

## File S1

### Materials and methods

#### Fly stocks

*w*<sup>1118</sup> and *yw* flies were used in this study, and all flies were cultured at 25°C.

#### Cas9/gRNA design

For the construction of pSP6-2sNLS-spcas9 (Figure S1B), genomic DNA of Cas9 was amplified by standard PCR protocol from *S. pyogenes* genome, and then inserted into pMD18-T vector. An N-terminal XhoI restriction enzyme site and C-terminal NLS sequence were included in the above PCR product. The N-terminal CMV-SP6-NLS-3xFLAG fragment was cut from TALEN expressing vector (HUANG *et al.* 2011) by Sall and XhoI and inserted into the XhoI site described above. Target DNA binding sequence of customized gRNA was selected according to the previous studies in Zebrafish (CHANG *et al.* 2013; HWANG *et al.* 2013). To simplify the gRNA design and the following *in vitro* transcription procedure, we follow the target sequence principle:

5'GGA/G-N<sub>17/18</sub>-NGG-3'. For the convenience of molecular identification of indel mutations in our interested loci except for *yellow*, a restriction enzyme site was chosen around the cutting site of the target sequence. The sequences of gRNAs are listed in Table S2.

#### ***In vitro* synthesis and microinjection of Cas9-coding mRNA and gRNA**

For Cas9 mRNA *in vitro* transcription, pSP6-2sNLS-spcas9 plasmids were linearized by XbaI and recovered as corresponding templates. Transcription was carried out following the instructions of the Sp6 mMACHINE mMACHINE Kit (Ambion, USA). The poly(A) signals were added to the 3' of capped mRNAs by *E. coli* Poly(A) polymerase Kit (New England BioLabs, USA). For customized gRNA *in vitro* transcription, the DNA templates were obtained from the pMD19-T gRNA scaffold vector (kindly provided by Dr. J. XIONG, peking university) by PCR. The transcription was carried out with RiboMAX Large Scale RNA Production Systems-T7 Kit (Promega, USA). The

purified Cas9-coding mRNA and gRNA were mixed to a final concentration of 1 $\mu$ g/ $\mu$ l and 50ng/ $\mu$ l respectively, followed by injection into *w*<sup>1118</sup> embryos according to the standard procedure. The sequences of all primers for different genes are listed in Table S2.

#### **Cas9/gRNA-mediated mutation screens and statistical analyses**

For the case of the *yellow* locus, the mosaic *yellow* phenotype in F<sub>0</sub> (injected animals) and indel yielders in F<sub>1</sub> were assessed according to the previous described methods (Liu *et al.* 2012). For detection of F<sub>0</sub> mutants of the other 6 loci, targeted genomic region was amplified by PCR from single F<sub>0</sub> fly genome using primers designed to anneal at approximately 100 to 300 base pairs upstream and downstream from the expected DSB site. The corresponding PCR products were sequenced and/or digested by corresponding enzymes (Table S3). For detection of F<sub>1</sub> mutants, single F<sub>1</sub> fly (5-10 flies randomly picked from each fertile F<sub>0</sub> cross) genomic DNA were amplified using primers described above. The resulting PCR products were sequenced. Mutated alleles were identified by comparison with the wild-type sequence. For *kl-3* and *RpL15*, targeted PCR products from an individual F<sub>0</sub> animal (*kl-3*) or pooled genomic DNA isolated from ten dying larvae (*RpL15*) were cloned into the pEASY-T vector for DNA sequencing. The genomic DNA sequences with modifications mediated by Cas9/gRNA were scored as indel yielders. A list of the primers is provided in Table S3.

CHANG, N. N., C. H. SUN, L. GAO, D. ZHU, X. F. XU *et al.*, 2013 Genome editing with RNA-guided Cas9 nuclease in Zebrafish embryos. *Cell Research* **23**: 465-472.

HUANG, P., A. XIAO, M. G. ZHOU, Z. Y. ZHU, S. LIN *et al.*, 2011 Heritable gene targeting in zebrafish using customized TALENs. *Nature Biotechnology* **29**: 699-700.

HWANG, W. Y., Y. F. FU, D. REYON, M. L. MAEDER, S. Q. TSAI *et al.*, 2013 Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnology* **31**: 227-229.