Genome engineering uncovers 54 evolutionarily conserved and testis-enriched genes that are not required for male fertility in mice

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Gene-expression analysis studies from Schultz et al. estimate that more than 2,300 genes in the mouse genome are expressed predominantly in the male germ line. As of their 2003 publication [Schultz N, Hamra FK, Garbers DL (2003) *Proc Natl Acad Sci USA* 100(21):12201–12206], the functions of the majority of these testis-enriched genes during spermatogenesis and fertilization were largely unknown. Since the study by Schultz et al., functional analysis of hundreds of reproductive-tract–enriched genes have been performed, but there remain many testis-enriched genes for which their relevance to reproduction remain unexplored or unreported. Historically, a gene knockout is the "gold standard" to determine whether a gene's function is essential in vivo. Although knockout mice without apparent phenotypes are rarely published, these knockout mouse lines and their phenotypic information need to be shared to prevent redundant experiments. Herein, we used bioinformatic and experimental approaches to uncover mouse testis-enriched genes that are evolutionarily conserved in humans. We then used gene-disruption approaches, including Knockout Mouse Project resources (targeting vectors and mice) and CRISPR/Cas9, to mutate and quickly analyze the fertility of these mutant mice. We discovered that 54 mutant mouse lines were fertile. Thus, despite evolutionary conservation of these genes in vertebrates and in some cases in all eukaryotes, our results indicate that these genes are not individually essential for male mouse fertility. Our phenotypic data are highly relevant in this fiscally tight funding period and postgenomic age when large numbers of genomes are being analyzed for disease association, and will prevent unnecessary expenditures and duplications of effort by others.

spermatozoa | genetically modified mice | genome editing

Spermatozoa are haploid cells whose role is to convey their genetic information to oocytes (reviewed in refs. 1–4). Because of this special role, spermatozoa possess highly specialized morphology and function. For example, spermatids compact their haploid genome into a small head during spermatogenesis and are equipped with a flagellum for motility. The mitochondrial sheath surrounding the midpiece of the flagellum provides energy for movement. Ejaculated spermatozoa undergo physiological processes called capacitation and the acrosome reaction that confer fertilizing ability. Only acrosome-reacted spermatozoa are capable of fusing with an oocyte.

Based on the microarray studies of Schultz et al. (5), many genes are believed to be expressed predominantly in male germ cells (>2,300 genes). In addition to housekeeping genes, many of these testis-enriched genes are thought to play a unique role during spermatogenesis and/or sperm function. However, the functions of most of these genes are still unclear because of the lack of an in vitro system to generate fully functional spermatozoa.

In mammals, gene knockout (KO) mice have been pivotal in studying gene function in vivo. Hundreds of genes are essential or important for reproduction, as revealed by various gene manipulation strategies (reviewed in refs. 1–4). There are several advantages to using KO mice in reproductive biology: (*i*) because reproductive organs are not essential for viability, genes predominantly expressed in these tissues can be studied without conditional KO methodology; (*ii*) genes essential for reproductive

Significance

In the mouse genome, thousands of genes are predominantly expressed in the testis, where these genes are thought to play important roles in spermatogenesis and fertilization. However, in this study, we report that 54 evolutionarily conserved and testis-enriched genes are not essential individually for male mouse fertility. Because the recent development of the CRISPR/Cas9 system has made it faster and easier to produce knockout mice, our results suggest that one should determine whether a gene of interest is essential for male fertility in vivo before spending significant effort to analyze the molecular function of the gene in vitro.

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functions can be screened by examining breeding pairs; and (*iii*) once infertility is confirmed in vivo, there are assisted reproductive technologies (e.g., germ-line stem cells, seminiferous tubule transplantation, in vitro fertilization and embryo transfer, and intracytoplasmic sperm injection) to analyze their defects. Many KO mice are fertile even though the targeted genes were thought essential for fertilization, based on in vitro experiments [e.g., *Acr*, *Zp3r*, *Zan*, *B4galt1*, *Adam1b*, and others. (6)]. Alternatively, without a preceding functional assay in vitro, many testis-enriched genes proved to be essential, by KO, for male reproduction. For example, TEX14 is required for intercellular bridge formation and progression through meiosis (7), GASZ functions in the piRNA pathway and is required for suppression of retrotransposons (8), CATSPER1 is a voltage-dependent calcium channel required for sperm hyperactivated motility (9), CLGN is an endoplasmic reticulum chaperone protein essential for sperm migration into the oviduct and fertilization (10), and PPP3R2 is a testis-specific regulatory subunit of calcineurin required for normal motility of the sperm midpiece (11). Taking these facts into account, it would be better to know first whether a gene of interest is essential in vivo before deciding to invest valuable research dollars and experimental time and personnel efforts into the project. In addition, with the advances made in using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to manipulate the mouse genome (12–15), it is likely as inexpensive and quicker to create genetic mutations and test for phenotypes as it is to test by in vitro experimentation.

If a KO mouse consistently sires pups, that gene is classified as not essential for male fertility. Whereas research involving KO mice with phenotypes are published, studies of KO mice without phenotypes are usually ignored and rarely published unless the gene is well recognized. The lack of publicity of a fertile mouse KO may mislead other researchers into making the same KO mouse and thereby wasting valuable resources and research staff time. Once these KO mice become available to academia as bioresources, researchers with differing areas of expertise are able to examine their hypothesis using these mice.

In this report, we describe our KO of 54 genes that exhibit testis-enriched expression, based on bioinformatic and/or experimental analysis. Using various gene manipulation tools, we discovered these evolutionarily conserved genes (present in at least the mouse and human genomes) are dispensable for male fertility. Thus, our findings indicate that, individually, these testis-enriched genes do not perform critical roles in spermatogenesis or fertilization in mice.

Results

Experimental Strategies to Uncover Testis-Enriched Genes for Functional Analysis. To identify genes that play a role in male reproduction, we used PubMed searches and multiple bioinformatic searches. The focus of these searches was to uncover genes that are testis-enriched and possess ORFs conserved between mouse and human. We predicted that these evolutionarily conserved genes would be expressed predominantly during spermatogenesis and would play a role in sperm formation or function. For the current publication, we focused on 54 genes (*SI Appendix*, Table S1) that are reported to be expressed in a testis-enriched manner or are bioinformatically predicted to be expressed predominantly in the testis, based on the abundance of expressed sequence tags (ESTs) in the UniGene (www.ncbi.nlm.nih.gov/unigene) and The Jackson Laboratory informatics (www.informatics.jax.org/expression.shtml) databases. We identified 4 genes from published reports, and we identified an additional 50 genes from the bioinformatic predictions. The genes identified from the published reports are *Capn11*, *Lyzl4*, *Pkdrej*, and *1110017D15Rik* (16–19). The genes identified from our bioinformatic predictions include genes with nomenclature based on sequencing at RIKEN (e.g., *1700007G11Rik*, *1700019B03Rik*, *1700034J05Rik*, *1700125H20Rik*, *1700015F17Rik*, *2900092C05Rik*, *4930522H14Rik*, *1700011E24Rik*, and *4933417A18Rik*), based on domains (e.g., *Ccdc178*, *Efcab3*, *Ms4a13*, *Smim23*, and *Tmem247*), or based on groupings within conserved families (e.g., *Syngr4*, *Ube2e*, and *Fam217a*).

RT-PCR Analysis Confirms Testis-Enriched Expression in Mouse. For our initial studies, we picked 16 genes (*1700093K21Rik*, *Adam18*, *Capn11*, *Efcab3*, *Lrrc36*, *Lyzl4*, *Lyzl6*, *Pkdrej*, *Spaca3*, *Spata4*, *Spata9*, *Syngr4*, *Tepp*, *Tex22*, *Tsga13*, and *Zcchc13*) that were predominantly expressed in testis based on previous reports [*Capn11* (16), *Lyzl4* (19), *Pkdrej* (17)] or analysis of the UniGene EST database. To confirm that the candidate genes were expressed in testis, we performed RT-PCR for each gene using testicular cDNAs obtained from 1-, 2-, 3-, 4-, and 5-wk-old mice. As a control, adult liver and kidney cDNAs were used. As expected, all of the genes were enriched in their expression in testis (Fig. 1*A*). Some genes (*Adam18*, *Capn11*, *Spata4*, *Syngr4*, and *Tex22*) started to express in 2-wk-old testes, suggesting that these genes could participate in early spermatogenesis. Other genes

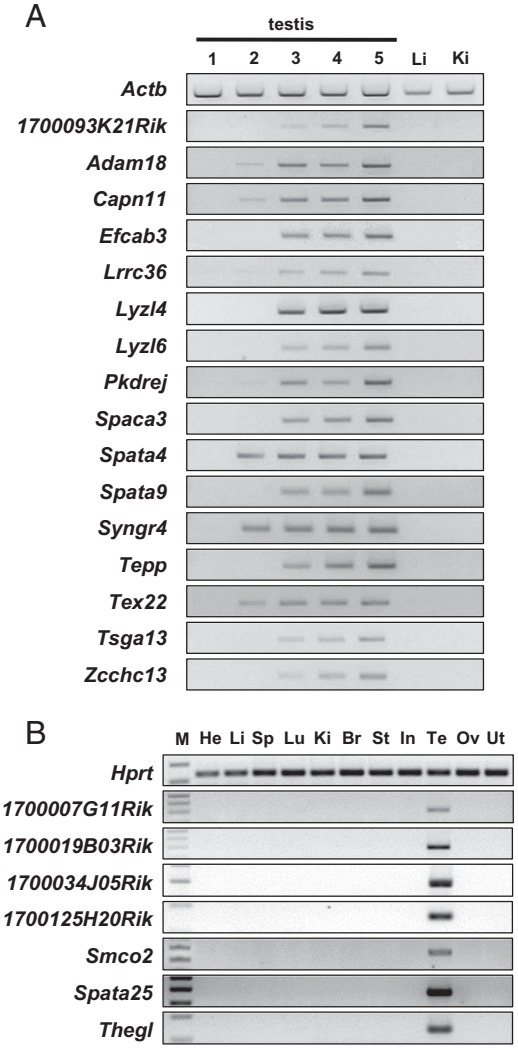

Fig. 1. RT-PCR analysis of genes that were mutated using homologous recombination in ES cells. (*A*) Expression of each gene was analyzed with RT-PCR using testicular cDNAs obtained from 1-, 2-, 3-, 4-, or 5-wk-old mice. Liver (Li) and kidney (Ki) cDNAs were also used. *Actb* (actin beta) as control. (*B*) cDNA from various tissues were used (Br, brain; He, heart; In, intestine; Ki, kidney; Li, liver; Lu, lung; Ov, ovary; Sp, spleen; St, stomach; Te, testis; Ut, uterus). M, molecular marker. *Hprt* (hypoxanthine guanine phosphoribosyl transferase) as control.

were expected to play a role in late spermatogenesis (spermiogenesis) or fertilization because they began expression in 3-wk-old testes. None of the genes was expressed in liver or kidney.

For our second analyses, we selected seven genes that were predicted to be testis-enriched and for which mutant mice were available from Knockout Mouse Project (KOMP). These genes are *1700007G11Rik*, *1700019B03Rik*, *1700034J05Rik*, *1700125H20Rik*, *Smco2*, *Spata25*, and *Thegl*. We performed RT-PCR for these genes using cDNA from adult mouse tissues including testis (Fig. 1*B*) as well as postnatal testis at specific time points (*SI Appendix*, Fig. S1*A*). We confirmed that all of the genes were expressed predominantly in the testis. Several genes were strongly expressed beginning at day 15 (e.g., *1700125H20Rik*), whereas others were expressed as late as day 30 postnatally (e.g., *1700019B03Rik*).

Because many studies in the laboratories of M.I. and M.M.M. have been focused on the analysis of transmembrane proteins that function during acrosome formation, sperm transit in the reproductive tract, and fertilization (1–3), we also analyzed bioinformatically predicted testis-enriched genes for transmembrane domains. Using the Simple Modular Architecture Research Tool (SMART) Web-based resource (smart.embl-heidelberg.de) for the genes that we analyzed in Fig. 1, we identified five genes predicted to have a single transmembrane domain (*1700093K21Rik*, *Adam18*, *Lyzl4*, *Spata9*, and *Smco2*). The transmembrane proteins with a single transmembrane helix are classified as type I (N terminus extracellular, in the endoplasmic reticulum, or Golgi) or type II (C terminus extracellular, in the endoplasmic reticulum, or Golgi) (20). We also identified two genes (*Pkdrej* and *Syngr4*) that encode type IV transmembrane proteins, which have multipass transmembrane domains.

In addition to the genes analyzed in Fig. 1, we also identified 12 additional genes that were predicted to have transmembrane-spanning domains. RT-PCR analysis of 10 of these genes (*1700015F17Rik*, *2900092C05Rik*, *4930522H14Rik*, *4931406B18Rik*, *BC051142*, *Fmr1nb*, *March11*, *Smim23*, *Tmco5*, and *Tmem247*) confirmed that these genes were enriched in their expression in the testis (Fig. 2*A* and *SI Appendix*, Fig. S1*B*). Based on UniGene, *Ms4a13* is predominantly expressed in the testis, and *Spink8* is predominantly expressed in the epididymis, testis, and prostate.

Among the genes analyzed in Fig. 1*A*, two genes (*Lyzl6* and *Spaca3*) encode an N-terminal signal peptide but no transmembrane domain, and therefore were predicted to be secreted proteins. We also identified five other genes predicted to be secreted proteins that are either testis-enriched (i.e., *Lyzl1* and *Spaca7*) or expressed in the testis and other tissues (*C1qtnf4*, *Col20a1*, and *Pinlyp*), based on UniGene.

Last, we identified an additional 14 genes that encoded conserved proteins with no signal peptide or transmembrane domain, predicted to be expressed in the testis, and for which mice were unavailable through KOMP. RT-PCR analysis confirmed that eight of these genes (*1110017D15Rik*, *1700011E24Rik*, *1700019N19Rik*, *4933417A18Rik*, *BC051628*, *BC089491*, *Ccdc178*, and *Fam217a*) were expressed predominantly in the testis (Fig. 2*B* and *SI Appendix*, Fig. S1*C*). Gene *1110017D15Rik* has multiple other names (*Smrp1*, *Cbe1*, *NYD-SP22*) and was previously reported as testis-enriched, localized to the spermatid manchette (18), present in cilia (21), and in multiple cell types including the cilia of bronchial epithelium (22). *Tktl1* is predicted to be predominantly expressed in uterus as well as testis. *Gm5617*, *Kif2b*, and *Ube2u* are testis-enriched based on UniGene. *Ubqlnl* and *Ubqln3* encode testis-enriched, ubiquitin-like proteins based on UniGene and a recent paper by Yuan et al. (23).

Functional Analysis of Genes Using Traditional ES Cells and KOMP Resources. To study the functions of these testis-enriched genes in vivo, we produced KO mouse models using traditional strategies and KOMP resources. Eight genes in Fig. 1*A* were knocked

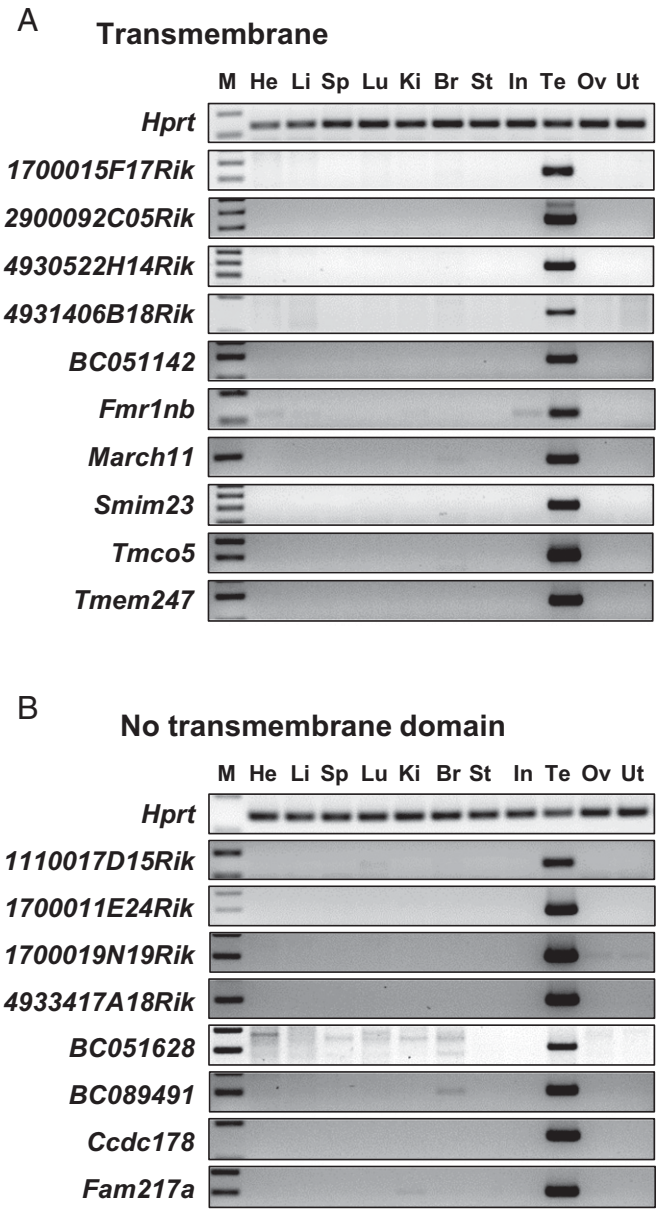

Fig. 2. RT-PCR analysis of the genes that were mutated using CRISPR/Cas9 system. (*A*) Genes with transmembrane domains. cDNA from various tissues were used. *Hprt* as control. (*B*) Genes without transmembrane domains.

out using the vectors from the International Knockout Mouse Consortium (IKMC) (knockout-first allele system, tm1a) (24). If the vectors were not available from IKMC, pNT1.1 vector (www.ncbi.nlm.nih.gov/nuccore/JN935771) (*SI Appendix*, Fig. S2*A*) (25) was used as a targeting vector. To knock out *Syngr4*, the targeting vector was produced with BAC recombineering (*SI Appendix*, Fig. S2*B*). The range of gene-targeting efficiency ranged from 8.3% to 52% for IKMC vectors and from 0.69% to 34% for pNT1.1 vectors (*SI Appendix*, Table S2). EGR05 (129S2), G01 (129S2 × C57BL6), and G101 (C57BL6) were used as ES cells (25, 26). Before we analyzed mice produced with IKMC vectors, the mice were crossed with CAG-Cre transgenic mice (B6D2F1) (27) to remove the regions flanked by loxP and generate null alleles.

For the seven genes shown in Fig. 1*B*, we obtained floxed allele mice (C57BL6) from the KOMP repository at Wellcome Trust Sanger Institute. We first excised the key exons between

the loxP sites using Gdf9-iCre transgenic mice (C57BL6; 129SvEv; BALBc) (28) to create null alleles before testing their fertility.

We confirmed the deletion of targeted regions in the mouse genome with PCR (*SI Appendix*, Fig. S3). The primers and amplification conditions for each gene are summarized (*SI Appendix*, Table S3). No overt developmental abnormalities were observed in any of the KO mouse lines produced. To examine fertility, adult KO males were mated with two wild-type females for several months. All of the mutated males showed normal fecundity (Table 1). We further analyzed the KO mice obtained from KOMP except for *Smco2*. Although slight differences were observed in the testis weights of *1700007G11Rik* and *1700034J05Rik* KO mice, there were no significant differences in the sperm counts or sperm motility or the fertility of these mice (*SI Appendix*, Table S4).

Functional Analysis of Testis-Enriched Genes Using CRISPR/Cas9. To analyze the function of the remaining 31 genes, we used the CRISPR/Cas9 system, which enabled us to produce KO mice efficiently in a short span of time (12, 13). We designed single guide RNAs (sgRNAs) and selected those that had fewer predicted off-target hits using Bowtie software (12) or the CRISPRdirect website (https://crispr.dbcls.jp) (29). The sgRNAs that exhibited greater activity in an in vitro assay (12) were injected into the pronuclei of fertilized embryos (B6D2F1 × B6D2F1) (*SI Appendix*, Table S5). The nucleotide immediately before the PAM sequence has been shown to influence cutting efficiency, with a G conveying greater activity (30). Indeed, our data show the presence of G immediately before PAM seems to be more efficient (A = 44 ± 24%, T = 30 ± 18%, G = 59 ± 28%, C = 34 ± 16%), but not significantly so ($P = 0.09$). Furthermore, there was no significant difference in the cutting efficiency of sgRNAs that started with a G compared with other nucleotides (A, T, or C) ($P = 0.50$), suggesting that sgRNA can begin with any nucleotide (13).

Homozygous mice that have insertion or deletion mutations resulting in frameshift mutations yielding prematurely truncated proteins were obtained by selective breeding of F_0 mice (*SI Appendix*, Fig. S4 and Table S6). No overt developmental abnormalities were observed in any of the KO mouse lines produced. Adult KO males were mated with two wild-type females for several months, and all of the homozygous mutant males showed normal fecundity (Table 2).

Discussion

Because more analyses on the mouse genome and transcriptome have been performed since the study by Schultz et al. (5), there may be some differences regarding the genes that were predicted to be expressed predominantly in the testis. Using the UniGene EST database, we found that about 1,000 genes were expressed predominantly in the testis. For example, when we searched EST profiles that resemble *Pgk2* or *Prm2* expression in mice using "show more entries with profiles like this," we found 1,059 genes (date of March 14, 2016). We did not search the genes that showed "testis-restricted" expression in the UniGene database because some genes such as *1700007G11Rik*, *Fmr1nb*, *March11*, and *Fam217a* are expressed predominantly in the testis (Figs. 1*B* and 2) but not included in the "testis-restricted" genes in the UniGene database. Thus, our gene expression search result is lower than the number of genes that Schultz et al. (5) suggest, but it is still ∼4% of the mouse genome, as Schultz et al. (5) estimated.

We generated homozygous mutant KO mice for 54 testis-enriched genes and analyzed the fertility of the males in vivo. All of the KO males were fertile. We cannot exclude the possibility of alternative translation initiation sites producing functional proteins. However, if this were the case, transmembrane and secreted proteins would not function properly because of the

Table 1. Average litter size of control and KO mice (homologous recombination in ES cells)

	Control				KO			
Gene	Genotype	Average litter size ± SD	No. of males	No. of litters	Average litter size ± SD	No. of males	No. of litters	Mating period
Generated in this study								
1700093K21Rik	Hetero	9.0 ± 1.5	2	10	8.9 ± 1.7	2	9	b
Adam18					9.2 ± 2.5	2	14	c
Capn11	Hetero	10.5 ± 1.8	3	15	9.5 ± 2.2	3	15	b
Efcab3	Hetero	10.3 ± 1.7	2	8	10.7 ± 1.9	2	9	b
Lrrc36					9.2 ± 1.5	2	11	c
Lyzl4					9.1 ± 1.7	2	9	b
Lyzl6					11.0 ± 1.4	1	6	c
Pkdrej	WT	7.7 ± 1.2	2	12	8.3 ± 2.1	3	15	b
Spaca3					8.2 ± 2.6	3	16	b
Spata4					9.3 ± 1.8	3	16	c
Spata9	Hetero	9.2 ± 1.8	2	12	9.2 ± 1.3	2	11	b
Syngr4	Hetero	8.9 ± 1.7	3	14	9.8 ± 1.5	3	12	b
Tepp	Hetero	7.5 ± 2.1	2	4	8.1 ± 2.1	3	15	b
Tex22					10.5 ± 1.8	2	10	c
Tsga13					9.1 ± 1.7	2	14	c
Zcchc13	WT	7.5 ± 1.0	2	4	7.5 ± 2.0	2	11	c
Control	WT	9.0 ± 1.5	3	15				
Obtained from KOMP								
1700007G11Rik	Hetero	6.7 ± 2.8	3	23	5.7 ± 3.5	4	19	d
1700019B03Rik	Hetero	6.8 ± 2.5	3	18	7.9 ± 2.1	2	15	d
1700034J05Rik	Hetero	8.7 ± 2.7	3	19	6.8 ± 2.0	4	32	d
1700125H20Rik	Hetero	8.1 ± 2.4	3	20	7.5 ± 2.4	4	31	d
Smco2	Hetero	8.1 ± 3.5	5	43	8.0 ± 3.7	4	54	d
Spata25	Hetero	7.4 ± 1.8	3	20	6.6 ± 2.6	4	29	d
Thegl	Hetero	7.0 ± 2.1	4	31	7.1 ± 3.1	3	21	d

The mating periods of KO mice were as follows: a, 5–8 wk; b, 9–12 wk; c, 13–16 wk; d, ≥17 wk.

Table 2. Average litter size of control and KO mice (CRISPR/Cas9 system)

	Control				KO				
Gene	Genotype	Average litter ± SD	No. of males	No. of litters	Genotype	Average litter ± SD	No. of males	No. of litters	Mating period
1110017D15Rik	WT	8.0 ± 3.0	1	2	−6+2/−6+2	9.2 ± 0.8	3	6	a
1700011E24Rik					−28/−28	10.8 ± 0.5	3	6	a
1700015F17Rik					−2/−2	9.3 ± 1.5	2	12	c
1700019N19Rik					−50/−50	8.6 ± 1.6	3	15	b
2900092C05Rik					+1/+1	10.9 ± 2.2	2	9	c
4930522H14Rik					−7/−7	8.2 ± 2.8	2	13	c
4931406B18Rik	Hetero	12.3 ± 1.0	3	6	+1/+1	10.5 ± 1.0	3	6	a
4933417A18Rik	Hetero	10 ± 1.9	1	9	−4/−4	8.9 ± 2.0	3	8	a
BC051142	Hetero	10 ± 1.4	3	6	−5/−5	8.5 ± 0.3	3	6	a
BC051628	Hetero	9.9 ± 2.5	2	8	−82/−82	8.3 ± 0.8	3	10	a
BC089491					−8/−8	10.2 ± 1.7	3	11	a
C1qtnf4					−40/−40	9.3 ± 1.7	2	11	b
Ccdc178					−7/−7	6.9 ± 2.0	3	17	c
Col20a1					+4/+4	10.1 ± 1.6	2	13	b
Fam217a	Hetero	9.3 ± 1.4	3	6	−4/−4 and −35/−35	9.3 ± 1.7	4	8	a
Fmr1nb					−1/Y	8.1 ± 0.9	3	9	a
Gm5617					−1/−2	10.3 ± 1.1	3	7	a
Kif2b	Hetero	8.8 ± 1.3	2	8	−5/−5	9.8 ± 1.1	3	12	a
Lyzl1					−13/−13	9.0 ± 2.0	3	12	b
March11	Hetero	8.6 ± 3.0	3	14	−50/−50	9.8 ± 2.6	5	9	a
Ms4a13					−5/−5	10.1 ± 1.5	2	7	a
Pinlyp					−7/−7	10.0 ± 2.0	2	5	a
Smim23					−2/−2	9.6 ± 1.2	2	9	c
Spaca7					−4/−4	10.3 ± 2.5	3	14	b
Spink8	Hetero	10.3 ± 2.0	2	6	+1/+1 and +1/−5	10.0 ± 1.9	4	12	a
Tktl1					−11+4/Y	7.4 ± 2.7	3	6	a
Tmco5					+1/+1	9.5 ± 0.7	2	11	b
Tmem247					−13/−13	11.6 ± 0.8	2	10	c
Ube2u					−2+4/−2+4	10.4 ± 1.2	3	11	a
Ubqln3					−1,858/−1,858	11.3 ± 2.0	2	7	a
Ubqlnl	Hetero	10.0 ± 1.4	1	4	+1/+1	10.0 ± 2.0	2	13	b
Control	WT	9.0 ± 1.5	3	15					

The mating periods of KO mice were as follows: a, 5–8 wk; b, 9–12 wk; c, 13–16 wk.

absence of the signal peptide. We will deposit all mice generated in this study to the RIKEN BioResource Center (Tsukuba, Japan) or the Center for Animal Resources and Development at Kumatomo University (Kumamoto, Japan), where they will be made available to researchers interested in these genes. KO mice obtained from KOMP are available from International Mouse Phenotyping Consortium (www.mousephenotype.org).

Fig. 3 shows the conservation of these genes among species. The conservation ranges from genes present in nearly all eukaryotic branches (*1700007G11Rik* and *Kif2b*) to genes that are specific to eutherians (*1700015F17Rik*, *2900092C05Rik*, *4930522H14Rik*, *4931406B18Rik*, *BC051142*, *Gm5617*, *Spaca7*, *Spink8*, and *Tex22*). The evolutionary rates of these genes are also summarized (*SI Appendix*, Table S7). There are several highly evolving genes (31, 32) such as *BC051142* and *Ccdc178*. As a reference, the evolutionary rates of several highly conserved genes among metazoans are listed. Among these 54 genes, there are several genes that were thought to play roles in male fertility such as *Lyzl4* (19) in reproduction, *Spaca3* in sperm–egg binding (33), *Spaca7* in cumulus dispersal (34), and *Spata4* in Sertoli cell proliferation (35). However, this study suggests that these genes are not essential for male fertility.

Because we investigated only litter size, there may be subtle phenotypes that we overlooked in these KO males. For example, *Pkdrej* KO mice have been produced previously, and their litter size was normal, consistent with the present study (36). However, detailed analyses showed that *Pkdrej* KO males exhibit lower reproductive success compared with WT males in sequential mating trials, suggesting that *Pkdrej* is important in postcopulatory sexual selection (36). In the case of *Cd59b* KO mice, males showed decreased fertility with age (37). Detailed studies may find subtle phenotypes in the KO mice that are described in our paper; however, we have shown that these genes are dispensable for male fertility under normal laboratory mating conditions, and the genetic backgrounds of our transgenic mice when breeding was initiated at 6–8 wk of age.

One reason that many KO males exhibit normal fertility may be due to functional redundancy. For example, CAPN11 is a calcium-dependent protease (calpain) that is predominantly expressed in testes, and calpain might play a role in the acrosome reaction (38). Although our study reveals that *Capn11* KO males are fertile, other calpain paralogs may work to complement the acrosome reaction. For example, *Capn1* and *Capn2* are also expressed in mouse testis (38). Another example involves members of the glycoside hydrolases *Lyzl1*, *Lyzl4*, and *Lyzl6*. Although KO males for each of these genes are fertile, other paralogs may compensate. The same may apply to *Ubqln3* and *Ubqlnl* that contain an N-terminal ubiquitin-like domain and a C-terminal ubiquitin-associated domain. To overcome this problem of functional redundancy, it may be necessary to mutate several genes to eliminate the chance of compensation from paralogs. However, traditional mouse mutagenesis using homologous recombination in ES cells is time and

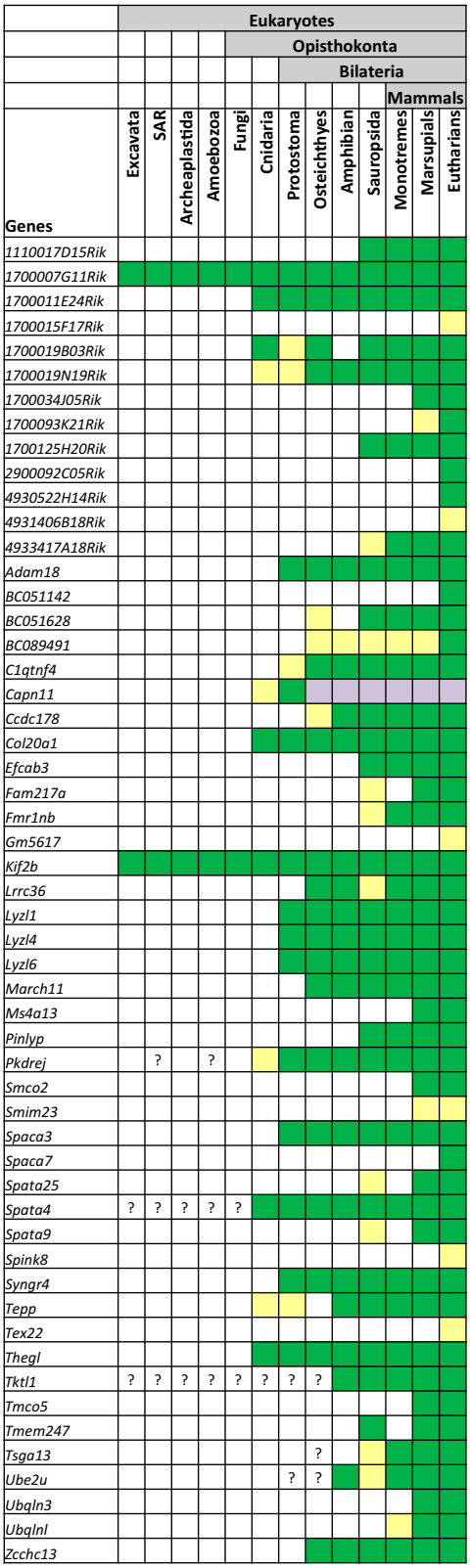

Fig. 3. Conservation of all genes in this study. The presence of orthologs indicated by shaded square. Orthologs within several species in a taxon are indicated with solid green. Yellow indicates loss of ortholog within several species within a taxon. Light purple represent gene duplication within a taxon. Question marks indicate potential orthologs in species within a taxon.

resource consuming. This problem may be solved using a CRISPR/Cas9 system that makes it possible to mutate multiple genes in one step (14).

In summary, we generated KO mice for 54 genes and demonstrated that these male mice display normal fertility. Although all of the genes are conserved in humans, some genes such as *C12orf71*(*1700034J05Rik*), *C2orf74*(*1700093K21Rik*), *COL20A1*, *EFCAB3*, *SMCO2*, and *SPINK8* show decreased expression in human testis compared with mouse testis, based on the UniGene database, suggesting that these genes may play different roles in humans. We could not identify essential functions of these 54 genes in male reproduction, but we believe that it is important information to disseminate to the scientific community. Our study suggests that an abundance of conserved and testis-enriched genes are not required for male fertility and we should prioritize our efforts to focus on genes that are essential for male reproduction.

Materials and Methods

Animals. Wild-type mice for experiments in the laboratory of M.I. were purchased from CLEA Japan or Japan SLC or were produced from intercrosses of C57BL6 and 129S6/SvEv mice generated in the laboratory of M.M.M. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan), or the Institutional Animal Care and Use Committee at Baylor College of Medicine (Houston, TX).

RT-PCR. For Fig. 1*A*, mouse cDNA was prepared from adult ICR liver, kidney, and testes from 1- to 5-wk-old males. For Figs. 1*B* and 2, and *SI Appendix*, Fig. S1, mouse cDNA was prepared from multiple adult tissues of C57BL6/129SvEv hybrid mice and testes of 5- to 60-d-old mice. The primers and amplification conditions for each gene are summarized (*SI Appendix*, Table S8).

KO Mice Production with pNT1.1 or IKMC Vector. KO mice were produced as described previously (25). The targeting vectors were from IKMC (24) or pNT1.1 (www.ncbi.nlm.nih.gov/nuccore/378747675) (25). The primers used to amplify long arms and short arms for pNT1.1 are listed (*SI Appendix*, Table S9). The homologously recombined ES cells (*SI Appendix*, Table S2) were injected into ICR eight-cell embryos. The obtained chimera mice were mated with B6D2F1 females for germ-line transmission.

***Syngr4* KO Mice Production with BAC Recombineering.** Primer 5306 (5′-AAAGAAGAGCAGCCACTATGCACCTCCCTGAAAGCCTC CACGACCTGGCA-3′) and Primer 5307 (5′-ATCCTTGAGATGCTCTTTGGTCTCCTCAGAAAG CGCACAGTCTCATTGTC-3′) were used to amplify FRT-PGK-gb2-neo-FRT-loxP cassette with PCR. This DNA fragment was homologously recombined into RP24-79J24 (BAC PAC) in *Escherichia coli*. Exon2 of *Syngr4* was replaced with this cassette. Linearized pMCS-tk was homologously recombined to produce the targeting vector. KO mice were produced using this targeting vector in the same way as pNT1.1 and IKMC vectors.

KO Mice Production with CRISPR/Cas9 System. KO mice were produced by pronuclear injection of circular pX330 plasmid as described previously (12). Off-target analysis was performed using CRISPRdirect software (https://crispr.dbcls.jp) (29) or Bowtie software (12). sgRNA sequences used for injection are listed (*SI Appendix*, Table S5). Primer sequences that were used for EGXXFP plasmid (12) construction and genotyping are listed (*SI Appendix*, Table S10). Genotyping was performed by direct sequencing following PCR.

Male Fertility Test. For the genes listed in Table 1 (KO mice generated in this study) and Table 2, sexually mature KO male mice were caged with two 2-mo-old B6D2F1 females for several months, and the number of pups was counted at the day of birth. For seven genes listed in Table 1 (KO mice obtained from KOMP; *1700007G11Rik*, *1700019B03Rik*, *1700034J05Rik*, *1700125H20Rik*, *Smco2*, *Spata25*, and *Thegl*), sexually mature KO males were paired with two 6-wk-old wild-type females for several months. The number of pups were counted the day after birth or at 10 d after birth. Average litter sizes are presented as the number of total pups born divided by the number of litters for each gene.

Testis Weights, Sperm Counts, and Sperm Motility. After breeding studies, males were killed by cervical dislocation following anesthesia. Testes were weighed individually. Epididymides from one male were minced in 1 mL of prewarmed HTF media, and then incubated at 37 °C for 20 min (tubes were inverted every 5 min). After incubation, sperm samples were diluted 1:50 in 1 mL of warmed HTF media. Motility percentages and velocities (average path velocity) were then analyzed using Hamilton Thorne's CEROS II system.

Conservation of the Genes Among Species. Identification of conserved genes was performed using Ensembl Compara, PSI-Blast, tBlastN, and OrthoDB searches. Potential orthologs (e.g., predicted genes from draft genomes) were individually paired with the mouse gene using Align Sequence Protein Blast. Evolutionary rates among vertebrates were obtained from OrthoDB (cegg.unige.ch/orthodb7/) (39).

Statistical Analysis. Statistical analyses were performed using Student's *t* test or analysis of variance. Differences were considered significant at $P < 0.05$.

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