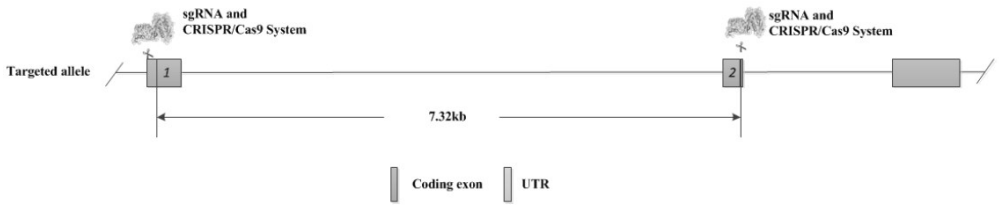
Procedures for Construction of Glrx1 $^{-/-}$mouse model

Step 1 Selection and design of gRNA of target Glrx1 gene

(1)Glrx-1-Cas9-KO mice were designed as supplementary Figure 1.



Supplementary Figure 1 A strategy for constructing Glrx-1-Cas9-KO mice

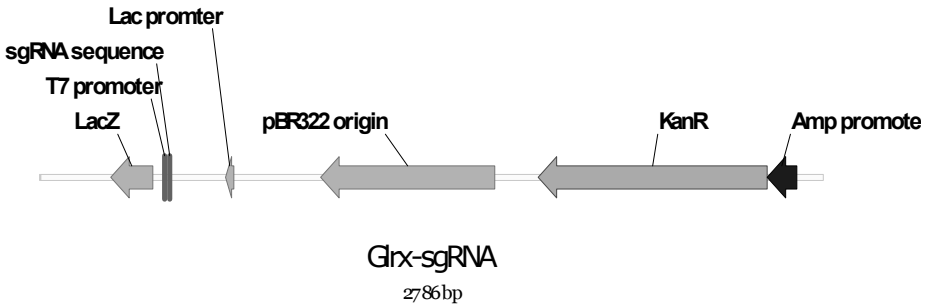
(2) According to this strategy, sgRNA sequence was designed (Supplementary table 1). The sgRNA sequence was inserted in the intron of Glrx1 gene.

Supplementary table 1 sgRNA sequence

sgRNA name	Sequences	PAM
Glrx-3S1(forward)	CGGAGATGACACTTACTGAT**GGG**（SEQ ID NO.1）	**GGG**
Glrx-5S1(forward)	GCTAAGCGCCGCTGCATTAC**CGG**（SEQ ID NO.2）	**CGG**

Step 2 Construction of SgRNA vector

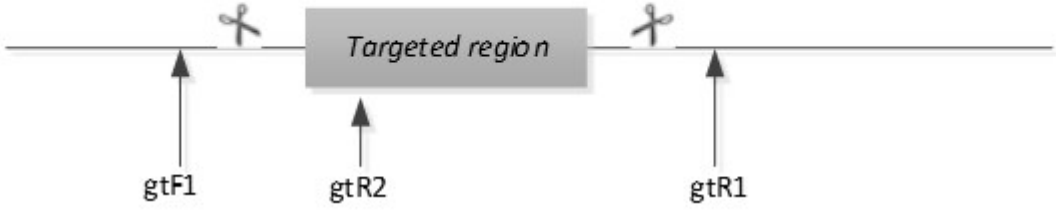
A pUC57-sgRNA vector was cut by BsaI enzyme in a 37°C water bath for 1 h. The products were checked on 1% agarose gel and recovered. The sgRNA primers were then annealed. The annealed products were connected with the recovered digestion products, and then transformed into E. coli was. Monoclonal genes were selected for PCR. If the PCR results were positive, the products were sequenced for verification. The sgRNA vector was obtained (Supplementary Figure 2).



Supplementary Figure 2 sgRNA vector

Step 3: In vitro transcription of sgRNA and Cas9 mRNA

The sgRNA and Cas9 mRNA were transcribed in vitro using a commercial kit (AM1354, AM1908, purchased from Ambion). And the strategy was shown in



Supplementary Figure 3 Cas9 knockout genotyping strategy (Fragment deletion)

Step 4: Microinjection of fertilized eggs

(1) Preparation of single cell fertilized eggs

Equine chorionic gonadotropin (5 IU) was intraperitoneally injected in mice on the first day, and then human chorionic gonadotropin was injected after 46-48 hours. Then 2 female mice were mixed with a male mouse in a cage. Thrombolysis was detected on the morning of the 4th day.

The fertilized eggs were obtained at 0.5 day of thrombolysis by sacrifice after cervical dislocation. The fallopian tubes were cut out, and the ovum was taken out with microscopic tweezers. After hyaluronidase digestion, plump embryos with uniform cytoplasm were selected and cultured in M16.

(2) Microinjection of fertilized eggs

The selected fertilized eggs were transferred into M2 strip in a row (30 to 50 pieces). An injection dish was placed on the loading table of an inverted microscope so that the direction of

the M2 droplet was perpendicular to the operator, that is, on the Y-axis. The injection tube was inserted into the cytoplasm and the Cas9 sgRNA system (sgRNA and Cas9 mRNA) was injected. The plasmid Cas9 D10A (plasmid # 42335, Addgene) was expressed. When loose cytoplasm was observed, the needle was rapidly withdrawn. After the injection, embryo was transferred to a petri dish containing M16 nutrient solution and recovered in a 37 °C and 5% CO_2 incubator for 0.5 to 1.0 h. The fertilized eggs were transplanted into E0.5 - day pseudopregesis recipients. F0 generation mice were born about 19-21 days after transplantation.

Step 5: Birth and identification of F0 generation mice

A total of 39 pups were born and 38 survived. Tail cutting identification was conducted on mice of F0 generation 1 week after birth, and 7 positive mice of F0 generation were obtained, with black hair color and gender of 5 females and 2 males. The PCR conditions were listed in supplementary Table 2

Supplementary table 2 Primers and PCR Amplification Program

Ordinal Number	**Primer Name**	**Primer Sequence**	**Amplified Fragment Length**
1	2074-Glrx-gtF1	GTGGCAAAGTTCAGTCACAA	Wild-type=8603 bp
	2074-Glrx-gtR1	TCCTCTTCTGGGCAACTGTC	Gene knockout：~ 1 kb

Routine PCR Program				**PCR Program**				
Primer used：				**Primer used：1**				
Step	**Temp.**	**Time**	**Cycles**	**Step**	**Temp.**	**Time**	**Cycles**	**±Temp./Cycles**
1	95°C	5min		1	95°C	5min		
2	95°C	30sec		2	98°C	30sec		
3	58°C	30sec		3	65°C	30sec		-0.5
4	72°C	45sec	2-4,35×	4	72°C	45sec	2-4,20×	
5	72°C	5min		5	98°C	30sec		
6	10°C	Hold		6	55°C	30sec		
				7	72°C	45sec	5-7,20×	
				8	72°C	5min		
				9	10°C	Hold		

Step 6: F0 mice were sexually mature and bred, and F1 mice were identified

F0 generation mice were sexually mature at about 8 weeks old and bred with C57BL/6J

backcross. F1 generation mice were identified by tail clippings at 1 week old, and 6 positive F1 generation heterozygotes were obtained as follows:

61#, 62#, 64# :

GCCCTTTAAAACTGAAGCATCCTACTTGGTAACTCCTCCTCCAAGGAGGTTCCTTATT
AAATGAGAGCTGCTGGCTAAGCGCC----------7588bp----------
ATACACATAGTTCTAGACATAAATACACAAAAAGATAACGT

73#, 74#, 75# :

CCAGTGTGCAATGGTAGGCCTAGGAAGTACTGACTCATACCAA----------7898bp----------TAGCTAAGGATGGAAATTTGGGAAGTAT

Supplementary table 3 $Glrx^{+/-}$ F1 heterozygotes

Sequence Number	Sex	Color	Genotype	Male/Female	Generation
61	♂	Black	-7588bp/wt,E1-E2 (whole coding region) deleted	♂14	F1
62	♂	Black	-7588bp/wt,E1-E2(whole coding region) deleted	♂14	F1
64	♀	Black	-7588bp/wt,E1-E2(whole coding region) deleted	♂14	F1
73	♀	Black	-7898bp/wt,E1-E2(whole coding region) deleted	♀7	F1
74	♀	Black	-7898bp/wt,E1-E2(whole coding region) deleted	♀7	F1
75	♀	Black	-7898bp/wt,E1-E2（(whole coding region) deleted	♀7	F1

Step 7 Propagation

Breeding these mice together leads to the production of F2 mice normally comprising 50% of heterozygotes, 25% of mutated homozygotes and 25% of WT mice. The mouse lineage was established and maintained for phenotypic characterization and further functional work.

3. Mendelian genetics.

- Steps to generate desired numbers of pups/mice;

1. Heterozygous x heterozygous mating generates 25% homozygous offspring
2. To generate 20 homozygous mice, need 80 offspring (20= 25% of 80)
3. We assume a breeding female averages 5 pups/litter

4. Need 14 heterozygous breeding pairs (80 pups/5 pups per litter = 16 litters) or 16 female and 16 male heterozygous mice minimum to generate one experimental cohort in one round of breeding (3 months)

➢ **Here is how we generate these 16 breeder pairs, again working backwards:**

- Mendelian genetics predict that a wild type (WT) x heterozygous mating generates 50% heterozygous offspring
- To generate 32 het. mice, need 64 offspring (32=50% of 64)
- Need ~11 WT x het. breeding pairs (64 pups/5 pups per litter = 12.8 litters)
- To generate 11 het. mice, need 22 offspring (11=50% of 22)
- Need ~4 WT x het breeding pairs (22 pups/5 pups per litter = 4.4 litters)
- To generate 5 het mice breeding pairs, need 10 offspring (10 pups/5 pups per litter = 2 litters)

❖ Therefore, starting with 2 WT x het breeder pairs, the 20 homozygous mice were ready in about 9 months (from 3 rounds of breeding).