Multiplex Conditional Mutagenesis Using Transgenic Expression of Cas9 and sgRNAs

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ABSTRACT Determining the mechanism of gene function is greatly enhanced using conditional mutagenesis. However, generating engineered conditional alleles is inefficient and has only been widely used in mice. Importantly, multiplex conditional mutagenesis requires extensive breeding. Here we demonstrate a system for one-generation multiplex conditional mutagenesis in zebrafish (*Danio rerio*) using transgenic expression of both *cas9* and multiple single guide RNAs (sgRNAs). We describe five distinct zebrafish *U6* promoters for sgRNA expression and demonstrate efficient multiplex biallelic inactivation of *tyrosinase* and *insulin receptor a* and *b*, resulting in defects in pigmentation and glucose homeostasis. Furthermore, we demonstrate temporal and tissue-specific mutagenesis using transgenic expression of *Cas9*. Heat-shock-inducible expression of *cas9* allows temporal control of *tyr* mutagenesis. Liver-specific expression of *cas9* disrupts *insulin receptor a* and *b*, causing fasting hypoglycemia and postprandial hyperglycemia. We also show that delivery of sgRNAs targeting *ascl1a* into the eye leads to impaired damage-induced photoreceptor regeneration. Our findings suggest that CRISPR/Cas9-based conditional mutagenesis in zebrafish is not only feasible but rapid and straightforward.

KEYWORDS CRISPR/Cas9; conditional mutagenesis; glucose homeostasis; retinal regeneration; zebrafish

CONDITIONAL gene inactivation is necessary for determining physiological functions of genes whose conventional mutation causes embryonic lethality or multiorgan defects. It has been widely used in mice because of the availability of embryonic stem (ES) cells and large collections of both ES cell lines and animals that carry conditional alleles. The conditional alleles usually contain strategically placed Cre or FLP target sites in introns that permit deletion of the intervening exon(s) (Gu *et al.* 1994). Some conditional alleles harbor a Cre and/or FLP invertible gene trap in an intron that allows an on/off switch of transcription (Schnutgen *et al.* 2005; Ni *et al.* 2012). The function or expression of these alleles is “switched” off in the presence of Cre or FLP activity. In *Drosophila*, conditional gene inactivation can be achieved

using RNA interference (RNAi), and genome-wide libraries of RNAi transgenes are available (Dietzl *et al.* 2007; Ni *et al.* 2008). For the increasingly popular zebrafish model, however, only limited conditional alleles generated from invertible gene trapping are available (Ni *et al.* 2012). The lack of ES cells and the inefficiency of RNAi in zebrafish necessitate new approaches for targeted conditional gene inactivation.

CRISPR/Cas9 mutagenesis has been applied to many model systems from cultured cells to whole organisms (Doudna and Charpentier 2014; Hsu *et al.* 2014). The system only requires a two-component RNA–protein complex (RNP): a single guide RNA (sgRNA) that identifies the target through base pairing and the Cas9 endonuclease that generates double-strand breaks (DSBs) at the target site upon sgRNA–target base pairing. CRISPR/Cas9 mutagenesis has been widely used in zebrafish (Chang *et al.* 2013; Hwang *et al.* 2013; Jao *et al.* 2013), where mutagenesis is usually achieved by injecting zygotes with *in vitro* synthesized Cas9 messenger RNA (mRNA) and sgRNA, or sgRNA–Cas9 RNP (Gagnon *et al.* 2014; Sung *et al.* 2014). This generates germline mutations as well as mosaic somatic mutations in all

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tissues. The mutagenesis in somatic cells is sufficient to cause extensive biallelic mutations and phenotypes in injected animals (Jao *et al.* 2013). Given the effectiveness of this system, we set out to determine whether transgenic expression of *cas9* and sgRNA can generate biallelic mutations and whether the CRISPR system can be adopted for targeted conditional mutagenesis. Here we report tissue-specific and inducible multiplex mutagenesis in zebrafish using transgenic lines expressing Cas9 and sgRNA.

Materials and Methods

Zebrafish strains and maintenance

Zebrafish were raised in an Aquatic Habitats system on a 14-hr/10-hr light/dark cycle. Embryos were obtained from natural crossing and raised at 28.5° in an incubator with lights on a 14-hr/10-hr light/dark cycle. Animals were staged by days postfertilization (dpf) or in months of age. At 5 dpf, fish were placed on the normal rearing diet and maintained as per standard protocols until appropriate age. All procedures have been approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Plasmid construction

Transgenes were generated using the Tol2-based Multisite Gateway system (Kwan *et al.* 2007). For Cas9-expression transgenes, the codon optimized *nls-cas9-nls* (Jao *et al.* 2013) was cloned into pME-MCS to generate pME-cas9. The broadly expressed promoters *actb2* and *ubi*, and the HotCre promoter were cloned into p5E-MCS. pDestTol2LR was made by inserting a 1.95-kb *Bgl*III fragment containing *cryaa:tagRFP* into the *Bgl*III site of pDestTol2pA2. To generate the sgRNA expression cassette, the U6a promoter was obtained by using U6aF1 and U6aR1 and inserted into the *Hind*III/*Pst*I sites of pT7-gRNA to replace the T7 promoter (Jao *et al.* 2013). The sgRNA core was then replaced with sgRNA^{EF} to facilitate U6 guided transcription (Chen *et al.* 2013). A transcription stop for U6, TTTT, was then added at the 3' end of sgRNA core by PCR. The cassette was then inserted into a miniTol2 vector to generate pT2-U6a:sgRNA (Urasaki *et al.* 2006). Other U6 promoters (*U6b*, *U6c*, *U6d*, and *U6e*) were amplified using primers listed in [Supporting Information, Table S1](#) and inserted into the *Apa*I/*Xho*I sites in pT2-U6a-sgRNA to replace U6a. The protospacer for *tyr* was inserted into pT2-U6a:sgRNA by annealing *tyr*-F and *tyr*-R and ligated in the presence of BsmBI as described (Jao *et al.* 2013). Protospacers for EGFP, *insra*, and *insrb* were ligated in the presence of BsmBI after annealing the primer pairs listed in [Table S1](#). To allow orderly assembly using Golden Gate ligation, *Bsa*I sites were added via PCR on both ends of the *U6:sgRNA* cassettes using primers listed in [Table S1](#). PCR products were inserted into the *Apa*I/*Xba*I sites of pLR-NN from the Golden Gate TALEN kit obtained from Addgene (Cermak *et al.* 2011). The destination vector for these *U6x:sgRNA* cassettes was made first by eliminating

the *Bsa*I site in pDestTol2CG2 (Kwan *et al.* 2007), followed by replacing the *cmcl2:EGFP* (*Aat*II/*Kpn*I) with a *cryaa:mCerulean* (*Sac*II/*Kpn*I) from phsp70l-loxP-mCherry-STOP-loxP-H2B-GFP-*cryaa*-cerulean to generate pDestTol2LC (Hesselson *et al.* 2009). Two *Bsa*I site-containing adaptors with appropriate overhang were sequentially inserted into the *Nsi*I/*Xho*I and *Kpn*I sites of pDestTol2LC, resulting in the pGGDestTol2LC series. All relevant plasmids have been deposited to Addgene for distribution ([File S1](#)).

Transgenesis

To generate transgenic fish, the mixture of Tol2 RNA (25 pg) and plasmid (20 pg) were injected into the cell of one-cell-stage embryos. Then injected embryos were cultured with egg water in a 28° incubator for 5 days. After prescreening for fluorescence, the positive larvae were raised in Aquatic Habitats system on a 14-hr/10-hr light/dark cycle until adult stage.

Semiquantitative RT-PCR analysis

Total RNA of transgenic larvae was isolated using TRIzol (Invitrogen). After treated with DNase (New England Biolabs) for 30 min, reverse transcription was performed using Moloney Murine Leukemia Virus reverse transcriptase (Promega), RNase inhibitor (New England Biolabs), 2 µg total RNA, oligo dT (for *actin* and *cas9*), or sgRNA specific oligo (5'-TTGCACCGACTCGGTG-3'). To detect the gene expression level, PCR reactions were prepared according to GoTaq Flexi DNA Polymerase instructions (Promega). *β-Actin*, *cas9*, and sgRNA were amplified from cDNA by PCR with the primers listed in [Table S1](#). The PCR conditions were 95° for 5 min; 22 (*β-actin*), 28 (*sgRNA*), or 30 (*cas9*) cycles of 30 sec at 95°, 30 sec at 60°, and 30 sec at 72°, followed by 72° for 5 min.

Heteroduplex mobility assay

DNA was isolated with 50 mM NaOH and then neutralized with 1 M Tris-HCl. After amplification with GoTaq Flexi DNA Polymerase (Promega) using genotyping primers, heteroduplex DNA was formed using the following parameters: 95° for 5 min, 95°–85° at –2°/sec, 85°–25° at –0.1°/sec, and hold at 16°. Samples were separated on a nondenaturing 10% polyacrylamide gel [10% acrylamide (29:1), 1× TBE, 0.05% ammonium persulfate, 0.05% TEMED] and run in 1× TBE buffer. After running, the gel was stained with ethidium bromide (0.5 µg/ml) and imaged by Gel Doc EZ System (Bio-Rad). The percentage of signal from the unmutated amplicon in each lane was quantified by Image Lab Software (Bio-Rad) and normalized to that of wild-type lanes. The mutation rate is the complement of the normalized percentage.

Assessing mutagenesis rate using quantitative PCR analysis

To determine the mutagenesis rate using qPCR (Yu *et al.* 2014), a fraction of the *tyr* PCR product used for the heteroduplex mobility assay (HMA) was diluted 50,000 × *g*. The diluted products were subjected to two sets of qPCR analyses: one set with primers >40 bp away from the

Cas9 cut site to amplify both wild-type and mutant alleles, and the other with one primer encompassing the Cas9 cut site, which should only amplify the wild-type allele. qPCR was performed by Bio-Rad CFX96 Real-Time system with SYBR Green Supermix (Bio-Rad) using the following program: 40 cycles of 10 sec at 95° and 30 sec at 60° after initial denaturing for 3 min at 95°. The primer pairs are listed in Table S1.

Feeding and glucose measurement

For 5% egg yolk feeding, chicken egg yolk was separated and then diluted to 5% with 0.3× Danieau buffer. The egg yolk solution was shaken every 30 min to maintain suspension. Before glucose measurement, fish were rinsed with 0.3× Danieau buffer three times. Glucose level of individual larvae was measured using Amplex Red Glucose/Glucose Oxidase Assay kit (Invitrogen). For blood glucose in adult animals, fish were either fasted overnight or fed as normal and blood was collected 30 min after the meal. Blood was collected from the dorsal aorta along the trunk of the fish as indicated in Zang *et al.* (2013). Blood glucose was determined as described (Li *et al.* 2014).

Imaging

Larvae were anesthetized with 0.01% MESAB (MS-222 or tricaine) in 0.3× Danieau and then mounted in 3% methylcellulose. Images were taken by a Zeiss Axio Imager Z1 microscope equipped with a Zeiss AxioCam MRm digital camera.

sgRNA injection and immunohistochemistry

sgRNA was synthesized by annealing a T7 promoter-containing gene-specific primer *ascl1a*^{sgRNA1} and *ascl1a*^{sgRNA2} with sgRNA scaffold primers. The anneal oligos were filled in with T4 DNA polymerase in the presence of 50 μM dNTP (New England Biolabs). The resultant double-stranded DNA (dsDNA) was used as template for T7 RNA polymerase using a MaxiScript T7 kit (Invitrogen). The sgRNA(*ascl1a*)s were combined and the mixture was injected into one eye (50 ng per eye) of adult *Tg(actb2:cas9; LR)* fish followed by electroporation prior to light lesioning as described (Thummel *et al.* 2008). Collection, fixation, embedding, sectioning, and immunohistochemistry (IHC) of injected *Tg(actb2:cas9; LR)* eyes were performed as described (Rajaram *et al.* 2014).

High-throughput sequencing

Genomic DNA from individual larvae was extracted using 50 mM NaOH and then neutralized with 1 M Tris-HCl. The targeted regions of *tyr*, *insra*, and *insrb* were amplified using gene-specific primer pairs with the forward primer containing M13 forward promoter sequence (Table S1) and a third M13 forward promoter-based barcode primer. After amplification, PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and products pooled together. The sequencing library was made from 100-ng PCR products using the Ovation Ultraflow Library system (NuGen), quan-

tified by qPCR (KAPA BioSystems). Around twenty-five million 300-bp reads were generated and the sequence data were processed using RTA version 1.18.54 and CASAVA version 1.8.2. Only about one million reads have a barcode.

Sequencing analysis

The detection of variants within 20 bp of the predicted cut site (or between two predicted cut sites) was performed by version 1.1 of the ampliconDIVider software (<https://github.com/mlafave/ampliconDIVider>). Briefly, ampliconDIVider first trims off nongenomic DNA and then uses novoalign to align the reads to amplicons of interest (chr15:42572556–42572836 for *tyr*, chr2:37298862–37298953 for *insra*, and chr22:11114676–11114957 for *insrb*) in version Zv9 of the zebrafish genome assembly. It loops through each sample and uses the programs bam2mpg and mpg2vcf to identify any deletions or insertions in the sample (<http://research.nhgri.nih.gov/software/bam2mpg/>) (Teer *et al.* 2010). As ampliconDIVider was designed for germline samples, the intermediate BAM files were analyzed manually for variant detection in somatic samples. The scripts can be found at https://github.com/mlafave/ampliconDIVider/blob/master/sh/yin_targeted_crispr_frameshift_count.sh. The number of wild-type reads and reads with mutations in the aforementioned distance from the predicted cut site were counted for mutation rate calculation.

Results

Transgenic expression of Cas9 and sgRNA(*tyr*) results in hypopigmentation

We set out to test whether transgenic expression of Cas9 and sgRNA targeting a gene of interest (*goi*) can cause biallelic mutagenesis (Figure 1A). As a pilot experiment, we tested whether ubiquitous expression of Cas9 would cause a pigmentation defect in the presence of sgRNA targeting *tyrosinase*. Tyrosinase is essential for vertebrate pigmentation and its loss of function leads to an easily recognizable albino phenotype (Searle 1990). Using Gateway assembly and Tol2 transgenesis (Urasaki *et al.* 2006; Kwan *et al.* 2007), we generated multiple transgenic lines expressing nls-Cas9-nls (hence referred to as Cas9 or *cas9*) (Jao *et al.* 2013). The zebrafish *ubiquitin* or *beta2-actin* promoter was used to drive *cas9* expression because of their high and ubiquitous activity (Higashijima *et al.* 1997; Boonanuntanasarn *et al.* 2008; Mosimann *et al.* 2011). Each transgene also carried a cassette for cardiac-specific EGFP (CG), or lens-specific tagRFP (LR), respectively, for genotyping. We compared the levels of *cas9* expression in seven lines of *Tg(ubi:cas9; CG)* using semiquantitative RT-PCR and raised F₁'s from two lines with high expression for additional experiments. We used a zebrafish *U6* promoter (*U6a*) to drive transcription of an sgRNA targeting *tyrosinase* [referred as *sgRNA(tyr)* henceforward] (Jao *et al.* 2013) and marked it with lens-specific mCerulean (LC) for genotyping. Ten germline-transmitting *Tg(U6a:sgRNA(tyr); LC)* founders were identified. They were crossed

Figure 1 Efficient disruption of *tyr* by transgenic Cas9 and sgRNA(*tyr*). (A) Experimental schematic of transgenic CRISPR mutagenesis. Transgenic lines with various *cas9* expression patterns were generated, including *Tg(ubi:cas9; CG)* and *Tg(actb2:cas9; LR)* with ubiquitous expression, *Tg(HotCre:cas9; CG)* with heat-shock-inducible expression, and *Tg(fabp10:cas9; CG)* with liver-specific expression. *Tg(U6:sgRNA(goi); LC)* lines were generated to ubiquitously express sgRNAs targeting the gene of interest. The fluorescent markers allowed noninvasive genotyping of the progeny. CG, *cmlc2:EGFP*, green heart; LR, *cryaa:tagRFP*, red lens; and LC, *cryaa:mCerulean*. (B–G) Images of 3-day-old embryos indicating biallelic mutations evidenced by loss of pigmentation in the retinal pigment epithelium. Representative images from three transgenic genotypes are shown from *Tg(ubi:cas9) × Tg(U6a:sgRNA(tyr))* (B–D) and *Tg(actb2:cas9) × Tg(U6a:sgRNA(tyr))* (E–G). (H) HMA showing efficient *tyr* mutagenesis in *cas9/sgRNA* double transgenic larvae at 5 dpf. Controls were sibling *Tg(U6a:sgRNA(tyr))* larvae at the same age. Both *Tg(ubi:cas9; CG)* and *Tg(actb2:cas9; LR)* were able to induce efficient mutagenesis. The numbers below each lane are the fraction of heteroduplex quantified by densitometry. (I) Comparison of HMA and qPCR-based assessment of mutagenesis efficiency. The same PCR products in H were analyzed by qPCR using two sets of primers, one amplifying both wild-type and mutant alleles while the other only amplifying the wild-type allele.

to F_1 's from a *Tg(ubi:cas9; CG)* line to identify suitable *Tg(U6a:sgRNA(tyr); LC)* lines. Double transgenic larvae from *Tg(ubi:cas9; CG) × Tg(U6a:sgRNA(tyr); LC)* had various degrees of pigmentation in the retinal pigment epithelium (RPE) at 2 dpf (Figure 1B), while single transgenic progeny, *Tg(U6a:sgRNA(tyr); LC)* or *Tg(ubi:cas9; CG)* did not show any abnormal pigmentation phenotypes (Figure 1, C and D). The line with the most severe phenotype was propagated. The *Tg(U6a:sgRNA(tyr); LC)* F_1 's were used to identify the best *Tg(actb2:cas9; LR)* line from 12 founders for additional experiments (Figure 1E). Again, single transgenic progeny, *Tg(U6a:sgRNA(tyr); LC)*, or *Tg(actb2:cas9; LR)* did not show any abnormal pigmentation phenotypes (Figure 1, F and G). To verify mutagenesis, we estimated the degree of mutagenesis in the double carrier progeny of the selected lines using an HMA where the PCR product encompassing the target region was separated on a 10% nondenaturing polyacrylamide gel followed by densitometry (Figure 1H). Heteroduplexes have

a slower mobility due to an open single-strand configuration surrounding the mismatched region (Ota *et al.* 2013). HMA indicated that 61–90% of the *tyr* loci were mutated in double transgenic larvae at 5 dpf. To validate the HMA approach, we determined the mutagenesis rate in the same fish using a quantitative PCR approach that uses a primer encompassing the Cas9 cleavage site to measure a decreased presence of the wild-type allele (Yu *et al.* 2014). As shown in Figure 1I, there was an excellent agreement between the two assays. We used HMA to determine the mutagenesis efficiency henceforth. Thus, transgenically expressed CRISPR components are sufficient to cause biallelic gene inactivation in zebrafish.

Development of a system for multiplex CRISPR/Cas9 mutagenesis in transgenic fish

We next tested whether multiplex mutagenesis is possible using the same transgenic approach. Multiplex CRISPR/Cas9 mutagenesis is desirable for several reasons. First, it allows

Figure 2 Development of a system for multiplex CRISPR/Cas9 mutagenesis. (A) Pairwise sequence comparison of the identity of the five zebrafish U6 promoters. (B) Semiquantitative RT-PCR analysis showing lower U6e promoter activity in embryos at 24 hpf injected with *Tg(U6a:sgRNA(atp2a2a); U6b:sgRNA(atp2a2b); U6c:sgRNA(nf1a); U6b:sgRNA(nf1b); U6c:sgRNA(dmd); LC*). Controls were noninjected siblings at the same stage. (C) Semiquantitative RT-PCR analysis showing lower U6e promoter activity in F₁ *Tg(U6a:sgRNA(try); U6b:sgRNA(fms); U6c:sgRNA(mpv17); LC*) larvae at 5 dpf. Controls were nontransgenic siblings at the same stage. (D) Schematic showing Golden Gate assembly of transgenic lines, including five sgRNA-expressing cassettes using four distinct zebrafish U6 promoters. Color triangles indicate *BsaI* distinct overhangs.

the expression of multiple sgRNAs to target the same gene, likely increasing mutagenesis. Second, it may facilitate gene–gene interaction studies. Third, by targeting related genes, it can overcome functional redundancy, a concern particularly applicable in zebrafish because of the common presence of duplicated genes. To minimize potential instability of tandem sgRNA-expressing transgenes, we sought to use distinct U6 promoters (Figure 2A). In addition to the U6a cluster on chromosome 21, three other U6 clusters have been previously described on chromosome 3 (U6e), 9 (U6b), and 11 (U6c) in zebrafish (Halbig *et al.* 2008; Clarke *et al.* 2012). We identified a fifth U6 cluster on chromosome 6 (U6d). The promoters for these U6 genes are divergent, with sequence identity <68% in the 400-bp upstream region (Figure 2A). We obtained U6b–U6e promoters using PCR.

We developed a Golden Gate ligation strategy to streamline the assembly of multiple sgRNA expression cassettes. This allowed ordered assembly of up to five U6x:sgRNA cassettes into a Tol2-based pGGDestTol2LC (Figure 2D, File S1). To compare the activity of the five U6 promoters, we used them to direct the expression of sgRNA targeting five genes, *atp2a2a*, *atp2a2b*, *nf1a*, *nf1b*, and *dmd*, whose somatic mosaicism is associated with human diseases (Biesecker and Spinner 2013). We assembled the expression cassettes in pGGDestTol2LC. RT-PCR analysis of each sgRNA indicated that the U6e promoter is less active while the other four promoters have similar activity (Figure 2B). To confirm that U6e promoter activity is lower, we used U6a, U6b, and U6e to

direct expression of sgRNA against *tyr*, *csf1r* (Parichy *et al.* 2000), and *mpv17* (Krauss *et al.* 2013), key genes responsible for the three pigment cell types in zebrafish. The three cassettes were placed in pGGDestTol2LC and the construct was injected to generate stable lines. RT-PCR analysis of the F₁ also indicated that U6e is less active (Figure 2C). We therefore chose the U6a–d promoters for multiplex sgRNA expression. Since U6a:sgRNA(*tyr*) cassette could serve as a mutagenesis indicator, it may be included in the multiplex sgRNA expression construct, leaving space for four additional cassettes (Figure 2D).

Inactivation of *insra* and *insrb* impairs glucose metabolism

As a test case for multiplex CRISPR mutagenesis, we chose *insulin receptor* genes as targets. Unlike mouse, zebrafish has two *insulin receptor* genes, *insra* and *insrb* (Toyoshima *et al.* 2008). We targeted a region with two adjacent high-quality sgRNAs in the 5' portion of each gene. The expression cassettes for the four sgRNAs, along with that of sgRNA(*tyr*), were placed in pGGDestTol2LC (Figure 3A). The targets for *insra* sgRNAs were located in exon 3, and those for *insrb* sgRNAs, exon 2 (Figure 3B). The construct was introduced into zebrafish and 14 germline transmitting founders were identified. For simplicity, we refer to these lines as *Tg(U6x:sgRNA(insra/b); LC)*. To make sure that the four U6 promoters had equivalent activity, we compared the levels of transcripts from each U6 promoter. Semiquantitative

Figure 3 Simultaneous inactivation of *insra* and *insrb* impairs glucose homeostasis. (A) Schematic of the sgRNA-expressing transgene. (B) Sequence of *insra* and *insrb* target regions. (C) RT-PCR analysis indicating that four distinct U6 promoters (U6a–U6d) have similar activity in directing *sgRNA(insra)* and *sgRNA(insrb)* expression in F₁ transgenic larvae at 5 dpf. Controls were nontransgenic larvae at the same stage. (D) HMA showing efficient mutagenesis at *tyr*, *insra*, and *insrb* loci in double transgenic larvae (samples 1–6) at 6 dpf. Arrows indicate smaller *insra* and *insrb* products that are from alleles with deletion between the two targets. (E) Positive correlation among the *tyr*, *insra*, and *insrb* mutation rate (*tyr* vs. *insra*, $r^2 = 0.72$; *tyr* vs. *insrb*, $r^2 = 0.71$). Mutation rate was calculated from sequencing data of 17 *Tg(actb2:cas9; U6x:sgRNA(insra/b))* fish from two founders. (F) *Tg(actb2:cas9; U6x:sgRNA(insra/b))* individuals share a set of highly prevalent alleles. The frequency of the top two *tyr* alleles and top three *insra* and *insrb* alleles in five siblings are shown. Del indicates the allele resulting from deletion of the sequence between the two DBSs in *insra* and *insrb*. #1 and #2 indicate the numbers 1 and 2 most frequent alleles other than the deletion. (G) Fasting and postprandial glucose levels (mean $\pm$ SE) in 6-day-old double transgenic larvae before and after incubation in 5% egg yolk solution for 2 hr. A significant increase was observed in double positive larvae ($n = 5$, t -test, $P < 0.01$) after feeding.

RT-PCR indicated they had similar activity in individual F₁'s (Figure 3C).

To determine whether multiplex mutagenesis occurs efficiently, we crossed the 14 germline transmitting *Tg(U6x:sgRNA(insra/b); LC)* founders to *Tg(actb2:cas9; LR)*. All *Tg(U6x:sgRNA(insra/b); LC); Tg(actb2:cas9; LR)* double carriers, as identified by having both CFP and RFP signal in the lens, had visible pigmentation phenotype in the RPE, although the severity of the phenotype varied. HMA analysis of 5 dpf double carriers from the line with the most severe pigmentation phenotypes confirmed efficient mutagenesis at the *tyr*, *insra*, and *insrb* loci (Figure 3D). Importantly, the mutation rates at *tyr* in hypopigmented *Tg(U6x:sgRNA(insra/b); LC);*

Tg(actb2:cas9; LR) larvae were comparable to those in *Tg(U6a:sgRNA(tyr); LC); Tg(actb2:cas9; LR)* (Figure 1H, Figure 3D), implying sufficient Cas9 activity even when multiple sgRNAs were expressed. The HMA indicated that about 70–90% of *insra* and *insrb* were mutated in larvae exhibiting hypopigmentation (Figure 3D).

We selected two lines for high throughput sequencing analysis to determine the mutation spectrum and to more accurately measure the mutation rates. One line had the most severe pigmentation phenotypes while the other had modest phenotypes but high germline transmission rate. Within these lines, the severity of the pigmentation phenotypes still varied somewhat within the same clutch. We

Figure 4 Temporal-specific CRISPR/Cas9 mutagenesis. (A) Heat-shock-inducible *tyr* inactivation. Cre RNA was injected into one-cell-stage embryos from *Tg(HotCre:cas9) × Tg(U6a:sgRNA(tyr); LC)*. Uninjected embryos were used as controls. The embryos were heat shocked at 6 or 24 hpf. Pigmentation phenotypes were induced in Cre RNA-injected double carriers (top) but not uninjected double carriers (bottom). Images were taken at 5 dpf. (B) HMA results of *tyr* locus in Cre RNA-injected, heat-shocked *Tg(HotCre:cas9);Tg(U6a:sgRNA(tyr); LC)* double carriers (mean $\pm$ SE, $n = 4$).

analyzed the amplicons encompassing the three targets from twelve double carriers from the line with weak pigmentation phenotypes and five double carriers from the line with severe pigmentation phenotypes (Table S2, Table S3, Table S4, and Table S5). The mutation rate at *tyr* determined by sequencing correlated with the severity of the pigmentation phenotypes. The group with severe pigmentation phenotypes had a mean mutation rate of 57.5%, while the group with modest pigmentation phenotypes had a mean mutation rate of 41.7%. In addition, the mutation rate is more variable in the group with modest phenotype, possibly due to subpopulations of independent germline insertions (Table S2). Nevertheless, the degree of mutagenesis in the 17 double carriers was positively correlated with each other in the same fish (*insra* vs. *tyr*, $r^2 = 0.72$; *insrb* vs. *tyr*, $r^2 = 0.71$) (Figure 3E), confirming our expectation that the severity of pigmentation phenotype may be a good predictor of the mutagenesis efficiency at the other targets. Sequence analysis in *insra* and *insrb* provided evidence for the benefit of using two sgRNAs. In addition to increasing the mutagenesis efficiency, two sgRNAs also resulted in deletion of the intervening sequence, leading to a prominent product in the PCR reaction that constituted close to 30% of the indels in *insra* and 15% of the indels in *insrb* (Figure 3F, Table S3, and Table S4). These deletions would be expected to result in complete loss of function. Sequencing analysis also revealed extremely high mosaicism, which was expected from transgenic expression of CRISPR/Cas9 since mutagenesis should occur after maternal–zygotic transition at the 1000-cell stage (Kane and Kimmel 1993). More than 3511 *insra* alleles were identified in an individual from which about ~75,000 reads were obtained. Despite the diversity, however, a set of highly frequent alleles was found in all the individuals. For example, the top three alleles of each locus were the same among the fish in the group with severe phenotypes and they constituted ~40% of all the reads in each individual (Figure 3F). The same three alleles were also identically ranked in the other group (Table S3, Table S4, and Table S5). As expected, one of the top alleles for *insra* and *insrb* was from precise deletion between the two DSBs. The prevalence of other common deletions suggested that the NHEJ repair is not random. Inspection of the DSB-flanking sequences suggested that the most frequent alleles may result from microhomology-mediated repair (Figure S1), supporting previous reports (Wang *et al.* 2013; Thomas *et al.* 2014).

Given the importance of insulin receptor in glucose homeostasis (Kitamura *et al.* 2003), we tested whether *Tg(U6x:sgRNA(insra/b); LC)*; *Tg(actb2:cas9; LR)* double carriers with severe pigmentation defects had defective glucose metabolism. At 6 dpf, the double carriers had similar fasting total free glucose content to single carrier siblings (Figure 3G). However, after feeding with 5% chicken egg yolk (Carten *et al.* 2011; Maddison and Chen 2012, the glucose levels increased almost twofold in double carriers, but remained unchanged in single carrier siblings ($n = 5$, $P < 0.01$) (Figure 3G). Thus, transgenic expression of *cas9* and sgRNA targeting *insra* and *insrb* resulted in impairment of glucose homeostasis.

Conditional CRISPR/Cas9 mutagenesis in transgenic fish

The primary impetus for the above experiments was to develop CRISPR/Cas9-based conditional mutagenesis. This can be achieved through regulated expression/availability of either *cas9* or sgRNA. To temporally control *cas9* expression, we generated *Tg(hsp70:loxP-mCherry-STOP-loxP-cas9)* lines [referred to as *Tg(HotCre:cas9)*], to allow heat-shock induction of *cas9* expression in a Cre-dependent manner (Hesselson *et al.* 2009). As a proof-of-principle experiment, we injected *in vitro* transcribed Cre mRNA into zygotes from *Tg(HotCre:cas9) × Tg(U6a:tyr(sgRNA); LC)* to remove the “STOP,” and heat shocked the progeny at 6 or 24 hr postfertilization (hpf) to induce *cas9*. All *Tg(HotCre:cas9); Tg(U6a:tyr(sgRNA); LC)* double carriers had hypopigmentation phenotypes (Figure 4A). In the absence of either Cre mRNA or heat shock, no pigmentation phenotypes were detectable (Figure 4A). The degree of mutagenesis in these embryos, as determined by HMA (Figure 4B), was slightly lower than in larvae with constitutive expression of *cas9*.

To spatially control Cas9 expression, we generated *Tg(fabp10:cas9, CG)* lines in which *cas9* expression is liver specific. These fish were crossed to *Tg(U6x:sgRNA(insra/b); LC)* to obtain double carriers. To determine whether liver-specific mutagenesis occurred in the double carriers, we assessed mutagenesis at *insra* and *insrb* loci in liver and muscle at 1 month of age. HMA indicated that close to 60% of the *insra* and *insrb* loci were mutated in the liver (Figure 5A). Considering that hepatocytes constitute ~75% of the liver at this stage (Ni *et al.* 2012), the fraction of mutant alleles in *cas9*-positive cells is close to 80%, similar to that in the ubiquitous *cas9* lines. We did not detect mutations in skeletal muscle, confirming tissue specificity of mutagenesis (Figure 5A).

Figure 5 Liver-specific CRISPR/Cas9 mutagenesis of *insra* and *insrb* impairs glucose homeostasis. (A) HMA results indicating liver-specific mutagenesis. Liver and muscle from 1-month-old *Tg(fabp10:cas9; CG); (U6x:sgRNA(insra/b); LC)* fish were analyzed. (B) Impaired glucose metabolism in *Tg(fabp10:cas9; CG); (U6x:sgRNA(insra/b); LC)* fish. Fasting and postprandial glucose levels (mean $\pm$ SE) were measured in 3-month-old transgenic fish and wild-type control fish. Transgenic fish ($n = 8$) had lower fasting glucose and higher postprandial glucose than wild-type control fish ($n = 10$); *t*-test, * $P < 0.05$, ** $P < 0.01$.

Insulin signaling in liver promotes glycogen synthesis and suppresses gluconeogenesis. Loss of *insra/b* function in the liver impairs glucose homeostasis. In mice with liver-specific disruption of insulin receptor (LIRKO), fasting blood glucose levels are lower and fed blood glucose levels are higher at 6 months of age due to impaired glycogen storage and weakened suppression of gluconeogenesis, respectively (Michael *et al.* 2000). To test whether the CRISPR/Cas9-mediated liver mutagenesis is sufficient to impair glucose metabolism, we determined fasting and postprandial blood glucose in 3-month-old *Tg(fabp10:cas9; CG); Tg(U6x:sgRNA(insra/b); LC)* fish. Fasting and postprandial glucose levels were measured a week apart in the same group of fish. Compared to wild-type controls, the *Tg(fabp10:cas9; CG); Tg(U6x:sgRNA(insra/b); LC)* fish had lower fasting glucose levels (27.36 ± 4.15 vs. 37.53 ± 2.24 mg/dl, $P < 0.037$), but markedly higher postprandial glucose (190.97 ± 29.70 vs. 59.01 ± 11.50 mg/dl, $P < 0.001$) (Figure 5B). Thus, the phenotypes in the Cre-mediated, conditional LIRKO mouse were recapitulated using liver-specific inactivation of insulin receptor genes in zebrafish using CRISPR/Cas9.

The tissue specificity of mutagenesis may also be achieved by targeted delivery of sgRNAs. To test this possibility, we targeted *ascl1a* that is essential for Müller glia dedifferentiation and retina regeneration in zebrafish (Ramachandran *et al.* 2010). Two sgRNAs targeting *ascl1a* were synthesized *in vitro* (Figure 6A) and delivered into one eye of dark-adapted *Tg(actb2:cas9; LR)* adults by injection followed by electroporation. After a 48-hr exposure to constant, high-intensity light to induce photoreceptor degeneration and Müller glia proliferation, the number of proliferating cells in both eyes was assessed using PCNA immunofluorescence. The number of PCNA⁺ progenitor cells in the inner nuclear layer of the sgRNA(*ascl1a*)-exposed retina was decreased by fivefold, to a mean of <9 compared to an average of >45 in the sgRNA(*EGFP*)-injected control retinas ($P < 0.01$) (Figure 6, B and C). The data indicate that spatial- and temporal-specific mutagenesis can be achieved by controlling either *cas9* expression or sgRNA delivery.

Discussion

Spatially and temporally controlled gene inactivation is critical for understanding detailed gene function. We show

here that the recently developed CRISPR mutagenesis can be adopted for controlled mutagenesis in zebrafish.

Our data indicate that transgenically expressed *cas9* and sgRNA can cause biallelic gene inactivation in somatic cells in a controllable fashion. Although CRISPR mutagenesis using stable transgenic lines expressing both *cas9* and sgRNA have been used for generating both germline and somatic mutations in nonvertebrate models such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Port *et al.* 2014; Shen *et al.* 2014; Xue *et al.* 2014), it has not been reported in vertebrates until our manuscript was being revised (Ablain *et al.* 2015). A Cas9 knock-in mouse line has been used to achieve tissue-specific mutagenesis (Platt *et al.* 2014; Swiech *et al.* 2015). However, in those reports, sgRNA was delivered by virus or nanoparticles rather than a transgenic approach. Although viral and nanoparticle delivery of sgRNA has many advantages, not every tissue/organ is accessible by this approach. Even in accessible tissues/organs, not all cells are equally targeted. For zebrafish, a reliable method of virus- or nanoparticle-mediated gene expression is not available. However, we achieved retinal-specific inactivation of *ascl1a* using targeted injection of sgRNA followed by electroporation. Other methods, such as lipofection (Ando and Okamoto 2006; Chang *et al.* 2014), may also be used for targeted delivery of sgRNA, though specificity and accessibility remain a concern. Transgenic lines with spatial and temporal control of *cas9* expression are a better choice for achieving desired control of mutagenesis in somatic cells. Although it requires the availability of suitable *cas9* lines, the high efficiency of transgenesis and the availability of many characterized tissue-specific promoters in zebrafish make this a straightforward and efficient process. The collection of Gal4 enhancer trap lines may also be used to direct UAS-driven tissue-specific expression of *cas9* (Scott *et al.* 2007; Kawakami *et al.* 2010). Several inducible expression systems, such as heat shock (Halloran *et al.* 2000), tetracycline (Knopf *et al.* 2010), and RU468 (Emelyanov and Parinov 2008) have been successfully adapted into zebrafish and could be used to achieve spatial and temporal control of *cas9* expression. Our *Tg(HotCre:cas9)* lines should provide both spatial and temporal control (Hesselson *et al.* 2009).

Conditional CRISPR mutagenesis may be a viable alternative to Cre/Flp-dependent conventional approaches. The

Figure 6 Retinal-specific CRISPR/Cas9 mutagenesis of *ascl1a* by targeted delivery of sgRNA suppresses damage-induced regeneration. (A) Sequence of *ascl1a* target regions. (B) Fewer proliferative cells were detected in *Tg(actb2:cas9; LR)* retinas after *sgRNA(ascl1a)* injection than *sgRNA(EGFP)*-injected retinas. Bar, 60 μ m. (C) Quantification of B. A significant decrease of PCNA⁺ cells was observed (mean $\pm$ SE, nonparametric Mann–Whitney *U*-test, *P* < 0.01).

observed indel frequency caused by cas9 is comparable to the recombination frequency induced by Cre/Flp. A major advantage of conditional CRISPR mutagenesis is that it does not require a preengineered conditional allele. Although a large collection of conditional alleles is available in mice (Skarnes *et al.* 2011), in general a conditional allele is time consuming to generate. Another advantage of conditional CRISPR mutagenesis is that only two elements, *cas9* and sgRNA, need to be bred together for conditional CRISPR mutagenesis, compared to three (two mutant alleles and a Cre/Flp transgene) for the conventional approach. This will decrease at least one generation of breeding. Finally, conditional CRISPR mutagenesis can target multiple genes simultaneously, while conventional conditional mutagenesis requires extensive breeding to achieve multiplex mutagenesis. Although conditional CRISPR mutagenesis does require the generation of sgRNA-expressing lines, this is much easier than generating an engineered conditional allele. The disadvantage of conditional CRISPR mutagenesis is that the mutations are very heterogeneous, while precise mutations are generated with the conventional approach. This can lead to a significant decrease of biallelic inactivation if the targeted region is tolerant of in-frame deletions. In such a scenario, only about two of three indels may be loss of function, resulting in biallelic inactivation in only four of nine cases. An advantage of our system is that it allows for targeting multiple sites in a single gene. If two distant sites are chosen, independent mutagenesis at each site would decrease the frequency of potentially hypomorphic small in-frame indels from one in three to one in nine. The deletion of the intervening sequence should further lower the frequency of hypomorphic alleles. If only one sgRNA is used for a gene, it is critical to target functionally important regions of a gene for conditional CRISPR mutagenesis so that even in-frame deletions result in severe loss of function.

We found that the phenotype severity induced by CRISPR/Cas9-mediated somatic mutagenesis is not uniform among siblings. The major cause may be individual difference in mutation rates. This could be due to different levels of cas9 or sgRNA expression since some of our transgenic fish carry more than one transgene insertion. Nevertheless, similar variation has been observed in flies when each of the transgenes is a single insertion at a defined genomic locus

(Port *et al.* 2014). Another cause may be the heterogeneity of the mutations. The mutated fish are highly mosaic at the target sites with some indels impairing function more than others. Individual animals likely have a different complement of mutations from each other, leading to phenotypic variation.

There is a high degree of correlation among the indel frequencies at different targets within the same fish. This suggests that the level of mutagenesis of one target can serve as an indicator for the rest. For ubiquitous mutagenesis, *tyr* is a good indicator target because of its restricted expression in pigmented cells and its easily visible phenotype due to biallelic inactivation. This allows easy identification of mutant fish. In addition, hypopigmentation minimizes the interference of fluorescence detection, facilitating fluorescent marker-based genetic or chemical screens for modifiers of mutant phenotype. For tissue-specific gene inactivation, however, other easily scorable markers need to be developed.

Taken together, our findings demonstrate that transgenic expression of cas9 and sgRNA is sufficient to generate extensive biallelic somatic mutations for functional analysis. The mutagenesis may be multiplexed for increased efficiency, eliminating redundancy, and/or analyzing gene–gene interaction. Multiplex conditional mutagenesis can be achieved by inducible or tissue-specific expression of cas9 or by tissue-specific delivery of sgRNAs.

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Supporting Information

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Multiplex Conditional Mutagenesis Using Transgenic Expression of Cas9 and sgRNAs

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Tyr
WT CAGCTCGTCTCTCCAGCAGTTCCCCGAGTCTGCA**CCTCCCAGAAAGTCTCCAGTCC**AAACGCTGCTGTCCAGTCTGG
D_143_13 CAGCTCGTCTCTCCAGCAGTTCCCCGAGTCTGCACCTCC-----AGTCCAAACGCTGCTGTCCAGTCTGG
D_146_1 CAGCTCGTCTCTCCAGCAGTTCCCCGAGTCTGCACCTCC--CAGAAGTCTCCAGTCCAAACGCTGCTGTCCAGTCTGG
D_146_2 CAGCTCGTCTCTCCAGCAGTTCCCCGAGTCTGCACCTCC--AGAAGTCTCCAGTCCAAACGCTGCTGTCCAGTCTGG
D_149_2 CAGCTCGTCTCTCCAGCAGTTCCCCGAGTCTGCACCTCC--GAAGTCTCCAGTCCAAACGCTGCTGTCCAGTCTGG
D_135_18 CAGCTCGTCTCTCCAGCAGTTCCCCGAGTCTGCACCTCC-----CTCCAGTCCAAACGCTGCTGTCCAGTCTGG
D_147_9 CAGCTCGTCTCTCCAGCAGTTCCCCGAGTCTGCACCTCC-----CTCCAGTCCAAACGCTGCTGTCCAGTCTGG

Insra
WT **CCACGCATTTGTTAGACGAGTGG**GGCTCCAAACAGCCGCCAGACACTGGTCTGTCGTCAGTGGCTGTCTTTAGTGCAG**GCGCGGTGCTTGCA****TGGA****TGG**
D_91_80 CCACGCATTTGTTAGAC-----GGATGG
D_88_3 CCACGCATTTGTTAGAC---GTGGGGCTCCAAACAGCCGCCAGACACTGGTCTGTCGTCAGTGGCTGTCTTTAGTGCAGGCGCGGTGCTTGCA**TGGA****TGG**
D_87_5 CCACGCATTTGTTAGAC---TGGGGCTCCAAACAGCCGCCAGACACTGGTCTGTCGTCAGTGGCTGTCTTTAGTGCAGGCGCGGTGCTTGCA**TGGA****TGG**
D_169_3 CCACGCATTTGTTAGACGAGTGGGGCTCCAAACAGCCGCCAGACACTGGTCTGTCGTCAGTGGCTGTCTTTAGTGCAGGCGCGGTGCTTGCA**TGGA****TGG**
D_90_76 CCACGCATTTGTTAGAC-----ATGAGGATGG

Insrb
WT CCACCGAATCCAAGATCTTGGACCA**GATCTTGGACCA****GAGTGG**ACAAATAGCACAGGTTTGGGTTCTTCTCGATGCGCA**CCGCTCCCCGCGTGATGTTGGTCA**AACTG
I_111_1 CCACCGAATCCAAGATCTTGGACCA**GATCTTGGACCA****GAGTGG**ACAAATAGCACAGGTTTGGGTTCTTCTCGATGCGCA**CCGCTCCCCGCGTGATGTTGGTCA**AACTG
D_112_48 CCACCGAATCCAAGATCTTGGACCA-----CCCGCGTGATGTTGGTCAAACTG
D_106_7 CCACCGAATCCAAGATCTTGGACCA-----GGACAAATAGCACAGGTTTGGGTTCTTCTCGATGCGCA**CCGCTCCCCGCGTGATGTTGGTCA**AACTG
D_112_1 CCACCGAATCCAAGATCTTGGACCA**GATCTTGGACCA****GAGTGG**ACAAATAGCACAGGTTTGGGTTCTTCTCGATGCGCA**CCGCTCCCCGCGTGATGTTGGTCA**AACTG
D_100_15 CCACCGAATCCAAGATCTTGGAC-----AAATAGCACAGGTTTGGGTTCTTCTCGATGCGCA**CCGCTCCCCGCGTGATGTTGGTCA**AACTG

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 AMMCN₁₈C (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 (1 µl 100 µM stock each in a 20 µl 1x NEB buffer 2) by incubating the mixture at 95°C for 5 min, ramping down to 50°C at 0.1°C/sec, incubating at 50°C for 10 min, and chilling to 4°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, 0.25 µl U6 plasmid (about 100ng), 1 µl annealed oligos, 0.3 µl T4 DNA ligase, 0.3 µl BsmBI, 0.2 µl PstI (optional), 0.2 µl Sall (optional), 5 µl H₂O] and incubating 3x(37°C for 20 min, 16°C for 15 min), then 37°C for 10 min, 55°C for 15 min, followed by 80°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 (50 µg/ml) plates.

Construction of pGGDestTol2LC-sgRNA vectors

1. Ligate the U6x:sgRNA cassettes into a pGGDestTol2LC vector by mixing the components (2 µl 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), 1 µl BsaI, 1 µl T4 DNA ligase, adjust volume to 20 µl with H₂O).
2. Incubate mixture 3x(37°C for 20 min, 16°C for 15 min), followed by 80°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

B

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)