Supplementary Materials and Methods

Whole Mount X-Gal Staining

Whole tissues were collected, rinsed with PBS and fixed with 4% PFA. Tissues were then rinsed in rinse buffer (100 mM Sodium Phosphate pH 7.3, 2 mM $MgCl_2$, 0.01% Sodium Deoxycholate, 0.02% NP-40) 3 times for 30 minutes each with agitation. Next, tissues were incubated in X-Gal Staining Solution (Rinse buffer supplemented with 5 mM Potassium Ferricyanide, 5 mM Potassium Ferrocyanide, and 1 mg/ml X-Gal) for 24 hr at 37°C with gentle rotation. Tissues were finally post-fixed in formalin (10%) and dehydrated in 70% ethanol prior to embedding and sectioning. Nuclei were counterstained with Nuclear Fast Red Solution according to manufacturer instructions.

Terminal Deoxynucleotidyl Transferase-Mediated Digoxigenin-dUTP Nick End Labeling (TUNEL) Assay

The presence of apoptotic fragmentation of DNA in frozen testis cross sections was determined by TUNEL analysis using the ApopTag kit (EMD Millipore Billerica, MA). The apoptotic index (AI) was calculated as the percentage of essentially round seminiferous tubules containing more than three TUNEL-positive germ cells in each cross section. For each condition, at least 5 cross sections and at least 100 seminiferous tubules were analyzed.

Oocyte collection and In vitro maturation

MII oocytes were obtained by superovulation. 6~8-week-old females received 5 IU of Pregnant Mare's Serum (PMSG, Sigma) through intraperitoneal injection (IP), followed by injection with 5 IU of human Chorionic Gonadotropin (hCG, Sigma) at 48 hr later. Oocytes were collected from the oviducts the next morning. Fully grown germinal vesicle (GV) oocytes were recovered from the ovaries of female mice at ~6~8 weeks of age, 48 hr post-IP injection of 5 IU of PMSG. GV oocytes were released by puncturing antral follicles with a fine needle in M2

medium (Invitrogen), supplemented with 200 μM of 3-isobutyl-1-methylxanthine (IBMX, Sigma) to prevent oocytes from undergoing meiosis. For in vitro maturation, oocytes were washed and cultured in IBMX-free M16 medium (Millipore) for 20 hr at 37 ºC in a 5% CO_2 incubator.

Embryo collection and in vitro development

Female mice (FVB) were superovulated through IP injections of PMSG and hCG prior to mating with WT males (FVB). Fertilized oocytes (zygotes) were collected from the oviducts at E0.5 and released into a hyaluronidase/M2 solution for dissociation. For study of in vitro embryo development, zygotes were cultured in KSOM medium at 37°C in a 5 % CO_2 incubator for 24~72 hr.

Implantation site examination

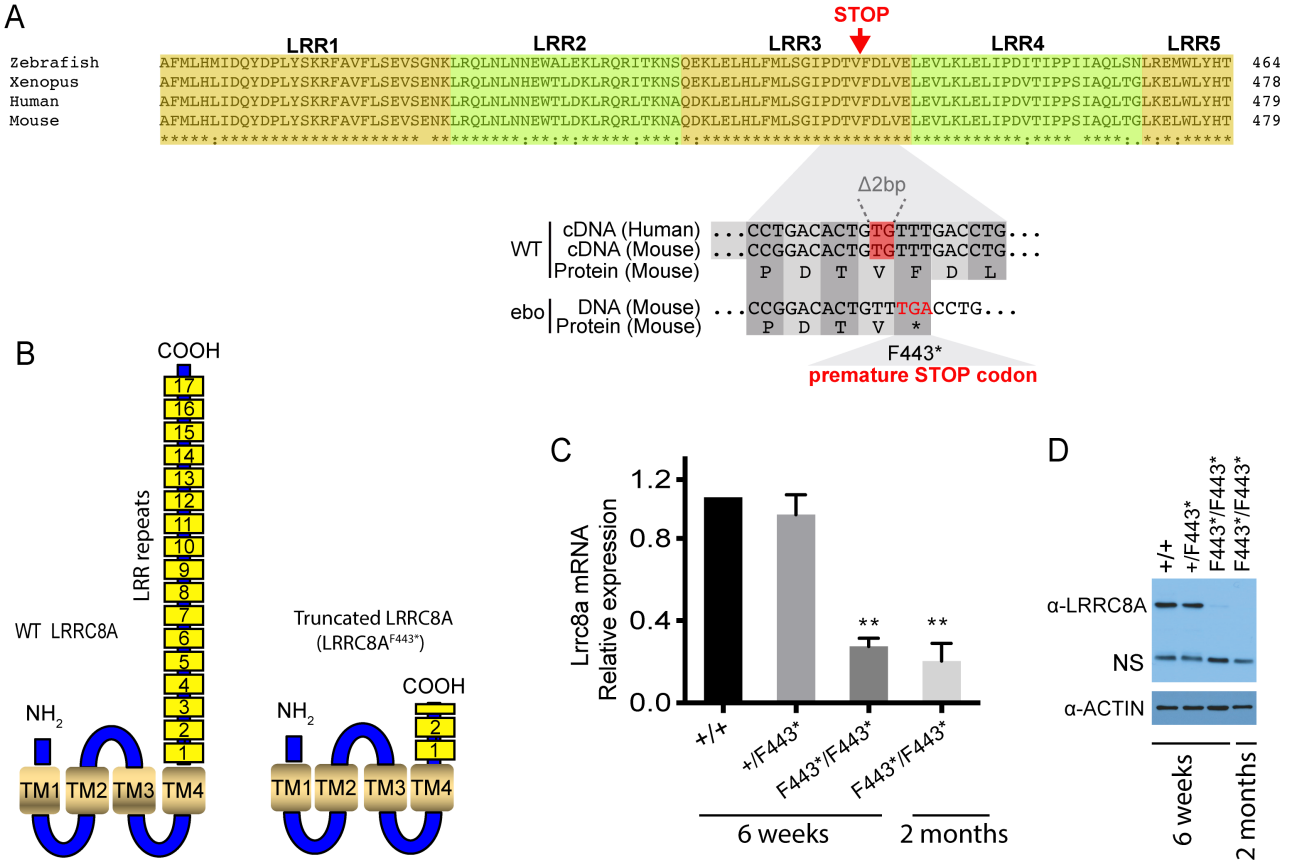
Virgin *Lrrc8a*$^{F443*/F443*}$ and WT littermates (FVB/N) females were mated 1:1 with WT adult males, and sacrificed by CO_2 inhalation on gestational day 18. The uterine horns along with any contents were completely removed and documented. The uterine horns were then completely submersed in a 10% ammonium sulfide solution (Fisher, Nazareth, PA) for 1 hr. Sites of fetal implantation, which appear as black spots, were quantified.

RNA-seq

Total RNA was extracted from the whole testis of WT and F443*/F443* mice at 6 weeks of age. 2 μg of total RNA was treated with DNase I prior to library preparation using TruSeq stranded mRNA-seq kit (Illumina) according to the manufacturer's protocol. The library was sequenced on a HiSeq2000 using 75 bp pair-end approach. Two biological replicates were prepared for each genotype. The reads were mapped to mouse genome (mm10) using TopHat (v2.0.10). Genes represented on fewer than 10 fragments among all samples were removed prior to differential gene calling analysis. The differentially expressed genes were identified using the R/Bioconductor package Edge. Genes with a

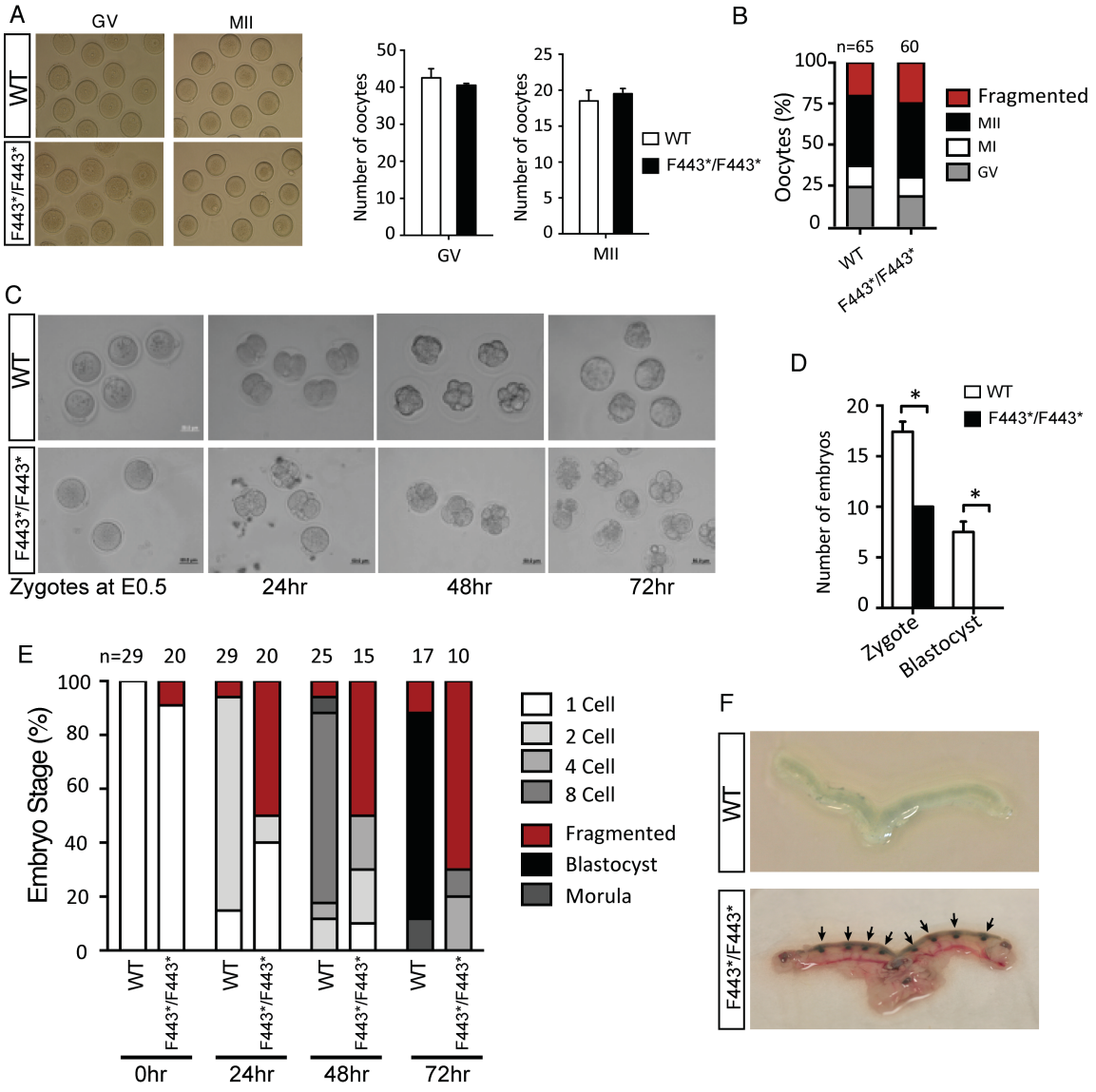
false discovery rate (FDR)<0.05 were called as differentially expressed. RNA-seq data was deposited in the public repository GEO database (GSE112730).

Supplementary Figure 1



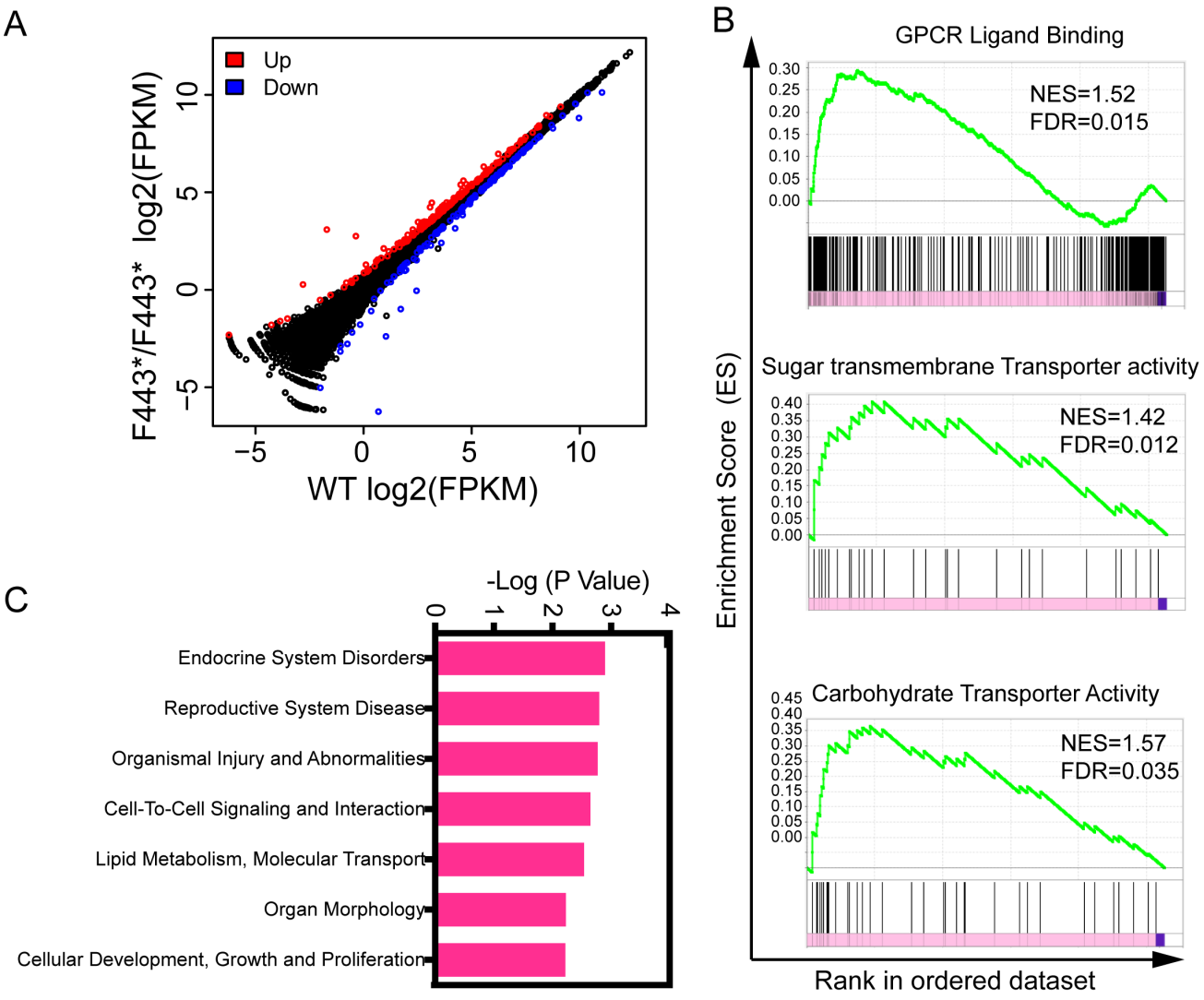
Supplementary Figure 1. The 2-bp (TG) deletion mutation (*Lrrc8a*, c.1325delTG, p.F443*) in exon 3 of *Lrrc8a* gene in the *ebo* mice. (A) Protein sequence alignment of LRRC8A orthologs. Note the position of the stop codon introduced by the 2-bp deletion (*Lrrc8a*^{1325delTG}) as identified by positional cloning and targeted sequencing in the conservative *Lrrc8a* locus in *ebo* mice. Sequence alignment was performed using Clustal Omega (DNAstar 11.0) (Top). LRRC8A amino acids sequences are highly conserved from zebrafish to mammals. Yellow and green rectangles highlight the domain of short Leucine-rich repeats (LRR). The deletion of two nucleotides "TG" occurred spontaneously in the *ebo* mice and resulted in the de novo formation of a novel downstream premature STOP codon (nonsense mutation) in the third LRR domain, as indicated (bottom). (B) Schematic diagrams illustrating the domain structures of the LRRC8A protein, which harbors four transmembrane helices and 17 LRR domains. The nonsense mutation (*Lrrc8a*^{F443*}) identified in the *ebo* mice led to the loss of C-terminal LRRs 4~17 in LRRC8A protein. (C) qRT-PCR quantification of relative *Lrrc8a* mRNA levels in the testes of 6-week- and 2-month-old mice from three male replicates among different genotypes as labeled. While the levels of *Lrrc8a* mRNA were comparable in the testes between WT and heterozygous (*Lrrc8a*$^{+/F443*}$) littermates, the *Lrrc8a* mRNA levels in *Lrrc8a*$^{F443*/F443*}$ testis were significantly reduced to less than one third of the levels in WT. **, p<0.01 (Student's t test, n=3). (D) Western blot analysis of LRRC8A protein expression in the testes of littermates at 6 weeks and 2 months of age among different genotypes using a monoclonal LRRC8A antibody raised against a C-terminal region. The levels of LRRC8A protein were comparable in the testes from WT and heterozygous mice. Full-length LRRC8A protein was not detected in the testis of homozygous *Lrrc8a*$^{F443*/F443*}$ mice. NS, non-specific band.

Supplementary Figure 2



Supplementary Figure 2. The *Lrrc8a*^{F443*} nonsense mutation does not affect oogenesis, but causes defective early preimplantation embryonic development. (A) Gross morphology of oocytes and average number of fully-grown GV and MII oocytes retrieved from superovulated WT and *Lrrc8a*$^{F443*/F443*}$ females at 6 weeks of age. n=120 for each genotype. (B) GV oocytes retrieved from the ovaries of PMSG-primed female mice at 48 hr post-injection were subjected to in vitro meiotic maturation. The percentages of oocytes at different stages (identified by morphology) were calculated. (C) Comparisons of preimplantation development from embryos derived from WT and mutant *Lrrc8a*$^{F443*/F443*}$ oocytes, fertilized by WT sperm. Superovulated WT and *Lrrc8a*$^{F443*/F443*}$ female mice at 6-8 weeks of age were intercrossed with WT males at 10 weeks of age, independently. Vaginal plugs were monitored to assess mating. Embryos were flushed from the oviducts the next day morning (E0.5) and were cultured in vitro for 24~72 hr. n=80 for each genotype. (D) The average number of embryos that reached the zygote and blastocyst stages. n=30. *, $p<0.05$. (Student's t test). (E) Statistical comparisons of percentages of embryos at different stages during preimplantation development. (F) When *Lrrc8a*$^{F443*/F443*}$ and WT females were intercrossed with fertility-proven WT males, no embryo implantation sites could be identified by blue dye staining in the *Lrrc8a*$^{F443*/F443*}$ females, whereas multiple sites were seen in WT females (n=5 for each genotype).

Supplementary Figure 3



Supplementary Figure 3. RNA-seq analyses identified differentially expressed genes and impacted molecular pathways in F443* mutant testes. (A) Scatter plot showing the genome-wide distribution of differentially expressed genes in testes of WT and F443* mice at 6 weeks of age (FDR<0.05). FPKM, fragments per kilobase of transcript per million mapped reads. (B) Gene Ontology (GO) analysis illustrates the top over-represented pathways impacted by the F443* mutation. (C) Gene Set Enrichment Analysis (GSEA) indicates the molecular functions affected by the F443* mutation in testes. FDR<0.05. NES, normalized enrichment score.