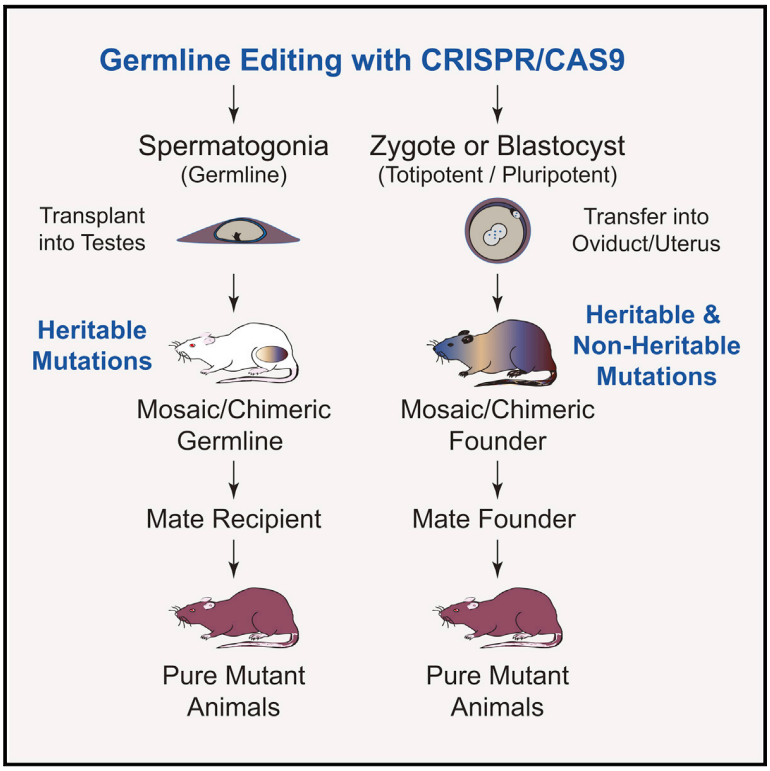
Report

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Targeted Germline Modifications in Rats Using CRISPR/Cas9 and Spermatogonial Stem Cells

Graphical Abstract



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In Brief

Chapman et al. now use CRISPR/Cas9 to target heritable mutations within spermatogonia, fully functional rat germline stem cells. Clonally enriched target alleles were vertically transmitted from donor spermatogonia to rat progeny. Direct germline editing in spermatogonia prevented CRISPR/Cas9-catalyzed animal mosaicism by avoiding totipotent and pluripotent states of embryogenesis.

Highlights

- CRISPR/Cas9 targets heritable germline modifications in donor spermatogonia
- Direct germline editing eliminates CRISPR/Cas9-catalyzed animal mosaicism
- In vitro and in vivo recessive genetic screens for spermatogenesis

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Targeted Germline Modifications in Rats Using CRISPR/Cas9 and Spermatogonial Stem Cells

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SUMMARY

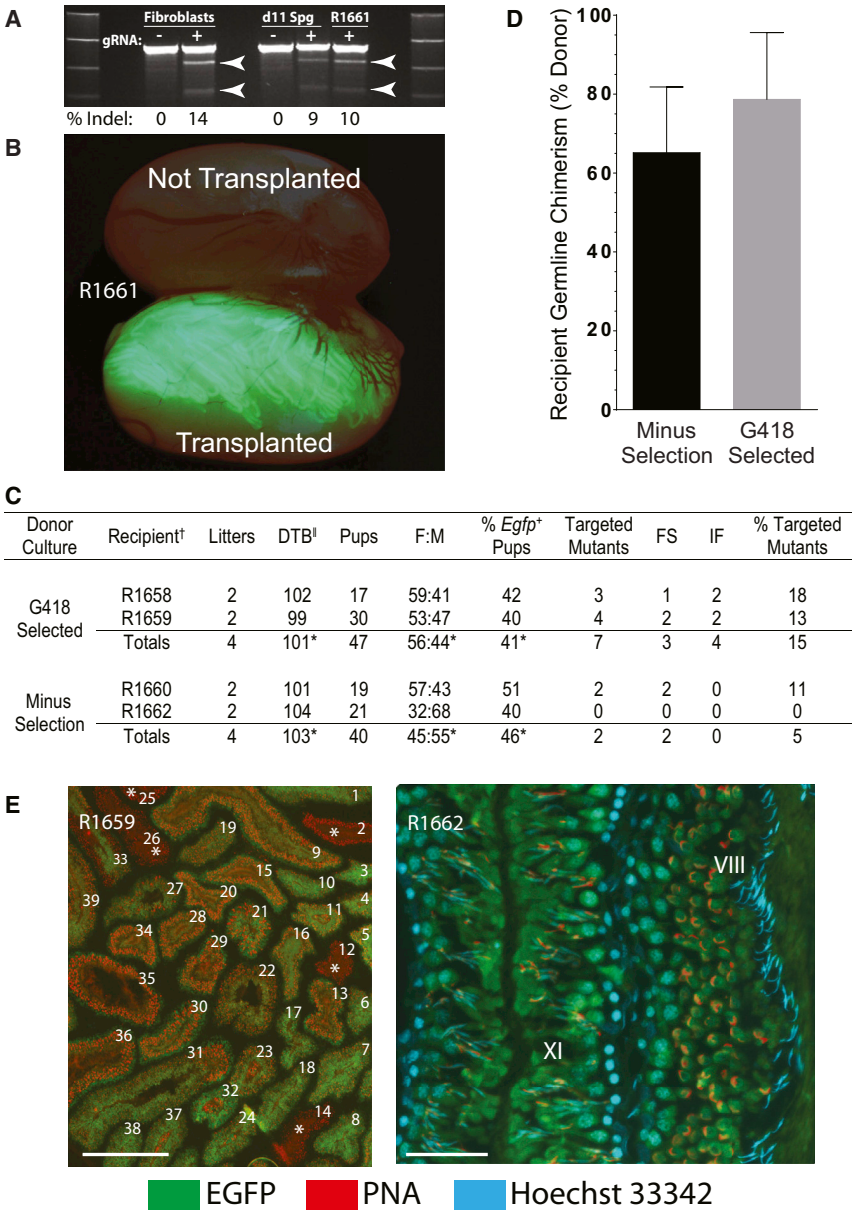
Organisms with targeted genomic modifications are efficiently produced by gene editing in embryos using CRISPR/Cas9 RNA-guided DNA endonuclease. Here, to facilitate germline editing in rats, we used CRISPR/Cas9 to catalyze targeted genomic mutations in rat spermatogonial stem cell cultures. CRISPR/Cas9-modified spermatogonia regenerated spermatogenesis and displayed long-term sperm-forming potential following transplantation into rat testes. Targeted germline mutations in *Epsti1* and *Erbb3* were vertically transmitted from recipients to exclusively generate "pure," non-mosaic mutant progeny. *Epsti1* mutant rats were produced with or without genetic selection of donor spermatogonia. Monoclonal enrichment of *Erbb3* null germlines unmasked recessive spermatogenesis defects in culture that were buffered in recipients, yielding mutant progeny isogenic at targeted alleles. Thus, spermatogonial gene editing with CRISPR/Cas9 provided a platform for generating targeted germline mutations in rats and for studying spermatogenesis.

INTRODUCTION

CRISPR/Cas9 RNA-guided DNA endonuclease technology is being widely utilized to generate targeted genomic mutations in diverse cell types and organisms to study their biological processes (Harrison et al., 2014). Gene editing with CRISPR/Cas9 in mammals yields high rates of donor-embryo-derived progeny harboring targeted gene mutations in rodents, pigs, goats, and monkeys (Hai et al., 2014; Li et al., 2013; Ni et al., 2014; Niu et al., 2014; Wang et al., 2013; Yang et al., 2013). Mutant rodents can be produced in <1 month upon injection of constructs expressing *gRNA*s and *Cas9* that direct cleavage of target gene sequences in donor zygotes (Li et al., 2013; Wang et al., 2013; Yang et al., 2013). CRISPR/Cas9 is so efficient, both alleles for multiple target genes can be disrupted in animals produced by co-injecting zygotes with respective g*RNA*s (Li et al., 2014; Ma et al., 2014; Ni et al., 2014; Wang et al., 2013; Yang et al., 2013).

CRISPR/Cas9 also efficiently catalyzes target allele mosaicism in animals, which reflects independent gene editing events made during early embryonic cleavage stages as the totipotent zygote undergoes pre-implantation development (Yen et al., 2014). CRISPR/Cas9 is typically delivered into mammalian zygotes on embryonic day (E)0.5 to E1, which is ~5–6 days before the germline is specified in rodents. As an example, the mouse germline is specified within a small population of 10–20 proximal epiblast cells between E6 to E6.5 (Ohinata et al., 2005). Taken together, this explains why CRISPR/Cas9-target allele mosaicism is observed in tissues of first-generation mutant animals and why targeted alleles in somatic tissues can differ from those specified in the germline. Target allele heterogeneity in mosaic animals must be outcrossed to generate colonies of pure, non-mosaic germline mutants isogenic for a given targeted allele in all cells of their body (Jaenisch et al., 1981; Soriano and Jaenisch, 1986; Wilkie et al., 1986). Sorting out new mutant strains by experimentally outcrossing allelic mosaicism takes months in rodents but can require years in some species due to longer life cycles and/or low fecundity (Niu et al., 2014; Pursel et al., 1989).

Target allele mosaicism is also generated when host embryos are reconstituted with pluripotent stem cells genetically modified using CRISPR/Cas9 (Wang et al., 2013). As with the early embryo, CRISPR/Cas9 holds potential to distinctly modify multiple target alleles within a stem cell clone as it divides (Wang et al., 2013). In addition to this variation, reconstitution of wild-type blastocysts with pluripotent donor cells further generates "chimeric" animals with organ systems and germlines developing from mixtures of host and donor embryonic cells (Tarkowski, 1961). Mosaicism and chimerism was substantially reduced when clonally enriched donor cells modified with CRISPR/Cas9 were used to produce pigs and goats by somatic cell nuclear transfer into enucleated oocytes (i.e., cloning) (Ni et al., 2014). Reconstituting tetraploid embryos with pre-screened, clonally expanded pluripotent lines would also be predicted to minimize mosaicism and chimerism in epiblast-derived tissues (Nagy et al., 1990). Alternatively, direct germline editing in donor



C

Donor Culture	Recipient†	Litters	DTB‖	Pups	F:M	% *Egfp*⁺ Pups	Targeted Mutants	FS	IF	% Targeted Mutants
G418 Selected	R1658	2	102	17	59:41	42	3	1	2	18
	R1659	2	99	30	53:47	40	4	2	2	13
	Totals	4	101*	47	56:44*	41*	7	3	4	15
Minus Selection	R1660	2	101	19	57:43	51	2	2	0	11
	R1662	2	104	21	32:68	40	0	0	0	0
	Totals	4	103*	40	45:55*	46*	2	2	0	5

Figure 1. CRISPR/Cas9-Mediated Gene Targeting in Donor Stem Spermatogonia

(A) CRISPR/Cas9 cleavage of *Epsti1* in rat fibroblast and spermatogonial (Sg day 11) cultures on day 11 post-transfection and in flow-sorted EGFP⁺ spermatogenic cells (R1661) on day 56 after transplantation into the right testis of recipient R1661: – and + symbols indicate transfection without and with *Epsti1* gRNA. Arrows indicate predicted ~461 base pairs (bp) plus ~226 bp Surveyor products.

(B) Spermatogenesis (green fluorescence) in testis of R1661 on day 56 after transplantation that developed from donor EGFP⁺ rat spermatogonial cultures containing CRISPR/Cas9-catalyzed *Epsti1* mutations.

(C) Targeted mutagenesis of *Epsti1* exon 2 in rats by CRISPR/Cas9 spermatogonial gene editing. Donor spermatogonia were hemizygous for tg*GCS-EGFP*. DTB, days from transplantation to first litter containing *Epsti1* mutant animals; FS, frame shift mutation; IF, mutation in frame. †R(n), recipient identifier, ‖litters born 99–128 days post-transplantation, %, percentage of total F1 progeny per recipient; ⋆average value.

(D) Donor germline chimerism (%) in wild-type recipient rat testes. Ratio of seminiferous tubules containing EGFP⁺ elongating spermatids/total tubules containing elongating spermatids plotted/donor culture condition (minus selection and G418 selected). Error bars indicate SD.

(E) Acrosome (PNA) and nuclear (Hoechst 33342) labeling marking donor-derived elongating spermatids (EGFP⁺) in tubule cross-sections from recipient R1659 and R1662. Left: 39 of 39 tubules contained elongating spermatids (EGFP⁺ or EGFP⁻). Five EGFP⁻ tubules are marked with an asterisk. Scale bar, 500 μm. Right: higher magnification image of donor spermatogenesis at stages VIII and XI in the rat seminiferous epithelial cycle (Hess, 1990). Scale bar, 100 μm.

See also Figures S1 and S2 and Tables S1 and S2.

spermatogonial stem cells would avoid both the totipotent and pluripotent states of embryogenesis (Brinster and Avarbock, 1994), thus eliminating production of mosaic/chimeric mutant progeny. Here, we report efficient production of mutant rats by spermatogonial gene editing with CRISPR/Cas9.

RESULTS

Spermatogonial gene editing at *Epsti1* loci was conducted using CRISPR/Cas9 as proof of concept for efficient gene targeting in rats. *Epsti1* encodes epithelial-stromal interaction, and *Epsti1* variants in men are associated with alterations in sperm function and family size (Kosova et al., 2012). Guide *RNA*s demonstrating highest specificity to exon 2 of rat *Epsti1* in silico were first tested for activity in rat fibroblast cultures (Figure 1A). A plasmid encoding an effective *Epsti1 gRNA* and *Cas9* (p*gEpsti1-330*) was transfected (neon electroporation) into rat spermatogonia hemizygous for a germline-specific *Egfp* marker (Cronkhite et al., 2005), with and without a second plasmid encoding a selectable marker (p*NeoΔtk*) (Hamra et al., 2005). Spermatogonia from each transfection, with and without p*NeoΔtk*, were plated onto fibroblast feeder layers and selected with and without G418 in growth medium (days 3–9). Spermatogonia were then sub-cultured for two passages (days 11 and 23) (Chapman et al., 2011) and transplanted into rat seminiferous tubules (day 34) (Figure 1B). Predicted cleavage of targeted *Epsti1* alleles in donor germlines by the Surveyor assay was similar in spermatogonial cultures harvested at day 11 post-transfection (~9% insertions or deletions [indels]) and in the total population of flow-sorted EGFP⁺ spermatogenic cells produced in recipient R1661 by day 56 post-transplantation (~10% indels) (Figure 1A). This provided

evidence that CRISPR/Cas9-dependent modifications to *Epsti1* were maintained in stem cells during sub-culture and persisted during spermatogenesis in recipient rats.

Based on robust colonization by stably modified donor spermatogonia in R1661 (Figures 1A and 1B), four remaining recipient rats (n = 2 transplanted with G418 selected cells and 2 transplanted with unselected cells) were paired with wild-type females at ~65 days post-transplantation until each pair produced two litters (n = 8 total litters) (Figure 1C). F1 progeny harbored ~10% total *Epsti1* mutants (n = 9/87 F1 pups) within an estimated 87% donor-derived progeny (38 $EGFP^+$/87 F1 pups = 43.6% × 2; hemizygous marker) (Figure 1C). Spermatogonia selected in G418-containing medium yielded ~3× more mutant progeny than spermatogonia from unselected cultures (5% mutants, minus selection; 15% mutants, G418 selected) (Figure 1C). Fifty-six percent of *Epsti1* mutations in progeny were frame shifts (five of nine) (Figures 1C and S1A). The remaining *Epsti1* variants (four of nine) were in-frame deletions (Figures 1C and S1A). Thus, cultures of CRISPR/Cas9-mutagenized spermatogonial stem cells were successfully applied to produce pure *Epsti1* mutant rats ~100 days post-transplantation (101.5 ± 2.1 days; n = 4 recipients) (Figure 1C).

Histological analysis of recipient testes at day 178 post-transplantation revealed robust spermatogenic potential of donor stem cells under each culture condition tested (Figures 1D and 1E). Most seminiferous tubules in recipients contained $EGFP^+$ elongating spermatids (65.5 ± 14%, minus selection; 79 ± 17%, G418 selected; n = 4 testes from two rats per culture condition, 117–176 tubules scored per testis) (Figure 1D; Table S1). Crosses between recipient male and wild-type female rats produced normal-sized litters (10.9 ± 3.4 pups per litter, n = 8) (http://www.harlan.com), with testes at approximately half the normal weight (Table S2) but approximately three times heavier than reported for non-transplanted, busulfan-treated testes (Hamra et al., 2002). $EGFP^+$ germ cells flow-sorted from recipient rat testes (Figure S2) revealed 5% (1/20 amplicons) and 36% (8/22 amplicons) distinct mutant *Epsti1* alleles derived from unselected and G418-selected spermatogonia, respectively (Figure S1B). Thus, intra-recipient donor haplotype frequencies were consistent with germline transmission rates for *Epsti1* mutations (Figure 1C; Table S1).

Although non-mosaic mutant rat strains were efficiently generated by spermatogonial gene editing with CRISPR/Cas9, the targeted *Epsti1* alleles varied considerably between strains (Figure S1). This appeared as random repair of cleaved template in distinct donor stem cells. Monoclonal enrichment of donor spermatogonial stem cells in culture following genome editing with CRISPR/Cas9 would, theoretically, reduce variation of targeted alleles transmitted to progeny by recipients. Clonal enrichment of CRISPR/Cas9-modified stem spermatogonia in culture prior to transplantation would also facilitate studying the effects of recessive mutations on spermatogenesis (particularly, in cases of embryonic lethality). As an example, *Erbb3* is critical for embryogenesis in mice (Erickson et al., 1997) and encodes a receptor tyrosine kinase activated by the polypeptide ligand NRG1 (Carraway et al., 1994). Furthermore, NRG1, GDNF, and serum were required for the clonal development of differentiating spermatogenic cells in vitro on laminin (Hamra et al., 2007). To define a germline receptor for NRG1 in rats, we analyzed CRISPR/Cas9-targeted *Erbb3* mutations in spermatogonial stem cell lines derived from individually picked colonies (Figure 2A).

Six of 26 picked colonies analyzed (~23%) were enriched with targeted *Erbb3* mutations after clonal expansion on fibroblast feeder layers (Figure 2A and Figure S3A). It is interesting that all six mutant colonies were classified as harboring isogenic (three of six colonies) or heterogeneous (three of six colonies) biallelic targeted mutations (Figure S3A). Mutant colonies with isogenic targeted alleles (i.e., B9, C7, D3) were, initially, falsely classified as wild-type germlines by the Surveyor assay (Figure S3B) but subsequently defined as biallelic *Erbb3* mutant germlines by sequencing (Figures S3A and S3B). This is consistent with the Surveyor assay becoming less accurate at estimating percent modified alleles as target allele variation decreases (Guschin et al., 2010).

Biallelic mutant spermatogonial lines derived from picked colonies were deficient in ERBB3 (Figure 2B) and proliferated at similar rates over multiple passages in culture compared to clonally expanded wild-type lines (Figure 2C). Unlike wild-type lines, six of six clonally enriched *Erbb3*-deficient germlines were severely compromised in their ability to support development of $ZBTB16^-$ spermatogenic colonies in culture on laminin in a serum-free, spermatogonial differentiation medium (SD medium) supplemented with NRG1, GDNF, FGF2, and all-*trans*-retinoic acid (Figures 2D and 2E). All wild-type germlines picked from the same transfection effectively developed into syncytia containing 8–32 differentiating spermatogenic cells negative for ZBTB16 labeling (tgGCS-$EGFP^+$, $ZBTB16^-$; n = 5 lines) (Figures 2D and 2E). ZBTB16 (or PLZF) is a marker for type A spermatogonia in mammalian testes and is critical for spermatogonial stem cell self-renewal in mice (Buaas et al., 2004; Costoya et al., 2004). In contrast to their in vitro phenotypes, *Erbb3*-deficient spermatogonial lines (B9 and C7) robustly regenerated spermatogenesis in recipient rats when analyzed 2.5–3.5 months post-transplantation (Figure 3A; Figure S4A). Thus, the ability of *Erbb3*-deficient germlines to regenerate spermatogenesis in recipient testes did not reflect NRG1's requirement for clonal development of differentiating spermatogenic cells in culture (Figures 2D and 2E).

To assess the sperm-forming potential of *Erbb3*-deficient spermatogonial lines longer term, recipients of clonally expanded lines C7 (R1697) and C8 (R1699) were paired with wild-type females at day 207 post-transplantation (~6.8 months; both testes transplanted per recipient). The recipient of line C8 was highly enriched with +C and ΔC frame shift mutations (Figures S3A and S3B) and fathered progeny inheriting each *Erbb3* germline mutation (~46% of progeny were +C or ΔC targeted alleles; 6 of 13 pups) (Figure 3B; Figure S3C). All $Egfp^+$ pups (five of five) harbored one of the two targeted alleles (Figure 3B; Figures S3C and S3D), consistent with colony C8 representing a homogeneous biallelic mutant germline (Figures S3A and S3B). Similarly, the recipient of isogenic line C7 (enriched with +C frame shift mutations; Figures S3A and S3B) fathered a litter of ten pups, four

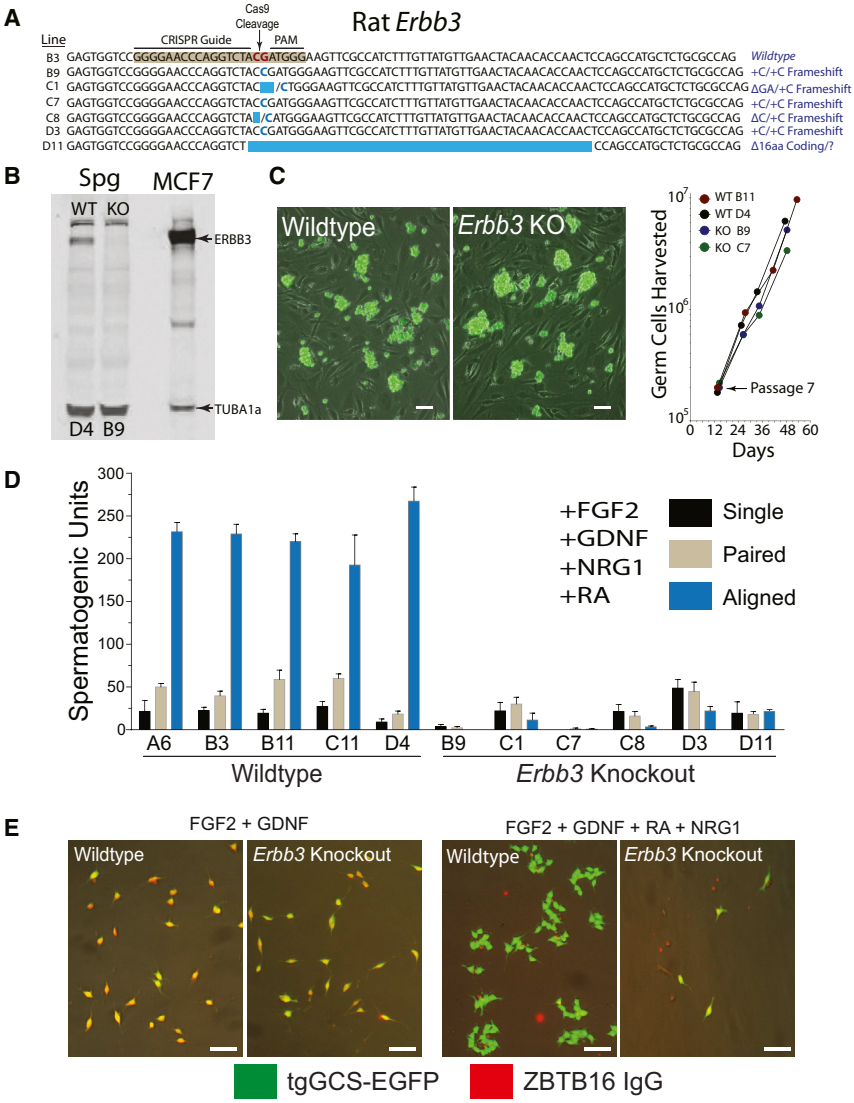


Figure 2. Monoclonal Enrichment of *Erbb3*-Deficient Stem Spermatogonia

(A) CRISPR/Cas9-targeted *Erbb3* mutations (exon 2) in clonally expanded rat spermatogonial lines.

(B) Relative abundance of ERBB3 (upper arrowhead) and TUBA1a (lower arrowhead) in wild-type (colony D4; WT) and *Erbb3*-deficient (colony B9; KO) spermatogonial lines.

(C) Left: cultures of clonally expanded wild-type (colony D4) and *Erbb3* knockout (colony B9) spermatogonia expressing tg*GCS-EGFP*. Right: Growth rates of clonally expanded wild-type (WT) and *Erbb3* knockout (KO) spermatogonial lines. Scale bars, 50 μm.

(D) NRG1-dependent development of differentiating spermatogenic cells from wild-type and *Erbb3* knockout spermatogonial lines. Error bars indicate SD.

(E) Spermatogenic cells from colonies D4 (wild-type) and B9 (*Erbb3* knockout) analyzed in (D). tg*GCS-EGFP* is indicated in green, and ZBTB16 antibody is indicated in red. IgG, immunoglobulin G. Scale bars, 40 μm.

See also Figure S3.

DISCUSSION

Here, we demonstrate how CRISPR/Cas9-mediated germline editing in donor spermatogonia can be used to produce "pure," non-mosaic mutant animals and to study the effects of gene mutations on spermatogenesis. In doing so, we provide proof of concept for targeted mutagenesis directly in fully functional rat donor germlines. This included polyclonal and monoclonal enrichment of rat spermatogonial lines harboring heritable, CRISPR/Cas9-targeted alleles. High colonization efficiency (Hamra et al., 2005; Wu et al., 2009), transfection efficiency, and sperm-forming potential of donor germlines translated into robust mutant rat production by spermatogonial gene editing with CRISPR/Cas9.

of which inherited the +C mutation in *Erbb3* (Figure S3C). Again, all *Egfp*$^+$ pups (three of three) harbored the targeted *Erbb3* allele (Figures S3C and S3D), which would be expected in progeny derived from a monoclonal donor germline harboring two targeted *Erbb3* alleles.

Analysis of recipient testes at day 239 post-transplantation (7.8 months) revealed donor-derived spermatogenesis from lines C7 and C8 (Figure S4B) but at visibly reduced levels of colonization compared to wild-type germlines (Figure 3C). Neither recipient testis weights (Figure 3D) nor the percent seminiferous tubules containing both EGFP$^+$ type A spermatogonia (ZBTB16$^+$) and spermatids (PNA$^+$) were significantly different between recipients of wild-type and *Erbb3* mutant germlines (Figure S4B). Donor-derived spermatids were present in >90% of seminiferous tubule sections colonized by EGFP$^+$ spermatogenic cells, independent of the clonal line transplanted (n = 3 wild-type lines and 3 biallelic mutant lines) (Figure S4B).

An estimated 15%–30% of donor-cell-derived progeny was obtained by breeding recipients transplanted with G418-selected donor spermatogonia (200,000 per testis) carrying targeted *Epsti1* alleles (Figure 1C). Similarly, ~43% of pups (10/23, n = 2 litters, 1 litter per donor strain) were derived from monoclonally enriched donor spermatogonial lines (60,000 per testis) harboring *Erbb3* null mutations (Figure 3B; Figure S3). Actual transmission rates from targeted germlines would be contingent on how mutated alleles affect sperm development or function and on rates at which biallelic null mutations were generated and maintained per transfected sperm-forming spermatogonium. In the two examples reported here, even if *Epsti1* or *Erbb3* was found essential for male fertility, the phenotypically diploid nature of clonally derived spermatids imparted by ring canals (Braun et al., 1989; Wilkie et al., 1991) would enable

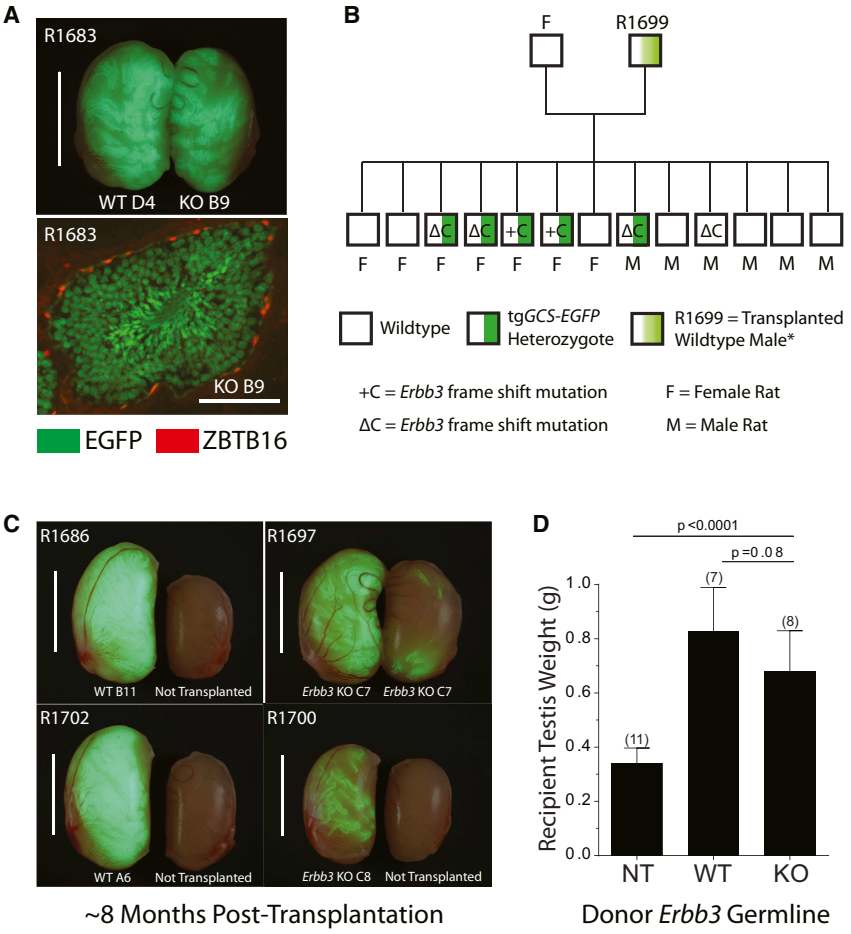


Figure 3. Transmission of Isogenic CRISPR/Cas9-Targeted Alleles to Rat Progeny

(A) Top: spermatogenesis (green) derived from wild-type (WT) colony D4 and *Erbb3* knockout (KO) colony B9 in testes from R1683 at ~3.5 months post-transplantation. Scale bar, 1 cm. Bottom: seminiferous tubule cross-section from R1683 illustrating spermatogenesis derived from colony B9 marked by EGFP (green). ZBTB16^{+} spermatogonia are indicated by red nuclei. Scale bar, 100 μm.

(B) Genotypes of rat progeny derived from clonally expanded spermatogonial line C8. tg*GCS-EGFP* is a hemizygous marker.

(C) Recipient rat testes ~7.8 months post-transplantation. Clonally expanded lines were transplanted into the right testes of R1686, R1702, and R1700 (EGFP^{+}), but contralateral left testes were not transplanted. Note that the right and left testes of R1697 were transplanted. Scale bar, 1 cm.

(D) Mean testis weights from recipient rats analyzed ~7.8 months after transplanting with wild-type (WT) spermatogonial lines (D4, n = 2; B11, n = 3; A6, n = 2) and *Erbb3*-deficient (KO) spermatogonial lines (B9, n = 2; C7, n = 3; C8, n = 3). NT, untransplanted testes, n = 11 (error bars indicate SD; p values are from multiple t tests). The ns are shown in parentheses above the bars.

See also Figures S3 and S4.

spermatogonial stem cells containing at least one functional copy of the targeted allele (wild-type or in-frame mutant) to vertically transmit recessive loss-of-function donor haplotypes.

Here, monoclonally enriched spermatogonial lines harboring biallelic, CRISPR/Cas9-targeted *Erbb3* null mutations were also applied to study spermatogenesis during culture in vitro and in recipient rat testes (Figures 2 and 3). Notably, the *Erbb3*-deficient germlines were analyzed in vitro using highly simplified, serum-free culture media that effectively promoted spermatogonial stem cell renewal (SG medium) or differentiation (SD medium) (Figure 2). Unknown factors in recipient testes, which were apparently absent or inactive in vitro, were sufficient to support spermatogonial differentiation on the *Erbb3*-deficient background. Looking ahead, the ability to "multiplex" with CRISPR/Cas9 holds the potential to study such redundant or polygenic processes contributing to the genetic robustness of spermatogenesis (Archambeault and Matzuk, 2014). Thus, recessive genetic assays in fully functional rat spermatogonial stem cells were established by these studies, providing an experimental platform to biochemically and genetically define in vitro spermatogenesis-stimulating factors, such as NRG1 and ERBB3.

Based on the germline transmission rates of targeted *Epsti1* alleles that we obtained by breeding recipient rats (Figure 1), transplanting spermatogonial stem cells into seminiferous tubules more directly after delivering CRISPR/Cas9 constructs than in the current study holds a clear potential to expedite production of pure mutant rat strains by up to an additional month (Figure 4). Spermatogonial gene editing using CRISPR/Cas9 technology can readily be adopted in rodents on a scalable level (Izsvák et al., 2010; Kanatsu-Shinohara et al., 2005; Nagano et al., 2001a) and could be established to streamline pure mutant animal production in other applied species important for science, industry, conservation, and medicine (Arregui et al., 2013; Hermann et al., 2012; Nagano et al., 2001b; Zeng et al., 2013). Indeed, proof of concept for correcting genetic disease in mice by spermatogonial gene editing with CRISPR/Cas9 was reported during the revision of this article (Wu et al., 2015).

EXPERIMENTAL PROCEDURES

Spermatogonial Gene Editing with CRISPR/Cas9

Spermatogonial lines were derived from freshly isolated laminin-binding spermatogonia using individual heterozygous *SD-Tg(ROSA-EGFP)2-4Reh* rats (Hamra et al., 2005). *SD-Tg(ROSA-EGFP)2-4Reh* Sprague-Dawley rats are referred to as tg*GCS-EGFP* rats because they exhibit germ-cell-specific expression of EGFP (Cronkhite et al., 2005). Spermatogonial lines were propagated on feeder layers of irradiated mouse embryonic fibroblasts (MEFs) as previously detailed using SG medium containing 6 ng/ml basic fibroblast growth factor (bFGF) (PGF0023, Life Technologies) and 6 ng/ml GDNF (512-GF, R&D Systems) (Chapman et al., 2011; Wu et al., 2009). To generate *Epsti1* mutants, spermatogonia were harvested at passage 8 and co-transfected in suspension with plasmids p*Neo⊿tk* and/or pX330 (Cong et al., 2013) using the Neon Transfection System (Life Technologies) set for two pulses at 1,100 V, 20 ms. pX330 co-expressed *Cas9* and g*RNAs* 5′-tgatagcaccgaacgagacc-3′ (p*gEpsti1-330*; cloned

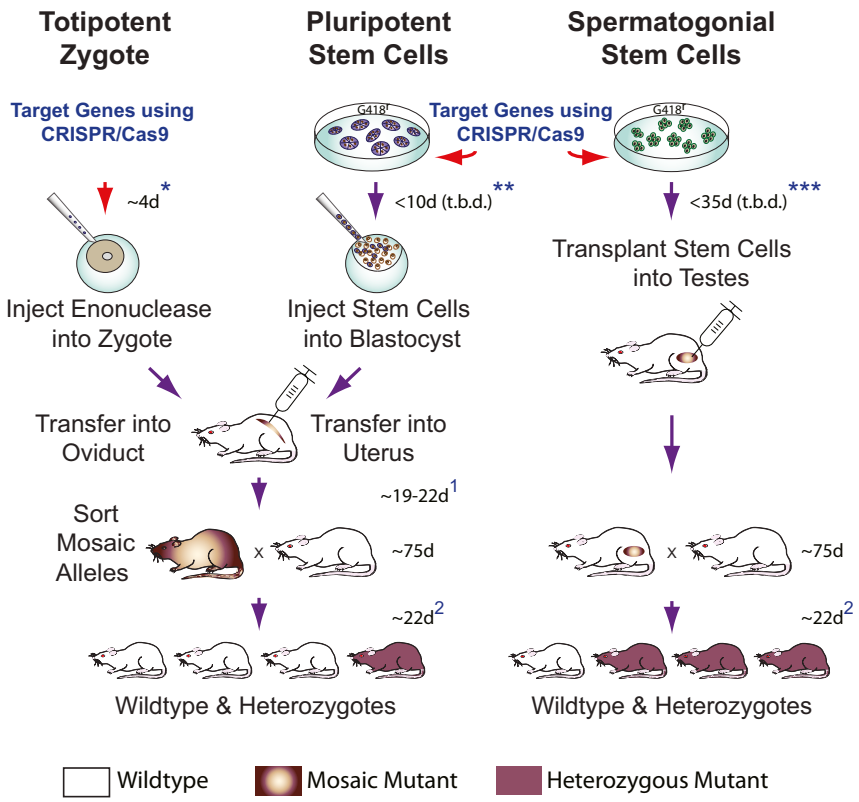


Figure 4. CRISPR/Cas9 Gene Targeting in Early Embryos and Spermatogonia

Left: zygotes are injected with CRISPR/Cas9 constructs and transferred into surrogate female oviducts to support donor embryo development into mutant progeny. Mosaic mutant progeny born ~19–22 days (d) post-transfer (depending on cleavage stage transferred) (superscript 1) display variation in targeted alleles in various tissues and must be crossed to wild-type stock to generate pure heterozygous mutants isogenic at respective targeted alleles in all cells of their body.

Center: rat pluripotent stem cell cultures could prospectively be genetically modified using CRISPR/Cas9. Rat embryonic stem cells with targeted mutations have been selected in culture prior to blastocyst injection (Tong et al., 2011). Injected blastocysts are transferred into uteri of surrogate females to produce mosaic/chimeric mutant animals ~19 days post-transfer (superscript 1). Mosaic/chimeric animals are crossed to wild-type stock to establish pure heterozygous mutants.

Right: spermatogonial stem cells can be genetically modified in culture using CRISPR/Cas9. Modified spermatogonia are injected into recipient rat testes to produce mutant spermatozoa that transmit targeted genomic modifications to heterozygous mutant progeny. Timelines for each approach listed above must consider ~75 days for rat breeder pairs to reach reproductive age, 21–23 days for rat gestation time, plus a 4- to 5-day estrus cycle in rats (Lohmiller and Swing, 2006) (superscript 2).

*Includes additional 4 days to establish pseudo-pregnant female recipients by paring with vasectomized males; does not include time needed to prepare vasectomized male rats. **Remains to be determined (t.b.d) using CRISPR/Cas9; estimate based on rat embryonic stem cell lines selected following transfection with classical DNA targeting constructs (Tong et al., 2011); includes an additional 4 days to establish pseudo-pregnant female recipients. ***Present study; minimum time required following delivery of CRISPR/CAS9 constructs to spermatogonia (with or without genetic selection) prior to transplantation was not studied and remains to be determined (t.b.d); includes 12 days to prepare recipient males.

into *BbsI* sites). p*NeoΔtk* was generated from parental plasmid, pKO1904 (Stratagene), by excising its thymidine kinase cassette and retaining the neomycin phosphotransferase open reading frame under control of the PGK1 promoter. Cultures not undergoing G418 selection were transfected using 10 μg p*gEpsti1-330*/10^6 spermatogonia; cultures undergoing G418 selection were co-transfected using 3 μg p*gEpsti1-330* + 7 μg p*NeoΔtk*/10^6 spermatogonia. Transfected spermatogonia for each respective condition were plated onto fresh MEFs for 3 days (day 3) and selected in SG medium containing 0 or 65 μg/ml G418 for 6 days (day 9) prior to harvesting and plating on fresh MEFs in SG medium on day 11 (Chapman et al., 2011). To generate clonally enriched *Erbb3*-deficient germlines, spermatogonial stem cells from passage 6 were similarly transfected with p*gErbb3-330* (*gRNA* 5′-ggggaacccaggtctacgat-3′) and diluted post-transfection by plating an equivalent of ~7.5 × 10^4 to 1.5 × 10^5 cells/9.5 cm^2 in SG medium to promote picking individual colonies for derivation of clonally enriched spermatogonial lines, as described elsewhere in detail (Chapman et al., 2011; Ivics et al., 2011; Izsvák et al., 2010). Individually picked colonies required a mean (± SD) of 81 ± 20 and 89 ± 21 days to expand wild-type (n = 5) and *Erbb3*-deficient (n = 6) spermatogonial lines to ~2 × 10^5 cells, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.02.040.

AUTHOR CONTRIBUTIONS

F.K.H., C.O., M.A.N., and J.M.H. designed the experiments and performed data analysis. K.M.C., G.A.M., P.J., J.C., A.E.W., and F.K.H. conducted the experiments. F.K.H. wrote the paper.

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