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Using CRISPR/Cas9 engineering to generate a mouse with a conditional knockout allele for the promyelocytic leukemia zinc finger transcription factor

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Abstract

The promyelocytic leukemia zinc finger (PLZF) transcription factor mediates a wide-range of biological processes. Accordingly, perturbation of PLZF function results in a myriad of physiologic defects, the most conspicuous of which is abnormal skeletal patterning. Although whole body knockout of *Plzf* in the mouse (*Plzf*^{KO}) has significantly expanded our understanding of *Plzf* function *in vivo*, a conditional knockout mouse model that enables tissue or cell-type specific ablation of *Plzf* has not been developed. Therefore, we used CRISPR/Cas 9 gene editing to generate a mouse model in which exon 2 of the murine *Plzf* gene is specifically flanked (or floxed) by *LoxP* sites (*Plzf*^{f/f}). Crossing our *Plzf*^{f/f} mouse with a global cre-driver mouse to generate the *Plzf*^{d/d} bigenic mouse, we demonstrate that exon 2 of the *Plzf* gene is ablated in the *Plzf*^{d/d} bigenic. Similar to the previously reported *Plzf*^{KO} mouse, the *Plzf*^{d/d} mouse exhibits a severe defect in skeletal patterning of the hindlimb, indicating that the *Plzf*^{f/f} mouse functions as designed. Therefore, studies in this short technical report demonstrate that the *Plzf*^{f/f} mouse will be useful to investigators who wish to explore the role of the *Plzf* transcription factor in a specific tissue or cell-type.

Keywords

promyelocytic leukemia zinc finger; mouse; CRISPR/Cas9; homology directed repair; conditional allele; cre recombinase; skeletal patterning; hindlimb; polydactyly

1 INTRODUCTION

Promyelocytic leukemia zinc finger (PLZF; also known as ZBTB16 or ZNF145) is a member of the evolutionary conserved POK (POZ and Kruppel) family of pleiotropic C2H2-type zinc finger transcription factors; reviewed in (Liu et al., 2016; Suliman et al., 2012). At the N-terminus, the BTB/POZ domain is responsible for homo- and hetero-dimerization,

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chromatin remodeling and epigenetic transcriptional control, formation of high-molecular weight DNA-protein complexes, and nuclear sub-localization. Positioned within the C-terminal region of PLZF, the nine C2H2 zinc finger motifs facilitate direct sequence-specific DNA binding to target genes. Cell and signaling context dependent, PLZF can serve as a transcriptional activator or repressor (Fahnenstich et al., 2003; Gaber et al., 2013; Ikeda et al., 2005; Kommagani et al., 2016; Labbaye et al., 2002; Liu et al., 2016; Mao et al., 2016; Singh et al., 2015; Suliman et al., 2012; Szwarc et al., 2018; Wang et al., 2012). Given its pleiotropic effects, PLZF drives a broad spectrum of developmental processes and physiological responses, including limb skeletal patterning, innate immune cell development, hematopoiesis, and spermatogenesis; reviewed in (Suliman et al., 2012). Underpinning these physiologies, PLZF modulates stem cell self-renewal, cell-cell communication, proliferation, differentiation, and apoptosis; reviewed in (Suliman et al., 2012).

The most conspicuous phenotype displayed by the *Plzf* knockout (*Plzf*^{KO}) mouse is a severe defect in normal skeletal patterning of the hindlimb (Barna et al., 2000; Barna et al., 2005). This skeletal defect is also reported for the Luxoid (lu/lu) mouse (Green, 1955), which carries a naturally occurring nonsense mutation in the *Plzf* allele as well as for the rat with a naturally occurring cis-regulatory *Plzf* mutation ((lx/lx) (Kren, 1975; Liska et al., 2016)). The *Plzf*^{KO} mouse exhibits homeotic-like anterior-to-posterior transformations in the axial and appendicular skeleton, causing hindlimb abnormalities such as fused or extra digits, loss of the tibia or conversion of the fibula to a tibia-like structure, and kinked tails (Barna et al., 2000). Subsequent analysis also revealed that these mutant mice exhibit impairments in the development of hematopoietic stem cell and neural progenitors (Gaber et al., 2013; Vincent-Fabert et al., 2016), in spermatogonial self-renewal (Buaas et al., 2004; Costoya et al., 2004), and in development of innate-like features of invariant natural killer T (iNKT) cells (Kovalovsky et al., 2008; Savage et al., 2011; Savage et al., 2008; Xu et al., 2009). Important from a translational perspective, many of these phenotypes also arise in humans with loss-of-function *PLZF* mutations (Fischer et al., 2008), highlighting the evolutionary conserved role of PLZF.

Despite tremendous advances in our understanding of *Plzf* function using the *Plzf*^{KO} mouse, a conditional knockout mouse model that enables tissue or cell-type specific ablation of *Plzf* has yet to be developed. To address this issue, we describe in this short technical report the generation—through the use of Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR associated (Cas)9 genome editing—of a *Plzf* floxed (*Plzf*^{f/f}) mouse that is engineered to facilitate cre-mediated *Plzf* abrogation in a tissue or cell-type specific manner.

2 RESULTS AND DISCUSSION

We previously created conditional null alleles using a traditional homologous recombination strategy in mouse embryonic stem cells (Fernandez-Valdivia et al., 2010; Hashimoto-Partyka et al., 2006). However, due its speed, simplicity, precision, and significantly lower cost, the CRISPR/Cas9 genome engineering approach was used to generate a conditional *Plzf* knockout allele in the mouse. The strategy to create the conditional *Plzf* knockout allele was

to flox exon 2 of the murine *Plzf* gene since exon 2 encodes greater than 50% of the protein, which includes the initiating ATG, the N-terminal BTB/POZ domain, and the first two C2H2 zinc fingers of the DNA binding domain (Barna et al., 2000). Exon 2 was also chosen because knockout of this exon using conventional embryonic stem cell gene targeting methods results in a null *Plzf* allele (Barna et al., 2000). To insert LoxP sites to flank exon 2 of the mouse *Plzf* gene, Cas9 mRNA, two separate and validated single guide RNAs (sgRNAs) targeted to intronic sequences flanking exon 2, two single-stranded oligonucleotide (ssODN) donors, each containing the 34 base-pair LoxP consensus site flanked asymmetrically by homology arms to each of the desired insertion sites (Figure 1 (Richardson et al., 2016)) were co-injected into approximately 200 single-cell C57BL/6NJ pronuclear-stage zygotes before their transfer into ICR recipient pseudopregnant females. To minimize the probability of off-target events, only sgRNAs predicted to have off-target sites with three mismatches or more were used to target Cas9 endonuclease activity to intronic sequences flanking exon 2 (Figure 2a); the location of the 5' and 3' sgRNAs in relation to exon 2 of the mouse *Plzf* gene is shown in Support Information Figure S1.

Thirty-five pups were delivered by ICR dams and were PCR screened for the insertion of the 5' and 3' LoxP insertion into the *Plzf* allele. Table 1 lists the PCR primers used to genotype potential founder pups (F0). For genotyping each LoxP insertion site, one PCR primer in the primer-pair is located outside the sequences of the homology arms in each of the ssODNs; the position of these primers in relation to exon 2 is shown in Support Information Figure S2. From thirty-five pups screened, one potential male founder ((F0) #9950) carried both the 5' and 3' LoxP sites. One F0 pup (#9959) was positive for only the 5' LoxP insertion whereas three pups (F0 #9958; #9960; and #9964) were positive for only the 3' LoxP insertion (data not shown). To determine whether the 5' and 3' LoxP sites in the F0 #9950 mouse are located on the same *Plzf* allele (in cis) and can pass through the germline, the #9950 mouse was bred with C57BL/6NJ females. Screening progeny from this breeding clearly showed that the 5' and 3' LoxP sites are transferred through the germline in *cis* (Figure 2b). Sanger sequencing of the 5' LoxP and 3' LoxP sites using genomic DNA isolated from progeny of the F0 #9950 mouse confirmed that each of the LoxP sites is intact (Support Information Figure S3). Analysis of the top 5 potential off-target sites as scored by the CRISPR Finder tool (<https://www.sanger.ac.uk/htgt/wge/>) for Cas9 cleavage activity revealed no off-target activity (Support Information Figure S4). Importantly, mice homozygous for the conditional *Plzf* allele (*Plzf^{f/f}*) are a phenocopy of wild type siblings.

To determine whether cre-mediated excision of exon 2 can occur at the *Plzf^{f/f}* allele, we crossed our *Plzf^{f/f}* conditional mouse with a global cre-driver transgenic mouse in which the cytomegalovirus (CMV) promoter drives the expression of cre recombinase (CMV^{cre}) in all cell types (including germ cells) (Schwenk et al., 1995) to generate the *Plzf^{d/d}* bigenic (Figure 3a). Genotyping with PCR primers: P1, P2, and P3 (Table 1 and Support Information Figure S2), demonstrated absence of exon 2 in the *Plzf^{d/d}* mouse (Figure 3a, b). Because we have previously demonstrated that Plzf protein is significantly induced in uterine tissue of ovariectomized wild type mice following injection of progesterone hormone (Kommagani et al., 2016), we injected ovariectomized *Plzf^{d/d}* females and corresponding monogenic controls with either hormone vehicle or progesterone hormone. Western analysis clearly showed that while uterine Plzf protein is not expressed in the vehicle-treated

ovariectomized control and *Plzf^{fl/d}* group, Plzf protein expression is only induced in the control uterus six hours following progesterone administration (Figure 3c). The absence of uterine Plzf protein induction by progesterone in the ovariectomized *Plzf^{fl/d}* mouse confirms that cre-mediated excision of exon 2 in the *Plzf^{fl/f}* conditional allele results in abrogation of Plzf protein expression.

The tetrapod hindlimb of vertebrates consists of three regions: the proximal stylopod (femur), the intermediate zeugopod (fused tibia and fibula), and the distal autopod (the foot consisting of the tarsals, metatarsals, and phalanges) (Niswander, 2003). As reported for the *Plzf^{KO}* mouse (Barna et al., 2000), the *Plzf^{fl/d}* bigenic exhibits similar hindlimb abnormalities (Figure 4a, b). Unlike the control (Figure 4c), the *Plzf^{fl/d}* hindlimb develops only rudimentary tibia and fibula structures in the severely shortened zeugopod region (Figure 4d (bracket)). Because of the significant reduction in zeugopod length, the heel bone (calcaneum) and knee are often brought close together, resulting in the plantar surface of the foot twisted away from the ground (Figure 4d), rendering the afflicted hindlimb ineffective for ambulation. While pentadactyl is the normal digit configuration in tetrapods (Figure 5a), the *Plzf^{fl/d}* bigenic—as previously reported for the *Plzf^{KO}* mouse (Barna et al., 2000)—exhibits a pre-axial polydactyly foot phenotype, where supernumerary digits (in many cases fused) occur at the anterior margin of the autopod (Figure 5b-e). An additional abnormal skeletal feature of the *Plzf^{fl/d}* mouse is the presence of a kinked tail (Figure 6), a phenotype also reported for the *Plzf^{KO}* mouse (Barna et al., 2000). Although beyond the scope of the current study, further phenotypic analysis has demonstrated that *Plzf^{fl/d}* male and female mice are infertile (also reported previously (Barna et al., 2000)).

In conclusion, we have successfully generated a new conditional knockout *Plzf^{fl/f}* mouse model using CRISPR/Cas9 genome editing. We predict that the *Plzf^{fl/f}* mouse will serve as an invaluable tool with which to further elucidate *Plzf* function in a tissue and cell-type specific manner. For example, we recently demonstrated that PLZF is essential for human endometrial stromal cell decidualization (Kommagani et al., 2016; Szwarc et al., 2018), a cellular process required for embryo implantation. Crossing the *Plzf^{fl/f}* mouse with our previously reported progesterone receptor promoter-driven cre (*Pgr^{cre}*) knockin mouse (Soyal et al., 2005) will now allow us to functionally validate Plzf action specifically in the mouse endometrium, without interference of phenotypes contributed by other target tissues due to this transcription factor's loss-of-function.

3 MATERIALS AND METHODS

3.1 Generation of the *Plzf^{fl/f}* mouse using CRISPR/Cas9 gene editing

Two separate sgRNAs and two ssODNs with LoxP sequences were used to insert (or knock-in) a LoxP site 5' and 3' of exon 2 of the mouse *Plzf* gene. Encoding more than 50% of murine Plzf protein, exon 2 of the *Plzf* gene was selected based on the targeting strategy previously reported by the Pandolfi group (Barna et al., 2000) and on the conditional knockout vector design detailed by the International Mouse Phenotyping Consortium/ Knockout Mouse Project (IMPC/KOMP (*Zbtb16^{tm405544a(L1L2_Bact_P)}*; www.informatics.jax.org/marker/MGI:103222)). Established methods were used to co-microinject 100ng/μl Cas9 mRNA, 20ng/μl validated sgRNA (each) and 100ng/μl of

ssODNs (each) into the cytoplasm of 200 C57BL/6NJ embryos (Lanza et al., 2018). Following micro-injection, zygotes were transferred into pseudopregnant ICR recipient females at approximately 25–32 zygotes per recipient. Both C57BL/6NJ and ICR mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Established methods of genotyping potential founder mice were followed (Lanza et al., 2018). Also, Sanger sequencing of cloned LoxP sites from mouse genomic DNA described previously were followed in these studies (Lanza et al., 2018).

3.2 Analysis of off-target Cas9 activity

The top five potential off-target sites for each sgRNA in the conditional targeting of the mouse *Plzf* gene were identified using the Wellcome Trust Sanger Institute Genome Editing website (<http://www.sanger.ac.uk/htgt/wge/>). Flanking PCR primers designed to amplify 80–180 base pair amplicons are listed in Support Information Figure S4 with the location, sequence, and number of mismatches from the original sgRNA. Genomic DNA from F2 mice was prepared as previously described (Lanza et al., 2018) and assayed by High Resolution Melt Analysis using the MeltDoctor HRM Master Mix (ThermoFisher Scientific Inc., Waltham, MA (#4415440)) according to the manufacturer's protocol and run on the Applied Biosystems QuantStudio 7 Flex Real-Time PCR System (ThermoFisher Scientific Inc.). At least two wild-type samples were analyzed concurrently with the test samples. For any sample with a HRM analysis result deviating from the wild-type sample, suggesting a mutagenesis event, PCR products were cloned and Sanger sequenced as described above.

3.2 Generation of the *Plzf^{d/d}* bigenic mouse

To generate the *Plzf^{d/d}* bigenic mouse, the *Plzf^{f/f}* mouse was crossed with the *CMV^{cre}* mouse in the C57/BL6 inbred strain (The Jackson Laboratory, Bar Harbor, ME (stock number: 006054; strain name B6.C-Tg(CMV-cre)1 Cgn/J (Schwenk et al., 1995))). Through cre-mediated excision in this bigenic, the floxed *Plzf* gene is ablated in all *Plzf^{d/d}* tissues, including germ cells. Note: in the *CMV^{cre}* mouse, the cre gene is X-linked and therefore transgene transmission through males is restricted to female progeny.

Mice were housed in an AAALAC accredited *vivarium* at Baylor College of Medicine. In temperature-regulated rooms (22 ± 2°C) with a cycling 12-hour lights-on:12-hour lights-off schedule, mice were fed irradiated Tekland global soy protein-free extruded rodent diet (Harlan Laboratories, Inc., Indianapolis, IN) and fresh water *ad libitum*. Pre-approved experimental protocols applied to mice were conducted in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals ("The Guide" (Eighth Edition 2011)), published by the National Research Council of the National Academies, Washington, D.C. (www.nap.edu). For all animal procedures described in this study, prior approval from the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine was obtained. As with our previous mouse models, the *Plzf^{f/f}* mouse will be made freely available to the scientific community upon request.

3.4 Whole-mount skeletal staining

To assess skeletal patterning, an established Alcian blue and Alizarin red staining protocol was followed (Rigueur and Lyons, 2014). Following euthanasia, skin, internal organs, and

brown fat were removed from monogenic control and *Plzf^{d/d}* mice. After a rinse with 1XPBS, tissues were dehydrated and fixed in two changes of 95% ethanol over a 16-hour period. Tissues were then placed in 100% acetone for further fixation and removal of adipose tissue. Following 48 hours of acetone incubation, Tissues were transferred to 0.03% Alcian blue solution (dissolved in 80% ethanol and 20% glacial acetic acid) for 24 hours to stain cartilage. For de-staining purposes, tissues were washed in two changes of 70% ethanol before incubation in 95% ethanol overnight. Following ethanol washes, soft tissue was hydrolyzed in 1% potassium hydroxide (KOH dissolved in distilled water) overnight to pre-clear the tissue and allow visualization of stained skeletal structures. Skeletal specimens were then incubated in 0.005% Alizarin red solution (in 1% KOH) for 2–5 days for bone staining. Following bone staining, skeletal specimens were transferred to a 1% KOH clearing solution. For long-term storage, stained skeletons were stored in 100% glycerol.

3.5 Hormone treatment and immunoblot analysis

To assess uterine Plzf protein induction by short-term progesterone exposure, female mice were ovariectomized at six weeks-of-age and rested for two weeks to void remaining circulating levels of ovarian steroid hormones (Kommagani et al., 2016). Following two weeks of rest, ovariectomized mice were then subcutaneously injected with 1mg of progesterone (Sigma-Aldrich, St. Louis, MO; in 100µl sesame oil) or 100µl vehicle. Mice were euthanized six hours later before uteri were removed for protein isolation using standard approaches (Kommagani et al., 2016). As detailed previously (Kommagani et al., 2016), the following primary antibodies were used for western analysis: a rabbit polyclonal anti-Plzf H-300 (Santa Cruz Biotechnology Inc., Dallas, TX (# SC-22839)) and a mouse monoclonal anti-β-actin AC-74 (Sigma-Aldrich; #A2228). Corresponding horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology Inc.) were used as secondary antibodies and the chemiluminescent signal was visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (ThermoFischer Scientific Inc., #34580).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a

5' LoxP single stranded oligonucleotide design

Target site

```

2501 CTCATGGTGG AACTGGCCT TATCCAGGAA GGCTCTGCTC TCAGGCTCGA
      GAGTACCACC TGTGACCGGA ATAGGTCCTT CCGAGACGAG AGTCCGAGCT
                        5' target site
2551 AGAATGCCTA GGATTCATTG CAATCATCAT TTCGCTTTAT CCGATGGATG
      TCTTACGGAT CCTAAGTAAC GTTAGTAGTA AAGCGAAATA GGCTACCTAC
                        Distal Arm (36)
2601 CGTGCTTCAG AGCAGTGGGG GAAAGAGTGT GGGGAAGCGC AAACCTTCCT
      GCACGAAGTC TCGTCACCCC CTTTCTCACA CCCCTTCGCG TTTGGAAGGA
                        Proximal Arm (91)
2651 TTTCTCTCA GCGTGTGGA AAGGATGATG GTACCTTCCT AGGAAGCCCA
      AAAGGAGAGT CGCACAACT TCTCTACTAC CATGGAAGGA TCCTTCGGGT
  
```

Asymmetric ssODN sequence (shown 5' to 3')

```

1  TACCATCATC CTTTCCAACA CGCTGAGAGG AAAAGGAAGG TTTGCGCTTC
      Proximal Arm (91)
51 CCCACACTCT TTCCCCACT GCTCTGAAGC ACGCATCCAT CATAACTTCG
      LoxP
101 TATAATGTAT GCTATACGAA GTTATGGATC CCGATAAAGC GAAATGATGA
      Distal Arm (36)
151 TTGCAATGAA TCCTAGGC
  
```

b

3' LoxP single stranded oligonucleotide design

Target site

```

4551 AGTGGGGTTC AGGCTGAGGG TCTCCTCTGA TCTGGATCTA GCCATGGTAT
      TCACCCCAAG TCCGACTCCC AGAGGAGACT AGACCTAGAT CGGTACCATA
                        3' target site
4601 CAGTCTAGCT TTTTCTTTGC CCACTGTGG CAGCCTATGA TGAAGCAGG
      GTCAGATCGA AAAAGAAACG GGTGACAGCC GTCGGATACT ACTTTCGTCC
                        Distal Arm (36)
4651 GGTGTTGGGAT CGTTTAAGTC CTGTATGTAC CCATAGGTCT CAAGTGCTTC
      CCAACCCCTA GCAAAATTCAG GACATACATG GGTATCCAGA GTTCACAGAG
                        Proximal Arm (91)
4701 TTTTGGCAGG CACATTGTGA GGCAGAGAAA GCTTTAGTTA AATTTAAGGA
      AAAACCGTCC GTGTAACACT CCGTCTCTTT CGAAATCAAT TTAATATCCT
  
```

Asymmetric ssODN sequence (shown in 5' to 3')

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1  TAAAGCTTTC TCTGCCTCAC AATGTGCCTG CCAAAAGAGA CACTTGAGAC
      Proximal Arm (91)
51 CTATGGGTAC ATACAGGACT TAAACGATCC CCAACCCCTG CATAACTTCG
      LoxP
101 TATAATGTAT GCTATACGAA GTTATGGATC CTTTCATCAT AGGCTGCCGA
      Distal Arm (36)
151 CAGTGGGCAA AGAAAAA
  
```

FIGURE 1.

Design and location of the 5' and 3' ssODNs used to generate the mouse *Plzf* conditional knockout allele. (a) Sequence of the 5' target site in intron 1 of the mouse *Plzf* allele. Sequences highlighted in gray (36 base pairs (bp)) and blue (91bp) represent the asymmetric homology arms that are distal and proximal relative to the location of the 3' protospacer adjacent motif (PAM). The ssODN is complementary to the non-target strand. The sequence of the 5' target site is highlighted in green and underlined; the 3' PAM (TGG) site is in red. Box includes the sequence (in the 5' to 3' direction) of the ssODNs for CRISPR/Cas9 initiated homology directed repair. The LoxP sequence is highlighted in yellow along with a novel *BamHI* restriction site in red; the underlined text shows the target sequence disrupted

by the LoxP sequence/*Bam*HI site in the ssODN donor. The proximal and distal homology arms are highlighted in blue and gray respectively. (b) Sequence of the 3' target site in intron 2 of the mouse *Plzf* allele. The distal and proximal asymmetric homology arms are highlighted in gray and green respectively. The location and sequence of the 3' target site is highlighted in blue and underlined with the 3' PAM in red (AGG). Box includes the sequence of the ssODN donor used to insert the 3' LoxP site into intron 2. The underlined target sequence is disrupted by the LoxP sequence is in yellow and with an additional 3' *Bam*HI restriction site in red, which is flanked by the proximal and distal homology arms (green and gray respectively).

FIGURE 2.

Generation of the mouse *Plzf^{fl/fl}* conditional knockout allele. (a) Quality scores for the 5' and 3' Cas9 guide sequences (sense strand) used to create the murine *Plzf* conditional knockout allele are shown. Note: *in vitro* transcribed sgRNAs were microinjected into C57BL/6NJ zygotes. (b) Schematic of the CRISPR/Cas9-mediated targeting strategy to simultaneously insert Lox P sites (open triangles) into intron 1 and 2 to flank *Plzf* exon 2. The location of the forward and reverse PCR primers to amplify the 5' and 3' LoxP sites is indicated. The restriction sites: *Xba*I, *Bam*HI, *Sma*I, and *Eco*RI are denoted by Xb, B, Sm, and E respectively. Using forward and reverse PCR primers (F1 and R1 to amplify the 5' LoxP site (200bp) and F2 and R2 to amplify the 3' LoxP site (200bp)), the gel shows a typical

genotype result for wild type (lane 1 (160bp band)), wild type/ LoxP (lane 2 (heterozygote (*Plzf^{f/+}*) 200 and 160bp bands)), and LoxP/LoxP (lane 3 (homozygous (*Plzf^{f/f}*) for the 5' and 3' LoxP insertion) 200 bp band)). This PCR result is from genotyping F2 generation mice derived from the F0 #9950 chimeric male mouse. Sequences are shown for 5' and 3' LoxP sites carried by these mice (also see Support Information S3).

FIGURE 3.

Generation of the *Plzf*^{d/d} bigenic mouse. (a) Schematic which details the breeding scheme to generate the *Plzf*^{d/d} bigenic mouse. The *CMV*^{Cre} transgenic mouse was crossed with *Plzf*^{f/f} mice to generate the *CMV*^{Cre}:*Plzf*^{f/f} (or *Plzf*^{d/d} bigenic). The location of the P1, P2, and P3 PCR primers to detect loss of *Plzf* exon 2 in the *Plzf*^{d/d} bigenic is shown (also see Support Information Figure S2). (b) Genotyping results using P1 and P2 forward and reverse PCR primers and P1 and P3 forward and reverse PCR primers demonstrate absence of exon 2 in the *Plzf*^{d/d} bigenic mouse. (c) Western analysis confirms that uterine *Plzf* protein is not produced in the *Plzf*^{d/d} mouse. Lanes 1 and 2 represent protein isolated from the uterus of untreated and progesterone-treated ovariectomized wild type mice respectively. Note the

significant induction of Plzf protein (75 kDa) expression (after 6-hours) in response to progesterone administration (Kommagani et al., 2016). Lanes 3 and 4 represent uterine protein isolates from untreated and progesterone-treated *Plzf^{fl/d}* mice respectively. Note the clear absence of Plzf protein induction by progesterone in the *Plzf^{fl/d}* mouse (lane 4). Each lane represents uterine protein pooled from four female mice per genotype; the experiment was performed in triplicate (β -actin (43 kDa) served as a loading control).

FIGURE 4.

The *Plzf^{d/d}* bigenic displays hindlimb abnormalities. (a) Alcian blue and Alizarin red stained skeleton of a monogenic control mouse. (b) Similarly stained skeleton of *Plzf^{d/d}* mouse. Note the hindlimb defect in the *Plzf^{d/d}* mouse (arrow and bracket). (c) Larger view of hindlimb of control mouse, showing the femur (stylopod), the patella (knee cap), the fused tibia and fibula (intermediate zeugopod), the calcaneum (heel bone), and the foot (distal autopod); the plantar surface of the foot is indicated by a straight black line. (d) The *Plzf^{d/d}* hindlimb displays a severe shortening of the zeugopod (bracket), with either an absence or the presence of a rudimentary tibia or fibula. Because the zeugopod region of the *Plzf^{d/d}*

hindlimb is significantly curved, the plantar surface of the foot is twisted away from the ground.

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FIGURE 5.
The *Plzf^{d/d}* hind foot displays fused digits and polydactyly. (a) Macro view of hind foot of monogenic control mouse. (b) Similar view of the *Plzf^{d/d}* hind foot. Note the fusion of three digits (arrowheads and bracket). Compared to control, the *Plzf^{d/d}* interdigital space is significantly larger (region encompassed by curved bracket). (c-e) Distal autopod skeletal region stained with Alcian blue and Alizarin red. Compared to control (c), note examples of fused digits (arrowhead) and polydactyly in the *Plzf^{d/d}* hind foot (d and e respectively).

FIGURE 6.

The *Plzf^{d/d}* mouse exhibits kinked tails. (a) Macro view of a control tail (1) and *Plzf^{d/d}* mutant tails (2 and 3). Note the obvious kink in the *Plzf^{d/d}* tail (arrowhead). (b-d) Corresponding Alcian blue and Alizarin red stained tailbone structure from control (c) and *Plzf^{d/d}* mutant mice (c and d); arrowheads indicate location of the tail kink.

Table 1.

List of PCR primers used for genotyping.

F1 Primer	5'-TTGTGAGCCTCATGGTGGAC-3'
R1 Primer	5'-AAAGGAAGGTTTGCCTTCC-3'
F2 Primer	5'-AGGGTCTCCTCTGATCTGGA-3'
R2 Primer	5'-TCTGCCTCACAATGTGCCTG-3'
P1 Primer	5'-GAAGATGAGGGCTTGCCCAT-3'
P2 Primer	5'-TTCTCTGCTCTGCAAGGTGG-3'
P3 Primer	5'-GAGATGGCATGAGAAGAGGCT-3'