Endonuclease G is required for early embryogenesis and normal apoptosis in mice

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Endonuclease G (EndoG) is a nuclear-encoded mitochondrial protein reported to be important for both nuclear DNA fragmentation during apoptosis and mitochondrial DNA replication. To evaluate the *in vivo* function of EndoG, we have investigated the effects of EndoG deficiency in cells and mice. We found that EndoG homozygous mutant embryos die between embryonic days 2.5 and 3.5. Mitochondrial DNA copy numbers in ovulated oocytes from EndoG heterozygous mutant and wild-type mice are similar, suggesting that EndoG is involved in a cellular function unrelated to mitochondrial DNA replication. Interestingly, we found that cells from EndoG heterozygous mutant mice exhibit increased resistance to both tumor necrosis factor α- and staurosporine-induced cell death. Moreover, spontaneous cell death of spermatogonia in EndoG heterozygous mutant mice is significantly reduced compared with wild-type mice. DNA fragmentation is also reduced in EndoG$^{+/-}$ thymocytes and splenocytes compared with wild-type cells, as well as in EndoG$^{+/-}$ thymus *in vivo* compared with that of the wild-type mice, on activation of apoptosis. These findings indicate that EndoG is essential during early embryogenesis and plays a critical role in normal apoptosis and nuclear DNA fragmentation.

Apoptosis is a physiological process critical for normal mammalian development and tissue homeostasis. Dysregulation of apoptosis can affect development and results in diseased conditions, including neural degeneration, autoimmunity, and cancer (1). Cleavage of chromatin into oligonucleosomal-sized fragments is a biochemical hallmark of apoptosis (2). Biochemical and genetic studies have identified at least two endonucleases important for mammalian DNA fragmentation during apoptosis, the DNA fragmentation factor (DFF) and endonuclease G (EndoG) (3–10).

DFF is a dimeric protein consisting of the DFF40 and DFF45 subunits [also termed CAD (caspase-activated DNase) or CPAN (caspase-activated nuclease), and ICAD (inhibitor of caspase-activated DNase) (3–7)]. DFF40 contains an endonuclease activity, and its synthesis and folding critically depend on DFF45 (3–7). On induction of apoptosis, DFF45 is cleaved by caspase-3. DFF40 is then activated to cleave DNA into nucleosomal-sized fragments and to induce chromatin condensation (3–7). We previously made a DFF45 mutant ($-/-$) mouse and found that DNA fragmentation in DFF45$^{-/-}$ thymocytes exposed to various apoptotic stimuli is profoundly impaired (11–14). Moreover, DFF45$^{-/-}$ thymocytes and mouse embryonic fibroblasts (MEFs) exposed to several apoptotic agents are partially resistant to apoptosis and chromatin condensation (11, 15, 16). DFF45$^{-/-}$ mice also exhibit reduced natural killer T cell death after certain viral infections (17) and are more resistant to neuronal cell death after excessive excitatory stimulation, compared with wild-type mice (18). Finally, DFF45$^{-/-}$ mice exhibit increased numbers of granule cells in the dentate gyrus and enhanced spatial learning capabilities compared with wild-type mice (19). These observations, together with biochemical studies, established that DFF is a major endonuclease responsible for DNA degradation after activation of apoptosis (14). Moreover, ineffective DNA fragmentation can have physiological consequences (14). During the course of these studies, we noticed residual DNA fragmentation in DFF45$^{-/-}$ splenocytes and MEFs (8, 11), suggesting the existence of additional endonucleases.

EndoG is an evolutionarily conserved, nuclear-encoded endonuclease reported to be involved in mtDNA replication, recombination, and degradation (20–22). Biochemical work showed that, on activation of apoptosis, EndoG is released from the mitochondrial intermembrane space and translocates to the cell nucleus to induce DNA fragmentation (8–10). In DFF45$^{-/-}$ MEFs, in response to specific apoptotic stimuli, EndoG cleaves chromosomal DNA in a caspase-independent manner (8). DNA fragmentation in DFF45$^{-/-}$ MEFs can be blocked by an anti-EndoG antibody, indicating that the residual DNA fragmentation activity in these cells is at least partially attributable to EndoG (8). Unlike DFF, which generates double-stranded DNA breaks, EndoG degrades single-stranded DNA more rapidly than duplex DNA (14). Genetic studies showed that an EndoG mutation in *Caenorhabditis elegans* results in less effective DNA degradation and delayed apoptosis *in vivo* (9). These biochemical and genetic studies suggest that EndoG participates in a DNA fragmentation pathway originating in mitochondria.

Despite these studies, the role of EndoG in mammalian development and apoptosis *in vivo* remained unknown. Because EndoG is primarily located in the mitochondrial intermembrane space (8, 23), it is an unlikely participant in mtDNA replication *in vivo* although direct evidence for this hypothesis is lacking. To address these issues, we investigated the consequences of EndoG mutation in cells and mice. We found that EndoG is required for early embryogenesis and normal apoptosis and is not required for maintenance of normal levels of mtDNA.

Materials and Methods

Embryos and Mice Carrying EndoG and/or DFF45 Mutations. A targeting construct for the EndoG gene was made by standard molecular cloning and was transfected into 129 embryonic stem cells as described (11). Genomic Southern analyses identified 15 homologous recombinants by using both the 5′ and the 3′ probes. Extensive breeding of the chimeric mice resulted in EndoG$^{+/-}$ mice and EndoG$^{-/-}$ embryos. EndoG$^{+/-}$DFF45$^{-/-}$ embryos and mice were obtained by crossing DFF45$^{-/-}$ mice (11) with EndoG$^{+/-}$ mice.

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Abbreviations: EndoG, endonuclease G; DFF, DNA fragmentation factor; MEF, mouse embryonic fibroblast; AIF, apoptosis-inducing factor; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; TNF, tumor necrosis factor; ICM, inner cell mass; DAPI, 4′,6-diamidino-2-phenylindole; E*n*, embryonic day *n*.

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Western Blotting. Mitochondrial-enriched fractions from wild-type and EndoG$^{+/-}$ MEFs and heart were probed with an anti-EndoG antibody (1:1,000), and nuclear extracts from the wild-type and EndoG$^{+/-}$ spleen and thymus were analyzed with an anti-DFF45 antibody, with enhanced chemiluminescence (Amersham Pharmacia).

Major Organ System Surveys. Histological examinations were performed on sections from all major organ systems as described (11). Tissues were fixed, dehydrated, and embedded in paraffin. Sections were cut at a thickness of 4–5 μm and stained with hematoxylin and eosin. Age-matched EndoG$^{+/-}$, EndoG$^{+/-}$DFF45$^{-/-}$, and wild-type littermates were used in the study.

Immune System Analyses. Single cell suspensions from blood, spleen, thymus, inguinal and mesenteric lymph nodes, and bone marrow were prepared (11). After hypotonic lysis of erythrocytes, cells were stained with fluorescently labeled anti-CD4, -CD8, -B220, -TCRβ (-T cell antigen receptor β), -CD3, -TCR$\gamma\delta$, -CD44, -CD69, -NK1.1, -CD11b, and -CD11c monoclonal antibodies (Pharmingen) to detect expression of different surface markers (11). Flow cytometric analysis was performed by using a FACSCalibur system and CELLQUEST 3.1 software (Becton Dickinson). Serum Ig isotype levels were measured (24). Mice were immunized with hen egg lysozyme suspended in complete Freund's adjuvant, and antigen-specific T and B cell responses were measured as described (24).

Embryonic Day (E) 2.5–3.5 EndoG Embryo Culture and Genotyping. E2.5 and E3.5 embryos from timed EndoG$^{+/-}$ breeding were harvested and singly cultured in microdrops of medium covered with mineral oil. Photographs were taken every 24 h by using a phase contrast microscope before genotyping by nested PCR. The following external primers were used: A-7 (TGCTCTTGTGTTCAGTCCCTGC), A-9 (ATGTTTTGTCTGCGTGTGCTTG), and PCR-neo (ACGAGACTAGTGAGAC GTGC). The following internal primers were used: A-34 (GTTGGCTGTGGTAAAGATCCC), A-35 (ATGAGGAGAGGGCATCGGA), and A-36 (CTTCCATTTGTCACGTCCTGC).

mtDNA Copy Number Analysis. Quantitative real-time PCR was performed by using a LightCycler-DNA Master SYBR Green I kit (Roche) using 5′-CCCCAGCCATAACACAGTATCAAAC and 5′-GCCCAAAGAATCAGAACAGATGC as primers for the mtDNA-encoded cytochrome oxidase subunit I gene. PCR contained 2.5 ng/μl primers in a 20-μl volume. Cycling conditions were as follows: 95°C for 150 s, followed by 40 cycles at 95°C for 10 s, 58°C for 15 s, and 72°C for 20 s. Unfertilized eggs from EndoG$^{+/-}$ and wild-type female mice were used.

Histological Analyses of Testes and Spermatogonia Cell Death. Testes were dissected from wild-type, EndoG$^{+/-}$, DFF45$^{-/-}$, and EndoG$^{+/-}$DFF45$^{-/-}$ mice. Paraffin-embedded sections were incubated with an anti-EndoG antibody (1:1,000) or a preimmune antibody. These sections were counterstained with hematoxylin. Separate sections were stained by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL). TUNEL-positive cells were counted at ×20 objective lens, and multiple areas were used for each mouse.

Apoptosis, Clonogenesis, and DNA Fragmentation Assays. Splenocytes, thymocytes, and MEFs were cultured at 1×10^6 per ml (11). These cells were treated with tumor necrosis factor (TNF)α (2 ng/ml plus 10 μg/ml cyclohexamide) or staurosporine (2 μM), or subjected to serum starvation. Cell death was examined by trypan blue and 4′,6-diamidino-2-phenylindole (DAPI) staining, and by using a caspase-3 activation kit (Clontech). Clonogenesis assays were performed by using MEFs. After TNFα treatment, MEFs were cultured in serial dilutions in fresh medium in the absence of TNFα, and live colonies were counted after 3 weeks. DNA fragmentation was assayed as described (11).

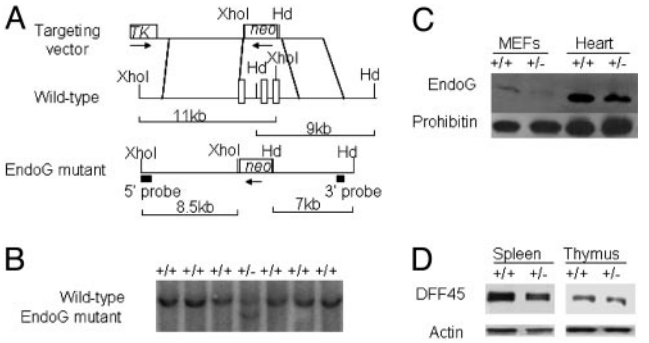

Fig. 1. Disruption of the EndoG gene. (*A*) Restriction maps of the wild-type EndoG gene locus, the targeting vector, and the mutant EndoG allele. Open boxes show exons 1–3 of EndoG. *neo* and *TK* encode selectable markers for G418 resistance and ganciclovir sensitivity, respectively. The locations and the expected sizes of the DNA fragments are indicated. *Hin*dIII (Hd) and *Xho*I were used for analyzing genomic DNA with 5′ and 3′ probes, the locations of which are indicated by the black boxes. (*B*) Identification of EndoG heterozygous mutant mice by Southern blotting using the 3′ probe. (*C*) Western analysis of EndoG expression by using extracts from MEFs or heart ($n = 3$ mice each) with prohibitin as a loading control. (*D*) Western blotting for DFF45 by using extracts from the spleen and thymus with actin as a control ($n = 3$ mice each). +/+, wild types; +/−, heterozygous mutants.

Results

Generating Mice Carrying an EndoG Mutation. We generated EndoG$^{+/-}$ mice by homologous DNA recombination in embryonic stem cells. The targeting construct was designed to delete most of the three exons of the EndoG gene (Fig. 1*A*). After identifying embryonic stem cell clones harboring the desired homologous recombination, we generated male chimeric mice that were bred with C57BL/6 females. Genomic Southern blotting using both the 5′ (data not shown) and 3′ probes (Fig. 1*B*) identified EndoG$^{+/-}$ mice. EndoG protein expression level was reduced in EndoG$^{+/-}$ mice compared with wild-type mice (Fig. 1*C*). The expression of DFF45 (Fig. 1*D*), apoptosis-inducing factor (AIF), cytochrome *c*, DNaseI, and DNaseII in EndoG$^{+/-}$ tissues is comparable to that in wild-type tissues (data not shown).

EndoG$^{+/-}$ Mice Develop Normally Whereas EndoG$^{-/-}$ Embryos Die During Early Embryogenesis. EndoG$^{+/-}$ mice appeared normal. An extensive histological survey of all of the major organ systems of EndoG$^{+/-}$ mice at 6 weeks of age did not reveal any obvious developmental abnormalities compared with age-matched wild-type littermates ($n = 3$ each, data not shown). There was no difference in the distribution of macrophages, dendritic cells, and distinct lymphocyte subsets, the activation status of lymphocytes, serum Ig levels, and antigen-specific T and B cell responses between EndoG$^{+/-}$ and wild-type mice ($n = 5$ mice each; data not shown). No reproductive abnormalities were found in either EndoG$^{+/-}$ males or females (data not shown).

When we intercrossed EndoG$^{+/-}$ mice, we failed to obtain live EndoG$^{-/-}$ mice. Of the first 313 pups analyzed, 64% were EndoG$^{+/-}$ and 36% were wild type (Table 1). From those isolated between E6.5 and E14.5, no EndoG$^{-/-}$ embryos were identified (Table 1). The latest developmental stage in which EndoG$^{-/-}$ embryos could be identified was between E2.5 and E3.5. However, fewer EndoG$^{-/-}$ embryos were identified than expected from the Mendelian segregation for E2.5 and E3.5

Table 1. The EndoG mutation is early embryonic lethal

	EndoG$^{+/+}$	EndoG$^{+/-}$	EndoG$^{-/-}$
Postnatal	113	200	0
E8.5–14.5	16	30	0
E6.5–7.5	18	33	0
E3.5	47	54	8
E2.5	57	70	9

Postnatal mice and E8.5–14.5 embryos were genotyped by Southern blotting and PCR, respectively. E6.5–7.5, E3.5, and E2.5 embryos were genotyped by nested PCR. Number of mice and embryos of each genotype are listed.

embryos (Table 1), suggesting that embryonic lethality was occurring at this time.

To determine how the EndoG mutation affects early embryogenesis, we isolated E2.5 and E3.5 embryos (Fig. 2*A*) and observed their growth and differentiation in culture. In sharp contrast to the wild-type and EndoG$^{+/-}$ embryos, EndoG$^{-/-}$ embryos lost normal morphology, failed to form a normal blastocoel and inner cell mass (ICM), and died in culture. These results indicate that the EndoG$^{-/-}$ mutation leads to early embryonic lethality.

Ovulated Unfertilized Oocytes from EndoG$^{+/-}$ and Wild-Type Mice Have Similar mtDNA Copy Numbers. mtDNA amplification occurs during oogenesis before the first meiotic division, and no further replication occurs until the blastocyst stage (25–27). To investigate whether EndoG functions in mtDNA replication, we performed quantitative real-time PCR using ovulated unfertilized oocytes from wild-type and EndoG$^{+/-}$ mice. We found that the relative copy numbers of mtDNA per cell are similar in oocytes from EndoG$^{+/-}$ mice compared with wild-type mice (Fig. 2*B*), suggesting that EndoG is not crucial for mtDNA replication. No selective loss of EndoG mutant oocytes from EndoG$^{+/-}$ females was evident because normal litter sizes and equal numbers of wild-type and EndoG$^{+/-}$ offsprings (23 wild-type and 25 EndoG$^{+/-}$ offsprings) were present from breedings between EndoG$^{+/+}$ males and EndoG$^{+/-}$females. Quantitative Southern analysis of mtDNA from the lung, brain, heart, liver, kidney, ovary, spleen, and thymus from EndoG$^{+/-}$ and wild-type mice and real-time PCR examination of wild-type and EndoG$^{+/-}$ blastocysts indicated that relative copy numbers of mtDNA per cell are also comparable in all samples examined between the two genotypes (data not shown).

EndoG$^{+/-}$ Cells Exhibit Increased Resistance to Cell Death. Although the level of EndoG protein expression is reduced in EndoG$^{+/-}$ mice, the mtDNA copy number is normal. Mitochondrial function, as measured by the activities of succinate dehydrogenase that is encoded by nuclear genes and cytochrome *c* oxidase that is encoded in part by mitochondrial genes, is also normal (data not shown). Thus, EndoG$^{+/-}$ tissues and cells can be used to measure whether reduced EndoG expression affects apoptosis. We measured the responses of EndoG$^{+/-}$ MEFs to both TNFα and staurosporine. Trypan blue staining indicated that EndoG$^{+/-}$ MEFs exhibit reduced cell death compared with wild-type MEFs 4 h after the treatment (Fig. 3*A*). EndoG$^{+/-}$ MEFS also exhibit reduced caspase-3 activation (Fig. 3*B*) and chromatin condensation (Fig. 3*C*) compared with wild-type MEFs after the apoptosis induction. These results indicate that apoptotic stimuli induce less cell death in EndoG$^{+/-}$ MEFs than wild-type MEFs. To discern whether EndoG$^{+/-}$ cells exhibit delayed cell death or more resistance to cell death compared with wild-type cells, we performed clonogenesis assays after treating MEFs with TNFα. Three weeks after the treatment, EndoG$^{+/-}$ cells form more colonies than wild-type control cells (Fig. 3*D*), indicating that reduced EndoG expression renders cells more resistant to cell death.

Effects of Combined EndoG and DFF45 Mutation on Cell Death. To investigate whether EndoG functions together with DFF in apoptosis, we generated EndoG$^{+/-}$DFF45$^{-/-}$ mice. Similar to those observed for EndoG$^{+/-}$ and DFF45$^{-/-}$ mice, a detailed histological survey of all of the major organ systems of EndoG$^{+/-}$DFF45$^{-/-}$ mice at 6 weeks of age did not reveal any obvious developmental abnormalities compared with age-matched wild-type littermates (n = 3 each, data not shown).

Spontaneous apoptosis occurs during spermatogenesis, and many molecules in the cell death program function during this process (28). EndoG is expressed ubiquitously, including in the mouse testes (Fig. 4*A*). Histological examinations indicate that testes and epididymis of EndoG$^{+/-}$ mice appear normal with mature sperm (data not shown). Moreover, the percentage of mobile sperm in EndoG$^{+/-}$ epididymis is similar to that in wild-type mice (data not shown). However, similar to results obtained with MEFs, TUNEL staining in the testes of EndoG$^{+/-}$ mice is significantly reduced as compared with wild-type mice (Fig. 4 *B* and *C*). Moreover, DFF45$^{-/-}$ and EndoG$^{+/-}$DFF45$^{-/-}$ testes exhibited less TUNEL staining than those of EndoG$^{+/-}$ (Fig. 4 *B* and *C*). However, the reduced spontaneous cell death in spermatogenesis did not obviously affect the reproductive

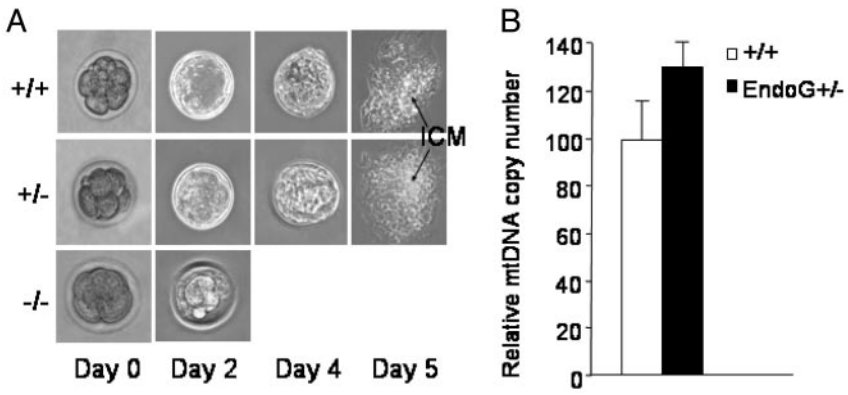

Fig. 2. EndoG is essential for early embryogenesis. (*A*) E2.5 embryos were cultured in microdrops *in vitro* for 5 days (n = 31 wild-type, 49 EndoG$^{+/-}$, and 5 EndoG$^{-/-}$ embryos, respectively). Arrows point to the ICM. (*B*) EndoG is not essential for mtDNA replication. mtDNA copy numbers in unfertilized eggs from wild-type and EndoG$^{+/-}$ females (n = 3 mice each with eight or more from each mouse) were determined by using real-time PCR. The mtDNA-encoded cytochrome oxidase subunit 1 locus was examined. Data represent mean + SEM. Differences were not significant ($P > 0.05$; Student's t test).

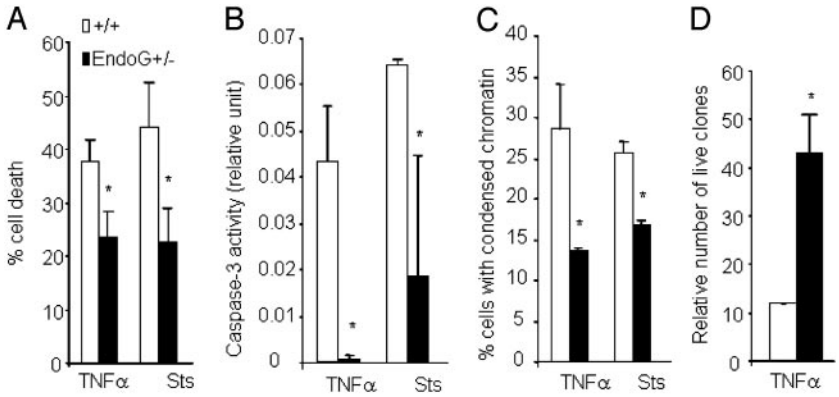

Fig. 3. Resistance to cell death in EndoG heterozygous mutant MEFs compared with wild-type cells in response to TNFα or staurosporine (Sts) treatment. (*A*) Percentage of cell death assayed by trypan blue 4 h after the treatments. (*B*) Caspase-3 activities 4 h after the treatments. (*C*) Percentage of cells with condensed chromatin assayed by DAPI staining 4 h after the treatments. (*D*) Clonogenesis assays of live colonies 3 weeks after TNFα treatment. Each experiment was repeated two to four times by using $n = 3$ independent MEFs each, and parallel results were obtained. Representative results are shown. Data are mean + SEM. *, $P < 0.05$ compared with the wild types by Student's *t* test.

capacity of the EndoG$^{+/-}$ mice because crosses between EndoG$^{+/-}$ males and wild-type females produced normal litter sizes compared with those from wild-type crosses, and equal numbers of EndoG$^{+/-}$ and wild-type offspring were obtained (71 wild type and 68 EndoG$^{+/-}$).

We also compared cell death in splenocytes and thymocytes from the above mice. After TNFα or staurosporine treatment, splenocytes (Fig. 5*A*) and thymocytes (data not shown) from EndoG$^{+/-}$, DFF45$^{-/-}$, and EndoG$^{+/-}$DFF45$^{-/-}$ mice exhibit comparable levels of cell death that are reduced compared with wild-type cells. DAPI staining further showed that chromatin condensation is reduced in EndoG$^{+/-}$ cells and essentially eliminated in DFF45$^{-/-}$ and EndoG$^{+/-}$DFF45$^{-/-}$ cells (Fig. 5*B* and data not shown). These results suggest that efficient apoptosis requires both DFF and EndoG.

Effects of EndoG and/or DFF45 Mutation on DNA Fragmentation. After treating various splenocytes and thymocytes with different apoptotic stimuli, we found that DNA fragmentation is reduced in EndoG$^{+/-}$ splenocytes and thymocytes, compared with wild-type cells, and essentially eliminated in DFF45$^{-/-}$ and EndoG$^{+/-}$DFF45$^{-/-}$ cells (Fig. 6*A* and data not shown). This result suggests that, whereas DFF is the predominant DNA fragmentation factor, EndoG also facilitates DNA fragmentation. To further test this notion *in vivo*, we treated wild-type, EndoG$^{+/-}$, DFF45$^{-/-}$, and EndoG$^{+/-}$DFF45$^{-/-}$ mice with dexamethasone for different periods of time. Again, we found that DNA fragmentation in the thymus is reduced in EndoG$^{+/-}$ mice, compared with wild-type mice, and essentially eliminated in DFF45$^{-/-}$ and EndoG$^{+/-}$DFF45$^{-/-}$ (Fig. 6*B*), suggesting a role of EndoG in DNA fragmentation *in vivo*.

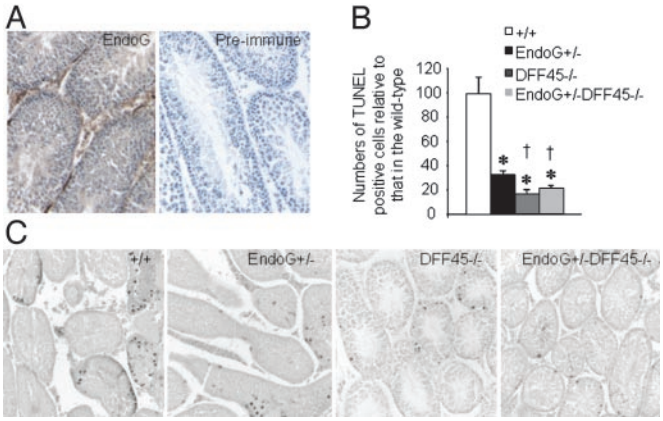

Fig. 4. Spontaneous cell death of differentiating spermatogonia is reduced in EndoG heterozygous mutant mice compared with that in wild-type mice. (*A*) EndoG expression in wild-type testes stained with an anti-EndoG antibody (*Left*) and stained with a preimmune antibody (*Right*). (*B*) Bar graph of the numbers of TUNEL-positive cells relative to those in the wild type ($n = 3$ mice each genotype). Data are mean + SEM. *, $P < 0.05$ compared with the wild types; †, $P < 0.05$ compared with EndoG$^{+/-}$ (Student's *t* test). (*C*) Examples of TUNEL staining.

Discussion

We have investigated the role of EndoG in apoptosis and mammalian development *in vivo* by using cells and mice carrying an EndoG mutation. Our findings suggest that EndoG is required for early embryogenesis and normal apoptosis. The critical role of EndoG in mammalian embryogenesis may be an evolutionarily acquired function because EndoG defects do not affect survival in yeast and *C. elegans* (9, 29). Apoptosis plays critical roles in normal development (30–33). At the blastocyst stage in mammalian development, the main site of apoptosis is in the ICM, to eliminate cells that retain the potential to form trophectoderm and to control for total ICM cell number (33). Apoptosis also occurs during cavitation, to convert a solid embryo into a hollow, two-layered egg cylinder (32). Because yeast does not normally go through apoptosis or exhibit morphogenesis and apoptosis in *C. elegans* seems to be a cell lineage-invariant event, the requirement for EndoG in mammalian embryogenesis suggests an evolutionarily acquired function that came with non-lineage-specific apoptosis during blastocoel and hollow egg cylinder formation. Despite much progress in identifying components of the cell death machinery, little is known about the apoptosis pathways that are essential for early mammalian embryogenesis. Apaf-1, caspase-9, and caspase-3 have all been shown to be necessary for nervous system development at later stages in mice (34), and cytochrome *c*-mediated cell death is essential at E8.5 to E9.5 stages (35). Notably, AIF, Mcl-1, and Fen1 participate in apoptotic regulation (36–38), and their expression is essential for early embryogenesis (39–41). EndoG may play a critical role in embryogenesis by directly regulating early developmental apoptosis or by regulating the function of other essential proteins, such as AIF or Fen1, as in *C. elegans* (38, 42).

We found similar mtDNA copy numbers in ovulated, unfertilized oocytes from EndoG$^{+/-}$ and wild-type female mice despite the EndoG mutation. Although more investigation is needed, our current results are consistent with the observation

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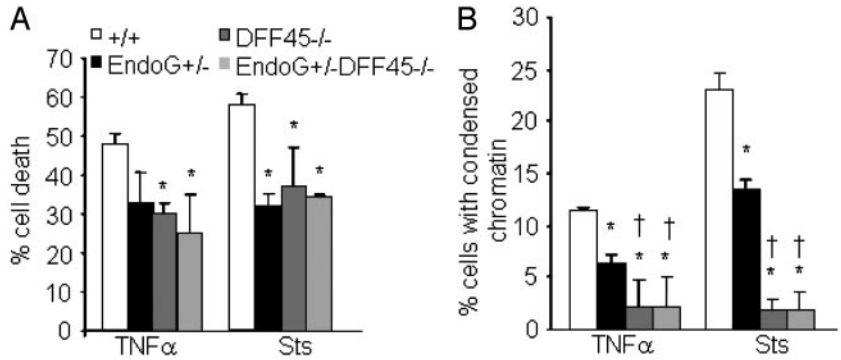

Fig. 5. Reduced cell death in DFF45$^{-/-}$EndoG$^{+/-}$ and DFF45$^{-/-}$ splenocytes compared with wild-type (+/+) and EndoG$^{+/-}$ cells in response to TNFα or staurosporine (Sts) treatment. (*A*) Percentage of cell death assayed by trypan blue staining 16 h after the treatment. (*B*) Percentage of cells with condensed chromatin assayed by DAPI staining 16 h after the treatment. Data represent mean + SEM. *, $P < 0.05$ compared with the wild types; †, $P < 0.05$ compared with EndoG$^{+/-}$ (Student's *t* test).

that EndoG is primarily present in the intermembrane space of the mitochondria (8, 23) and suggest that, although EndoG may show some specificity in cleaving mitochondrial nucleic acids *in vitro* (20), the wild-type amount of EndoG is not essential for mtDNA replication, at least during oogenesis. The endoribonuclease activity required for mtDNA replication may be provided by RNase mitochondrial RNA processing (MRP) (43) and/or RNase H (44). Our observation is also consistent with previous findings (22) that disruption of the EndoG homolog in *Saccharomyces cerevisiae* does not interfere with mtDNA propagation.

By analyzing overall cell death, dying cells with chromatin condensation, and the proliferation potential of cells surviving a previous apoptotic exposure, we found that a reduction in EndoG expression results in partial resistance to apoptosis in EndoG$^{+/-}$ MEFs, splenocytes, and thymocytes. Our findings are consistent with prior studies (9) indicating that EndoG disruption in *C. elegans* results in delayed apoptosis. A delay in DNA degradation due to the EndoG deficiency may provide an opportunity for cells to repair protein and organelle damage generated by the initial apoptotic stimulation and to ultimately resist death. EndoG may act alone to degrade DNA or function together with other endonucleases (45). In *C. elegans*, EndoG interacts with a degradeosome containing AIF and Fen1 (38, 42, 46). Similar degradeosomes may also exist in mammalian cells.

We found that, although EndoG$^{+/-}$ and DFF45$^{-/-}$ cells are both partially resistant to apoptosis, EndoG$^{+/-}$DFF45$^{-/-}$ cells exhibit a similar resistance to apoptosis as that of the DFF45$^{-/-}$ cells. Moreover, EndoG$^{+/-}$DFF45$^{-/-}$ mice develop normally without confounding phenotypes, compared with wild-type and EndoG$^{+/-}$ mice. Furthermore, EndoG$^{+/-}$ cells exhibit reduced DNA fragmentation compared with wild-type cells whereas DFF45$^{-/-}$ and EndoG$^{+/-}$DFF45$^{-/-}$ cells exhibit no obvious DNA fragmentation. These observations suggest that a mild initial delay of DNA fragmentation may be all it takes to inhibit the extent of cell death. Alternatively, EndoG may function in apoptosis independently of its role in DNA fragmentation. Overall, our observations are consistent with the previous findings (8, 9, 11, 12) that both EndoG and DFF contribute to DNA fragmentation and apoptosis. Furthermore, these results suggest that DFF plays the more prominent role in DNA fragmentation and apoptosis in mammals and that EndoG likely

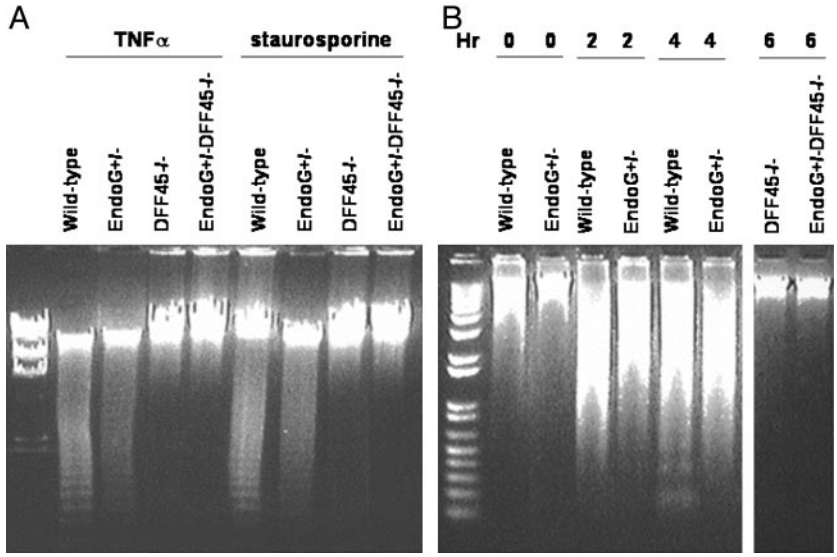

Fig. 6. EndoG plays a role in DNA fragmentation. (*A*) DNA fragmentation comparisons in splenocytes after 16 h of TNFα or staurosporine treatments. Each measurement was performed at least three times by using three to five mice, and parallel results were obtained. Representative results are shown. (*B*) DNA fragmentation in wild-type, EndoG$^{+/-}$, DFF45$^{-/-}$, and DFF45$^{-/-}$EndoG$^{+/-}$ thymi after i.p. dexamethasone treatment (12.5 μg/kg) for 0, 2, 4, and 6 h, respectively. This experiment was repeated at least three times by using three to five mice, and parallel results were obtained.

facilitates DFF function in DNA fragmentation and apoptosis *in vivo*. Together, our results indicate that EndoG is required for both embryogenesis and normal apoptosis in mammals.

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