Supplementary information, Data S1 Materials and Methods

Animals

Mice were housed in standard cages in an Assessment And Accreditation Of Laboratory Animal Care credited SPF animal facility on a 12-h light/dark cycle. Wild type zebrafish used for laying were maintained at 28.5℃ in our zebrafish facility. Injected zebrafish embryos were cultured at 28.5℃ in an incubator. All animal protocols are approved by the Animal Care and Use Committee of the Model Animal Research Center, the host for the National Resource Center for Mutant Mice in China, Nanjing University.

Cell culture, transfection, and immunostaining

293T cells were cultured in DMEM/High glucose (HyClone, SH30022.01B) with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Before transfection, 293T cells were cultured on cover slips coating with poly-D-lysine (Sigma, P7280). Plasmid DNA was used for transfection of 293T cells by Lipofectamine 2000 (Invitrogen, 11668-019). For immunostaining, cells were fixed in 4% PFA (paraformaldehyde) for 15 minutes at RT (room temperature), washed with PBS twice, permeabilized by incubation with 0.2% Triton 100 in PBS for 5 minutes, washed with PBS twice, and then blocked with normal goat serum (BOSTER, AR0009) for 1 hour at RT. Anti-flag M2 (Sigma, F1804) was added to the blocking buffer and incubated with cells for 2 hours at RT. After washes in PBS, cells were incubated with cy3-conjugated-goat anti mouse IgG (Jackson, 115-165-146) secondary antibody and Hoechst (Sigma, 33258) in PBS for 1 hour at RT, followed by washing in PBS and mounting with Vectashield medium (Vector Laboratories, H-1000). All fluorescent pictures were taken by Olympus Flueview 1000 confocal microscope.

DNA constructs

Cas9 was synthesized with codon optimization and inserted into pST1374 vector with and without NLS-flag to construct pST1374-Cas9 and pST1374-NLS-flag-Cas9 vectors. Cas9 C-NLS Cla1 For and Cas9 C-NLS Apa1 Rev primers were used to construct pST1374-NLS-flag-Cas9-NLS. Triple NLS was amplified from oligonucleotides template by Cas9-N-3NLS For and Cas9-N-3NLS Rev primers. PCR product was digested with Nhe1 and Not1, and inserted into pST1374-NLS-flag-Cas9 cut with the same enzymes to construct pST1374-3NLS-flag-Cas9. Linker sequence was amplified from ZFN vector by Cas9-N-NLS-flag-linker For and Cas9-N-NLS-flag-linker Rev primers and inserted into the junction of flag and Cas9 to construct pST1374-NLS-flag-linker-Cas9. Sequence of plasmids and primers are listed in Supplementary information, Data S2 and Table S1.

Transcription

The pST1374-Cas9 vector was linearized by Age1 enzyme and in vitro transcribed using T7 Ultra Kit (Ambion, AM1345). Cas9 mRNA was purified by RNeasy Mini Kit (Qiagen, 74104). Chimeric RNA templates carrying a T7 promoter sequence were generated by PCR from synthesized oligonucleotides templates. T7 shortscript kit (Ambion, AM1354) was used to transcribe chimeric RNA in vitro. RNA was recovered by phenol-chloroform extraction and alcohol precipitation. To obtain pre-annealed chimeric RNA, incubate chimeric RNA at 95 ℃ for 1minute, and then let it slowly cool down to room temperature. The synthesized oligonucleotides and primers used for the preparation of RNA templates are listed in Supplementary information, Table S2.

T7EN1 cleavage assay

Injected zebrafish embryos were collected after 12 hours of culture and digested in lysis buffer (10 μM Tris-HCl, 0.4 M NaCl, 2 μM EDTA, 1% SDS and 100 μg/ml Proteinase K). DNA was extracted from lysate by phenol-chloroform. Mouse genomic DNA from tail of 5-day-old pup was extracted by the same way as above. T7EN1 cleavage assay was performed as described [1]. In brief, targeted fragments were amplified from extracted DNA, and purified with PCR cleanup kit (Axygen, AP-PCR-50). Purified PCR product was denatured and reannealed in NEBuffer 2 (NEB) using a thermocycler. Hybridized PCR products were digested with T7EN1 (NEB, M0302L) for 30 min and loaded into 2% agarose gel.

Cas9/RNA Injection of one-cell Embryos

Mouse zygotes obtained by mating of Pouf5-IRES-EGFP (008214, JAX lab) or CAG-EGFP (RBRC00267, Riken) males with superovulated C57BL/6J females, were injected with a mixture of NLS-flag-linker-Cas9 mRNA (20 ng/μl) and EGFP-A chimeric RNA (20 ng/ul). Microinjections were performed into the larger (male) pronucleus of fertilized oocytes whenever possible. For those zygotes in which the pronuclei showed no obvious size difference, one of the pronuclei was randomly selected. Injected zygotes were transferred into pseudopregnant CD1 female mice, and viable adult mice were obtained. Knock-in Pouf5-IRES-EGFP mice were genotyped with primers oIMR8134, oIMR8135 and oIMR8292.

oIMR8134:5’-CAAGGCAAGGGAGGTAGACA-3’;

oIMR8135:5’-TGCCAGACAATGGCTATGAG-3’;

oIMR8292:5’-CCAAAAGACGGCAATATGGT-3’. Transgenic CAG-EGFP mice were genotyped with For-A and Rev primers listed in Supplementary information, Figure S2.

Reference

1 Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. FLASH assembly of TALENs for high-throughput genome editing. *Nat Biotechnol* 2012; **30**:460-465.