GENOME RESEARCH

Supplemental Materials

Generation of mouse conditional knockout alleles in one step using the *i*-GONAD method

Renjie Shang[1,2], Haifeng Zhang[1], Pengpeng Bi[1,2,*]

[1]Center for Molecular Medicine, University of Georgia, Athens, GA 30602, USA

[2]Department of Genetics, University of Georgia, Athens, GA 30602, USA

*Correspondence: pbi@uga.edu

Corresponding Author: Pengpeng Bi

Email: pbi@uga.edu

Telephone: (706) 542-7768

This PDF file includes:

Materials and Methods

Supplemental Figs S1–S11

Materials and Methods

gRNA and ssODNA sequences

Fosl1 5’ gRNA: CCGGACGCTTGTCATCTCAT.

Fosl1 3’ gRNA: AGAGGGTCGCCCCATTCTTC.

Fosl1 5’ ssODNA:
AGGAAGATCAAAGTTTACAGAGAGGGAAAGCATTTCAGGAAAGAAACCAGCAGTAAAGCT
AGGTGTGTGTGAGCTGGAAATGTCACCAATGATAACTTCGTATAGCATACATTATACGAAGT
TATAGATGACAAGCGTCCGGTACAGAGAAGCAACTGTGG.

Fosl1 3’ ssODNA:
ACCATTTGGAACAGGAGTTCCAGGTTCCAGTCTCAGCATTGACCTTGAAGAAACTACCATAT
GTTTTTGGACTTCCATATTCTCACCTGAAATAACTTCGTATAGCATACATTATACGAAGTTAT
GAATGGGGCGACCCTCTGCTCTTCCTTGTCTCCTAT.

Plagl1 5’ gRNA: GATTGCAGGCTTGAATACGG.

Plagl1 3’ gRNA: ATGGAACCATCTATGAGACC.

Plagl1 5’ ssODNA:
CTGGGTATCTGCAGAGAGAAGAGGAACAGATTTCTCACATCAGCTATTGGAAAACTGTATCC
CCCCACCAGCACCAGCATCAGCACCACCGATAACTTCGTATAATGTATGCTATACGAAGTTA
TTATTCAAGCCTGCAATCAGTATATTCTTTTAAGAGG.

Plagl1 3’ ssODNA:
ATCTCAGAGCAGTGAATTTGATCCCGTGAGGCTATCTTGCTGCTTCATTGTCTACTCTAGGTC
AGGTCAACTGTGTCTTATGGGCCCTGGTATAACTTCGTATAATGTATGCTATACGAAGTTATC
TCATAGATGGTTCCATAGTGCAAGGTTTCCCCATT.

Ak040954 5’ gRNA: ACCCTTATAAAATAACCTCG.

Ak040954 3’ gRNA: GTATCTGGCGATGTGTTTAG.

Ak040954 5’ ssODNA:
CAAACTCTTGCCTGCTGGGTCCCCAGCAAGCCTTACAGTCCTGATGTCTGCTCTAACACATTT
GCACTCCACCTCTGGTTCCACACCTCTAATAACTTCGTATAGCATACATTATACGAAGTTATA
ACACATCGCCAGATACCCAAAGTCATCAGTGAACA.

Ak040954 3’ ssODNA:
TGTTTTTCTTTATGCATCTCAAAGGATCCTGTACTGCTTTCAAAGGAACCACTGCACCACACG
GTTCCTGTCTTCCTGTCTTTCTCCACGAATAACTTCGTATAATGTATGCTATACGAAGTTATG
GTTATTTTATAAGGGTAAAGCTCAGGTCTTACTTA.

Clcf1 5’ gRNA: GTCAGCACGTCCCACCCGAG.

Clcf1 3’ gRNA: GTACACAGGCCTTGGAGTAC.

Clcf1 5’ ssODNA:
ATCATTTGCATGGAGGAGACAGGTGAAGCAGTGGGGTGATGAGTTCTCCTATGGAGAAGTG
CAGAGAGAGAAAACCACGAAGGAGCCACTCATAACTTCGTATAATGTATGCTATACGAAGT
TATGGGTGGGACGTGCTGACTGTCTGGGTGGAGAATGCC.

Clcf1 3’ ssODNA:
AAACACACACAGCTATCTGGGAGGACCAAGGCATAGTGGGTGTGGCAAGGGAGTGGATGGG
TTGCTCCAGGGCCAGCAGTAAGAGCCTGTAATAACTTCGTATAATGTATGCTATACGAAGTT
ATCTCCAAGGCCTGTGTACCTCTATGGTATAAAGAGAC.

Gm44386 5’ gRNA: CACCCTCAAACTTGATACAT.

Gm44386 3’ gRNA: GACCACAATGCAATTTACAT.

Gm44386 5’ ssODNA:

TCAAAGCAATTACTATATTAATTATTACTATATTATCACCAAAGGGGTCAGGACCCAAAGGT
TGAGAACCCCTGTTCTGGGTCCTCCTATGATAACTTCGTATAATGTATGCTATACGAAGTTAT
TATCAAGTTTGAGGGTGGCTTGGGCTACATGAGACT.

Gm44386 3' ssODNA:
CATCATGAGTTCCAAGCCAGCCTGGACAGAAGGAGCCCCTGTTAGAGATGTAGAGCCATGC
AAGCATGAAGACCTGAGTTTATAACCCATGATAACTTCGTATAGCATACATTATACGAAGTT
ATTAAATTGCATTGTGGTCAGGCAGTGGTGGCACATGC.

Mouse genotyping primer sequences

Gene	Primer name		Sequence (5'–3')	Expected amplicon size
Fosl1	5' *loxP*	Forward	TCCACAGATGAGGAGGCTGA	WT 392 bp, *loxP* 426 bp
		Reverse	TGGGCTGATCTAGGTTGGGA	
	3' *loxP*	Forward	AGAAGCGCTTAGCTTCAGGG	WT 317 bp, *loxP* 351 bp
		Reverse	TGCTTGCACTCCAGAGCATT	
	Long-range PCR		5' *loxP*-F + 3' *loxP*-R	WT 2,115 bp
Plagl1	5' *loxP*	Forward	AGAAAGGGAGGATGGGTTGC	WT 278 bp, *loxP* 312 bp
		Reverse	TGAGGAACTGCTGTGCCATT	
	3' *loxP*	Forward	GGGTTGAAGCCAGTGACAGT	WT 200 bp, *loxP* 234 bp
		Reverse	TTTGGGGAGACTGCCTTTCC	
	Long-range PCR-F		GCAACTAGACTCCATGTCCCC	WT 8,441 bp
	Long-range PCR-R		AGTTGTGCTTGGTGCTGAGT	
Ak040954	5' *loxP*	Forward	CTCCCTCTTCCCTGACCAGA	WT 327 bp, *loxP* 361 bp
		Reverse	TTGCCCTCCACTGATTGTCC	
	3' *loxP*	Forward	GGCCACAGTTCTGCTGGTAT	WT 357 bp, *loxP* 391 bp
		Reverse	CAGGCCTCTCTTTGTCCTGG	
	Long-range PCR		5' *loxP*-F + 3' *loxP*-R	WT 3,755 bp
Clcf1	5' *loxP*	Forward	CTACCTCTCCAGCCTGGTCT	WT 355 bp, *loxP* 389 bp
		Reverse	AGTCAGGCTCGTTGAAAGGG	
	3' *loxP*	Forward	ACCCTAACCCTACCTGCCAT	WT 188 bp, *loxP* 222 bp
		Reverse	CCAGAGGCAAGGAAGACTGG	
	Long-range PCR		5' *loxP*-F + 3' *loxP*-R	WT 1,977 bp
Gm44386	5' *loxP*	Forward	GGAACAGGCTGTCACAGGAG	WT 397 bp, *loxP* 431 bp
		Reverse	GAAGCTGGAGATCAGAAGGCA	
	3' *loxP*	Forward	AGGAGCCCCTGTTAGAGATGT	WT 381 bp, *loxP* 415 bp
		Reverse	GTGTGGGGATATGCTGTCTGA	
	Long-range PCR		5' *loxP*-F + 3' *loxP*-R	WT 4,122 bp
Mecp2	5' *loxP*	Forward	CCCAGCTTGACCCAAGGATA	WT 287 bp, *loxP* 327 bp
		Reverse	GGCTGAAGGTTGTAGTGGCT	
	3' *loxP*	Forward	AGGTGGGTAGGAAGGCTAGG	WT 218 bp, *loxP* 258 bp
		Reverse	CTCCTCTGTACTCCCTGGCT	
	Long-range PCR		5' *loxP*-F + 3' *loxP*-R	WT 773 bp
Pax7^{CreER}	10221-*Pax7*^{CE}		ACTAGGCTCCACTCTGTCCTTC	WT 724 bp, *Pax7*^{CreER} 231 bp
	10222-*Pax7*^{CE}		GCAGATGTAGGGACATTCCAGTG	

Genotyping primers were designed to detect indels in predicted off-targeting sites. The primer sequences are listed in the table below.

Target gene	Off-target of gRNA	Sequence (5'–3')	WT size (bp)
Fosl1	#1	CGCTCACTTGGAGAAATGCC	370
		TGACAACATAGAACTGTGTAGGTTT	
	#2	CCAGCCTGTTTCTTTCCCCA	270
		GGTCATCTTCTGCCCCTGAC	
Plagl1	#3	AGCCCCATACAAACATCGGG	388
		ACCTTGCACTCTGTCTACGC	
	#4	ACAGAATGCATCAAGTGCTATG	369
		GTTCCTCAGATGCTGTGCCT	
Ak040954	#5	ACAGAGAGCTCAGGCATTGG	117
		TCACCGAGGTAACTTTGCCC	
	#6	CTACCCTGTGTCCCAAGCTG	206
		GCTTGAGTCCAAGACCAGCA	
Clcf1	#7	TGGGAAGTTAAGTTCGCGGG	293
		TACAGACCTCCGAACGTCCA	
	#8	TGGGGTACATTGCTGGTCAC	249
		AGCAAATGCAGGCTGAGCTA	
Gm44386	#9	ACACTATGGGGCACTGAAGC	282
		ATGCTAATGCCTGTCGTGCT	
	#10	CATTGGCACCCTTGTTGGTG	108
		GCTTACAGATGTAGCACTGGC	

Cell cultures and transfection

10T1/2 fibroblasts were maintained in 10% FBS with 1% penicillin/streptomycin in DMEM. Lentiviruses expressing Cas9 and gRNA were packed by co-transfections of pLenti-V2 (a gift from Feng Zhang, Addgene, 1000000052) with psPAX2 and pMD2.G plasmids into Lenti-X 293T cells (Takara Bio, 632180). Two days after infection, genomic DNA was extracted for genotyping. Primary myoblasts were isolated from adult muscle tissues by enzymatic digestions as previously described (Bi et al. 2016). Pre-plating was performed to enrich myoblast and separate fibroblasts. To activate CreER, 2 μM 4-hydroxytamoxifen (Cayman Chemical, 14854) was added to cell culture for 48 hrs before collecting RNA for gene expression analysis.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from mouse tissue or cells with TRIzol (Invitrogen). cDNA was synthesized using iScript™ Reverse Transcription Supermix (Bio-Rad, 1708841). Gene expression was assessed using standard qPCR approaches with KAPA SYBR FAST qPCR Master Mix (KK4605). Analysis was performed on a QuantStudio 3 PCR System (Thermo Fisher Scientific) with the following primers:

Fosl1-F1: 5'- ATGTACCGAGACTACGGGGAA -3' (in exon1),

Fosl1-R1: 5'- CTGCTGCTGTCGATGCTTG -3' (in exon2).

This pair of primers was used to generate Supplemental Fig. S1A.

Fosl1-F2: 5'- CCAGGACCCGTACTTGAACC-3' (in exon4),

Fosl1-R2: 5'- AGGAGTGTAGGAGAGCCCAG-3' (in exon4).

This pair of primers was used to generate Fig. 6D.

18s-F: 5'- ACCGCAGCTAGGAATAATGGA -3',

18s-R: 5'- GCCTCAGTTCCGAAAACCA -3'.

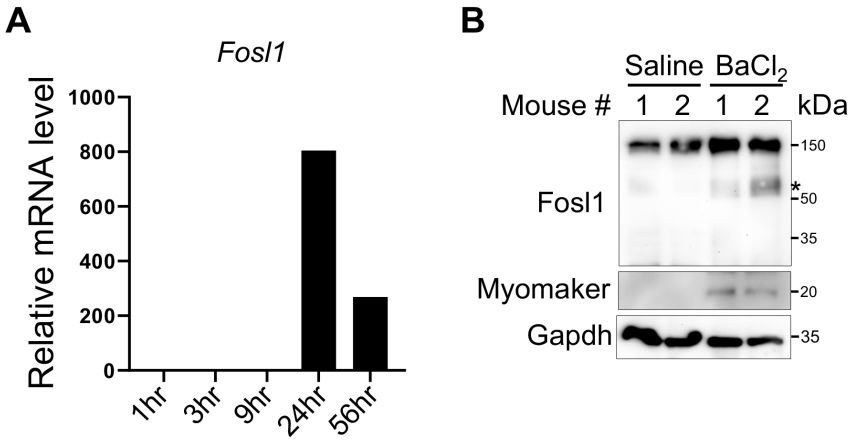
The $2^{\Delta\Delta Ct}$ method was used to analyze the relative changes in gene expression normalized against *18s* rRNA expression.

Western blotting analysis

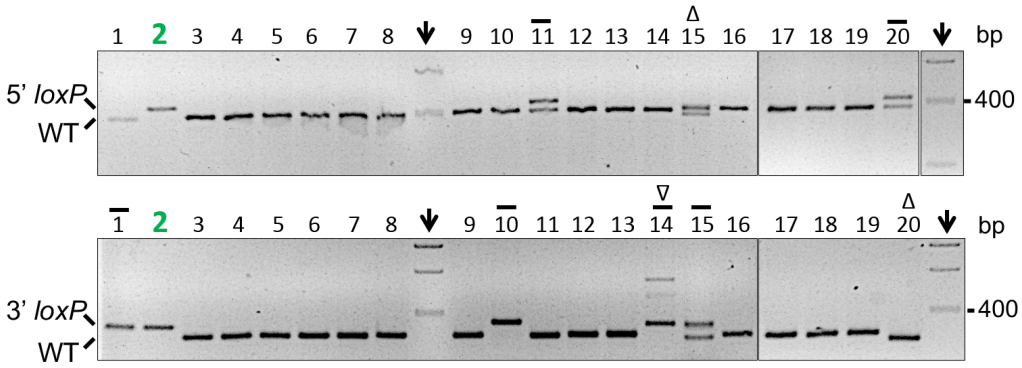
Protein was isolated from tissues using RIPA buffer (Sigma-Aldrich, R0278). Protein concentrations were determined using BCA Protein Assay Reagent (Thermo Fisher Scientific, 23225), followed by measurement with NanoDrop. Protein samples were mixed with 4X Laemmli sample buffer (Bio-Rad, 161-0747) and 20-40 μg protein was loaded and separated by SDS-PAGE gel electrophoresis. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore), blocked in 5% fat-free milk for 1 hour at room temperature, and then incubated with the following primary antibodies diluted in 5% milk overnight at 4°C: Gapdh (Thermo Fisher Scientific, MA5-15738), Myomaker (customized, mouse monoclonal), Fosl1 (Santa Cruz Biotechnology, sc-28310). The HRP-conjugated secondary antibodies: Donkey anti-sheep IgG-HRP (Santa Cruz Biotechnology, sc-2473), Goat Anti-Mouse IgG (H+L)-HRP Conjugate (Bio-Rad, 170-6516) and Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad, 170-6515) were diluted at 1:5,000. Immunodetection was performed using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, sc2048).

Requests for materials

Materials include mouse mutants generated in this study can be requested by contacting the corresponding author.

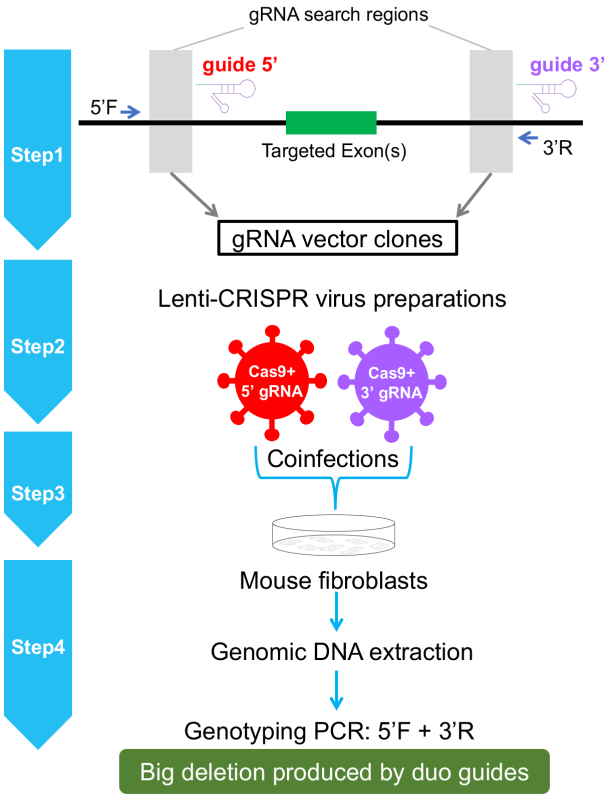


Supplemental Fig. S1. Induction of *Fosl1* expression in muscle tissue following $BaCl_2$ induced injury. (*A*) qPCR measurements of *Fosl1* mRNA levels at different hours (hr) following muscle injury. (*B*) Western blotting results of muscle tissues at 48 hrs post injury, * highlights the specific band. Myomaker blot serves as a positive control of injury response.

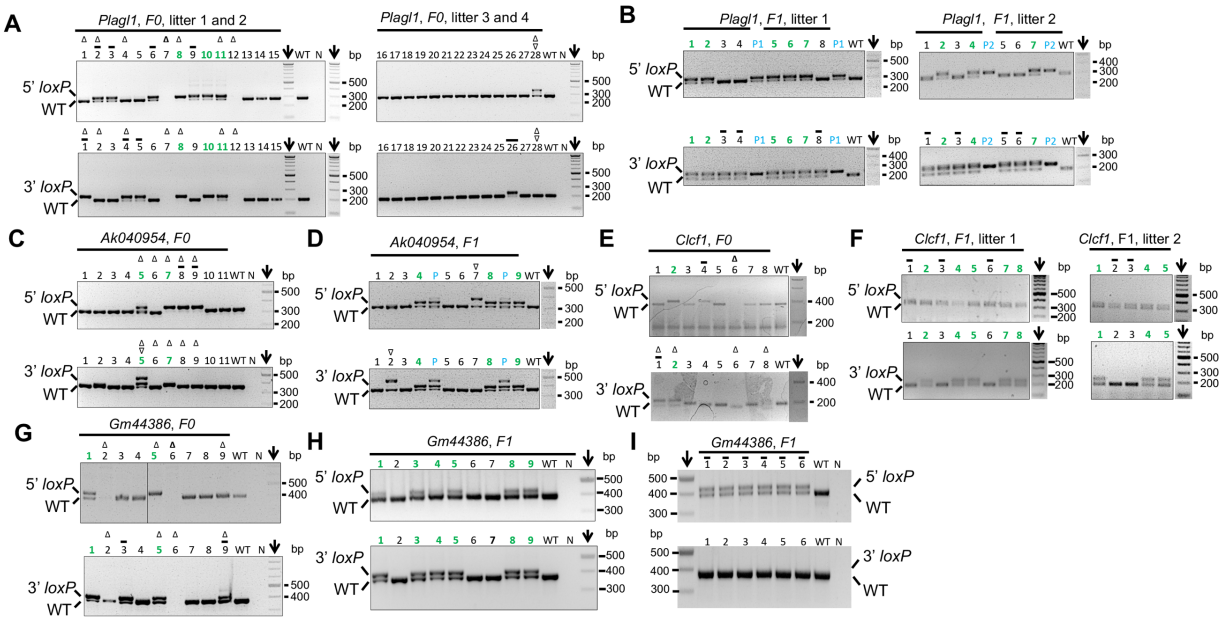


Supplemental Fig. S2. *Fosl1* genotyping results of the *F0* generation.

Detection of 5'–*loxP* (top row) and 3'–*loxP* (bottom row) insertions using primer pairs 1F&1R and 2F&2R shown in Fig. 1*B*. 5'– and 3'–*loxP* band sizes are 426 bp and 351 bp, respectively. #2 founder mouse that showed simultaneous 5'– and 3'–*loxP* insertions is highlighted in green; arrow, DNA marker lane; mouse with single *loxP* integration is indicated with a bar on top of ID; Δ, deletions; ∇, large insertions.

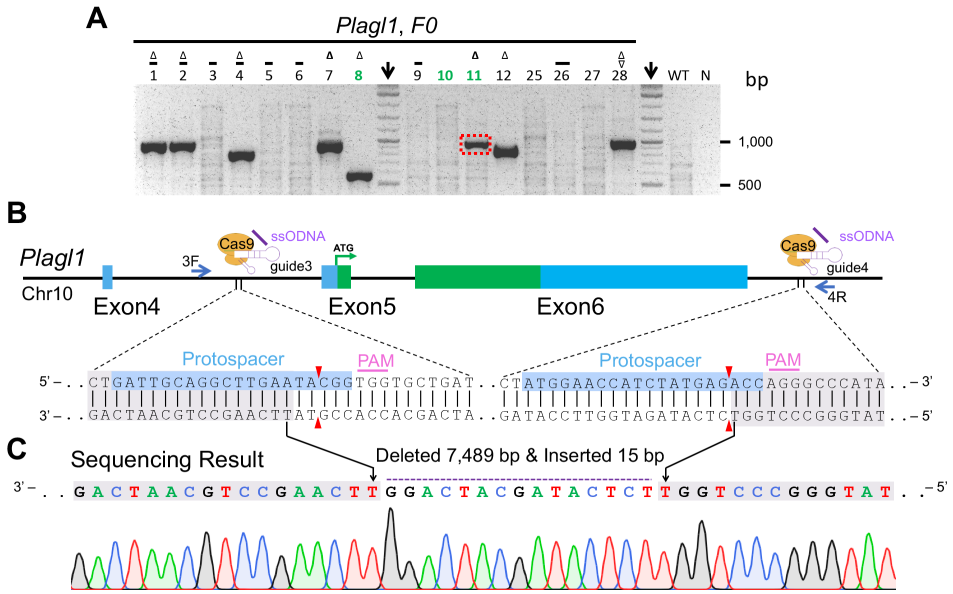


Supplemental Fig. S3. A workflow to test gRNA editing efficiency in mouse fibroblasts. Candidate gRNAs with high on-target scores and low risk of off-targeting were cloned into lenti-CRISPR vectors. Efficiency of each gRNA pair (one gRNA from each intron) was tested in fibroblasts. Two days after infections, genotyping PCR was performed with primers 5'F + 3'R. The gRNA pair that can efficiently generate large truncations were chosen for *in vivo i*-GONAD experiments for *loxP* insertions.

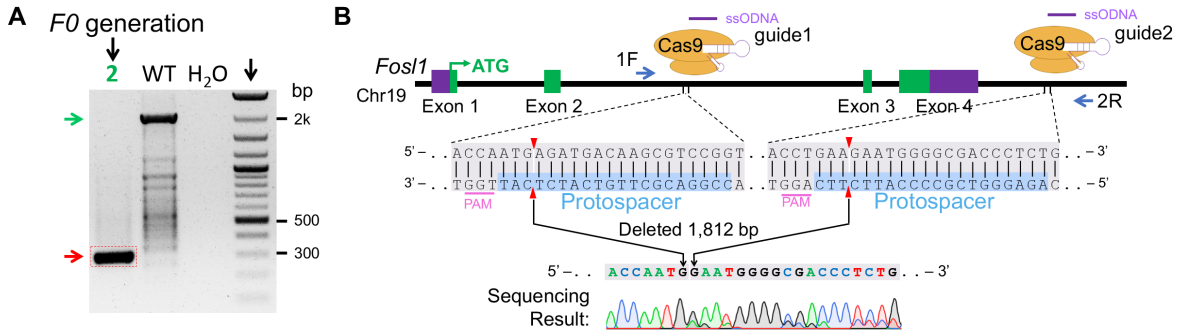


Supplemental Fig. S4. Genotyping results of *F0* and *F1* generations.

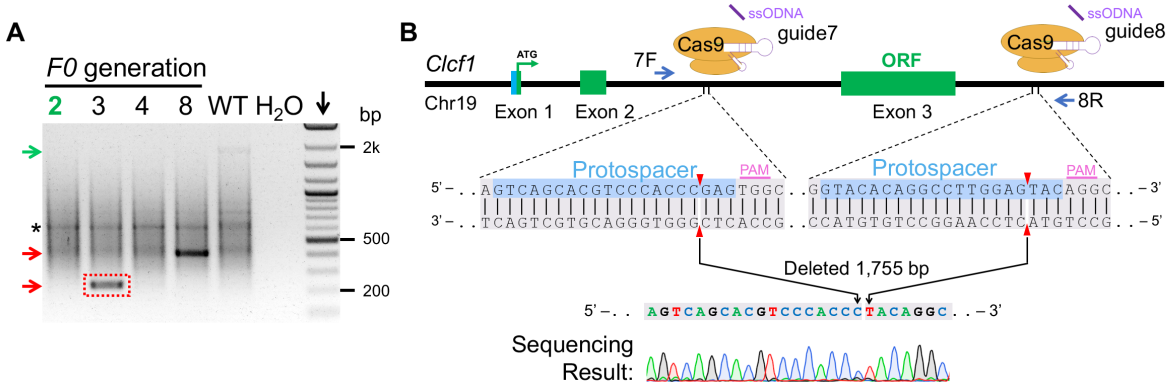
(*A*) *Plagl1* genotyping results of *F0* generation. The 5'– and 3'–*loxP* band sizes are 312 bp and 234 bp, respectively. (*B*) *Plagl1* genotyping results of *F1* generation from breeding of #10 mouse in *A*. P1: positive control (founder mouse #10); P2: positive control (founder mouse #8). (*C*) *Ak040954* genotyping results of *F0* generation. 5'– and 3'–*loxP* band sizes are 361 bp and 391 bp, respectively. (*D*) *Ak040954* genotyping results of *F1* generation from breeding of #5 founder mouse in *C*. P: positive control (founder mouse #5). (*E*) *Clcf1* genotyping result of *F0* generation. 5'– and 3'–*loxP* bands are 389 bp and 222 bp, respectively. (*F*) *Clcf1* genotyping results of *F1* generation from breeding of #2 founder mouse in *E*. (*G*) *Gm44386* genotyping results for *F0* generation. 5'– and 3'–*loxP* bands are 431 bp and 415 bp, respectively. (*H,I*) *Gm44386* genotyping results for *F1* generation from breeding of #1 founder (*H*) and #5 founder (*I*). For all panels, N: negative control (water); green ID highlight mice that showed simultaneous 5'– and 3'–*loxP* insertions; arrow, DNA marker lane; mouse with single-side *loxP* is indicated with a bar on top of ID; Δ, deletions; ∇, big insertions.



Supplemental Fig. S5. Deletions of exons 5 and 6 of *Plagl1* gene using *i*-GONAD method. (*A*) *Plagl1* genotyping of *F0* generation using primers shown in *B*. Note that WT and floxed alleles are too large thus not detected in current PCR condition. Mice #1, #2, #4, #7, #8, #11, #12, #28 showed specific bands of large deletions. WT: wild-type; N: negative control (water); green ID highlight founders that showed simultaneous 5'– and 3'–*loxP* insertions; arrow, DNA marker lane; mouse with single-side *loxP* integration is indicated with a bar on top of ID; Δ, deletions; ∇, large insertions. (*B*) *Plagl1* gene structure. (*C*) Sanger sequencing result for the null allele shown in *A* (red box).

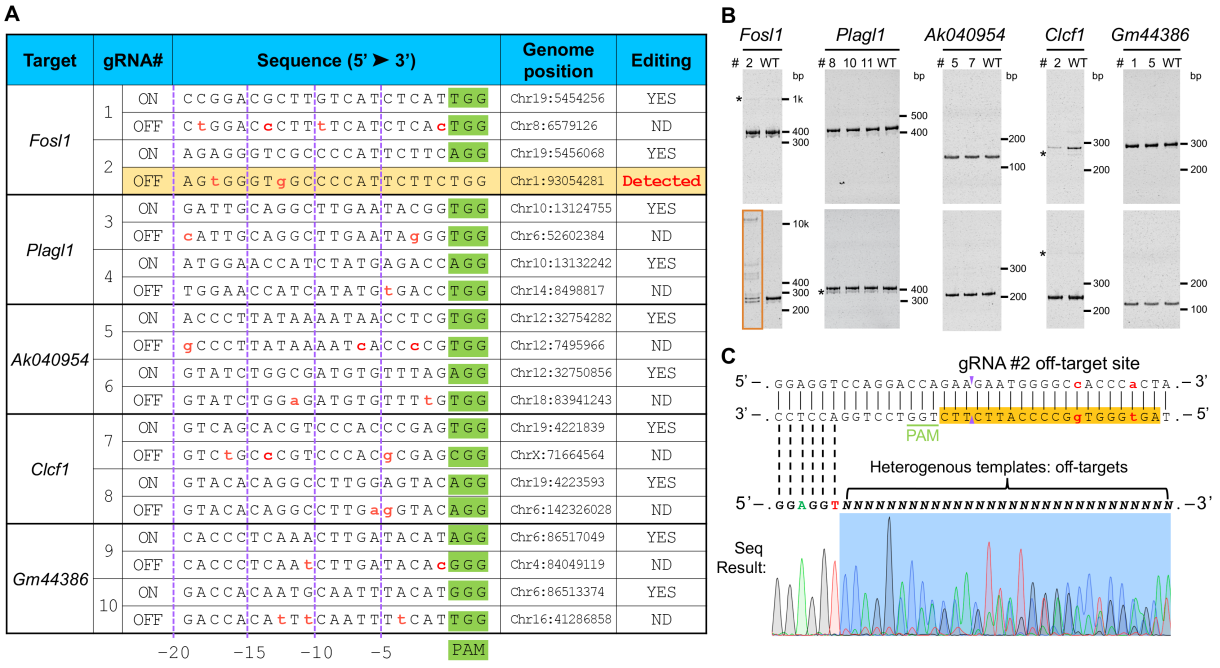


Supplemental Fig. S6. Deletions of exons 3 and 4 of *Fosl1* gene by *i*-GONAD method.
(*A*) *Fosl1* genotyping of *F0* generation using primers shown in *B*. WT allele is 2,115 bp (green arrow). Note the absence of the WT and *loxP* bands for #2 sample could be caused by biased amplification of the smaller truncated allele (red arrow). (*B*) *Fosl1* gene structure. Sanger sequencing result for the null allele shown in *A* (red box).



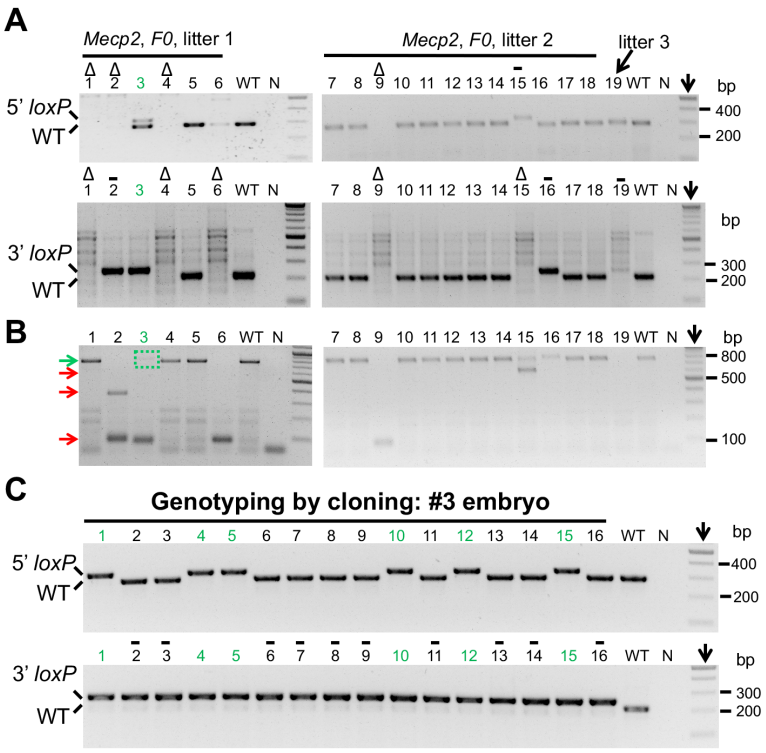
Supplemental Fig. S7. Deletion of exon 3 of *Clcf1* gene using the *i*-GONAD method.

(*A*) *Clcf1* genotyping of *F0* generation using primers shown in *B*. WT band should be 1,977 bp (green arrow). Red arrows point to the PCR amplifications of null alleles. Note that current PCR condition is not suitable to detect large-sized WT or floxed alleles. (*B*) *Clcf1* gene structure. Sanger sequencing results for the null allele shown in *A* (red box).



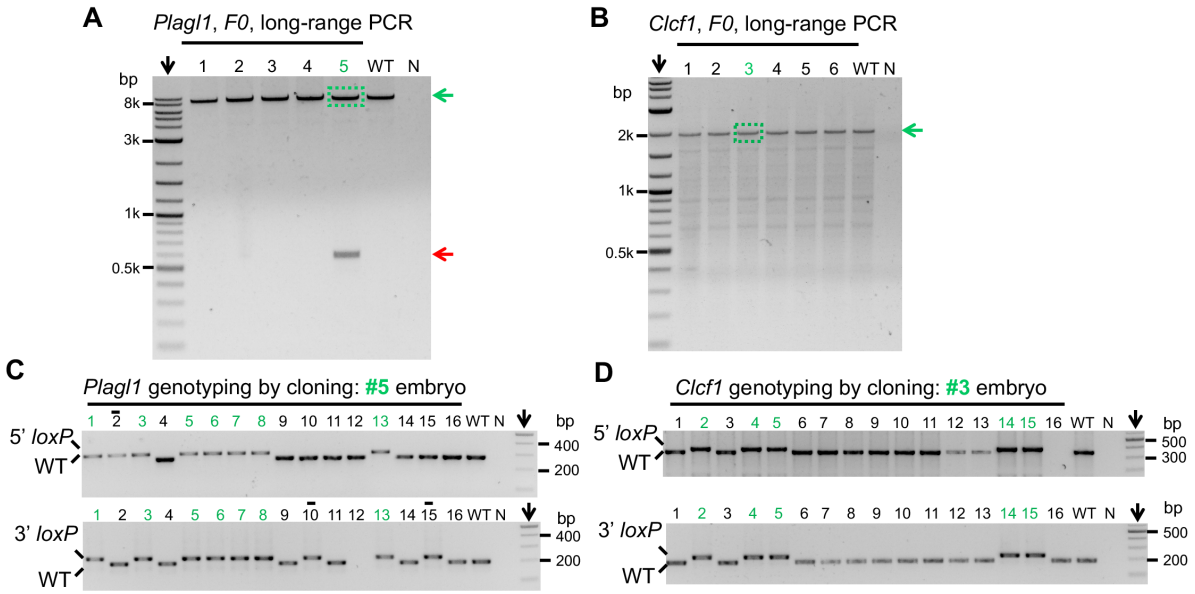
Supplemental Fig. S8. Examining off-targeting from *i*-GONAD experiments.

(*A*) Information about top-predicted off-targeting sites for gRNAs used to produce floxed alleles. ND: not detected. Mismatches were highlighted in red. Genome positions are coordinates of mouse GRCm38/mm10. (*B*) Polyacrylamide gel electrophoresis results for examinations of off-target editing. Top panels are predicted off-sites for 5' gRNAs; lower panels are predicted off-sites for 3' gRNAs. Founder mice that contained floxed alleles were selected for off-targeting evaluations. Asterisks highlight the DNA species that universally appeared in all samples including WT (a mixture of C57BL/6J and CD-1 genomic DNA, as a control of any artifact due to single nucleotide polymorphism). (*C*) Sanger sequencing result of the off-target region for gRNA #2.



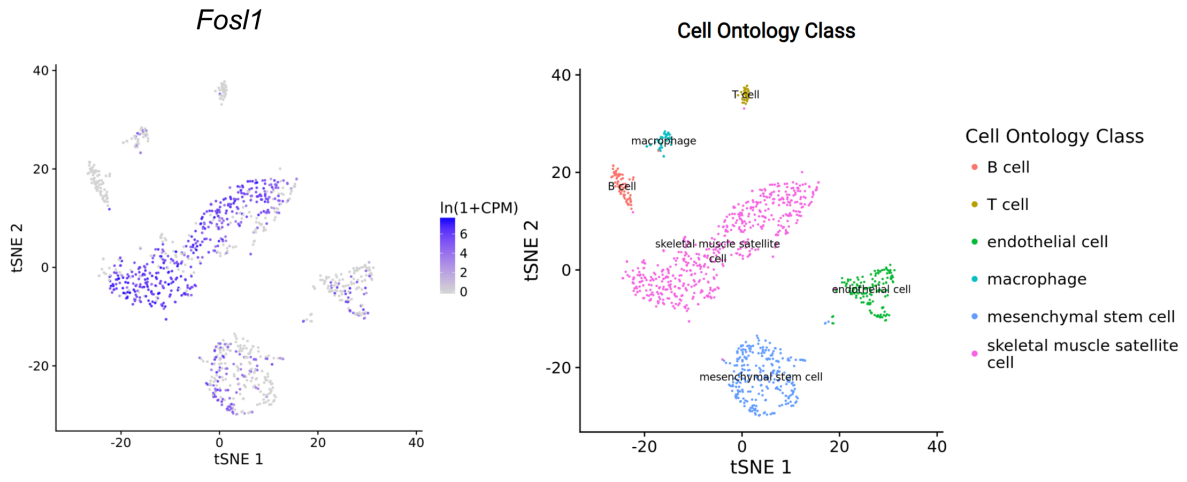
Gene Name	F0 founder	loxP in cis	Single loxP		WT	Other	Total
			5' loxP	3' loxP			
Mecp2	#3	**6**	0	10	0	0	16

Supplemental Fig. S9. Test of *i*-GONAD on *Mecp2* gene by using symmetric ssODNAs.
(*A*) *Mecp2* genotyping results of E12.5 mouse embryos. Top panels: 5' gRNA region (WT band is 287 bp, *loxP* band is 327 bp). Lower panels: 3' gRNA region (WT band is 218 bp, *loxP* band is 258 bp). Note only embryo #3 was positive for both 5'– and 3'–*loxP*. (*B*) Long-range genotyping PCR results. WT band is 773 bp, floxed-allele band is 853 bp. Green arrow points to the region of WT and floxed-allele amplicons. Red arrows point to amplicons of null alleles. Note the weak band enclosed by the green box appeared slightly larger than WT. (*C*) Genotyping results of bacterial colonies from cloning of the purified long-range PCR products (green box in *B*) for embryo #3. (*D*) Summary of bacterial colony genotyping results.



Supplemental Fig. S10. Genotyping analyses of conditional alleles produced from C57BL/6J mice.

(*A*) Long-range genotyping PCR results for *Plagl1* gene (WT band is 8,441 bp, floxed-allele band is 8,509 bp). Red arrow points to one null allele. (*B*) Long-range genotyping PCR results for *Clcf1* gene (WT band is 1,977 bp, floxed-allele band is 2,045 bp). (*C,D*) Genotyping results of bacterial colonies from cloning of the purified long-range PCR products enclosed by green boxes in *A* and *B*.



Supplemental Fig. S11. Expression of *Fosl1* gene in various cell types within mouse muscle tissue.

Fosl1 expression data in single-cell RNA sequencing dataset of adult mouse muscles (Tabula Muris et al. 2018). Note that in addition to myoblasts (labelled as satellite cell), *Fosl1* expression was also detected in fibroblasts (labelled as mesenchymal stem cell), endothelial cells and macrophages.