Figure S1 Alignment of top 5 alleles of the 3 loci in *Tg(actb2:cas9; (U6x:sgRNA(insra/b)* fish. The mutant alleles are arranged according to their frequency with the top being the most frequent. Protospacers and PAM are highlighted in yellow and cyan in the wild-type sequence, respectively. Micro-homology pairs are labelled by the same types of underline, or in bold in the wild-type sequence. Red letter in the mutant allele indicates insertion. Dashes in the mutant alleles indicate deletion.

Tables S1-S5
Available for download as Excel files at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.176917/-/DC1

Table S1. List of primers use in the study.

Table S2. Mutation rates at the three loci calculated based on high throughput sequencing data.

Table S3. Alleles and read counts of *insra* locus identified in the 17 double carriers. “D” denotes deletion. “I” denotes insertion. “C” denotes deletion and insertion. The first number indicates the position at which InDel occurs. The second number is the number of nucleotides of insertion or deletion in the allele.

Table S4. Alleles and read counts of *insrb* locus identified in the 17 double carriers.

Table S5. Alleles and read counts of *tyr* locus identified in the 17 double carriers.

File S1
Supplemental Methods

Construction of U6-based sgRNA expression vectors

1. Order a pair of U6 vector-specific, 23nt targeting primers for each target. For each target (GN19), order a forward primer TTCGN19 for the U6 promoter vectors; order a reverse primer AAACN19. Note that the N19 in the reverse primers is reverse complementary to that in the forward primer.

   To order primers compatible with both the pU6x-sgRNA vectors and pT7-gRNA (Addgene plasmid #4675; (JAO *et al.* 2013)), order degenerate primers WTMGGN18 (forward) and AMMCN_{18}C (reverse), where M=A or C, W=A or T. Again, the N18 in the reverse primers is reverse complementary to that in the forward primer.

2. Anneal the two primers (1µl 100µM stock each in a 20µl 1x NEB buffer 2) by incubating the mixture at 95^{o}C for 5 min, ramping down to 50^{o}C at 0.1^{o}C/sec, incubating at 50^{o}C for 10 min, and chilling to 4^{o}C at normal ramp speed.

3. Ligate the annealed oligos to the U6 vector of choice (pU6x-sgRNA #1-#5, see diagrams and plasmid list in the next pages) by mixing the components [1 µl 10x CutSmart buffer, 1 µl T4 DNA ligase buffer, 0.25µl U6 plasmid (about 100ng), 1µl annealed oligos, 0.3 µl T4 DNA ligase, 0.3 µl BsmBI, 0.2µl PstI (optional), 0.2 µl SalI (optional), 5µl H2O] and incubating 3x(37^{o}C for 20 min, 16^{o}C for 15 min), then 37^{o}C for 10 min, 55^{o}C for 15 min, followed by 80^{o}C for 15 min (optional).

4. The reaction is ready for transformation (use 2 µl of the ligation and plate 10% of the transformants). Transform and spread onto spectinomycin (50µg/ml) plates.

Construction of pGGDestTol2LC-sgRNA vectors

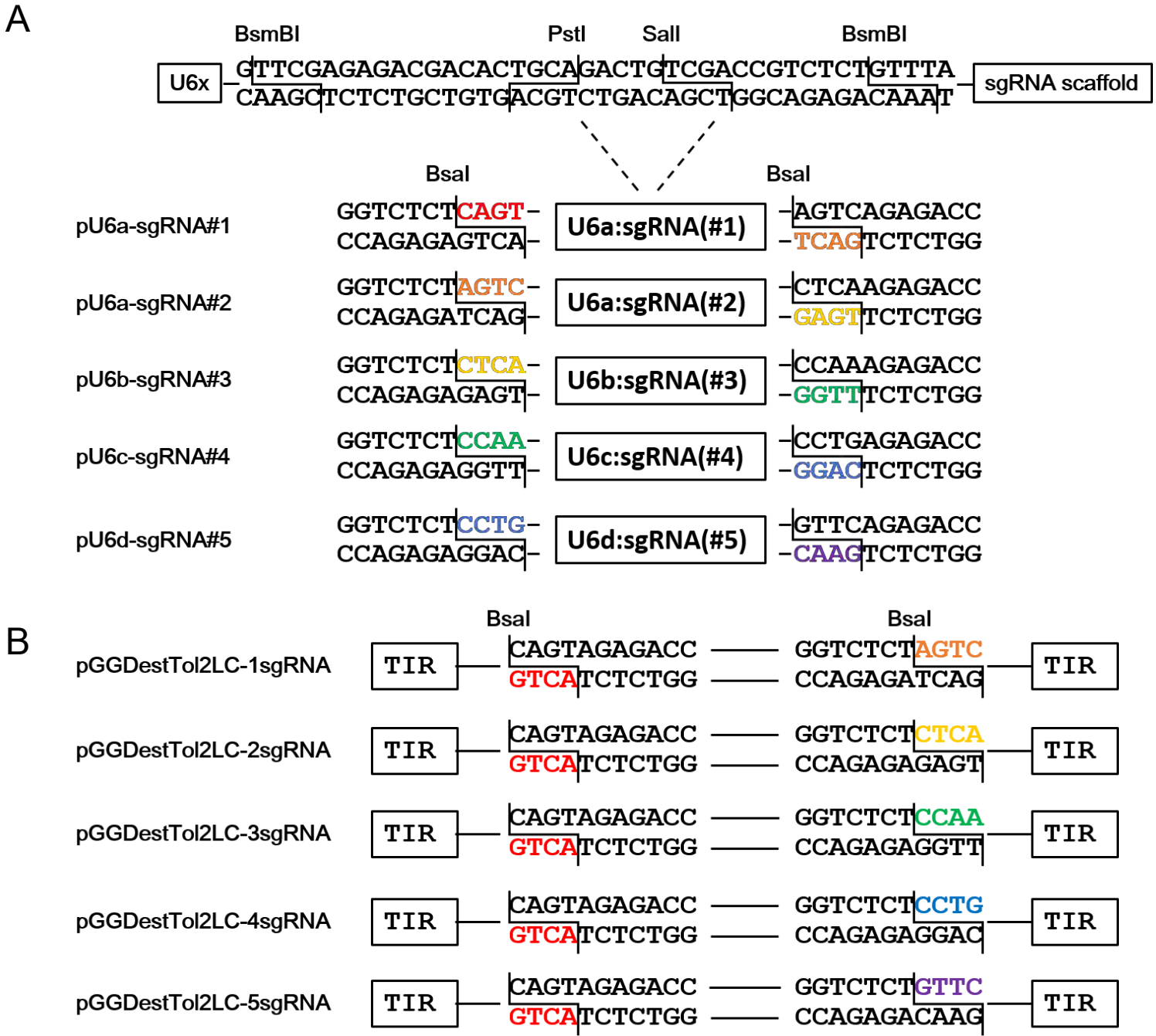
1. Ligate the U6x:sgRNA cassettes into a pGGDestTol2LC vector by mixing the components (2µl 10x CutSmart buffer, 2 µl T4 DNA ligase buffer, 100ng of each pU6x-sgRNA plasmid, 50ng empty pGGDestTol2LC-sgRNA vector of choice (see plasmid list and diagrams), 1µl BsaI, 1µl T4 DNA ligase, adjust volume to 20 µl with H2O).

2. Incubate mixture 3x(37^{o}C for 20 min, 16^{o}C for 15 min), followed by 80^{o}C for 15 min.

3. The reaction is ready for transformation (use 5 µl of the ligation and plate 50% of the transformants). Transform and spread onto ampicillin (100 µg/ml) plates.

Reference.

JAO, L. E., S. R. WENTE and W. CHEN, 2013 Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proc Natl Acad Sci U S A **110:** 13904-13909.


A
BsmBI
PstI
SalI
BsmBI
U6x
GTTCGAGAGACGACACTGCAGACTGTCGACCGTCTCTGTTTA
CAAGCTCTCTGCTGTGACGTCTGACAGCTGGCAGAGACAAAT
sgRNA scaffold
BsaI
BsaI
pU6a-sgRNA#1
GGTCTCTCAGT
CCAGAGAGTCA
U6a:sgRNA(#1)
AGTCAGAGACC
TCAGTCTCTGG
pU6a-sgRNA#2
GGTCTCTAGTC
CCAGAGATCAG
U6a:sgRNA(#2)
CTCAAGAGACC
GAGTTCTCTGG
pU6b-sgRNA#3
GGTCTCTCTCA
CCAGAGAGAGT
U6b:sgRNA(#3)
CCAAAGAGACC
GGTTTCTCTGG
pU6c-sgRNA#4
GGTCTCTCCAA
CCAGAGAGGTT
U6c:sgRNA(#4)
CCTGAGAGACC
GGACTCTCTGG
pU6d-sgRNA#5
GGTCTCTCCTG
CCAGAGAGGAC
U6d:sgRNA(#5)
GTTCAGAGACC
CAAGTCTCTGG
B
BsaI
BsaI
pGGDestTol2LC-1sgRNA
TIR
CAGTAGAGACC
GTCATCTCTGG
GGTCTCTAGTC
CCAGAGATCAG
TIR
pGGDestTol2LC-2sgRNA
TIR
CAGTAGAGACC
GTCATCTCTGG
GGTCTCTCTCA
CCAGAGAGAGT
TIR
pGGDestTol2LC-3sgRNA
TIR
CAGTAGAGACC
GTCATCTCTGG
GGTCTCTCCAA
CCAGAGAGGTT
TIR
pGGDestTol2LC-4sgRNA
TIR
CAGTAGAGACC
GTCATCTCTGG
GGTCTCTCCTG
CCAGAGAGGAC
TIR
pGGDestTol2LC-5sgRNA
TIR
CAGTAGAGACC
GTCATCTCTGG
GGTCTCTGTTC
CCAGAGACAAG
TIR

List of plasmids deposited at Addgene (Deposit 71794)

Plasmid ID	Plasmid Name
64237	pME-Cas9
64239	pGGDestTol2LC-1sgRNA
64240	pGGDestTol2LC-2sgRNA
64241	pGGDestTol2LC-3sgRNA
64242	pGGDestTol2LC-4sgRNA
64243	pGGDestTol2LC-5sgRNA
64245	pU6a:sgRNA#1
64246	pU6a:sgRNA#2
64247	pU6b:sgRNA#3
64248	pU6c:sgRNA#4
64249	pU6d:sgRNA#5
64250	pU6a:sgRNA(tyr)