5. FA deficiency leads to de-repression of germline TEs.
(A) Fancd2 is essential for TE repression in PGCs. qPCR analysis of TEs in FACS-sorted GFP+ SSEA1+ PGCs from E11.5 embryos of WT and *Fancd2–/–* females expressing the *Pou5f1*-eGFP reporter gene, using the primer sets described in Materials and Methods. Results are presented as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01. (B) De-repression of germline TEs in *Fanca*-deficient PGCs. qPCR analysis of TEs in FACS-sorted GFP+ SSEA1+ PGCs from E11.5 embryos of WT and *Fanca–/–* females expressing the *Pou5f1*-eGFP reporter gene, using the primer sets described in Materials and Methods. Results are presented as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01. (C) μChIP analysis for H2A/H4R3me2s at the promoter region of LINE1 TEs in FACS-sorted GFP+ SSEA1+ PGCs from E10.5 embryos of WT, *Fanca–/–* and *Fancd2–/–* females expressing the *Pou5f1*-eGFP reporter gene. Percentages of anti- H2A/H4R3me2s immunoprecipitated DNA compared to input are shown. w/o, without primary antibodies. Results are presented as mean ± SD of three independent experiments. *p < 0.05. (D) μChIP analysis for Fancd2 (FLAG) and Prmt5 at the promoter region of LINE1 TEs in FACS-sorted GFP+ SSEA1+ PGCs from E10.5 embryos of WT, *Fanca–/–* and *Fancd2–/–* females expressing the *Pou5f1*-eGFP reporter gene and the *Fancd2KI/KI* transgene. Percentages of anti-FLAG and anti-Prmt5 immunoprecipitated DNA compared to input are shown. w/o, without primary antibodies. Results are presented as mean ± SD of three independent experiments. *p < 0.05. (E) Western analysis of the Prmt5 and Fancd2 proteins in the in-vitro expanded PGCs from E13.5 WT (WT;*Fancd2-KI*) and *Fanca*-deficient (*Fanca–/–;Fancd2-KI*) embryos, using antibodies against the indicated proteins. (E) Western analysis of the Prmt5 and Fancd2 proteins in the E13.5 PGCs from WT (WT;*Fancd2-KI*) and *Fanca*-deficient (*Fanca–/–;Fancd2-KI*) mice, using antibodies against the indicated proteins. (F) Increased accumulation of DNA damage in *Fancd2*-deficient PGCs. Genomic DNA was stained with SYBR Gold and imaged after single cell electrophoresis (left). Quantification

for the percent of DNA in the comet tail is shown (right). Results are presented as mean ± SD of three independent experiments. *p < 0.05.