Targeted Genome Modification in Mice Using Zinc Finger Nucleases

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ABSTRACT

Homologous recombination-based gene targeting using *Mus musculus* embryonic stem cells has greatly impacted biomedical research. This study presents a powerful new technology for more efficient and less time-consuming gene targeting in mice using embryonic injection of zinc-finger nucleases (ZFNs), which generate site-specific double strand breaks, leading to insertions or deletions via DNA repair by the nonhomologous end joining pathway. Three individual genes, multi-drug resistant 1a (*Mdr1a*), jagged 1 (*Jag1*), and notch homolog 3 (*Notch3*), were targeted in FVB/N and C57BL/6 mice. Injection of ZFNs resulted in a range of specific gene deletions, from several nucleotides to over 1000 base pairs in length, among 20-75% of live births. Modified alleles were efficiently transmitted through the germline, and animals homozygous for targeted modifications were obtained in as short as four months. In addition, the technology can be adapted to any genetic background, eliminating the need for generations of backcrossing to achieve congeneric animals. We also validated the functional disruption of *Mdr1a* and demonstrated that the ZFN-mediated modifications lead to true knockouts. We conclude that ZFN technology is an efficient and convenient alternative to conventional gene targeting and will greatly facilitate rapid building of mouse models and functional genomics research.

INTRODUCTION

Conventional gene targeting technology in mice relies on homologous recombination in embryonic stem (ES) cells to target specific gene
sequences, most commonly to disrupt gene function (DOETSCHMAN 1987; KUEHN 1987; THOMAS 1987). Advantages of gene targeting in ES cells are selective target sequence modification, the ability to insert or delete genetic information, and the stability of the targeted mutations through subsequent generations. There are also potential limitations, including limited rates of germline transmission, and strain limitations due to lack of conventional ES cell lines (LEDERMANN 2000; MISHINA 2007). Moving the targeted allele from one strain to another requires ten generations of backcrosses that take two to three years. A minimum of one year is necessary for backcrossing if speed congenics is applied (MARKEL 1997).

Zinc Finger Nucleases (ZFNs) are fusions of specific DNA-binding zinc finger proteins (ZFPs) and a nuclease domain, such as the DNA cleavage domain of a type II endonuclease, FokI (BIBIKOVA 2001; KIM 1996; SMITH 1999). A pair of ZFPs provide target specificity, and their nuclease domains dimerize to cleave the DNA, generating double-strand breaks (DSBs) (MANI 2005), which are detrimental to the cell if left unrepaired (RICH 2000). The cell uses two main pathways to repair DSBs: high-fidelity homologous recombination and error-prone nonhomologous end joining (NHEJ) (HUERTAS 2010; LIEBER 1999; PARDO 2009). ZFN-mediated gene disruption results from deletions or insertions frequently introduced by NHEJ. Figure 1 illustrates the cellular events following the injection of a pair of ZFNs targeting the mouse Mdr1a (also known as Abcb1a) gene.

ZFNs have been successfully applied to generate genome modifications in plants (SHUKLA 2009; TOWNSEND 2009), fruit flies (BIBIKOVA 2002), C. elegans (MORTON 2006), cultured mammalian cells (PORTEUS 2003;
SANTIAGO 2008), zebrafish (DOYON 2008; MENG 2008), and most recently in rats (GEURTS 2009; MASHIMO 2010). The technology is especially valuable for rats because rat ES cell lines have only become available recently (BUEHR 2008; Li 2008), and successful homologous recombination-mediated genome modification has not been reported. Previously, ENU mutagenesis (ZAN 2003) or transposons (KITADA 2007) were the two main methods for generating gene knockout rats, both of which are random approaches and require labor-intensive and time-consuming screens to obtain the desired gene disruptions.

Although ES cell-based knockout technology is widely used in mice, ZFN technology offers three advantages: (i) high efficiency, (ii) drastically reduced timeline, both of which are comparable to, if not better than those of the creation of a transgene (GORDON 1980), and (iii) the freedom to apply the technology in various genetic backgrounds. In addition, no exogenous sequences need to be introduced because selection is not necessary.

Here, we created the first genome-engineered mice using ZFN technology. Three genes were disrupted in two different backgrounds: Mdr1a, Jag1, and Notch3 in the FVB/N strain and Jag1 also in the C57BL/6 strain. All founders tested transmitted the genetic modifications through the germline.

MATERIALS AND METHODS

In vitro preparation of ZFN mRNAs: The ZFN expression plasmids were obtained from Sigma’s CompoZr product line. Each plasmid was linearized at the Xbal site, which is located at the 3’ end of the FokI ORF. 5’ capped and 3’ polyA tailed message RNA was prepared using either MessageMax T7 Capped transcription kit and poly (A) polymerase tailing kit
(Epicentre Biotechnology, Madison, WI) or mMessage Machine T7 kit and poly (A) tailing kit (Ambion, Austin, TX). The polyA tailing reaction was precipitated twice with an equal volume of 5 M NH4OAc and then dissolved in injection buffer (1 mM Tris-HCl, pH 7.4, 0.25 mM EDTA). mRNA concentration was estimated using a NanoDrop 2000 Spectrometer (Thermo Scientific, Wilmington, DE).

**ZFN validation in cultured cells:** NIH 3T3 cells were grown in DMEM with 10% FBS and antibiotics at 37°C with 5% CO2. ZFN mRNAs were paired at 1:1 ratio and transfected into the NIH 3T3 cells to confirm ZFN activity using a Nucleofector (Lonza, Basel, Switzerland), following the manufacturer’s 96-well shuttle protocol for 3T3 cells. Twenty-four hours after transfection, culturing medium was removed, and cells were incubated with 15 µl of trypsin per well for 5 min at 37°C. Cell suspension was then transferred to 100 µl of QuickExtract (Epicentre) and incubated at 68°C for 10 min and 98°C for 3 min. The extracted DNA was then used as template in a PCR reaction to amplify 350-650 basepair amplicons around the target site with following primer pairs:

- **Mdr1a Cel-I F:** ctgtttcttgacaaaaacaacactaggctc
- **Mdr1a Cel-I R:** gggtcatgggaaagagtttaaaatc
- **Jag1 Cel-I F:** cttcggggcacttgtcttag
- **Jag1 Cel-I R:** gcgggactgatactccttga
- **Notch3 Cel-I F:** tttaaagtgggcgtttctgg
- **Notch3 Cel-I R:** ggcagaggtacttgtccacc
Each 50 μl PCR reaction contained 1 ul of template, 5 μl of buffer II, 5 μl of 10 μM each primer, 0.5 μl of AccuPrime High Fidelity (Invitrogen, Carlsbad, CA) and 38.5 μl of water. The following PCR program was used: 95°C, 5 min, 35 cycles of 95°C, 30 sec, 60°C, 30 sec, and 68°C, 45 sec, and then 68°C, 5 min, 4°C, forever. Three microliter of the above PCR reaction was mixed with 7 μl of 1x buffer II and incubated under the following program: 95°C, 10 min, 95°C to 85°C, at -2°C/s, 85°C to 25°C at –0.1°C/s, 4°C forever. One microliter each of nuclease S (Cel-I) and enhancer (Transgenomic, Omaha, NE) were added to digest the above reaction at 42°C for 20 min. The mixture is resolved on a 10% polyacrylamide TBE gel (Bio-Rad, Hercules, CA).

Microinjection and mouse husbandry: FVB/NTac and C57BL/6NTac mice were housed in static cages and maintained on a 14h/10h light/dark cycle with *ad libitum* access to food and water. Three to four week-old females were injected with PMS (5 I.U./per mouse) 48 h before hCG (5 I.U./mouse) injection. One-cell fertilized eggs were harvested 10-12 h after hCG injection for microinjection. ZFN mRNA was injected at 2 ng/μl. Injected eggs were transferred to pseudopregnant females (Swiss Webster (SW) females from Taconic Labs mated with vasectomized SW males) at 0.5 dpc.

Founder identification using mutation detection assay: Toe clips were incubated in 100-200 μl of QuickExtract (Epicentre Biotechnology) at 50°C for 30 min, 65°C for 10 min and 98°C for 3 min. PCR and mutation
detection assay were done under the same conditions as in ZFN validation in cultured cells using the same sets of primers.

**TA cloning and sequencing:** To identify the modifications in founders, the extracted DNA was amplified with Sigma’s JumpStart Taq ReadyMix PCR kit. Each PCR reaction contained 25 μl of 2x ReadyMix, 5 μl of primers, 1 μl of template, and 19 μl of water. The same PCR program was used as in ZFN validation in cultured cells. Each PCR reaction was cloned using TOPO TA cloning kit (Invitrogen) following the manufacture’s instructions.

At least 8 colonies were picked from each transformation, PCR amplified with T3 and T7 primers, and sequenced with either T3 or T7 primer. Sequencing was done at Elim Biopharmaceuticals (Hayward, CA).

**PCR for detecting large deletions:** To detect larger deletions, which removed the original Cel-I priming sites, another set of distal primers were used for each of the targets:

- **Mdr1a** 800F: ctgctgtgaagcagatacc
- **Mdr1a** 800R: ctgaaaactgaatgagacatttgc
- **Jag1** 600F: ggtgggaactggaagtagca
- **Jag1** 600R: ggagtctctctcccgctctt
- **Notch3** 800F: tctcaacaaacccacaacca
- **Notch3** 800R: gtcgctgcaagagcaagtg

Each 50 μl PCR contained: 1 μl of template, 5 μl of 10x buffer II, 5 μl of 10 μM of each 800F/R primer, 0.5 μl of AccuPrime Taq Polymerase High Fidelity (Invitrogen), and 38.5 μl of water. The following program was used: 95°C, 5 min, 35 cycles of 95°C, 30 sec, 62°C, 30 sec, and 68°C, 45 sec, and
then 68°C, 5 min, 4°C, forever. The samples were resolved on a 1% agarose gel. Distinct bands with lower molecular weight than the wt were sequenced.

**RNA preparation from tissues and RT-PCR:** Mdr1a-/- or Mdr1a+/+ littermates were sacrificed for tissue harvest at 5-9 weeks of age. Large intestine, kidney and liver tissues were dissected and immediately used or archived for later processing, tissue biopsies were placed in RNAlater solution (Ambion) and stored at -20°C. Total RNA was prepared using GenElute Mammalian Total RNA Miniprep kit (Sigma) following manufacture’s instructions. To eliminate any DNA contamination the RNA was treated with DNAsel (New England Biolabs, Ipswich, MA) before being loaded onto the purification columns.

*Mdr1a* RT-PCR analysis was carried out with 1 μl of total RNA, primers RT-F (5’-GCCGATAAAAGAGCCATGTTG) and RT-R (5’-GATAAGGAGAAAAGCTGCACC), using SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity kit (Invitrogen). Reverse transcription and subsequent PCR were carried out with 1 cycle of 55°C for 30 min. and 94°C for 2 min. for cDNA synthesis; and 40 cycles of 94°C for 15 sec, 56°C for 30 sec, and 68°C for 1 min for amplification. The PCR product was loaded in a 1.2% agarose gel and visualized with ethidium bromide. Nested PCR used primers RT-F2 (5’- CTGGAGGAAGAAATGACCACG) and RT-R2 (5’-GATAGCTTTCTTTATCCCCAGCC).

**Western blot analysis:** Mice were euthanized and the large intestine was immediately harvested and flushed with ice-cold PBS buffer, snap frozen on dry ice, and stored at –80°C. For protein preparation, tissue pieces
equivalent to approximately 200 μl were shaved off the frozen samples and placed into an ice-cold microcentrifuge tube. Four hundred microliter of ice-cold PBS with 4x protease inhibitors was added, and the sample was dounce-homogenized. The homogenate was pelleted at 20,000 x g for 5 min at 4°C, the supernatant (S1) was removed. The pellet, after being resuspended in 400 μl of ice-cold PBS with 4x protease inhibitors, was centrifugated at 4,000 x g for 5 min at 4°C. The supernatant (S2) was removed, and the pellet was resuspended in 500 μl lysis buffer (composition) (GERLACH 1987), dounce-homogenized, incubated on ice for 40 min with intermittent vortexing for 15 seconds per interval, and finally pelleted at 20,000 x g for 20 min at 4°C. The supernatant (S3) was collected, and the pellet was resuspended again in 250 μl of lysis buffer, dounce-homogenized, spun at 4,000 x g for 5 min at 4°C, and the supernatant (S4) was kept. The S3 and S4 fractions were diluted 1:1 with 2x Laemmli buffer (Sigma), and incubated at 37 °C for 5-10 min. Lysates (15ul, 10ul, or 5ul) were separated on a 4-20% Mini-PROTEAN TGX precast gel (BioRad), and transferred to nitrocellulose membrane using a semi-dry transblot (BioRad), at 25V for 1 hour. The transfer buffer contained standard tris-glycine salts, 18% MeOH and 0.25% SDS. Mouse anti-Mdr1a antibody C219 (Covance, Princeton, NJ) at 1:100 and mouse anti-Actin antibody at 1:1000 (Sigma) were incubated together with the blot overnight in 5% milk/TBST, rocking at 4°C, rinsed briefly in TBST, and the HRP-conjugated goat anti mouse secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) was incubated for 1 hour in 1% milk/TBST following a quick rinse with TBST, followed by 2 x 50 ml washes of 1% milk/TBST for how long.
HRP was detected using the SuperSignal West Pico substrate (Thermo) and a ChemiDocXRS+ Imaging system (BioRad, Hercules, CA).

RESULTS

**ZFN injection resulted in high-efficiency knockout at the Mdr1a Locus:** Validated Mdr1a ZFN mRNA (Figure S1 and Methods) was microinjected into fertilized FVB/N eggs, which were transferred into pseudopregnant females. Pups born from the injected embryos were tested using a DNA mismatch endonuclease (Cel-I) assay (see Methods) for modifications at the target site. Thirty out of the 44 live births contained deletions or insertions. Figure 2 shows the founders among wild-type littermates.

**Larger deletions generated by ZFN activity:** Some of the samples yielded no amplification product with the Cel-I primers. To detect potentially larger deletions that would have destroyed the priming sites used in Figure 2, a larger region spanning 800 bp on both sides of the cleavage site was PCR-amplified. Figure 3 shows that 15 out of the 44 pups indeed contain larger deletions, including four animals that were not identified as founders by the previous PCR assay. The PCR products for all founders were TA cloned and sequenced to reveal the exact sequences of modifications, and the deletions ranged between 3-731 bp in length as well as some small insertions (Table S1). Interestingly, three small deletions were each found in two or more founders: a 19 bp deletion in founders 7, 17 and 36, a 21 bp deletion in founders 17 and 20, and a 6 bp deletion in founders 34 and 44 (Figure S2). All
three deletions are flanked by 2 bp microhomology, which is predicted to create a common NHEJ junction (LIEBER 1999).

**High rate of germline transmission by Mdr1a founders:** Nine of the founders were chosen to backcross to the wild-type FVB/N mice to the F1 generation, all of which transmitted at least one mutant allele to their offspring. Seven founders transmitted more than two mutated alleles. Interestingly, in some cases, alleles that were not initially identified in the founders were also transmitted through the germline, and discovered in the next generation, such as by founders 6, 8, 13, 21, and 44 (Table S2), most likely due to incomplete sequencing of the TA clones (see discussion).

**Mdr1a Expression by RT-PCR and Western:** The Mdr1a protein is differentially expressed in tissues. Liver and large intestine predominantly express Mdr1a, and kidney expresses both Mdr1a and Mdr1b (SCHINKEL 1994). To verify that a deletion in the Mdr1a gene abolishes its expression, we performed RT-PCR on total RNA from liver, kidney and intestine of Mdr1a-/- mice established from founder 23, with a 396 bp deletion (Figure 4A), using a forward and a reverse primer located in exons 5 and 9, respectively. Samples from all the Mdr1a-/- tissues produced a smaller product with lower yield than those of corresponding wild-type samples, with a sequence correlating to exon 7 skipping and subsequent multiple premature stop codons in exon 8 in the mutant animals (Figure 4B). Furthermore, Western blotting with an anti-Mdr1a antibody showed absence of Mdr1a protein in the large intestine of
*Mdr1a*-/- animals (Figure 4C), demonstrating that the 396 bp deletion leads to a true knockout.

**High efficiency targeting and germline transmission in C57BL/6 strain:** Next, we microinjected *Jag1* ZFN mRNA into fertilized eggs from C57BL/6 strain and identified 24% founders among live births (Figure 5A). The *Jag1* ZFNs precisely target the junction of intron 1 and exon 2, therefore, even small deletions can destroy the recognition site for splicing. Deletions among *Jag1* founders range from 1 to 14 bp (Figure 5B). Five founders, 4, 19, 21, 28, and 37, carry deletions that mutated the conserved G residue at the end of intron and will likely lead to exon 2 skipping and deletion of 102 amino acids from the protein. Except for founders 28 and 37, both with two mutant alleles, the rest of the founders only bear one mutated allele. Like among *Mdr1a* founders, some *Jag1* founders carry the same deletions. Founders 7, 23, and 25 share the same 1 bp deletion. Founders 19 and 21 bear the same 4 bp deletion. Except for the mutant allele in founders 19 and 21, all the rest deletions are flanked by 1-2 bp microhomology (Figure 5B, also see Discussion). Founder 28 has a 2 bp deletion, both resulting in frameshift and premature stop codons shortly downstream. Founder 19 was backcrossed to wild-type C57BL/6 and achieved germline transmission in the first mating (three heterozygotes among eight F1 pups).

**Notch3 targeting in FVB/N mice:** We targeted a third gene, *Notch3*, again in FVB/N and obtained 20% founder rate (Figure 6A). Founders 1 and 2 have large deletions, 367 bp and 1121 bp, respectively (Figure 6B). Number 9
is the only founder carrying two different mutated alleles, a 1 bp deletion and an 8 bp deletion. Again, the same 8 bp deletion in founder 9 was also identified in founders 13 and 23, and founders 8 and 26 both carry an identical 16 bp deletion. All three deletions are flanked by a 2 bp microhomology (Figure 6C, also see discussion). All deletions are completely within exon 11, resulting in frameshift that introduces premature translational stop codons within the exon.

**Potential Off-target Sites Validation:** We identified 20 sites in the mouse genome that are most similar to the *Mdr1a* target site, all with 5 bp mismatches from the ZFN binding sequence, and top potential off-target sites for *Jag1* and *Notch3*, all with at least 6 bp mismatches from respective target site (Tables S3-5). To validate specificity of the *Mdr1a* ZFNs, we tested the site in *Mdr1b* gene, which is 88% identical to *Mdr1a*, in all 44 *Mdr1a* F0 pups using mutation detection assay. None of the 44 pups had an NHEJ event at the *Mdr1b* site (Figure S3). To further and fully characterize the *Mdr1a* mutant animals, we tested all the predicted potential off-target sites in four founder animals, and found no spurious mutations (Figure S4).

**DISCUSSION**

We generated mice with modifications at three loci by direct injection of ZFN mRNA into the pronucleus of one-cell mouse embryos. Comparing to conventional knockout mice technology, the ZFN technology offers a few obvious advantages. By bypassing ES cells, ZFN technology enables one to generate a mouse homozygous for the targeted allele in a matter of months,
with no need for selection. Highly efficient targeting (20-75%) allows one to identify founders by screening relatively small number of pups. Many founders carrying more than one mutant allele in addition to the wild-type allele, implying ZFNs remain active beyond one-cell stage. Every cell division doubles the number of wild-type allele, which is the only allele cleavable by ZFNs. Deletions or insertions change the space between ZFN binding sites, preventing FokI domains from dimerization. For those founders harboring up to five different alleles, ZFN-mediated cleavage likely did not happen before the first embryonic cell division. Thus, most founders are mosaics. All tested founders transmitted at least one mutant allele through the germline (Table S1).

Most Mdr1a founders transmitted more than one allele, as observed in rats as well (GEURTS 2009). Some alleles that were not identified in the founders were inherited in F1’s (Table S2), which was likely caused by PCR bias and incomplete sampling of the TA clones. PCR reactions for detecting large deletions, which favor amplification of smaller products resulting from larger deletions, were used to TA clone followed by sequencing to identify mutant alleles. We only sequenced 8-16 clones from each founder. Some of the small deletions, especially if they were also low representing, could be missed. Although all live births were tested with Cel-I assay (with a detection limit around 1%), some of the negative pups may carry low-representing alleles yet germline-competent. It is also possible that toe or tail clips do not necessarily have the same genotype as germ cells, which we observed only one confirmed example. Founder 23 did not have wild-type allele amplification in either toe or tail DNA. Yet when mated to wild-types, only 50% of its F1s
were heterozygous. The other half was wild type. So there was wild-type allele in the germline but was not represented in either toes or tails.

We looked at the effect of modifications on gene expression in one of the Mdr1a-/- strains. The RT-PCR results demonstrate that the samples from the Mdr1a-/- founder #23 produce a transcript missing the 172 bp exon 7 that causes exon skipping during mRNA splicing and immediately creates multiple premature translational stop codons in the message (Figure 4B). Such mutations often lead to nonsense mediated decay (NMD) of the mutant mRNA (Chang 2007), and this is supported by qRT-PCR and semi-quantitative RT-PCR analyses showing an estimated 70% decrease in abundance for the mutant Mdr1a transcript compared to the wild-type (Figure 4B and data not shown), implying mRNA degradation, likely provoked by NMD. In the Mdr1a-/- samples, there were faint bands at and above the size of the wild-type transcript, which are most likely PCR artifact because amplification of those bands excised from the gel yielded mostly the exon-skipped product (Figure S3B). The bands at the wild-type size in secondary rounds of PCR were mixtures that did not yield readable sequences (not shown). This conclusion is supported by Western blot analysis using an anti-Mdr1a antibody that detected abundant protein expression in the large intestine (the tissue mainly expresses Mdr1a) of wild-type littermates but no detectable Mdr1a protein in the same tissue of homozygous animals derived from founder #23. Thus, the Mdr1a-/- mice derived from founder 23 represent a functional knockout. Consistent with the theory of possible NMD, we obtained similar RT-PCR results on another animal, a compound homozygote from founder 11, harboring 417 and 533 bp deletions in respective alleles.
Smaller amplicon corresponding to exon skipping was detected at lower level than that of wild-type PCR product (not shown), just as in the case of \textit{Mdr1a}\textsuperscript{-/-} from founder 23. The observation extends to the rat as well. A 19 bp deletion in the rat \textit{Mdr1a} locus, greatly reduced the mRNA level, even though size wise, it was similar to the wild-type. And again, Western blots showed complete lack of Mdr1a expression in \textit{Mdr1}\textsuperscript{-/-} large intestine (ICD and XC, unpublished data).

The mouse \textit{Mdr1a} gene has 28 exons, and the encoded protein is composed of two units of six transmembrane domains (TMs 1-6 and TMs 7-12), each unit with an ATP binding site, and with a linker region in between the units (MITZUTANI 2005). All 12 TM domains as well as the two ATP-binding motifs are essential for Mdr1a function (PIPPERT 2001). The \textit{Mdr1a} ZFNs target exon 7, which encodes TMs 3 and 4. Based on previous work in this field, any partial protein that might result from the described frameshift and nonsense mutations we observed (assuming such protein fragments could be stable) should not be functional (PIPPERT 2001). Among the mutant alleles, 41\% causes exon skipping, 37\% results in frame-shift, and the rest of 22\% carries in-frame deletions (Table S1). It is safe to predict that the majority of the mutants obtained will be true knockouts.

Interestingly, large deletions were introduced in both targets, \textit{Mdr1a} and \textit{Notch3} in the FVB/N strain but not in \textit{Jag1} in C57BL/6, suggesting a possible difference in DNA repair that may be related to the host genetic background. However, injection of \textit{Jag1} ZFNs into FVB/N embryos resulted in similar founder rate and deletion sizes (not shown) as in C57BL/6, indicating the difference in deletion size might not have resulted from variation in genetic
background. The *Mdr1a* locus also has higher percentage of large deletions than *Notch3*, although both were targeted in FVB/N. It is possible that the target site per se contributes at least partially to the determination of modifications. Table S6 contains data from all the injections in both FVB/N and C57BL/6, including number of eggs injected, number of pups born from each injection and number of founders identified among live births. Due to procedural similarity between generation of a transgene and ZFN-mediated genome modifications, any background that is competent for traditional transgenesis should in theory be a good candidate for using to create a ZFN-mediated knockout. We have not accumulated enough data to analyze differences on targeting efficiency or types of modifications caused by different mouse background. However, we and others have observed similar targeting rates in various rat strains, and the size of deletions seem to also be target-dependent (SAGE Labs unpublished data) (MASHIMO 2010).

Another interesting observation was that for all three targets, some small deletions were identical in multiple founders (Figures 5, 6 and S2), assuming deletion occurs randomly during NHEJ. We considered the possibility that these deletions were merely PCR artifact caused by GC-rich microhomology flanking some of the deletions. However, several of the small deletions transmitted germline (Table S2), proving that these small deletions are true targeting events. Our data support the notion that microhomology of 1-4 bp at the ends of DSBs promotes, but is not necessary for, NHEJ (LIEBER 1999). We noticed that most of the deletions, regardless of whether identified in single or multiple founders, contain 1-4 bp microhomology at the deletion boundary (Figures 5, 6, and S2). In alleles such as that shared by founders 19
and 21 of Jag1, where microhomology is not present, we hypothesize that sequence-dependent DNA secondary structures might form around the target site that pause the resection of the ends by exonucleases before ligation (Huertas 2010), so that certain deletions resulted in multiple founders. Founder 11 contains an unusual allele with discontinuous deletions, a 417 bp deletion from –528 to –112, over 100 bp upstream of the cleavage site and flanked by a 5 bp microhomology GACAA, and a 19 bp deletion at the cleavage site, -14 to +5 (Table S1), and this complex allele was transmitted through the germline (Table S2). One explanation could be that two sequential ZFN cleavages occurred in the same chromatid. The repair of the first DSB was initiated as homologous recombination using the sister chromatid as template but was completed by NHEJ using the 5 bp microhomology, as observed previously (Richardson 2000), leading to 417 bp deletion upstream of the target site. The restored target site was cleaved again by ZFNs and repaired by NHEJ, resulting in 19 bp deletion.

We identified sequences in the mouse genome that are most similar to the Mdr1a, Jag1, and Notch3 target sites and tested the potential off-target sites for the Mdr1a ZFNs. No modifications were detected at the Mdr1b site in any of the 44 live births, and of 80 other off-targets tested (20 sites in four independent founders), none harbored modifications, illustrating the specificity of the Mdr1a ZFNs (See Figure S3). Being the best we could have done without performing costly whole genome sequencing, these data do not exclude that there are off-target sites that do not resemble the target site. Assuming hypothetical, unlinked off-target modifications will be diluted through breeding, an indirect way to detect potential off-target events could be
to compare phenotypically early-generation to later-generation homozygotes. The lack of difference in phenotypes implies the absence of off-target events. To include wild-type littermates as controls in phenotyping assays is another way to reduce the possible interference of off-target modifications on phenotype. In the mean time, we do realize that the ultimate proof of absence or presence of off-target events has to come from whole-genome sequencing, which will hopefully be affordable in the near future with continuous reduction in sequencing cost.

Altogether, we conclude that ZFN technology is a valuable alternative to conventional knockout technology for generating genome modifications in mice.

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FIGURE LEGENDS

Figure 1
The ZFN targeting mechanism. ZFN pairs bind to the target site, and FokI endonuclease domain dimerizes and makes a double strand break between the binding sites. If a DSB is repaired so that the wild type sequence is restored, ZFNs can bind and cleave again. Otherwise, non-homologous end joining (NHEJ) introduces deletions or insertions, which change the spacing between the binding sites so that ZFNs might still bind but dimerization or cleavage cannot occur. Insertions or deletions potentially disrupt the gene function.

Figure 2
Identification of genetically engineered Mdr1a founders using the Cel-I mutation detection assay. Cleaved bands indicate a mutation is present at the target site (see Methods). Bands are marked with respective sizes in base pairs. M, PCR marker. 1-44, 44 pups born from injected eggs. The numbers representing the mutant founder animals are underlined.

Figure 3
Large deletions in Mdr1a founders. PCR products were amplified using primers located 800 bp upstream and downstream of the ZFN target site. Bands significantly smaller than the 1.6 kb wild-type band indicate large deletions in the target locus. Four founders that were not identified in Figure 2 are underlined.
Figure 4

*Mdr1a* expression in homozygous knockout animals. (A) A schematic of *Mdr1a* genomic and mRNA structures around the ZFN target site in exon 7, marked with a solid black rectangle. Exons are represented by open rectangles with respective numbers. The size of each exon in base pairs is labeled directly underneath it. Intron sequences are represented by broken bars with size in base pairs underneath. The position of the 396 bp deletion in founder #23 is labeled above intron 6 and exon 7. RT-F and RT-R are the primers used in RT-PCR, located in exons 5 and 9, respectively. (B) *Mdr1a* expression in tissues. For RT reactions, 40 ng of total RNA was used as template. Normalization of the input RNA was confirmed by *GAPDH* amplification with or without reverse transcriptase. M, PCR marker; WT, wild-type mouse; F2, *Mdr1a* -/- mouse; K, kidney; I, large intestine; L, liver. Amplicon sizes are marked on the right. (C) Western blot analysis with large intestine. +, positive control, lysate from the human Mdr1-overexpressing SK-N-FI cells (ATCC, Manassas, VA). S3 (15 µl, 10 µl, and 5 µl loaded in each of the three lanes) and S4 (15 µl loaded), the third and fourth supernatant fractions of large intestine membrane preparations (see methods). Actin was used as a loading control. *Mdr1a*+/+, wild-type intestine; *Mdr1a*-/-, intestine from a homozygous knockout mouse derived from founder #23.

Figure 5

Identification and genotype of *Jag1* founders. (A) *Jag1* founders identified using the Cel-I mutation detection assay. M, PCR marker; 1-38, pups born from two injection sessions. The numbers of founders are underlined. The
sizes in base pairs of uncut and cut bands are labeled on the right. (B) Genotype of the Jag1 founders. Target site sequences of wild-type and founders are aligned. ZFN binding sites are in bold. A dash represents a deleted nucleotide. 1-4 bp of microhomology that was likely used by NHEJ is underlined. The frameshift (fs), exon skipping (es), or in-frame amino acid loss (if) resulting from each deletion is indicated to the right of each sequence.

Figure 6
Identification and genotype of Notch3 founders. M, PCR marker. (A) The Cel-I mutation detection assay was used to identify founders, whose numbers are underlined. (B) Large deletions were detected in founders 1 and 2. C. Genotype of the Notch3 founders. ZFN binding sites are in bold. A dash represents a deleted nucleotide. 1-4 bp of microhomology that was likely used by NHEJ is underlined. All deletions result in frameshift (fs), which is labeled to the right of each sequence.
Figure 1.

The diagram illustrates the process of ZFN (Zinc Finger Nuclease) binding to DNA, leading to double-strand breaks (DSB) at specific sites. The DSB can be repaired via two pathways: Correct Repair and NHEJ (Non-Homologous End-Joining).

- Correct Repair results in deletion or insertion of genetic material.
- NHEJ results in deletion or insertion of genetic material.

The DNA sequence shown is:

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5' - GCCATCAGUCCTGGTTGAGCTGCTGAGCGCAACATCAGUA-3'
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The FokI restriction enzyme recognition site is also indicated.
Figure 2.
Figure 3.
Figure 4.

a. gDNA:
RT-F | 5 6 | 7 8 | 9
1.7 kb 190 bp 172 bp 172 bp
52 bp 125 bp

mRNA (WT):
RT-F ← 5 6 7 8 9 9

mRNA (F2):
RT-F ← 5 6 6 9

b. b:

C.