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Supplemental Information

MFN2 Plays a Distinct Role from MFN1 in Regulating Spermatogonial Differentiation

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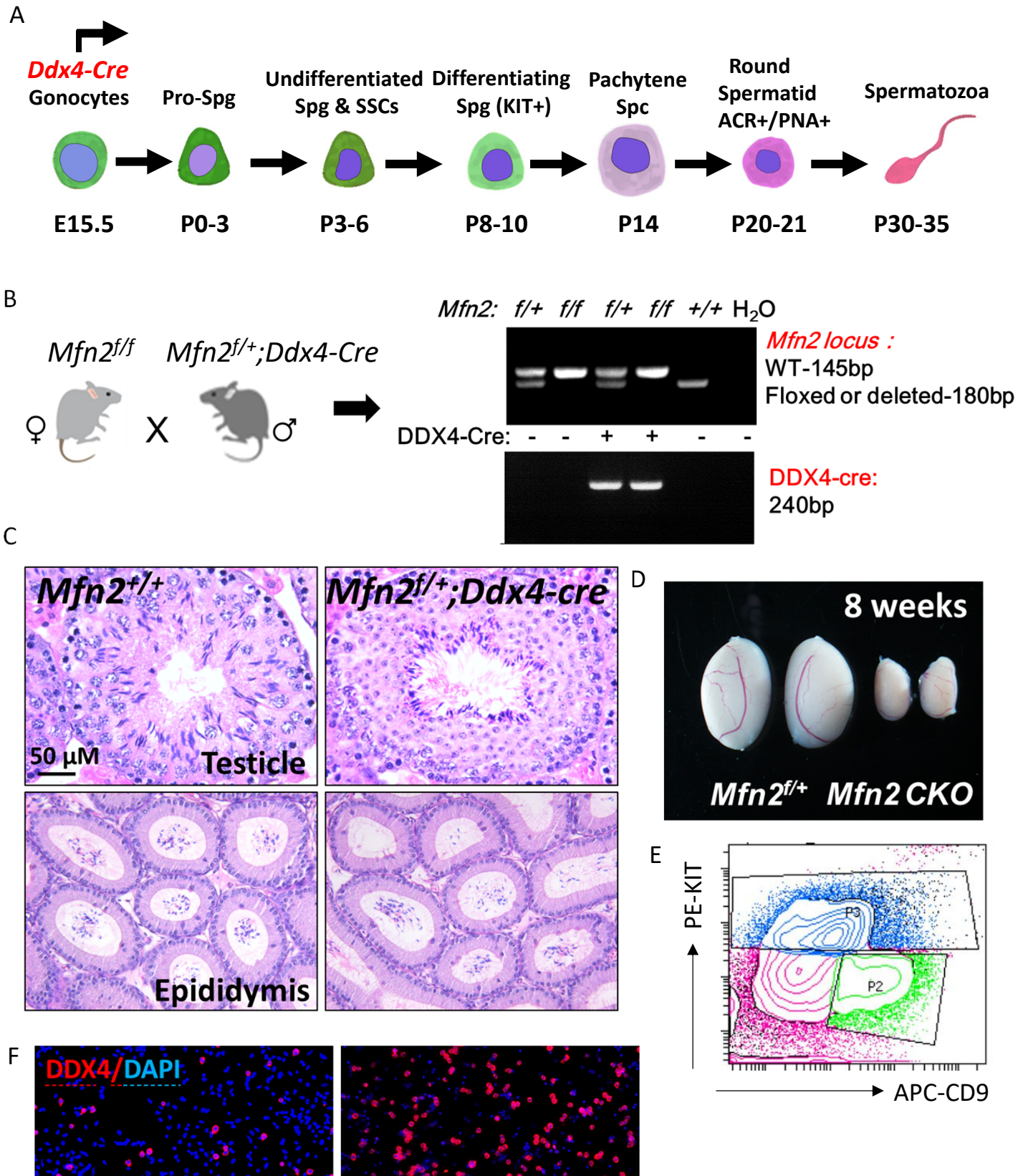
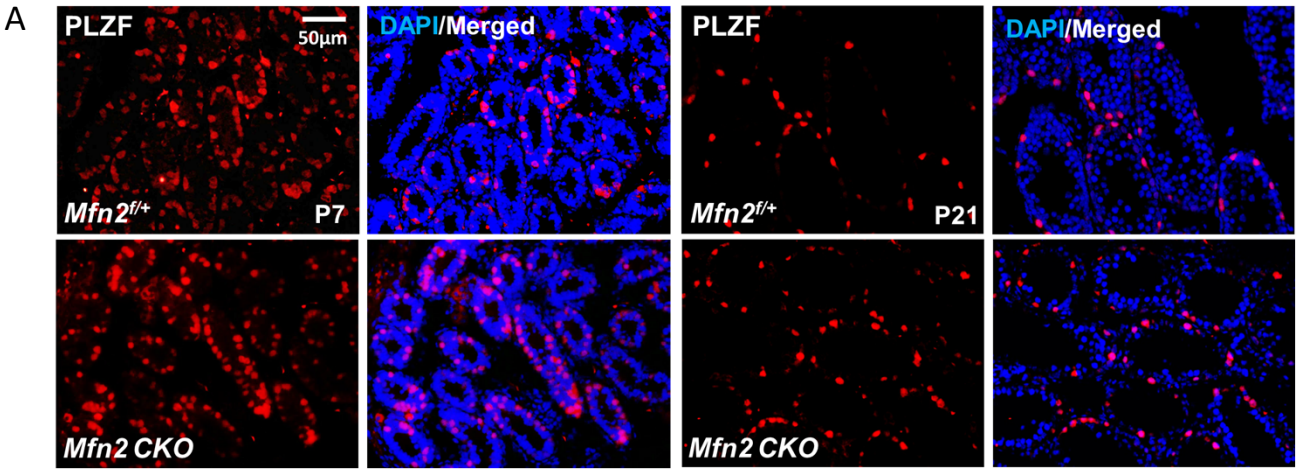
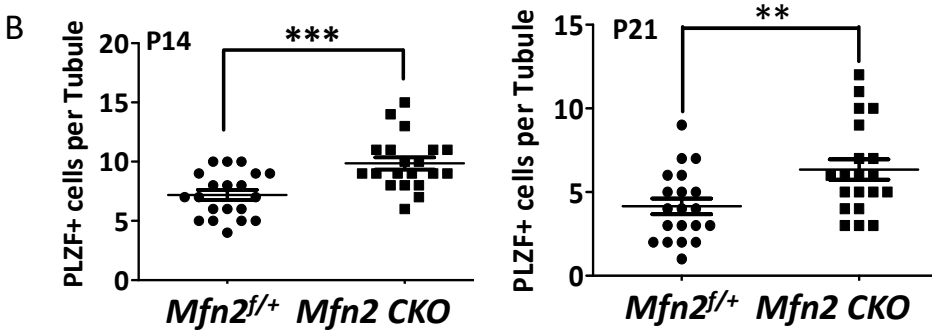
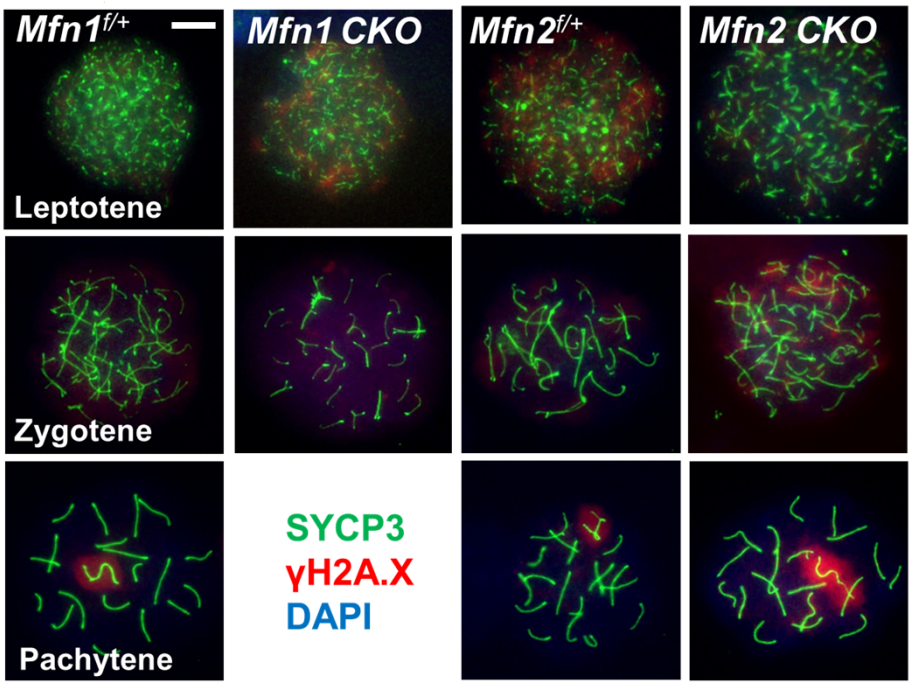
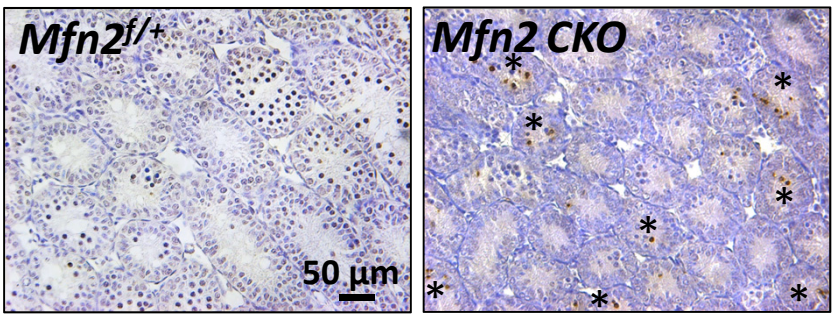


Chen et al., Supplemental Figure S1

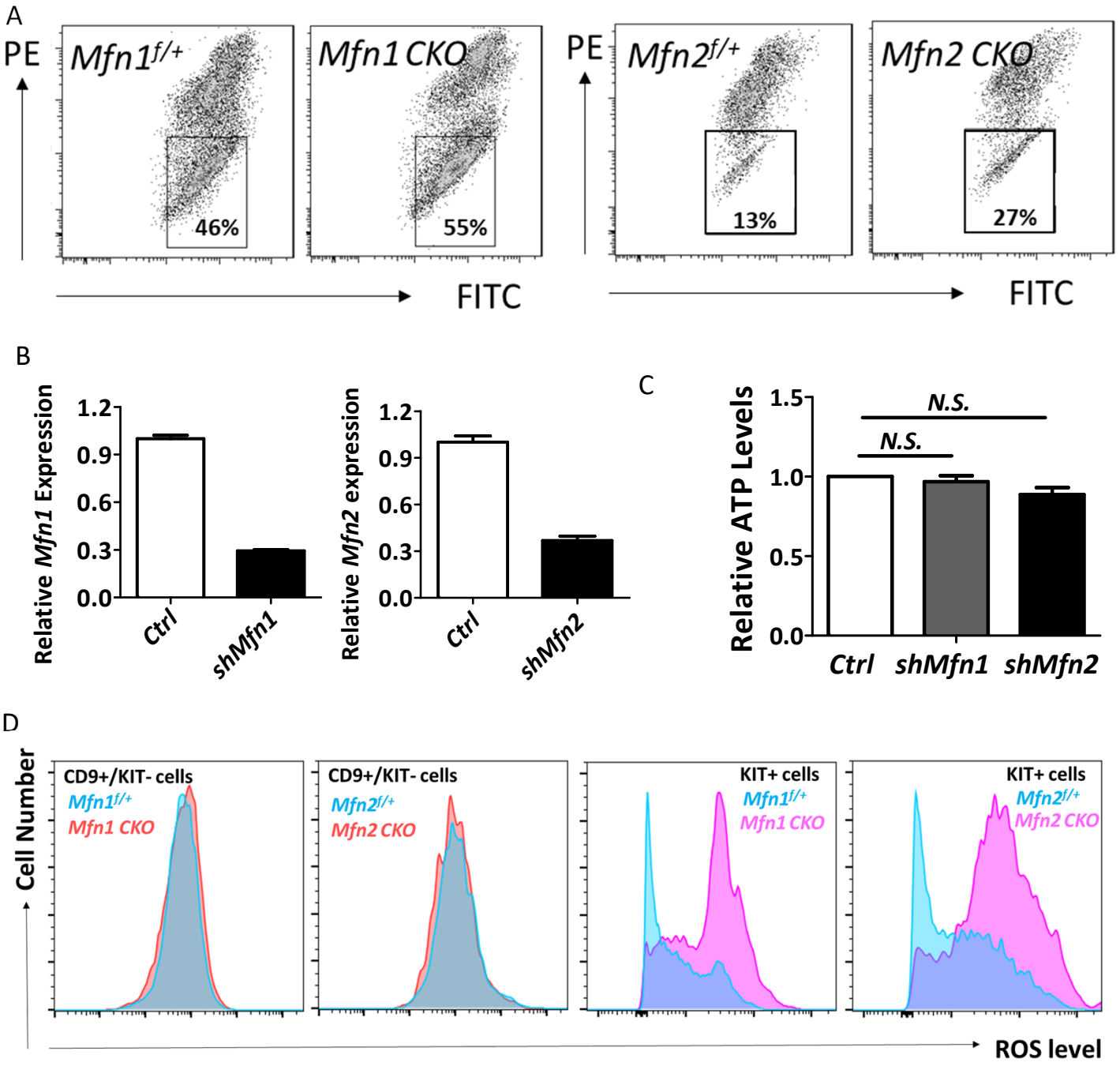








Chen et al., Supplemental Figure S2



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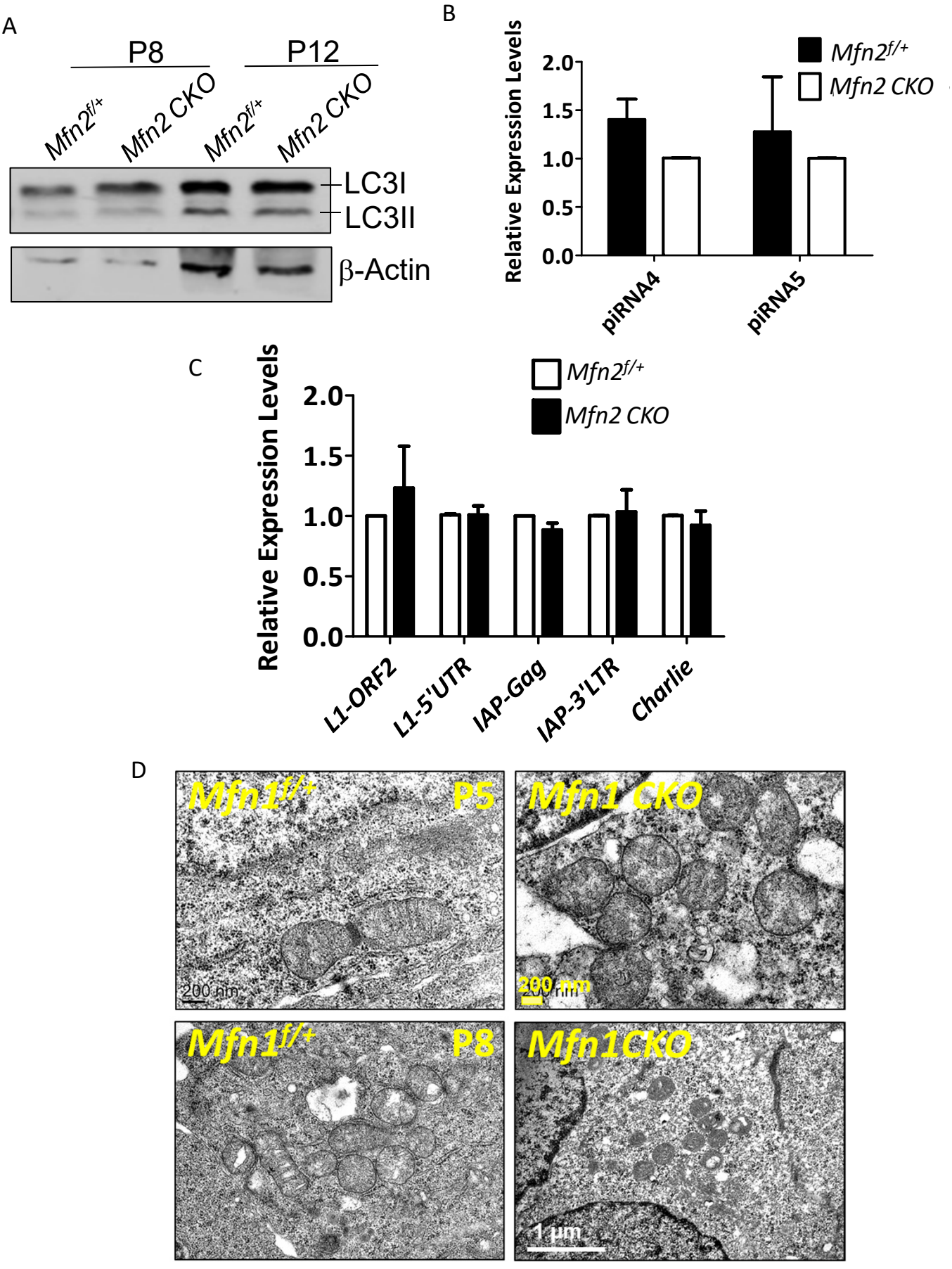

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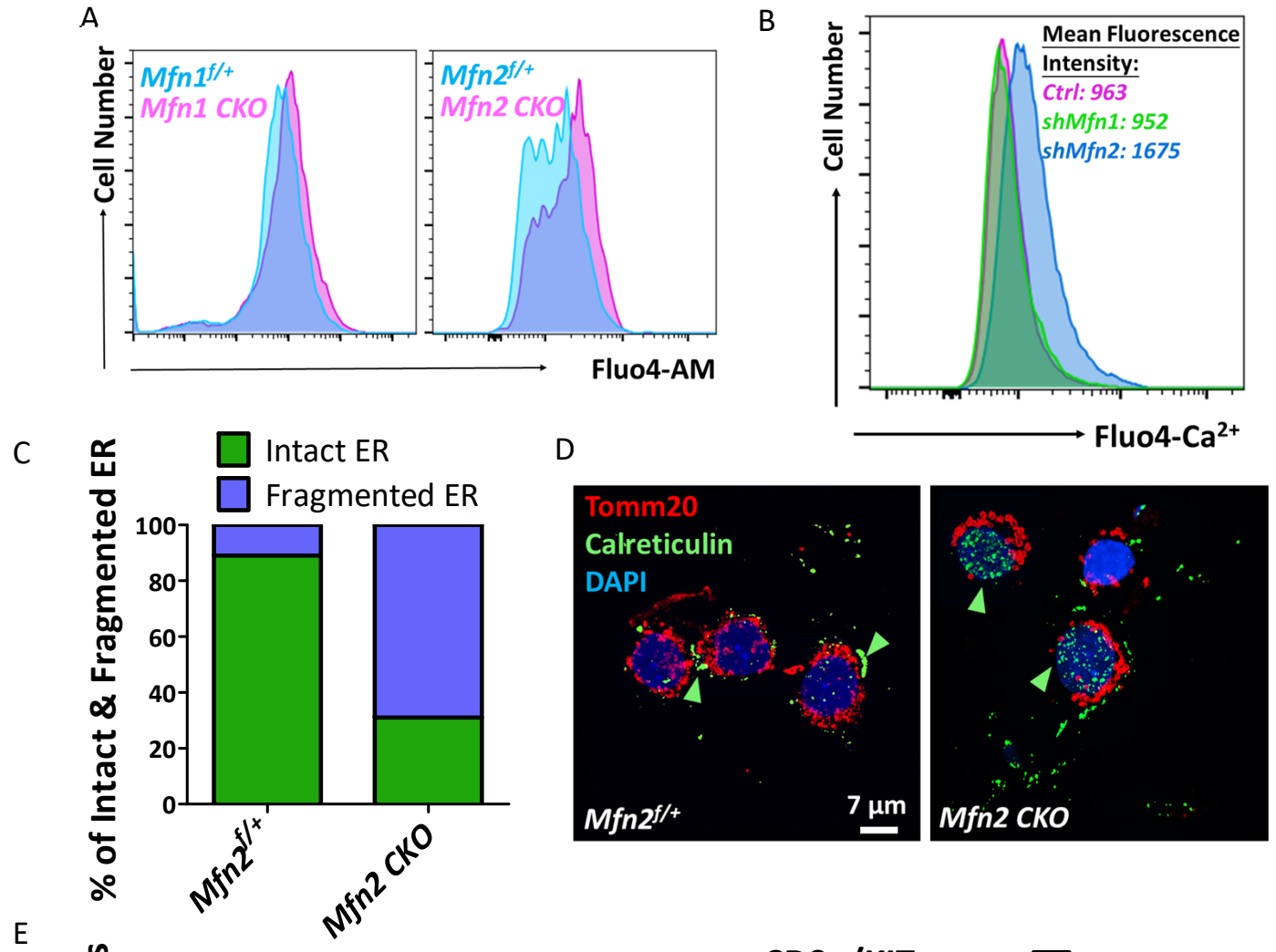
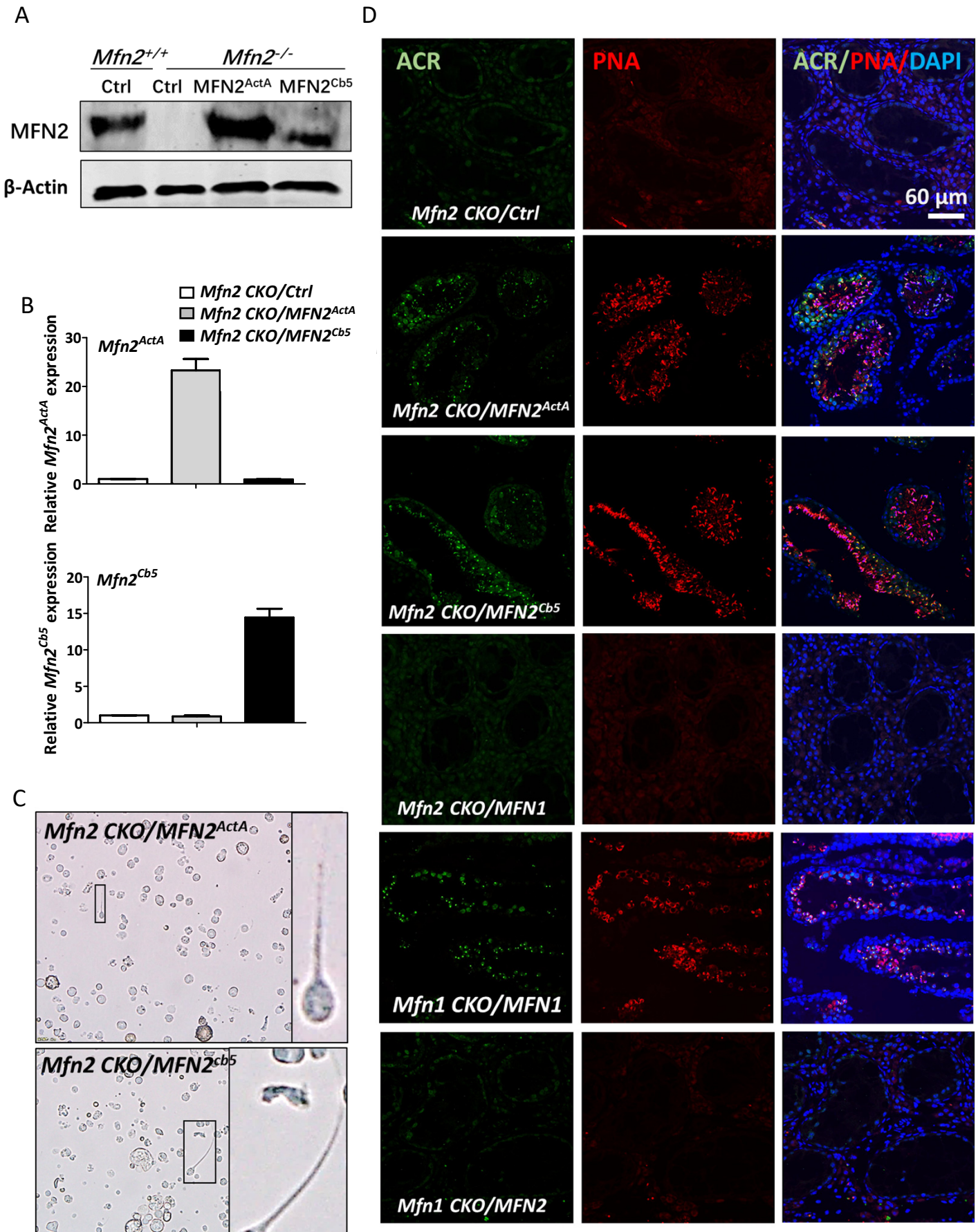


Chen et al., Supplemental Figure S5



Chen et al., Supplemental Figure S6

Supplemental Figure Legends

Fig. S1. Charactering MFN deficient testes. (**A**) Timeline of male germ cell development in mice. Differentiating spermatogonia start to appear at P8-10, with pachytene spermatocytes at P14, and haploid spermatid around P21. Therefore, in our study, we assessed undifferentiated and differentiating spermatogonia between P10-12, performed meiosis chromosome spreading assay to detect primary spermatocytes at P14, and examined testes with viral introduction at 30-40 days post injection to detect haploid spermatid formation from spermatogonia. (**B**) Breeding and genotyping strategies to obtain *Mfn2*$^{f/+}$ mice and *Mfn2*$^{f/f}$*; Ddx4-Cre* littermates. (**C**) Histological studies of testis and epididymis sections from wild-type adult mice or mice with heterozygote *Mfn2* deletion in germ cells. No obvious alteration in male reproductive organs was observed in mice with heterozygote *Mfn2* deletion in germ cells. (**D**) Gross morphology of testes from *Mfn2*$^{f/+}$ or *Mfn2*$^{f/f}$*; Ddx4-Cre* adult mice at 8 weeks. (**E**) An example of CD9 and KIT staining analyzed by flow cytometry. (**F**) An example of IF with DDX4 antibody to assess the percentage of germ cells collected using serial adhering procedures. Dissociated germ cells are loosely attached to the tissue culture plates compared to somatic and supporting cells which are tightly adhered to the plates. Using serial adhering procedures, the germ cells can be lifted easily and separated from somatic cells. The percentage of DDX4+ cells was ~8% in dissociated single cell population from testes (Before) and increased to >75% after serial adhering procedures (After).

Fig. S2. *Mfn2* conditional knockout in germ cells specifically impairs differentiating spermatogonia and spermatocytes. (**A**) IHF on testicle sections from *Mfn2*$^{f/+}$ or *Mfn2*$^{f/f}$*; Ddx4-Cre* mice at P7 and P21 with an antibody against PLZF. (**B**) Numbers of PLZF+ spermatogonia per seminiferous tubule section from *Mfn2*$^{f/+}$ and *Mfn2*$^{f/f}$*; Ddx4-Cre* mice at P7 and P21. Data are presented as mean ± one s.e.m. **: $P<0.01$; ***: $P<0.001$. (**C**) Meiosis chromosome spreading was performed on P14 testes with *Mfn1* or *Mfn2* conditional deletion in germ cells and those from their

littermate controls. The prophase I of primary spermatocytes was staged according to co-staining of SYCP3 with γ-H2A.X. (**D**) Apoptosis was examined by TUNEL assays on testis sections from *Mfn2*$^{f/+}$ and *Mfn2*$^{f/f}$*; Ddx4-Cre* mice at P14. The seminiferous tubules with brown apoptotic cells were labeled with *.

Fig. S3. MFN deficiency compromises mitochondrial functions in differentiating spermatogonia and spermatocytes. (**A**) Examples of flow cytometry analyses to examine mitochondrial membrane potential of non-adherent germ cells collected from mice at P12. (**B**) *Mfn1* and *Mfn2* transcript levels were analyzed by real-time RT-PCR upon shRNA knockdown of MFN1 and MFN2 respectively. (**C**) ATP levels were measured on GC1 cells infected with scrambled shRNA (*Ctrl*), shRNAs against MFN1 or MFN2 (shMfn1 or shMfn2). Relative ATP levels were calculated in comparison to scrambled shRNA control. (**D**) Examples of flow cytometry analyses to examine ROS levels in germ cells sorted from mice at P12.

Fig. S4. MFN deficiency does not affect autophagy or the expression of piRNA and transposable elements in germ cells. (**A**) Autophagy was examined by measuring LC3I/II ratio with Western Blotting on *Mfns*$^{f/+}$ and *Mfn2*$^{f/f}$*; Ddx4-Cre* testes at P12. (**B-C**) Expression levels of piRNAs (**B**) and transposable elements (**C**) were measured with real-time RT-PCR assays on *Mfn2*$^{f/+}$ and *Mfn2*$^{f/f}$*; Ddx4-Cre* P4 testes. (**B-C**) Data are presented as mean ± one s.e.m. N=3. No statistically significant difference in their expression levels between *Mfn2*$^{f/+}$ and *Mfn2*$^{f/f}$*; Ddx4-Cre* groups was observed. (**D**) Mitochondrial architecture was examined by TEM on testicle sections from control or *Mfn1* conditional knockout mice at P5 and P8. Fragmented and small mitochondria were observed in *Mfn1* knockout testes at P8.

Fig. S5. Deletion of *Mfn2* but not *Mfn1* leads to disturbed ER functions in germ cells. (**A**) An example of flow cytometry to measure Ca^{2+} levels (Fluo-3 AM intensity) in non-adherent germ cells collected from testes of P12 mice. (**B**) Ca^{2+} levels were measured in GC1 cells with scrambled shRNA (Ctrl) or shRNA against MFN1 or

MFN2. Mean Fluo-4 AM intensity from each group was shown. (**C**) The percentages of *Mfn2*$^{f/+}$ and *Mfn2*$^{f/f}$*; Ddx4-Cre* germ cells with intact ER or fragmented ER detected by TEM were calculated from 9-13 sample grids. (**D**) 3-D super-resolution imaging with Tomm 20 and Calreticulin co-staining was performed on *Mfn2*$^{f/+}$ and *Mfn2*$^{f/f}$*; Ddx4-Cre* germ cells. Green arrows point to ER. Swollen mitochondria and fragmented ER with increased Calreticulin expression were observed in *Mfn2*$^{f/f}$*; Ddx4-Cre* germ cells. (**E**) Transcript levels of ER chaperons were measured by real-time RT-PCR on non-adherent germ cells (left panel) or CD9+/KIT- spermatogonia (right panel) from P12 *Mfn2*$^{f/+}$ and *Mfn2*$^{f/f}$*; Ddx4-Cre* mice. Data are presented as mean ± one s.e.m. from three biological replicates. *: $P<0.05$. **: $P<0.01$. (**F**) Calreticulin expression was examined by Western Blots on GC1 cells with scrambled shRNA (Ctrl), shRNA against MFN1 or MFN2.

Fig. S6. MFN2 plays a non-redundant role in germ cell development from MFN1. (**A**) MFN2 expression was examined by Western Blots on wild-type or *Mfn2*$^{-/-}$ MEFs infected with empty vector control viruses (Ctrl) or viruses expressing MFN2 tagged with mitochondrion (MFN2^{ActA})- or ER (MFN2^{Cb5})-localization signaling peptides. β-actin served as a loading control. (**B**) Expression of MFN2^{ActA} or MFN2^{Cb5} were detected by real-time RT-PCR in non-adherent germ cells from *Mfn2*$^{f/f}$*; Ddx4-Cre* mice rescued with control viruses (Ctrl) or viruses expressing MFN2^{ActA} or MFN2^{Cb5} at day 40 post infection. Data are presented as mean ± one s.e.m. from three independent experiments. (**C**) Fully developed spermatozoa were detected in dissociated cells from *Mfn2*$^{f/f}$*; Ddx4-Cre* testes infected with viruses expressing MFN2^{ActA} or MFN2^{Cb5} at day 40 post injection. (**D**) IHF analyses with an antibody against acrosin (ACR) and stained with peanut agglutinin (PNA), counterstained with DAPI, on testis sections from mice at day 40 post viral infection with ActA- or Cb5-tagged MFN2, full-length MFN1, or MFN2 through seminiferous tubules. Ctrl: control virus produced with an empty vector.

	Supplemental Table S1 Primers used in this study
Primers for genotyping	
Primer Name	**Sequences (5'-3')**
Mfn1 or Mfn2 conditional knockout mice	
Mfn1-genotyping-F	TTGGTAATCTTTAGCGGTGCTC
Mfn1-genotyping-R	AGCAGTTGGTTGTGTGACCA
Mfn1-genotyping-exc-R	TTAAAGACACGGCTAATGGCAG
Mfn2-genotyping-F	GAAGTAGGCAGTCTCCATCG
Mfn2-genotyping-R	AACATCGCTCAGCCTGAACC
Ddx4-cre Mouse	
Transgene _MVH-Cre_For	CACGTGCAGCCGTTTAAGCCGCGT
Transgene _MVH-Cre_Rev	TTCCCATTCTAAACAACACCCTGAA
Primers for real-time PCR (mRNA and tranposons)	
Primer Name	**Sequences (5'-3')**
m-RT-Mfn1-F	ATGGCAGAAACGGTATCTCCA
m-RT-Mfn1-R	CTCGGATGCTATTCGATCAAGTT
m-RT-Mfn2-F	GTGGGCTGGAGACTCATCG
m-RT-Mfn2-R	CTCACTGGCGTATTCCACAA
M-RT-Grp78-F	ACTTGGGGACCACCTATTCCT
M-RT-Grp78-R	ATCGCCAATCAGACGCTCC
M-RT-Grp94-F	TCGTCAGAGCTGATGATGAAGT
M-RT-Grp94-R	GCGTTTAACCCATCCAACTGAAT
M-RT-Grp75-F	ATGGCTGGAATGGCCTTAGC
M-RT-Grp75-R	ACCCAAATCAATACCAACCACTG
M-RT-ATF4-F	AAGGAGGAAGACACTCCCTCT
M-RT-ATF4-R	CAGGTGGGTCATAAGGTTTGG
M-RT-Bip-F	ACTTGGGGACCACCTATTCCT
M-RT-Bip-R	ATCGCCAATCAGACGCTCC
M-RT-CHOP-F	CTGGAAGCCTGGTATGAGGAT
M-RT-CHOP-R	CAGGGTCAAGAGTAGTGAAGGT
M-RT-Xbp1-F	GACAGAGAGTCAAACTAACGTGG
M-RT-Xbp1-R	GTCCAGCAGGCAAGAAGGT
M-RT-Mfn2-ActA/Cb5-F	CCAGCAAGTTGACATCACCCG
M-RT-Mfn2-ActA-R	GATAAACGCCCCTAAAGAGAA
M-RT-Mfn2-Cb5-R	CAGGGCTGAGATGGCTGGGAT
M-RT-β-Actin-F	GGCTGTATTCCCCTCCATCG
M-RT-β-Actin-R	CCAGTTGGTAACAATGCCATGT
L1-ORF2-Q-F	GGAGGGACATTTCATTCTCATCA
L1-ORF2-Q-R	GCTGCTCTTGTATTTGGAGCATAGA
L1-5'UTR-Q-F	GGCGAAAGGCAAACGTAAGA

L1-5'UTR-Q-R	GGAGTGCTGCGTTCTGATGA
IAP-Gag-Q-F	AACCAATGCTAATTTCACCTTGGT
IAP-Gag-Q-R	GCCAATCAGCAGGCGTTAGT
IAP-3'LTR-Q-F	GCACATGCGCAGATTATTTGTT
IAP-3'LTR-Q-R	CCACATTCGCCGTTACAAGAT
Charlie-Q-F	TTGAGAATCGGATGGGAGAC
Charlie-Q-R	AAGAACTGTCTTATTCAGGC
Primers for real-time RT-PCR of 5S rRNA and piRNAs	
Primer Name	**Sequences (5'-3')**
5S-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAGCCTA
5S- Forward	ACACTCCAGCTGGGGCCTGGGAATACCGG
Universal-QPCR-Rev	CTCAACTGGTGTCGTGGAGTCGG
piRNA4-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTTCGTCTCCG
piRNA4-OPCR-Forward	ACACTCCAGCTGGGGTAATCCCAGCTCTTGG
piRNA5-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCCCAAACA
piRNA5-Forward	ACACTCCAGCTGGGGTCAGCCCTCGACACA
Primers for cloning shRNAs	
m-Mfn1-shRNA-F	CCGGCCCAGTGTACTGAAAGTGTATCTCGAGATACACTTTCAGTACACTGGGTTTTTG
m-Mfn1-shRNA-R	AATTCAAAAACCCAGTGTACTGAAAGTGTATCTCGAGATACACTTTCAGTACACTGGG
m-Mfn2-shRNA-F	TGCTGGACAGCTGGATTGATAATTCAAGAGATTATCAATCCAGCTGTCCAGCTTTTTTC
m-Mfn2-shRNA -R	TCGAGAAAAAAGCTGGACAGCTGGATTGATAATCTCTTGAATTATCAATCCAGCTGTCCAGCA
Scramble-sense	TGAGCGTGTAGTCACGCATAGCCCTGACCCAGCTATGCGTGACTACACGCTCTTTTTC
Scramble-antisense	TCGAGAAAAAGAGCGTGTAGTCACGCATAGCTGGGTCAGGGCTATGCGTGACTACACGCTCA

Supplemental Experimental Procedures and Materials

Cell Culture

GC1 (ATCC, CRL-2053), *Mfn2*$^{+/+}$ and *Mfn2*$^{-/-}$ MEFs (kind gifts from Dr. Quan Chen, Institute of Physics, Chinese Academy of Sciences), and 293T cells were maintained in DMEM/10% FBS (Fetal Bovine Serum, Sigma-Aldrich). Viruses were produced using

293T cells following standard procedures.

Collecting germ cells using serial adhering procedures, Immunofluorescence (IF) Assays, and 3D super-resolution imaging

Testes were mechanically disassembled into small pieces and incubated sequentially in 1 mg/ml collagenase IV/PBS (Thermo Fisher Scientific, 17104019) for 15 min and 0.05% trypsin/PBS (Thermo Fisher Scientific, 25200072) for 5 min with occasional agitation. Dissociated single cells were sequentially plated onto gelatin-coated plates with DMEM/10% FBS in 37°C/5% CO_2 incubator for ~1 hr and 3 hr. Non-adherent cells were carefully collected from supernatant and plated onto Matrigel (Corning, 354234)-coated glass slide or plates. For IF assays, cells were fixed in 4% paraformaldehyde after plating onto Matrigel for 4 hr, followed by staining with primary and fluorochrome-conjugated secondary antibodies. For other assays, after 1-2 hr incubation on Matrigel, loosely adherent germ cells were collected by gently pipetting and lifting them from residue tightly adherent somatic/supporting cells. IF was performed using the following antibodies, probes, or fluorescent dyes: 8-Oxoguanine (Merck Millipore, MAB3560-C), MitoTracker (Thermo Fisher Scientific, M22425), Fluo3-AM (S1056) and Hoechst 33342 (C1022) from Beyotime Biotechnology (Shanghai, China), Tomm 20 (Abcam, ab186735), Calreticulin (BD Biosciences, 612137). Alexa Fluor 488- or TRITC-conjugated anti-mouse, or anti-rabbit secondary antibodies are from Jackson ImmunoResearch. Images were obtained using a Leica confocal microscope. The 3D super-resolution imaging was performed using a high speed Andor dragonfly confocal platform (Oxford Instruments Company, Unite Kingdom).

Meiotic Chromosome Spreading Assays

Dissociated germ cells were incubated in hypo-extraction buffer (30 mM Tris, 50 mM Sucrose, 17 mM NaCitrate, 5 mM EDTA, pH 8.3) and Protease Inhibitor Cocktail (Santa Cruz Biotech) on ice for 90 min. Cells were then resuspended in 20 µl hypo-extraction buffer with 60 µl 100 mM sucrose, cytospined onto slide, fixed in 4 % PFA

for 15 min, and incubated with 0.04 % photoflo (KODAK) for 5 min. Air dried slides were blocked with 3% BSA/PBS for 30 min at room temperature and stained with SYCP3 (Abcam, ab15093) and γ-H2A.X (Merck Millipore, 05-636) antibodies at 4ºC and fluorescence-conjugated secondary antibodies (Jackson ImmunoResearch) with DAPI afterwards. Images were captured under a Leica microscope with oil immersion lens.

TUNEL assays

Mouse testes were fixed in 4% PFA in PBS at 4°C overnight and were embedded in paraffin. Testicle sections were digested with 20 μg/ml protease K at room temperature for 20 minutes before staining for apoptotic cells with a DeadEnd™ Colorimetric TUNEL System (VWR/Promega, PAG3250/G3250) following the manufacturer's instruction.

Measurement of ATP Levels

Cellular ATP levels were measured using an ATP Assay Kit (Beyotime Biotech， S0026) according to manufacturer's instructions. Briefly, dissociated germ cells were lysed and centrifuged at 12,000 rpm for 5 min. Supernatants (60 μL) were mixed with 60 μL of ATP detection buffer in a 96-well plate. Luminance (RLU) was measured using an Infinite™ M200 Microplate Reader (Thermo Fisher Scientific). Protein concentration for each sample was determined by Bradford protein assay, and ATP concentration (μM) per mg of protein was calculated. Three or more technical replicates were examined for each independent experiment.

Western Blots

Western blots were performed according to standard protocols and the final fluorescent signals against targeted proteins were detected using the Li-COR Odyssey system (LI-COR Biosciences). Primary antibodies used in this study: MFN1 (Absci, AB45059), MFN2 (Abcam, ab189862), β-Actin (Santa Cruz Biotech, sc-10731), Bip/GRP78

(Santa Cruz Biotech, 610978), CHOP (Santa Cruz Biotech, SC793), Calreticulin (BD Biosciences, 612137). For autophagy detection by Western Blotting, an antibody against LC3B from Sigma-Aldrich (L7543) was used to detect LC3B-I (18kDa) and LC3B-II (16kDa) with Western blotting analyses according to standard protocols.

Determination of the Expression Levels of piRNA

Total RNAs were extracted from testes using Trizol according to Manufacturer's instructions with a minor modification in the RNA precipitation step. Briefly, samples with isopropanol was centrifuged at 4℃ 12,000 rpm for a prolonged time (40 min) to ensure efficient precipitation of small size RNAs. Reverse transcription was carried out using PrimeScript® RT Kit with gDNA Eraser (Takara) and piRNA specific RT primers. Quantitative real-time PCR was performed on Stratagene MX3000P instrument using SYBR Premix Ex TaqTM II (Takara). The expression levels of piRNA were normalized to 5S rRNA. Primers used in this study were listed in Supplemental Table S1.

Construction of Knockdown Plasmids

The shRNA against *Mfn1* was cloned into *pLKO.1-puro* vector. The *Mfn2* shRNA and a scramble shRNA control were designed and cloned into pll3.7-U6-MCS-EF1α-EGFP vector. Inserted shRNA sequences were confirmed by DNA sequencing. The shRNA sequences were provided in Supplemental Table S1.