Efficient genome engineering approaches for the short-lived African turquoise killifish

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A central challenge in experimental aging research is the lack of short-lived vertebrate models for genetic studies. Here we present a comprehensive protocol for efficient genome engineering in the African turquoise killifish (*Nothobranchius furzeri*), which is the shortest-lived vertebrate in captivity with a median life span of 4–6 months. By taking advantage of the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 nuclease (CRISPR/Cas9) system and the turquoise killifish genome, this platform enables the generation of knockout alleles via nonhomologous end joining (NHEJ) and knock-in alleles via homology-directed repair (HDR). We include guidelines for guide RNA (gRNA) target design, embryo injection and hatching, germ-line transmission and for minimizing off-target effects. We also provide strategies for Tol2-based transgenesis and large-scale husbandry conditions that are critical for success. Because of the fast life cycle of the turquoise killifish, stable lines can be generated as rapidly as 2–3 months, which is much faster than other fish models. This protocol provides powerful genetic tools for studying vertebrate aging and aging-related diseases.

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INTRODUCTION

The African turquoise killifish (*N. furzeri*) has emerged as an exciting vertebrate model system for experimental aging because of its naturally compressed life span and short generation time[1–3] (**Fig. 1a**). These features are likely to be an important adaptation to its seasonal habitat—ephemeral water pools in southeast Africa, where water is present only during a brief rainy season[1,3,4] (**Fig. 1b**). The turquoise killifish maintains its compressed life span and short generation time in the laboratory, when water is in constant supply. The turquoise killifish was first collected in 1968 in the Gonarezhou National Park (Zimbabwe), and the inbred GRZ line, with a median life span of 4–6 months, is currently the shortest-lived vertebrate that can be bred in captivity—even when compared with other annual killifish[1,3–7].

Old turquoise killifish exhibit a comprehensive range of phenotypes that are typical of vertebrate aging, including decline in mitochondrial function[8], cognitive impairment[9] and increased incidences of neoplastic lesions[4,10]. Moreover, similar to other aging model systems, the life span of the turquoise killifish can be manipulated by conserved environmental interventions such as dietary restriction, temperature and drug treatments[9,11,12]. Finally, the turquoise killifish's naturally compressed life cycle and rapid sexual maturity of roughly 3–4 weeks are crucial features for genetic manipulations, because they allow the rapid generation of stable lines[2]. These unique life history characteristics allow the turquoise killifish to bridge a long-standing gap between traditional genetic models for experimental aging: the nonvertebrate models (yeast, worms and flies), with life spans of weeks or months, and vertebrate models (mouse and zebrafish), with life spans of several years (**Fig. 1a**).

Teleost fish have substantial advantages as laboratory animals, exemplified by the success of the zebrafish model system[13,14]. Fish share key organs and systems with human physiology, some of which are unique to vertebrates (such as blood, bones and spleen). Furthermore, their external fertilization provides increased accessibility for manipulation and a clear view of embryonic development. Many teleost fish also exhibit human-like short telomeres[15], remarkable regenerative capacity[16] and they are compatible with high-throughput approaches, specifically genetic and drug screens[17]. Because of their extreme adaptations to a wide range of habitats, fish have been successfully used to study interesting biological questions, including adaptive evolution in sticklebacks[18] and eye degeneration in cave fish[19]. Similarly, the turquoise killifish's adaptation to its extreme environment has yielded a compressed life span that provides a unique opportunity for studying vertebrate aging. The turquoise killifish has additional advantages when compared with other fish (such as zebrafish), including an XY-based sexual determination system[20,21] and fascinating developmental features (developmental dormancy during drought referred to as diapause[22] and active migration of blastomeres before gastrulation[23]). In addition, the highly inbred GRZ strain[1,24,25] facilitates reference genome assembly and genome engineering, and it provides a point of reference among different laboratories using the turquoise killifish. Finally, there exist several distinct wild-derived strains with different characteristics[10] that have allowed for the genetic mapping of traits, including color, sex and life span[6,20,21,26,27].

Efficient and reliable ways to generate precise changes to the genome of model organisms have been a long-standing goal of discovery-based and translational research. However, until recently, genetic experimental approaches have been entirely lacking in this species. Transgenesis using Tol2-based random integration was the first step in developing genome engineering approaches in the turquoise killifish[28–30], thus allowing for the expression of exogenous genes in a temporal and tissue-specific manner. More recently, a comprehensive genome-to-phenotype platform that allows precise genome editing in the turquoise killifish has been developed[2]. This platform includes the *de novo* assembled genome sequence (detailed analysis of the turquoise killifish genome was reported in refs. 6,21) and a rapid genome-editing pipeline in this organism using the CRISPR and Cas9 system[2]. The genome-to-phenotype platform in the turquoise killifish enabled efficient targeting of multiple disease- and aging-related genes[2]. For example, this pipeline, combined with the turquoise

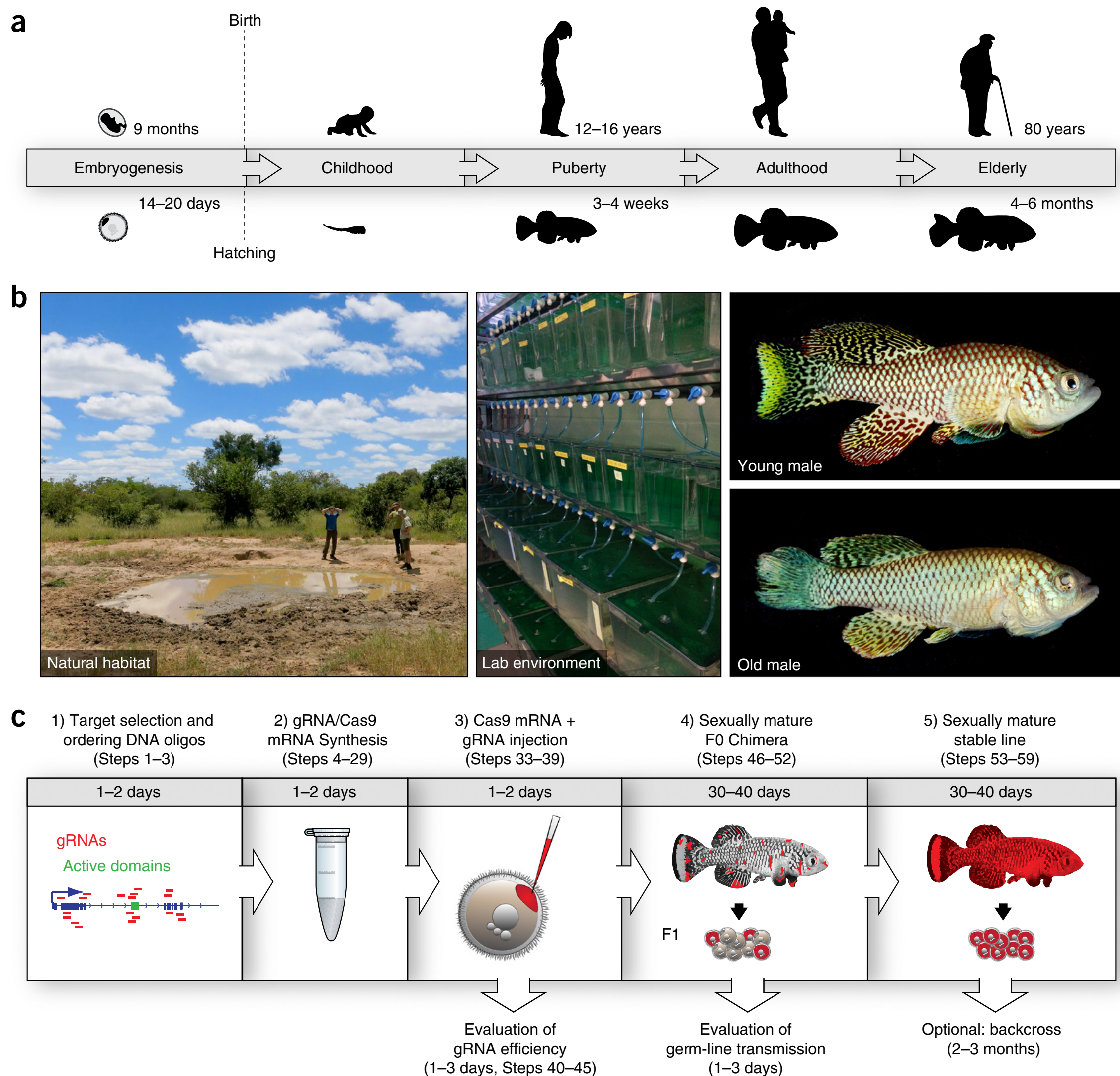

Figure 1 | The turquoise killifish model for experimental aging research. (**a**) Important landmarks in the life history of humans and the turquoise killifish, and their respective timeline. From fertilization to hatching (usually between 14 and 20 d post fertilization), animals are referred to as 'embryos'. Freshly hatched fish are referred to as 'fry' for the first few days, and shortly after they are referred to as 'juvenile fish' (until the animals reach sexual maturity). At the age of 20–30 d post hatching (d.p.h.), the animals reach sexual maturity and are considered adults. The life cycle (from 'egg to egg') of the turquoise killifish is 5–7 weeks. (**b**) The turquoise killifish's natural habitat (ephemeral ponds in Zimbabwe and Mozambique, left) and lab environment (housing using water recirculating systems, middle). Right: an example of a young and an old turquoise killifish male. (**c**) A genome-to-phenotype platform for generating stable mutant fish lines in the turquoise killifish, including a detailed timeline. Panel **b** (left and center images) photo credit: Itamar Harel. Right-hand images in panel **b** adapted with permission from ref. 1, Cold Spring Harbor Press. Panel **c** adapted with permission from ref. 2, Cell Press.

killifish's compressed life span, has allowed us to generate the fastest vertebrate model for a human age-associated telomere syndrome (e.g., dyskeratosis congenita), by knocking out the protein subunit of telomerase[1,2]. Genome engineering approaches, including transgenesis and genome editing, have transformed the naturally short-lived turquoise killifish into a promising genetic model system for experimental aging research.

CRISPR and *Cas* genes are essential components in bacterial adaptive immunity. A tool based on the bacterial Cas9 from *Streptococcus pyogenes* has provided a simple and efficient way to edit the genomes of multiple model and non-model organisms[31–33] (and reviewed in Hsu *et al.*[34]). In this tool, the Cas9 nuclease is guided by a gRNA molecule to a specific genomic locus, where, similar to earlier genome-editing strategies such as zinc-finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs; reviewed in Doudna and Sontheimer[35]), it generates a double-strand break (DSB). The DSB can then be repaired by either NHEJ or HDR (reviewed in Ran *et al.*[36]). NHEJ is frequently imprecise, and it can introduce indels (small deletions or insertions) in the genome. In cases in which the indels cause frameshift mutations, this can lead to a premature stop codon and a loss-of-function (i.e., knockout) mutation. HDR, on the other hand, uses a homologous DNA template for repairing the DSB, and thus it can be used to precisely introduce new sequences into the genome (i.e., knock-in)[35,37]. The CRISPR/Cas9 system has been instrumental for efficient genome editing in multiple model organisms, including the turquoise killifish, in which it has allowed the generation of knockout and

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a

PolG

5′ --> 3′
Exon
Intron
Target
Exon 2

Ranking	Target sequence	Genomic location	Exon	Strand	G+C content (%)	Off-targets		
						0	1	2
1	TGTTTCGCTGCCCTCTCCAG**AGG**	GapFilledScaffold_660:57362	2	+	61	0	0	0
2	GAGAAAATGATCCTCTGGAG**AGG**	GapFilledScaffold_660:57373	2	–	48	0	0	0
3	GTATCATTCGCTTCAGGTCG**AGG**	GapFilledScaffold_660:57435	2	+	52	0	0	0
4	GGATGTTAAGGGGGTTGATG**CGG**	GapFilledScaffold_660:57471	2	–	57	0	0	0
5	GACAGCATCTGGATGTTAAG**GGG**	GapFilledScaffold_660:57481	2	–	52	0	0	0
6	GGTTGTGCCGGAGTACAGAG**CGG**	GapFilledScaffold_660:57534	2	+	65	0	0	0
7	GGAGTACAGAGCGGCAGATG**TGG**	GapFilledScaffold_660:57543	2	+	61	0	0	0

Variable oligo (PolG target #6): 5′-TAATACGACTCACTATAGGTTGTGCCGGAGTACAGAGGTTTTAGAGCTAGAAATAGCAAG
Variable oligo (PolG target #7): 5′-TAATACGACTCACTATAGGAGTACAGAGCGGCAGATGGTTTTAGAGCTAGAAATAGCAAG
PolG-F PCR oligo: 5′-GCCCTCTCCAGAGGATCATT
PolG-R PCR oligo: 5′-ACCTCCCAGCTCCATTCCT

b

gRNA genomic target, Steps 4–6
T7 promoter
60 bp (variable)
80 bp (constant)

T4 DNA polymerase, Steps 7–8

Cleaning template, Step 9

T7 RNA polymerase, Step 10
PolG gRNAs (target #) #6 #7

c

PolG target #6 (6/10 with mutations)
Wild-type sequence:
GATGCTGCGCTCCACATCTG**CCG**CTCTGTACTCCGGCACAACCCCATG
Mutated sequences (examples):
GATGCTGCGCTCCACATCTG**CCG**-------CTCCGGCACAACCCCATG
GATGCTGCTCTCCTCATCTG**CCG**CTCCG------GGACAACCCCGTGG
GATGCTGCGCTCCACATCTG**CC**-----GTACTCCGGCACAACCCCATG

PolG target #7 (9/10 with mutations)
Wild-type sequence:
TGCTTGATGCTGCGCT**CCA**CATCTGCCGCTCTGTACTCCGGCACAACC
Mutated sequences (examples):
TGCTTGATGCTGCGCT---------CCGCTCTGTACTCCGGCACAACC
TGCTTGATG------------CTGCCGCTCTGTACTCCGGCACAACC
TGCTTGATGCTGCGCT**CCA**CAT-------------------------
TGCTTGATGCTGCGCT**CCA**CATGCTCCCTGCCGCTCTGTACTCCGGCA

HhaI digestion – + + +
gRNA injection (target #) – – #6 #7

d

TERT

exon 11

Genomic sequence
GGTCTGGTGGTCAACCCGCAGAAGG**TGG**TGGTGAACT

Co-injected ssDNA template containing K836R point mutations
...GGTCTGGTGGTCAACCCGCAGAGGG**T**C**G**TGGTGAACT....
Homology arm (~35 bp) Homology arm (~35 bp)

HDR-based genome engineering translates to precise protein mutations

YGLVVNPQKVVVNF WT
YGLVVNPQRVVVNF Precise K836R (10%)
YGLVVNPQRVVVSF Partial (10%)
YGLVSTRRGSW* **fs**
YGLVVNPQK-VVNF V837del

Figure 2 | CRISPR/Cas9-based genome editing. (**a**) NHEJ-based genome editing for an additional aging and a disease-related gene, the catalytic subunit of the mDNA (*PolG*). An output from CHOPCHOP (https://chopchop.rc.fas.harvard.edu) providing a list of suggested gRNA targets (top). The sequences of the variable oligonucleotide and oligonucleotides for PCR amplification used in this example (bottom). (**b**) A diagram for the synthesis of gRNAs used in CRISPR/Cas9-based genome editing (top), and a representative example for gRNAs (with targets sequences #6 and #7) resolved on an agarose gel (bottom). (**c**) Examples for direct sequencing (top) and enzymatic digestion (bottom), as classical options for downstream analysis. (**d**) HDR-based genome editing in the *TERT* gene (exon 11), for precise editing of new sequences into the genome (i.e., knock-in). The core sequence of the ssDNA template used to edit a genomic region encoding for a lysine residue (K836) in the turquoise killifish TERT protein (left), and the specific amino acid residues modified as a result (right). gRNA targets are in red, and PAM sequences are in bold. HhaI restriction site is highlighted with a yellow background, and mutated sequences are in blue. Oligo, oligonucleotide; fs, frameshift; del, deletion. Panel **d** adapted with permission from ref. 2, Cell Press.

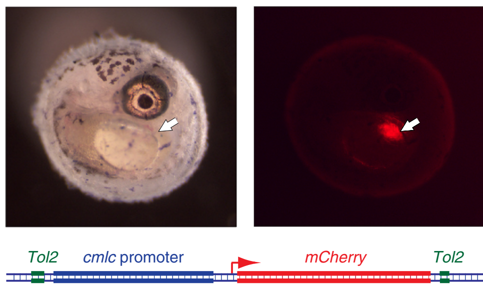

Figure 3 | Tol2-based transgenesis. A new transgenic line harboring the mCherry fluorescence reporter, under the zebrafish cardiac myosin light chain *(cmlc)* promoter, is specifically expressed in the heart. This gene cassette can be used as a surrogate marker for detecting successful germ-line transmission.

knock-in alleles in multiple genes[2]. Overall, our current protocol, which takes advantage of the turquoise killifish's short generation time, allows for the generation of stable lines in as quickly as 2–3 months, both for CRISPR/Cas9-based genome editing[2] and Tol2-based transgenesis[28].

Development of the protocol

Genome engineering approaches in fish have greatly benefited from microinjection protocols[38], transposase systems[39–41] (such as Tol1, Tol2 and Sleeping Beauty) and, more recently, genome-editing approaches, such as ZFNs and TALENs[42], and CRISPR/Cas9[43–46]. However, successful implementation of CRISPR/Cas9-based genome editing[2] (**Figs. 1c** and **2**), transgenesis[28–30] (**Fig. 3**) and microinjection[2,28] (**Figs. 4** and **5**) in the turquoise killifish required optimization of previously described protocols for other fish. Several husbandry protocols have been described for the African killifish[2,4,10,20], including a recent detailed laboratory breeding protocol (ref. 47). Adaptations to our initial housing protocol were also developed here to enable large-scale husbandry in commercial water recirculating systems (first described in ref. 2, and see **Fig. 1b**).

Injection apparatus. The egg of the turquoise killifish possesses a tough chorion (probably because of adaptation to its natural, harsh environment), which makes it largely inaccessible using an injection needle optimized for zebrafish microinjections. This obstacle was overcome by developing an injection mold of specific dimensions to hold the egg securely during injection and by optimizing the injection needle to enable it to penetrate the chorion more easily[2,28]. We continued to improve upon these key aspects to further increase efficiency and postinjection survival; the most up-to-date dimensions for the injection mold are provided in the **Supplementary Methods**, and new settings for the injection needle are described in 'Equipment Setup' and **Figure 5**.

Injection reagents. To achieve the highest rate of CRISPR/Cas9-based genome editing[2], we tested several Cas9 variants (e.g., codon-optimized and nuclear-localized variants) and found that the variant described in this protocol (nCas9n; see 'Reagents') was the most efficient. It was also necessary to optimize the injection volume (2.5–10 pl) and to determine the optimal concentration range for each component of the injection mix (see PROCEDURE Step 37).

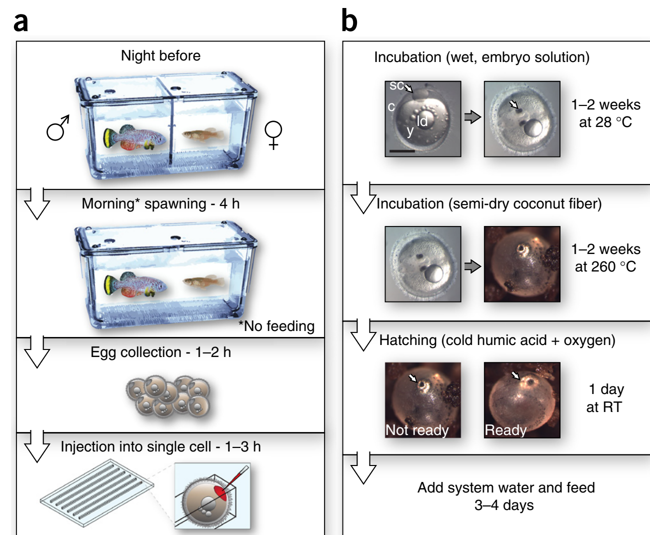

Figure 4 | Injection and hatching. (**a**) A diagram visualizing the key steps in the injection process described in Steps 33–39. (**b**) A diagram visualizing the key steps in the hatching process described in Steps 46–52. Top: white arrows point to the single cell (left), and early eye formation (right). Bottom: white arrows point to the size of the eyes in a non-ready embryo (left) and a ready-to-hatch embryo (right); Scale bar, 0.5 mm. Photo credit: Itamar Harel and Chi-Kuo Hu. RT, room temperature; sc, single cell; ld, lipid droplets; c, chorion, containing surface projections; y, yolk.

Embryo incubation. The embryonic development of the turquoise killifish differs from other fish models; it is largely nonsynchronous, and it requires aquatic and nonaquatic phases during development. In addition, embryos can enter a long phase of diapause[21,48] (for months and even years). These adaptations required the identification of incubation temperatures, thus allowing for stereotypical development, and the development of hatching protocols with optimal survival[2,28]. We previously incubated embryos at 26 °C, but we observed that development could be largely nonsynchronous, with some embryos developing in 14–20 d and others entering diapause for variable periods of time (for weeks or months). We have since found that development progresses in a more synchronous manner at 28 °C, and at this temperature the majority of embryos skip diapause and are ready to hatch by 14–20 d (for more details see 'Hatching', PROCEDURE Steps 46–52, and **Fig. 4b**). In general, the higher the temperature, the faster the development proceeds. For example, at 20–23 °C, development can naturally pause and the embryos can stay in a state of diapause for several months[21,48], which can be useful for the preservation of strains.

Fish growth and maintenance. The rapid growth, early sexual maturity and high fecundity of turquoise killifish leads to a requirement for frequent feeding with a rich diet, and previous studies have primarily depended on live, frozen and freeze-dried food. To facilitate standardized conditions of growth and maintenance, we tested many commercially available feeds, and we provide guidelines for feeding with dry pellets.

Overview of the procedure

We provide a detailed protocol for CRISPR/Cas9-based genome editing in the turquoise killifish[2], including generation of knockout

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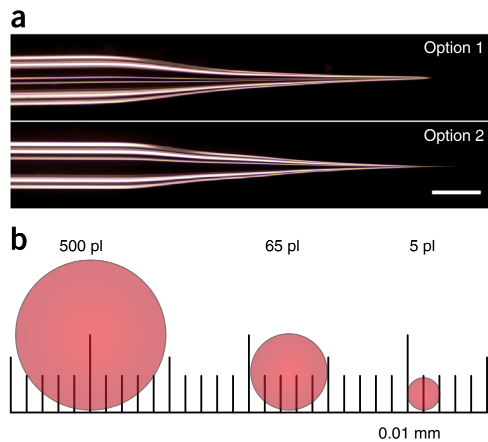

Figure 5 | Preparation of injection needles. (**a**) Two main options for injection needles. The tip of the needle is broken by gently rubbing the tip on a Kimwipe. Injections are made into the cell, not the yolk. (**b**) A diagram of a micrometer slide (with injection droplets in different sizes) as seen through a stereoscope. Each needle should be calibrated for reproducible injection volumes.

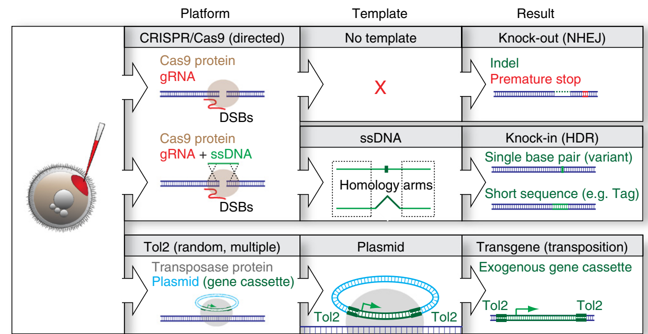

Figure 6 | Genome engineering in the turquoise killifish. The main overlaps and differences between the genetic approaches detailed in this protocol, highlighting their specific advantages and potential applications.

alleles via NHEJ and knock-in alleles via HDR (**Fig. 6**). The protocol contains optimized guidelines for gRNA target design, embryo injection and hatching, germ-line transmission and minimizing off-target effects. It also includes specific adaptations for Tol2-based transgenesis (**Box 1**, and see **Figs. 3** and **6**) and optimized husbandry conditions that are critical for the success of these genome engineering approaches (specifically concerning feeding and hatching). From our experience, this protocol is highly efficient (e.g., frequency of successful editing is typically 7–90%; ref. 2) and it can be easily adopted, including by laboratories with a small turquoise killifish colony.

The protocol for the generation of genome-engineered turquoise killifish lines consists of three main stages: target selection, injections and germ-line transmission.

Target selection and synthesis of gRNA and CRISPR/Cas9 for genome editing. Target selection for a specific gene can be done via CHOPCHOP (https://chopchop.rc.fas.harvard.edu/; ref. 49), or CHOPCHOP v2 (http://chopchop.cbu.uib.no; ref. 50), which contains the genome sequence and gene models for the turquoise killifish[2], and it provides essential information for successful target selection (including the ranking of gRNAs, predicted genome-wide off-targets, possible restriction sites and oligonucleotide candidates for PCR amplification). Target sequences for gRNAs are selected according to the following sequence criterion: 5′-(N)20-NGG-3′. The first 20 base pairs are considered as the core target sequence (and they are present in the gRNA molecule), and the 3′ ‘NGG’ represents the protospacer adjacent motif (PAM) site in the genome[31–33,35]. Depending on the promoter used for gRNA synthesis, the first two 5′ nucleotides should be limited to GG (when using the T7 promoter, as described in this protocol) or to GN (when using the *U6* promoter). For each gene, 2–5 gRNA target sequences should be designed for the evaluation of efficiency (**Fig. 1c**), and their position should be optimized depending on the desired approach. Generally, for a knockout approach, gRNA target sequences should be selected to be upstream of critical protein domains, and for a knock-in approach gRNA target sequences should be selected to overlap the desired knock-in locus.

Once gRNA target sequences are selected, the CRISPR/Cas9 system is established by synthesizing the gRNAs and the *Cas9* mRNA. The gRNAs can be easily synthesized by T7-mediated transcription using two annealed oligonucleotides as templates[2,46,51] (**Fig. 2b**). This method does not require any cloning or digestion steps (**Fig. 2b**), and therefore it is extremely quick and is compatible with high-throughput approaches. It is helpful to design target gRNA sequences that will facilitate verification of successful genome editing—for example, by disruption of a restriction site (see **Fig. 2** for an efficient example, which could also be used as a positive control). For synthesis of the *Cas9* mRNA, we use SP6-mediated transcription of a codon-optimized nuclear-localized version[45] (nCas9n) template.

Injection and evaluation of gRNA efficiency for genome editing. The components required for the injection setup are overall similar to other protocols developed for fish[38], but they require specific adaptations for the African turquoise killifish model. To generate the fertilized eggs that are required for injection, mating pairs need to be set up. A single female fish can produce between 0 and 50 eggs. The accelerated growth, rapid sexual maturity and frequent mating of the turquoise killifish require high food consumption using high-quality food pellets supplemented with live/frozen food. Fecundity is dependent on food quality and frequency of feeding, as well as on the overall age and size of fish (larger female fish yielding a larger number of eggs). After the injection setup is established, the genome-editing efficiency of individual (or pooled) gRNAs could be estimated using any of the recently developed approaches, such as high-resolution melting (HRM)[52] or CRISPR-STAT[53]. We provide details for two classical, alternative approaches (**Fig. 2c**) that can be used if the indel is small, or if the exact sequence of the edited region is required: (i) designing the gRNA target to overlap with a unique restriction site that could be disrupted upon successful editing[2]; (ii) PCR amplification of the genomic DNA region surrounding the gRNA target sequence, cloning and direct sequencing of single colonies[2] (or sequencing of purified plasmids grown from single colonies). Once an optimal gRNA is selected, additional injections should be performed to generate F0 chimeric founders (**Fig. 1c**).

Germ-line transmission and backcross. Once the F0 chimeric founders are sexually mature (3–4 weeks), they should be set up into mating pairs with wild-type fish, and germ-line transmission

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Box 1 | Tol2-based transgenesis ● TIMING 2–3 months

DNA transposons are efficient tools for the generation of transgenic animals[73]. The medaka *Tol2* element encodes a functional transposase protein that can catalyze transposition of a construct flanked by specific *Tol2* DNA sequences[74]. A large DNA insert (such as a gene cassette) can be cloned between these sequences, and in the presence of the transposase protein the cassette will randomly integrate into the genome. This system has been demonstrated to work in many vertebrate cells, including zebrafish, *Xenopus*, chicken, mice and humans[74,75].

Several steps described in the current protocol for CRISPR/Cas9 genome editing can be fully used for Tol2-based transgenesis. However, there are four main differences (**Fig. 6**): (i) there is no gRNA target selection and synthesis (Steps 1–18 do not apply); (ii) instead of nCas9n mRNA, Tol2 transposase mRNA is synthesized (the overall protocol for Tol2 mRNA synthesis resembles that of nCasn9 synthesis (Steps 19–29), with specific differences highlighted below); (iii) an exogenous gene is introduced, and therefore a plasmid containing an exogenous promoter and gene of interest (flanked by two Tol2 sequences) is co-injected (described below); (iv) the detection of germ-line transmission is different, because of the fact that integration is random (described below, and see **Fig. 6**).

1. For *in vitro* transcription of Tol2 transposase mRNA, use the PCS2FA plasmid[75], purified using a Maxiprep kit, according the manufacturer's instructions. The PCS2FA plasmid is available from Kawakami (kokawaka@lab.nig.ac.jp). Additional details, tools and alternatives can be found on the Tol2Kit website: http://tol2kit.genetics.utah.edu/index.php/Main_Page.
2. Follow Steps 19–29 of the main PROCEDURE to linearize the PCS2FA with NotI enzyme, and to synthesize and purify the Tol2 transposase mRNA.
3. Prepare a construct flanked by specific Tol2 DNA sequences. Many transgenic constructs (developed for zebrafish or *Xenopus*) are predicted to work in the turquoise killifish, as previously demonstrated with the β-actin promoter from *Xenopus*[28] and zebrafish[29], and the zebrafish heat-shock protein 70 (HSP70)[30] promoters. New transgenic constructs could be generated using the Tol2Kit[75] with endogenous regulatory elements from the turquoise killifish genome, recently available as a bacterial artificial chromosome (BAC) library[21]: http://www.genome.clemson.edu.

▲ CRITICAL STEP When trying a new promoter, it is recommended to use a small surrogate marker on the same plasmid, such as a fluorescent protein gene under the control of the cardiac myosin light chain (*cmlc*) promoter[76] (expression in the heart, see **Fig. 3**) or gamma-crystalin (*γ-cryst*) promoter[39] (expression in the lens; W. Wei, personal communication). Select hatched fish (fry) expressing the fluorescence marker as F0 founders, as well as for detecting germ-line transmission in F1.

▲ CRITICAL STEP The purity of the co-injected DNA plasmid (containing a transgene of choice) is important. Use Maxiprep (Qiagen), preferably an endotoxin-free kit. Similar to CRISPR/Cas9 injections, expect ~50% survival for injected embryo, and ~50% embryos chimeric for the transgene.

4. Prepare an injection mixture (35 μl recommended volume) as tabulated below:

Component	Amount	Final concentration
Tol2 transposase mRNA (0.5–1 μg/μl)	1–2 μl	30 ng/μl
DNA plasmid (0.75–1.5 μg/μl) (containing a transgene of choice flanked by Tol2 sites)	1–2 μl	40 ng/μl
KCl (2 M)	7 μl	400 mM
Phenol-red (0.5% (wt/vol))	7 μl	0.1% (wt/vol)
Nuclease-free water	Up to 35 μl	
Total	35 μl	

5. Perform injections as described in Steps 33–39 of the main PROCEDURE.
6. Assess germ-line transmission visually using a fluorescence stereoscope in the F1 (when fluorescence reporters are used), or according to Steps 53–59 of the main Procedure using a set of oligonucleotides unique for the transgene.

▲ CRITICAL STEP The transgene can randomly integrate into several places in the genome, and backcrosses should be conducted until the construct is inherited in a Mendelian manner.

? TROUBLESHOOTING

should be evaluated using their progeny (fertilized eggs) by either of the methods described in the previous paragraph. Multiple types of indels or HDR-based knock-in variants (partial or in-frame) could be present in an individual fish (heterozygous or possibly homozygous, see **Fig. 2c**), as their germ line could be chimeric. Once germ-line transmission has been confirmed for a breeding pair, adult F1 progeny from that successful pair should be genotyped via fin clips. Fish that exhibit a specific desired indel (or knock-in) at this stage will be considered to be part of a stable line, and they can be backcrossed to wild-type fish to eliminate potential off-target effects generated by the Cas9 nuclease. Note that each individual F0 founder will exhibit a unique set of indels, and the F1 progeny will be heterozygous for the desired allele. For efficient gRNA targets, 2–10 founders are usually sufficient to successfully generate a stable line using an NHEJ-based knockout approach, and 10–30 founders are usually sufficient for an HDR-based knock-in approach (**Fig. 6**).

Advantages and limitations

The main advantage of using the turquoise killifish for experimental approaches is its short generation time and compressed life span, which allows one to quickly generate stable lines, to

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backcross them and to study adult phenotypes. The lack of standardized husbandry (including dietary requirements, fish density, temperature and water quality) is a limitation that probably contributes to differences in reported life-span measurements[6,10,24]. Although this protocol provides detailed guidelines that are experimentally verified for genome engineering, further optimization will be needed, in particular for life-span studies. Compared with zebrafish and mice, another challenge is the limited number of tools (i.e., antibodies, mutants and so on) that are available for the turquoise killifish. Finally, fish do not have all the features and organs that are present in mammals, such as lungs, mammary glands and hair.

Several genome-editing technologies (e.g., ZFNs and TALENs) have been successfully used to generate targeted mutations in other organisms, but those approaches have not yet been established in the turquoise killifish. Unlike those other genome engineering approaches, which rely on modified DNA-binding domains to provide targeting specificity, the CRISPR/Cas9 system relies on an RNA molecule to guide the Cas9 nuclease. This aspect allows for a much faster and simpler experimental design, and it is therefore more compatible with high-throughput approaches[2,31,45,54,55]. However, one of the limitations of the CRISPR/Cas9 system is the specific requirement for target design and potential off-target effects. Off-target effects could be mitigated, at least in part, by backcrosses, which are relatively fast in the turquoise killifish. An additional way to test for potential off-target effects is to create several mutant alleles for the same gene and to assess whether they have the same phenotype.

Extending the applications of the protocol

Tol2-based transgenesis[28–30] and CRISPR/Cas9-based genome editing[2] have been successfully used in the turquoise killifish. In this protocol, we further developed and optimized key aspects related to husbandry and genome engineering, and thus we provide a standardized platform for adapting additional genetic approaches. 'Split systems' allow for conditional gene regulation *in vivo,* such as precise temporal and tissue-specific expression of genes, as well as for lineage tracing. Split systems that have been successfully used in zebrafish include Gal4/upstream activating sequence (UAS)[56], Cre/*loxP*[57–59] and Tet-ON[60]. These approaches could be introduced in the turquoise killifish model using both the CRISPR/Cas9 genome editing and Tol2-based transgenesis. For example, the CRISPR/Cas9 system, in combination with a single-stranded DNA (ssDNA) template, would allow the systematic knock-in of the short sequences required for these split systems, such as the *loxP* and UAS sequences.

Advances in the CRISPR/Cas9 system are continuously emerging[37], which should not only improve the efficiency of existing approaches but also open new possibilities. For example, key advances are CRISPR interference[61,62] and CRISPR activation[63,64], which allow reversible repression or activation of endogenous genes. Furthermore, the efficiency and specificity of the existing CRISPR/Cas9 protocol could be enhanced by faster methods for detecting successful editing[52,53], improved gRNA specificity and selection parameters[31,65–67], as well as expansion of the PAM site sequences[68] (which will increase the flexibility of target selection). All these advances could be quickly implemented and adapted for the killifish model system using this protocol. Finally, this protocol could be adapted for genome engineering approaches in other turquoise killifish strains[10], as well as other annual killifish species sharing similar life cycles[5].

Experimental design.

Several aspects have to be considered before initiating this protocol, specifically the establishment of a breeding turquoise killifish colony (see 'Reagent setup'). This protocol follows the ARRIVE guidelines for the reporting of animal experiments[69], and specific aspects are addressed in this section and the 'PROCEDURE' section. Furthermore, as it is a vertebrate model organism, care and experimental use of turquoise killifish must be approved by, and adhere to, relevant institutional ethics guidelines.

Large-scale husbandry and maintenance. The turquoise killifish can be efficiently grown in commercially available water recirculating systems[2], with an optimal temperature range of 25–28 °C, and ~10% water change per day. For optimal growth rate, the turquoise killifish (maximum length of 6.5–7 cm) should not be housed at high density (1–3 fish per 3-liter tank). Male fish that are housed at high density (without females) can demonstrate aggression, and they should be separated. To reduce the possibility of strain contamination, it is advised to use lidded tanks, as adult fish can occasionally jump (**Fig. 1b**), and to label each tank with all the required details (specifically date of birth and strain).

The turquoise killifish can withstand a wide range of water parameters that occur naturally in its habitat (including a range of temperature, pH, oxygen levels and salinity), which makes it a relatively hardy fish. It prefers dimmer lighting (subdued ceiling lighting), slow water flow (~5 liters/h) and higher salinity (800–4,000 μS). Higher salinity also inhibits velvet disease caused by *Oodinium*, which is a parasitic dinoflagellate. For further information regarding the diagnosis and treatment of common fish diseases in research facilities, see 'Diseases of Zebrafish in Research Facilities' from the Zebrafish International Resource Center (ZIRC): http://www.zebrafish.org/health.

Because of the accelerated growth, rapid sexual maturity and frequent mating, food consumption is higher compared with zebrafish, and high-quality food pellets (see 'Reagents') can be supplemented with live/frozen food such as *Artemia* (brine shrimp) or blood worms[48]. Fecundity is dependent on food quality and frequency of feeding, as well as on the overall age and size of fish, with larger female fish yielding a larger number of eggs. It is possible to squeeze sperm and eggs, which could be beneficial for *in vitro* fertilization or long-term preservation of gametes. However, sperm freezing and recovery protocols have not yet been optimized.

From fertilization to hatching, which normally occurs 14–20 d post fertilization (d.p.f.), animals are referred to as 'embryos'. Freshly hatched fish are referred to as 'fry' for the first few days (until the yolk sac has almost disappeared and swim bladder is fully operational), and shortly after they are referred to as 'juvenile fish' (until the animals reach sexual maturity). At the age of 20–30 d post hatching (d.p.h.), the animals reach sexual maturity and are considered adults (see 'TIMING' section and **Fig. 1**). Thus, the life cycle (from 'egg to egg') of the turquoise killifish is 5–7 weeks. Embryonic development in the turquoise killifish can be largely nonsynchronous, and it requires both aquatic and nonaquatic phases, and therefore more 'hands-on' time is needed at this stage for turquoise killifish compared with other fish models (see 'Development of the Protocol' section, 'Hatching', PROCEDURE Steps 46–52 and **Fig. 4b**).

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MATERIALS

REAGENTS

- African turquoise killifish (*N. furzeri*) GRZ strain. It is available from authors upon request **! CAUTION** As a vertebrate model organism, turquoise killifish care and use has to be approved by, and must adhere to, relevant institutional ethics guidelines. All turquoise killifish care and use in our lab was approved by the Stanford Subcommittee on Research Animal Care **▲ CRITICAL** A breeding turquoise killifish colony must be established before attempting this protocol (see 'Reagent Setup' section).
- Sea salt (Instant Ocean, cat. no. SS15-10)
- nCas9n expression plasmid[45] (Addgene, cat. no. 47929)
- Optional: Cas9 protein with NLS (1 mg/ml, PNA Bio). The Cas9 protein can be used instead of nCas9n mRNA (Procedure Steps 19–29)
- Custom DNA oligonucleotides (once annealed, it will serve as the gRNA template for the *in vitro* transcription), 200 µM stock concentration and standard desalted (Integrated DNA Technologies)
- mMessage mMachine SP6 Ultra kit (Life Technologies, cat. no. AM1340)
- mMessage mMachine T7 Ultra kit (Life Technologies, cat. no. AM1345)
- MEGAscript T7 kit (Life Technologies, cat. no. AM1334)
- DNase/RNase-free dH_2O (Invitrogen, #10977-015)
- 100-bp DNA ladder (Promega, cat. no. G2101)
- 1-kb DNA ladder (Promega, cat. no. G5711)
- Ethidium bromide (Thermo Scientific, cat. no. 15585011)
- Agarose (Life Technologies, cat. no. 16500500)
- UltraPure phenol:chloroform:isoamyl alcohol (25:24:1; Life Technologies, cat. no. 15593)
- Proteinase K solution (20 mg/ml; Ambion, cat. no. AM2546)
- High-fidelity PCR superMix (Invitrogen, cat. no. 12532-016)
- GoTaq green master mix (Promega, cat. no. M712)
- PCR lysis buffer (Viagenbiotech, 102-T)
- Phenol red solution, 0.5% (wt/vol; Sigma-Aldrich, cat. no. P0290)
- Humic acid (Sigma-Aldrich, cat. no. 53680) **▲ CRITICAL** Humic acid solution needs to be prepared in advance, at least a day before injections. Refrigerate the solution at 4 °C. It is good for several weeks if kept refrigerated (see 'Reagent Setup' section).
- Coconut fiber (ZooMed Item #EE-8, 8 dry quarts, loose coconut fiber substrate) **▲ CRITICAL** Coconut fiber needs to be prepared in advance, at least a day before it is required (see 'Reagent Setup' and 'Hatching' sections).
- Oxygen tablets (Pemble-Halverson, http://otabs.com/)
- Yamamoto's embryo solution[70] (10×; see Reagent Setup)
- NaCl (Sigma-Aldrich, ACS grade)
- KCl (Sigma-Aldrich, ACS grade)
- $CaCl_2$ (Sigma-Aldrich, ACS grade)
- $NaHCO_3$ (Sigma-Aldrich, ACS grade)
- Methylene blue (2.3% (wt/vol) stock solution; Kordon, cat. no. 37344)
- TOPO TA cloning kit (Life Technologies, cat. no. K4600)
- Zero Blunt TOPO PCR cloning kit (Life Technologies, cat. no. K2800-20)
- One Shot TOP10 competent *Escherichia coli* (Life Technologies, cat. no. C4040)
- Agarose (Life Technologies, cat. no. 16500-500)
- EndoFree Maxiprep kit (Qiagen, cat. no. 12362)
- NEB buffer 2 (NEB, cat. no. B7002S)
- BSA, molecular biology grade (NEB, cat. no. B9000S)
- T4 DNA polymerase (NEB, cat. no. M0203S)
- dNTP solution set (100 mM; NEB, cat. no. N0446S)
- PCR purification kit (Qiagen, cat. no. 28104)
- GlycoBlue Coprecipitant (15 mg/ml; Invitrogen, cat. no. AM9516)
- EDTA (0.5M; pH 8.0; Ambion, cat. no. AM9260G)
- Sodium Acetate (3M), pH 5.5 (Ambion, cat. no. AM9740)
- QIAquick nucleotide removal kit (Qiagen, cat. no. 28304)
- Miniprep Kit (Qiagen, cat. no. 27104)
- NotI (NEB, cat. no. R0189S)
- Tricaine (Sigma-Aldrich, cat. no. A5040) **! CAUTION** Tricaine is a hazardous chemical. When you are handling tricaine powder (or while handling fish that have been exposed to tricaine), protective gloves should be used. Please refer to the Material Safety Data Sheets (MSDS) for more details.
- Potassium chloride (KCl; 2 M; Affymetrix, cat. no. 75896)
- Otohime fish diet (Reed Mariculture)
- Premium grade brine shrimp eggs (Brine Shrimp Direct)
- Ethanol (100%; 200 proof; Rossville Gold Shield Chemical)
- Isopropanol, ACS plus (Fisher Scientific, cat. no. A416-500)
- TE buffer (1×; pH 8; Thermo Fisher, cat. no. AM9849)
- Sodium hypochlorite (NaClO; 10–15% available chlorine; Sigma-Aldrich, cat. no. 425044) **! CAUTION** Sodium Hypochlorite is a strong oxidizing agent. Avoid contact with eyes, skin and clothing, and wear protective gloves during handling. Exposure to the skin and eyes may cause irritation, and permanent eye damage can occur if eyes are not washed immediately. Please refer to the MSDS for more details.

EQUIPMENT

- Dumont tweezers #5 (WPI, cat. no. 500342)
- Gilson PIPETMAN (Fisher Scientific, cat. nos. F123600G, F144801G, F123601G and F123602G)
- Injection mold (well depth: 1.1 mm; well width: 0.95 mm, See the **Supplementary Methods** for 3D printing design, in STL or DWG file formats, ready to print) **▲ CRITICAL** The 3D-printed injection mold should be ordered in advance, depending on the shipping time of locally available providers.
- Phase lock gel tubes light (Eppendorf 5 Prime, cat. no. 2302820)
- LB agar plates with 100 µg/ml Ampicillin (Teknova, cat. no. L1004)
- Borosilicate microcapillaries with filament (Sutter, cat. no. BF100-58-10)
- Kimwipe (Kimtech Science)
- Barrier pipet tips, low binding (Genesee scientific, cat. nos. 24-401, 24-404, 24-412, 24-430)
- 1.7-ml Tubes (Genesee scientific, cat. no. 24-282)
- 0.22-µm Filter (Genesee scientific, cat. no. 25-227)
- Pestle for a 1.5-ml tube, RNase and DNase free (Argos, cat. no. P7339-901)
- Commercially available water recirculating systems with 2.8-liter tanks (Aquaneering) **▲ CRITICAL** Water recirculating systems are an efficient way to house a breeding turquoise killifish colony, and they must be established before attempting this protocol (see 'Reagent Setup' section).
- Thermal cycler (MJ Research PTC-200)
- Breeding tank sets (Pentair, SBTANK Tank-2)
- 60 × 15 mm Petri dishes (Fisher, cat. no. 0875713a)
- Sutter Instrument P-87
- Nikon C-PS stereoscope and Zeiss KL 1500 LCD optic fibers
- Injection apparatus (Applied Scientific Instrumentation), which includes an MHC model magnetic stand, an MMPI model pressure injector, a foot switch to pulse the injected solution into the embryos, an MM3 model micromanipulator and an M-PIP model micropipette holder (Applied Scientific Instrumentation), assisted by a back-pressure unit (Warner Instrument) **▲ CRITICAL** It is recommended that the injection apparatus be assembled according to the manufacturer's guidelines before attempting this protocol.
- 8-Inch fine mesh strainer (OXO Good Grips, cat. no. 38991)
- Stage micrometer, 1 mm divided into 0.01 mm units (Meiji Techno America, cat. no. MA285)
- Compact incubator for embryos (ThermoScientific, cat. no. 50125590)
- 30 Mesh sand (Homedepot, cat. no. 200000278)

REAGENT SETUP

Yamamoto's embryo solution (10×: 170 mM NaCl, 27 mM KCl, 25 mM $CaCl_2$, 0.2 mM $NaHCO_3$) Add 7.5 g of NaCl, 0.2 g of KCl, 0.2 g of $CaCl_2$ and 0.02 g of $NaHCO_3$ in 100 ml of H_2O; adjust the pH to 7.3 with $NaHCO_3$, and filter-sterilize the solution through a 0.22-µm filter. Methylene blue (100 µl/l) should be added to the 1× solution (2.3% (wt/vol) stock solution) to limit parasitic infection. The solution could be kept at room temperature (20–25 °C) for several months.

African turquoise killifish husbandry and maintenance Turquoise killifish are housed at 26 °C in a central filtration recirculating system, with a 12-h light/dark cycle[2], and conductivity of 3,500 µS. Fish are housed as pairs in a 2.8-liter tank. For spawning, fish are housed in a 9.8-liter tank with a single male with 4–5 females. Fish are fed twice a day with dry food pellets, supplemented with live *Artemia* (brine shrimp) or fed exclusively live blood worms[48].

Box 2 | Disinfecting embryos by bleaching ● TIMING 1 h

(i) Freshly prepare bleach solution: 400 µl of NaOCl (stock solution of 10–15% (vol/vol)) in 1 liter of autoclaved system water.
(ii) Transfer embryos into a 10-cm Petri dish, and wash them with 5–7 ml of autoclaved system water (in order to separate them from any remaining coconut fiber debris).
(iii) Transfer embryos to a clean 10-cm Petri dish, and incubate them in 5–7 ml of bleach solution for 5 min in the same plate.
(iv) Wash them once in 5–7 ml of autoclaved system water for 5 min in the same plate.
(v) Incubate the embryos in 5–7 ml of bleach solution for 5 min in the same plate.
(vi) Wash them twice with 5–7 ml of autoclaved system water for 5 min in the same plate.
(vii) Transfer the embryos into a new 6-cm Petri dish with 5 ml of 1× Yamamoto embryo solution.

Receiving or shipping fertilized eggs For initiating a colony or for the distribution of strains, embryos are usually shipped in 60-mm Petri dishes with moist coconut fiber when eyes are visible (**Fig. 4b**). Petri dishes are then sealed with Parafilm and, when weather conditions are not below freezing, they can be shipped via regular mail. Upon arrival, it is important to disinfect the embryos with bleach, to transfer them to a new Petri dish with moist coconut fiber and to incubate them at 26 °C. Monitor them every 3–4 d, and hatch only individuals that are fully ready (see 'Hatching', PROCEDURE Steps 46–52). Hatched fish should be kept separately from the colony (preferably in a quarantined area), and their health should be monitored (see 'Large-scale husbandry and maintenance'). If fish are healthy, their disinfected progeny can eventually be moved into the main recirculating system. Disinfecting or 'bleaching' of embryos is performed according to standard procedures widely used for fish (**Box 2**).
Coconut fiber preparation Presoak coconut fiber with dH_2O and autoclave. Once cooled, squeeze out water (keeping it moist, but not dripping), and keep it in closed containers (such as used tip boxes) at room temperature. Use it within a few weeks.
Humic acid preparation Dissolve 1 g/l humic acid in system water (water from the recirculating system). Mix it well and autoclave the mixture. Refrigerate this mixture at 4 °C. It remains good for several weeks if it is kept refrigerated.
EQUIPMENT SETUP
Preparation of injection needles A good needle is critical for successful injections. The injection needles designed for the turquoise killifish have a shorter and less flexible tip than the needles used for zebrafish injections, which allows easier penetration through the thicker chorion. The parameters for two different needle designs (**Fig. 5a**) are tabulated below, and they have been optimized on a Sutter Instrument P-87 (with a trough filament), and using filamented microcapillaries for both options.

Option 1 produces a strong needle and is great as a starting point. The tip is best broken by gently rubbing the tip on a Kimwipe, because breaking the tip using forceps usually results in an opening that is too big. Option 2 results in a similar-shaped needle with a slightly sharper tip that can be broken using either forceps or a Kimwipe. Although it is slightly harder to break, this needle can penetrate the chorion more easily. Pulled needles can be stored (for years) in a closed Petri dish with two strips of modeling clay to hold them.

Needle design	Line	Pressure	Heat	Pull	Velocity	Time
Option 1		450	Ramp value: 45 °C	30	40	200
Option 2	1	500	Ramp value: 14 °C	20	20	0
	2	500	Ramp value: 69 °C	10	20	0

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PROCEDURE

▲ CRITICAL Genome editing using the CRISPR/Cas system is described in the main PROCEDURE. Modifications that are required for adapting the Procedure for Tol2 transposon-based transgenesis are described in **Box 1**.

Target selection ● TIMING 1–2 d

▲ CRITICAL Steps 1–18 are omitted for Tol2-based transgenesis

1| Select a gene (or genes) of interest, and compare conserved regions between vertebrate orthologs using http://genome.ucsc.edu/ (conserved regions are less likely to be alternatively spliced). The turquoise killifish gene models are available at https://chopchop.rc.fas.harvard.edu/ (refs 2,48), and additional information is provided in two recent turquoise killifish genome browsers: http://africanturquoisekillifishbrowser.org/ (ref. 6) and http://www.nothobranchius.info/NFINgb (ref. 21).

2| Identify gRNA target sequences using CHOPCHOP (https://chopchop.rc.fas.harvard.edu/). CHOPCHOP accepts a wide range of inputs (gene identifiers, genomic regions or pasted sequences), and it can predict potential off-targets genome wide. Target sequences are color-coded according to quality scores, and they are displayed at their genomic positions. In addition, each target sequence is presented with possible restriction sites and suggested oligonucleotides for PCR amplification (flanking the sequence of interest). Selecting gRNA targets in close proximity to a unique restriction site can facilitate the identification of successful editing (a detailed example is provided in **Fig. 2a**).
▲ CRITICAL STEP In this protocol, T7 polymerase is used for gRNA synthesis; therefore, the first two 5′ nucleotides should be limited to Gs, as follows: 5′-GG-(N)18-NGG-3′ (as demonstrated for the selected gRNA targets in **Fig. 2a**).

3| By using either CHOPCHOP or Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), design oligonucleotides to amplify the genomic region containing the targeted sequence; the oligonucleotides should flank the sequence of interest and generate an amplicon size in the range 300–1,000 bp. Order standard desalted oligonucleotides, and verify the sequence by direct sequencing of the purified PCR product (**Fig. 2a**).
▲ **CRITICAL STEP** Naturally occurring genetic variation (even in the inbred GRZ strain) could reduce the efficiency of editing, or even completely block it. Thus, the step of verifying the desired target sequence by sequencing is essential (specifically when using other turquoise killifish strains).

Synthesis of gRNAs ● TIMING 1–2 d
▲ **CRITICAL** All steps involving RNA synthesis and handling should be done in a clean environment, using RNase- and DNase-free reagents.
4| Order the oligonucleotides (200 µM stock concentration, standard desalted) that, once annealed, will serve as the gRNA template for the *in vitro* transcription; see table below (see **Fig. 2a**, additional examples can be found in Figs. 6 and S4, and Table S1 in Harel *et al.*[2]).

Oligonucleotide	Sequence (5′ to 3′)	Purpose
Universal reverse oligonucleotide	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	Used for generation of all gRNA templates
Variable oligonucleotide	TAATACGACTCACTATA**[GG-(N)18]**GTTTTAGAGCTAGA AATAGCAAG	core target sequence in bold and unique for each gRNA

▲ **CRITICAL STEP** This universal reverse oligonucleotide will be used in all the gRNA template generations. Divide the oligonucleotide stock into aliquots, and freeze them at −20 °C to avoid freeze-thaw cycles.

5| Prepare the following annealing reaction in a PCR tube, and mix it by pipetting:

Component	Amount	Final concentration
Universal reverse oligonucleotide (200 µM)	1 µl	20 µM
Variable oligonucleotide (200 µM)	1 µl	20 µM
NEB 2 buffer 10×	1 µl	1×
Nuclease-free water	7 µl	
Total	10 µl	

6| Use the following program on a standard thermocycler to anneal the oligonucleotides:

Cycle number	Denature	Anneal	Final
1	95 °C 30 s		
2		72 °C 2 min	
3		37 °C 2 min	
4		25 °C 2 min	
5		12 °C 2 min	
6			4 °C Forever

7| After annealing, 5′ ssDNA overhangs will remain on both sides of the template (**Fig. 2b**, top panel), and T4 DNA polymerase can be used to catalyze the gap-filling (synthesis of DNA in the 5′ → 3′). Add the following to the reaction tube and mix by pipetting:

Component	Amount	Final concentration
dNTP mix (25 mM)	0.5 µl	1.25 mM
T4 DNA polymerase (3,000 units/ml)	0.5 µl	0.15 units/µl
BSA (20 mg/ml)	0.2 µl	0.4 mg/ml
Nuclease-free water	8.8 µl	
Total	10 µl	

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8| Use a standard thermocycler to incubate the reaction at 12 °C for 20 min.

9| Clean the annealed and gap-filled oligonucleotides using a PCR purification kit according to the manufacturer's protocol, and elute in 30 µl of Elution buffer (supplied in the kit). Measure the concentration using a standard NanoDrop instrument, and expect a concentration of roughly 0.5 µg/ml. The resulting product is used as a template for gRNA synthesis.
■ **PAUSE POINT** the template can be stored at −20 °C for several months.

10| For gRNA synthesis, use 1 µl of the eluted template in a 20-µl T7 MEGAscript transcription reaction, according to the manufacturer's instructions.

11| Add 1 µl of TURBO DNase (provided in the T7 MEGAscript kit) to the reaction to remove the DNA template, mix it gently and incubate it for an additional 15 min at 37 °C.

12| Precipitate the RNA by adding the following components in the order listed, and mix well before adding the ethanol:

Component	Amount	Final concentration
Reaction mix	20 µl	
Nuclease-free water	115 µl	
3M Na Acetate	15 µl	0.1 mM
GlycoBlue Coprecipitant	1 µl	
Ethanol 100% (molecular biology grade)	300 µl	
Total	451 µl	

13| Mix the contents well (or vortex briefly), and chill the mixture at −20 °C for at least 15 min.
▲ **CRITICAL STEP** The reaction can be placed at −20 °C overnight with no decrease in yield.

14| Pellet the gRNAs for 15 min in a chilled microcentrifuge (4 °C) at top speed (~11,000*g*).

15| Remove the supernatant, respin the tube for a few seconds and remove all the residual fluid with a fine-tipped pipette.
▲ **CRITICAL STEP** Do not let the pellet completely dry; otherwise, it will not dissolve.

16| Resuspend the pellet in dH_2O or 1× TE buffer (~30 µl), by letting the tube sit on ice for 30 min with occasional flicking. Measure the concentration using NanoDrop, and adjust it to a concentration of 0.5–1 µg/µl.

17| Run 2 µl of the reaction using a formaldehyde loading dye (provided in the T7 MEGAscript kit) in a 1% (wt/vol) agarose gel. A discrete band should be seen (**Fig. 2b**, bottom panel).

18| Prepare 2- to 3-µl aliquots and store them at −80 °C.
■ **PAUSE POINT** Aliquots can be stored at −80 °C for several months.

Synthesis of *Cas9* mRNA ● TIMING 1–2 d
▲ **CRITICAL** If Cas9 protein will be injected at Step 37 (instead of nCas9n mRNA), then Steps 19–29 can be omitted.
▲ **CRITICAL** For Tol2 transgenesis, transposase mRNA (rather than nCas9n mRNA) is synthesized as described in **Box 1**.
19| Purify the nCas9n expression plasmid DNA using a Maxiprep kit, by resuspending the DNA pellet according to the manufacturer's instructions.

20| Linearize 5 µg of the nCas9n expression plasmid DNA with 1–2 µl of NotI at 37 °C for 2 h, in a total volume of 40 µl using the supplied buffer (1× NEB buffer 3.1).

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21| Run 1 µl of the reaction alongside a comparable amount of an uncut plasmid (~125 ng) on a 1.5% (wt/vol) agarose gel to verify complete digestion of the plasmid.
▲ CRITICAL STEP The remaining undigested plasmid DNA can lead to very long RNA molecules (the full plasmid would be used as a template), thus reducing the overall efficiency of mRNA synthesis. Complete digestion should yield a single band that should be 'sharper' and usually higher than the uncut plasmid. If digestion is incomplete, repeat Step 20 and increase the incubation time or amount of enzyme.
? TROUBLESHOOTING

22| Purify the digested nCas9n expression plasmid using a PCR purification kit, according to the manufacturer's instructions, and elute in 50 µl of 1× Elution buffer.
▲ CRITICAL STEP DNA should be free of contaminating proteins and RNA, as these might affect the quality and yield of mRNA synthesis.

23| Transcribe capped and polyadenylated nCas9n mRNA from the purified plasmid template using the mMessage mMachine SP6 kit according to the manufacturer's instructions.
▲ CRITICAL STEP The nCas9n expression plasmid used in this protocol is cloned into the pCS2 backbone, which includes an SV40 polyadenylation (poly(A)) site. If an alternative Cas9 expression plasmid is chosen by the user, it is recommended to verify that it includes a poly(A) site. Otherwise, the mMESSAGE mMACHINE ULTRA (SP6 or T7) kits include additional reagents for poly(A) tailing.

24| Add 1 µl of TURBO DNase (provided in the mMessage mMachine SP6 kit) to the reaction mixture, mix it well and incubate the mixture for an additional 15 min at 37 °C.

25| Purify the resulting mRNA using lithium chloride precipitation according to the manufacturer's instructions in the mMessage mMachine SP6 kit.
▲ CRITICAL STEP Do not let the pellet fully dry; otherwise, it will not dissolve.

26| Resuspend the nCas9n mRNA pellet in dH_2O or TE buffer at a concentration of 1–3 µg/µl.

27| Incubate the resuspended nCas9n mRNA at 65 °C for 10 min to completely dissolve the mRNA.

28| Run 2 µl of the reaction using a formaldehyde loading dye (provided in the mMessage mMachine SP6 kit) on a 1% (wt/vol) agarose gel. A well-defined band should be seen, similar to that observed in Step 17.

29| Measure the concentration using NanoDrop, and adjust the concentration to ~1 µg/µl. Prepare 2- to 3-µl aliquots and store them at –80 °C.
▲ CRITICAL STEP If the concentration of mRNA is too low, transcribe several reactions (Step 23) and pool them together during purification steps (Steps 24–29).
■ PAUSE POINT Aliquots could be stored at –80 °C for several months.

Optional: ssDNA template for knock-in via HDR ● TIMING 1–2 d
▲ CRITICAL HDR uses a homologous DNA template for repairing the DSB, and thus it can be used to precisely introduce new sequences into the genome (i.e., knock-in). This optional section should be included only if a specific sequence is to be introduced (or removed) from the genome.
30| Design ssDNA with homology arms (i.e., 30–50 bases, each side) flanking the gRNA target site. It is recommended that the introduced mutations be as close as possible to the PAM site. The example shown in **Figure 2d** (previously published in Harel *et al.*[2]) displays the core sequence of the ssDNA template used to specifically edit a genomic region encoding for a lysine residue (K836) in the turquoise killifish TERT protein. Once it is successfully edited, the first mutation (on the left, A→G) will give rise to the K836R amino acid change. The second mutation (on the right, G→C) is silent, and it will change the PAM site. Changing the PAM site will prevent Cas9 from recutting the edited locus[2].

31| Order the ssDNA (standard desalted oligonucleotide) and purify it using the QIAquick Nucleotide Removal kit. Elute in dH_2O or 1× elution buffer (provided in the kit) at a final concentration of 100–200 µM.
▲ CRITICAL STEP The QIAquick Nucleotide Removal kit is optimized for oligonucleotides; therefore, it is advised to use this kit rather than a PCR purification kit.

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32| Prepare 2-µl aliquots and freeze them at −20 °C.
■ **PAUSE POINT** Aliquots could be stored at −20 °C for several months, with no observed decline in efficiency.

Injection ● TIMING 1–2 d
▲ **CRITICAL** For Tol2-based transgenesis, injection is performed as described in Steps 33–39, using the injection mix described in **Box 1**.
33| Set up several breeding groups per tank (one male with 2–3 female fish) of sexually mature (1–2 months old) wild-type fish the evening before injection, using a breeding tank (**Fig. 4a**), which contains a barrier to separate the male and female fish. Alternatively, sand trays can be used as previously described[28,29].
! CAUTION As the turquoise killifish is a vertebrate model organism, care and use of the turquoise killifish has to be approved, and relevant institutional ethics guidelines must be followed. Care and use of turquoise killifish in our lab is approved by the Stanford Subcommittee on Research Animal Care.
▲ **CRITICAL STEP** For injections, it is best to plan for a yield of 100–300 eggs according to the observed fecundity in the colony (see 'Reagent Setup'). Crossing 10–20 females is usually sufficient for producing a large clutch of eggs that are suitable for injections. Because of the relatively slow rate of cell division after fertilization (reaching two-cell stage at ~3.5 h.p.f.[71]), there is less urgency for injection in turquoise killifish compared with zebrafish. Therefore, eggs could be collected for a longer period of time (4–5 h), which helps generate larger clutches of eggs for injection. Fish can be set up for mating weekly, and as frequently as every 3–4 d[48,72]. Note that the first couple of mating events would be a 'training period' for the mating pair, and it might yield fewer eggs.
▲ **CRITICAL STEP** Mating using sand trays instead of a breeding tank (**Fig. 4a**) is more natural for the fish, and makes it easier to obtain larger clutches of eggs from the beginning. Note that it is important to thoroughly wash sand and to autoclave it before using it for the first time and before it is recycled for subsequent uses. However, once fish are accustomed to the breeding tanks, both methods can be equally efficient, and the choice between them depends on the user.

34| On the day of injection, remove the barrier and let the fish mate for a few hours (usually 4–5 h). Next, collect eggs using a fine mesh strainer and a standard glass Pasteur pipette. Keep the eggs in a 6-cm Petri dish with 5 ml of 1× Yamamoto embryo solution at room temperature. Injections should start within 1–2 h, in order for embryos to be at the single-cell or two-cell stage.
▲ **CRITICAL STEP** The average size (diameter) of eggs is 1.3–1.35 mm. Therefore, for retaining the eggs, the holes of the mesh should be ~1 mm (see Materials). If sand trays are used, for good separation between eggs and sand, the size of sand grains should be ~600 µm (corresponding to 'Mesh 30'; see 'Equipment' section).

35| Make a 1.5% (wt/vol) agarose plate, and while the agarose is still hot lay a plastic injection mold on the surface (it will float). This will produce six troughs that will hold eggs in position (**Fig. 4a**).
▲ **CRITICAL STEP** Use the supplied 3D-printing design (in STL or DWG file formats, ready to print) for an injection mold with optimized dimensions (**Supplementary Methods**). Using these optimized dimensions makes handling and positioning of fertilized eggs fairly straightforward. Different materials might vary in quality depending on the provider; therefore it is advised to accurately measure the final product. From our experience, Hi-Def Acrylate (http://www.shapeways.com/) works well.

36| Break the needle (see 'Equipment Setup' section) by gently rubbing the tip on a Kimwipe.

37| Prepare the injection mixture as tabulated below, and keep it on ice. Back-fill the needle with 2–5 µl of injection mixture, and wait for the mixture to migrate to the tip.
▲ **CRITICAL STEP** Alternatively, preliminary tests in our lab suggest that Cas9 protein (Cas9 protein with NLS, PNA Bio) can be used at a final concentration of 200 ng/µl instead of the nCas9n mRNA (I.H., data not shown). However, we primarily use nCas9n mRNA, as it works with high efficiency, and we have not directly compared the efficiencies of nCas9n mRNA and Cas9 protein.
▲ **CRITICAL STEP** The needles designed for turquoise killifish injection have a shorter and less flexible tip compared with zebrafish injection needles. This allows easier penetration through the thicker chorion (see 'Equipment Setup' and **Fig. 5a**). Thus, turquoise killifish injections might also require some practice.

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To facilitate piercing through the chorion, embryos can be stored at 4 °C for 10 min before injection. As the chorion reaches room temperature, it relaxes, facilitating needle penetration.

Component	Amount	Final concentration
Purified gRNA from Step 18 (150–300 ng/μl)	1–2 μl	30 ng/μl
nCas9n mRNA from Step 29 (1–3 μg/μl)	1–3 μl	200–300 ng/μl
Phenol red (0.5% (wt/vol))	2 μl	0.1% (wt/vol)
Optional: purified ssDNA template from Step 32 (for HDR experiments)		20 μM
Nuclease-free water	Up to 10 μl	
Total	10 μl	

▲ **CRITICAL STEP** Agarose plates can be prepared several days in advance and kept at 4 °C. If plates are sealed with Parafilm, they can be kept at 4 °C for several weeks. Needles should be filled immediately before injections, whereas the remaining injection mixture is kept on ice. A total volume of 10 μl of injection mixture should be enough for injecting 100–200 eggs. However, in case the needles break frequently (or a larger number of injected eggs are required), the total volume of injection mixture could be scaled up.

38| By using a stereoscope, place the fertilized eggs into the agar troughs, and orient the single cell (or the two cells) upward towards the injection needle (an example of a fertilized egg with a single cell can be seen in **Fig. 4b**). By using the injection apparatus, inject into the cytoplasm of the single- or two-cell stage embryos using the following parameters: 30 p.s.i. with 75-ms pulses—~2.5–10 pl of injection mixture, which corresponds to ~10% of the cell's volume.
▲ **CRITICAL STEP** Back-pressure should be adjusted, so the needle in the liquid medium looks like a 'smoking gun', with slight leaking of the red injection solution from the needle tip. Make sure that a small red dot (phenol red) is seen in the cell after injection.
▲ **CRITICAL STEP** Injection parameters may vary slightly between different needles. To adjust the parameters to achieve a reproducible injection volume, inject into mineral oil placed on top of a micrometer slide (see 'Materials' section and **Fig. 5b**), and measure the diameter of a droplet. Under the oil, the droplet is a sphere, and therefore the injection volume can be calculated according to the following formula:

$$\frac{4}{3}\pi r^3$$

Note the difference between the injection volume in zebrafish (usually between 0.5 and 2 nl) and turquoise killifish (usually between 2.5 and 10 pl). With the suggested concentrations, each injected picoliter should deliver ~30 fg of gRNA and 200–300 fg of nCas9n mRNA.

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39| After injection, monitor the embryos daily and remove dead embryos (opaque and blue in color).
▲ **CRITICAL STEP** Expect ~50% survival of injected embryos, and ~50% chimeric embryos when using efficient gRNAs.
? TROUBLESHOOTING

Evaluation of editing efficiency ● TIMING 1–3 d
▲ **CRITICAL** A detailed example is provided in **Figure 2**. Several robust approaches were recently developed for downstream assessment of targeting efficiency, including HRM[52] and CRISPR-STAT[53]. In cases in which the indel is very small (i.e., 1–2 bp), or identification of the exact modified sequence is required (specifically important in HDR-based knock-in experiments), we provide two additional alternative approaches: direct sequencing (Step 44A) and enzymatic digestion (Step 44B). Efficiency of Tol2-based transgenesis is evaluated as described in **Box 1**.
40| 48 h after injection, isolate genomic DNA from a 'test set' (10–15 embryos) from the total injected eggs (100–300 embryos) by adding the following to a 1.7-ml Eppendorf tube:

Component	Amount	Final concentration
10–15 Injected embryos		
PCR Lysis buffer	200 μl	
Proteinase K	5 μl	0.5 mg/ml
Total	205 μl	

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41| Crush the embryos with a pestle and incubate them overnight at 55 °C, followed by proteinase K inactivation for 10 min at 95 °C. The genomic DNA is now ready to use for a PCR.

42| Use oligonucleotides from Step 3, and 0.5–1 μl of the genomic DNA for PCR amplification with High Fidelity PCR SuperMix, according to the manufacturer's instructions, in a total volume of 50 μl.
▲ CRITICAL STEP Impurities in the genomic DNA can inhibit the reaction. Consider purifying the genomic DNA using a genomic DNA cleanup kit, or use a more robust (but less accurate) PCR mix (GoTaq Green Master Mix).

43| Clean the PCR product using a PCR purification kit. Elute in 30–40 μl of elution buffer.

44| There are 2 options for downstream analysis: option A should be used when the precise sequence of the edited region is required (specifically important for newly generated stable lines or in HDR-based knock-in experiments). Option B can be used for routine genotyping, only if the edited region resulted in a loss (or gain) of a unique restriction site. Although Option B works very well, it should be pursued mostly if newly developed downstream approaches (including HRM[52] and CRISPR-STAT[53]) have not been established in the lab.
? TROUBLESHOOTING

(A) Direct sequencing ● TIMING 2–4 d

(i) If high-fidelity PCR was used at Step 42, clone PCR products using the TOPO-Blunt kit; if GoTaq Green Master Mix was used at Step 42, clone PCR products using the TOPO-TA kit. Subject 20 colonies to direct sequencing (a service available through a sequencing company; MCLab, for example). Alternatively, purify DNA from single colonies using a Miniprep kit and sequence (see example in **Fig. 2c**, top panel).

(B) Enzymatic digestion ● TIMING 1–2 d

(i) If the PAM site was selected on the basis of its proximity (5–10 bp) to a unique restriction site, digest the purified PCR products from Step 42 using the selected restriction enzyme according to the manufacturer's instructions (see example in **Fig. 2c**, bottom panel).
▲ CRITICAL STEP Some restriction enzymes can be used directly in the PCR mix, which means that Step 42 (purification) can be omitted.

(ii) Resolve the restriction enzyme digest from Step 44B(i) on a 1–1.5% (wt/vol) agarose gel. Use genomic DNA from noninjected embryos as a negative control.

45| When successful editing is confirmed on the 'test set' of embryos (10–15), continue to Step 46 to hatch the remaining injected embryos. Efficiency can range between 0 and 90% in the test case, and it is advised to continue to Step 46 when the efficiency is above 10% (see example in **Fig. 2**).

Hatching ● TIMING 5–7 weeks

▲ CRITICAL These steps are identical for Tol2-based transgenesis (**Box 1**). This step requires only 3–4 d hands-on. However, it also includes a period of 14–20 d for the embryos to develop (d.p.f.) and be ready for hatching, and 3–4 weeks of posthatching (d.p.h.) period, for the animals to reach sexual maturity (**Fig. 1a**).

46| Keep the embryos in 60-mm Petri dishes with 5 ml of 1× Yamamoto embryo solution, at a density of up to 50 embryos per dish. Incubate the embryos at 28 °C to increase synchronous embryonic development. Replace with 5 ml of fresh 1× Yamamoto embryo solution weekly, and remove dead embryos (stained blue by methylene blue).
▲ CRITICAL STEP Dead embryos can increase the chances of contamination and affect the survival of healthy embryos. If increased (more then 1–5 eggs per week) death is observed, embryos should be monitored daily, and the embryo solution should be replaced more frequently.
? TROUBLESHOOTING

47| After 1–2 weeks, when eyes are visible (**Fig. 4b**), add moist coconut fiber to the bottom of the 60-mm Petri dishes, and compress it to a flat surface (~0.5 cm deep) using a paper towel.

48| By using a glass Pasteur pipette, transfer the embryos from the 1× Yamamoto embryo solution to the Petri dish containing coconut fiber, close the lid of the Petri dish and keep it at 26 °C. Keep the embryos spread apart, with up to ~50 embryos per plate.
▲ CRITICAL STEP Retain undeveloped embryos in 1× Yamamoto embryo solution, and monitor their development until they are ready to hatch.
? TROUBLESHOOTING

49| After a week, transfer all the developed embryos (**Fig. 4b**) using a Pasteur pipette into cold (4 °C) humic acid in a container with a lid (for example, a tip box, filled to a depth of ~1 cm); ensure that the embryos are completely submerged. This allows for synchronized hatching.
▲ **CRITICAL STEP** Synchronized hatching is achieved when embryos are wetted. To increase hatching survival, embryos should be hatched only when they are fully ready, using the size of the eye as a marker (**Fig. 4b**). When embryos are hatched prematurely, the majority could end up as 'belly sliders' (i.e., embryos that have not successfully inflated their gas bladder), and many will die.

50| To improve oxygen supply, add approximately half a teaspoon of oxygen tablet to the humic acid (rather than crushing the tablet, we scrape the surface to obtain sufficient quantities of powdered tablet). Leave the container at room temperature. Alternatively, aeration can be used instead of oxygen tablets—for example, with a standard aquarium air pump connected to a tube with an airstone.
▲ **CRITICAL STEP** If embryos are hatched without sufficient oxygenation, the majority could end up as 'belly sliders'.

51| A day after transfer, fill the lidded container with system water (approximately an additional 2-cm depth, on top of the humic acid), and feed fry daily with freshly hatched brine shrimp (*Artemia salina*).
▲ **CRITICAL STEP** Using water with a salinity of ~1 g of sea salt per liter will allow brine shrimp to survive longer, and serve as available food. Unhatched embryos can be transferred back to coconut fiber, and hatching can be attempted again a week later (Steps 47–52)

52| 2–4 d after the addition of system water, transfer hatched fry to the system and feed them 1–2 times per day with brine shrimp until 3 weeks of age. After 3 weeks, feed adult fish twice a day with dry food supplemented with live/frozen food (such as brine shrimp or blood worms). These fish are F0 chimeric 'founders' and could be heterozygous or homozygous for the desired mutation (or many could be wild type, depending on the editing efficiency).

Germ-line transmission and backcross ● TIMING 1–3 d
▲ **CRITICAL** These steps are identical for Tol2-based transgenesis (**Box 1**).
53| Set up mating pairs between sexually mature F0 founders and wild-type fish. Fish are usually sexually mature after 3–4 weeks, once male fish start showing tail color. Sexual maturity is easily visible in male fish, initially by the longer base of the dorsal fin and ultimately by their distinct colors.

54| Collect eggs using a sand tray[28] or by using a breeding tank, as described in step 33. After 48 h, evaluate germ-line transmission on 10–15 pooled embryos (F1), as described in Steps 40–45 for the evaluation of editing efficiency. A fish will be considered a 'stable line' when the mutation is detected in the F1. Heterozygous stable lines should be genotyped every generation. Homozygous stable lines could be genotyped every several generations.

55| (Optional) Backcross heterozygous stable lines with wild-type GRZ fish for 2–3 generations to eliminate potential off-target effects. Genotype each generation as described in Steps 56–59.

56| For genotyping, anesthetize adult fish in 200 mg of Tricaine in 1 liter of system water at room temperature. After a few minutes, fish will stop moving.
▲ **CRITICAL** Tricaine solution should be freshly prepared before starting the experiment.

57| Use a plastic spoon to gently place fish on a sponge (moistened with system water), and quickly trim 2–3 mm from the tail fin (most caudal fin) with a sterile razor blade. Alternatively, remove 2–3 scales with forceps (which is less invasive, but might require some practice).

58| Place the tissue in 1.7-ml Eppendorf tubes and process it as described in Steps 40–45.

59| Gently put the fish in 1 liter of fresh system water at room temperature to recover for 10 min, before returning to the system.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

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TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
21 and **Box 1**	Poor nCas9n or *Tol2* mRNA synthesis or degraded product	Problem with the RNA synthesis kit	The synthesis buffer is sensitive to repeated freeze-thaw cycles. Consider making aliquots of the buffer. Verify the date of expiration of RNA synthesis
		Incomplete digestion of the DNA template	Remaining undigested plasmid DNA can lead to very long RNA molecules (using the full plasmid as a template), and thus it can reduce the overall efficiency of mRNA synthesis. In these cases, gel purification after digestion is recommended
		Concentration and quality of the DNA plasmid	Lower amounts or impure DNA plasmid can result in lower yield of RNA synthesis. Purify the plasmid DNA before RNA synthesis, and follow the instructions in the mMESSAGE mMACHINE kit for the amount of DNA plasmid used as a template
		RNase contamination	To prevent RNA degradation during and/or after RNA synthesis, use RNase-free reagents and RNase decontamination solutions to clean the working surface
39, 46, 48 and **Box 1**	Low survival or abnormal development of injected embryos	Too much DNA or RNA injected	High concentrations of DNA (and RNA to a lesser extent) are toxic to the cell, and they lead to increased mortality. In our trials, up to 100 ng/µl of DNA and 330 ng/µl of Tol2 RNA work efficiently. If needed, adjust the concentration of DNA and *Tol2* RNA according to our guidelines
		Too high injection volume	The injection solution must be lower than 1/10th of the cell's volume (~2.5–10 pl, measured by micrometer). Possible solutions include lowering the injection volume; decreasing the diameter of the needle opening; lowering the air pressure; or decreasing the injection pulse of the injector
		Impure injection solution	Impure injection solution increases mortality of the embryos after injection. Purify DNA and RNA before use
44 and **Box 1**	Little or no genome editing (in CRISPR/Cas9) or expression (in Tol2) detected in injected embryos	Inefficient delivery of injection mixture	To prevent clogging, centrifuge the injection mixture at 11,000*g* in a chilled centrifuge for 5 min. If the injection volume is too small (or concentration of DNA and RNA is very low), injection efficiency decreases accordingly. Adjust DNA/RNA concentration and injection volume
		Decreased nCas9n/Tol2 mRNA integrity	nCas9n and *Tol2* mRNA may be degraded. Check mRNA integrity by electrophoresis using agarose gel before making the injection solution
		Accidental injection into the yolk	Make sure that the injection occurs into the embryo (single cell or two cells at this stage; see **Fig. 4b**) and not into the yolk or lipid droplet. If the single cell is too difficult to see after egg collection, incubation of the fertilized eggs at 28 °C for 10–30 min helps visualize the single cell. Eggs without a visible single cell are probably unfertilized

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● TIMING

Steps 1–3, target selection: 1–2 d
Steps 4–18, synthesis of gRNAs: 1–2 d
Steps 19–29, synthesis of *Cas9* mRNA: 1–2 d
Steps 30–32 (optional), ssDNA template for knock-in via HDR: 1–2 d. Synthesis of gRNAs, *Cas9* mRNA and preparation of ssDNA template can be set up in parallel.
Steps 33–39, injection: 1–2 d. Inexperienced users will need more time to increase injection efficiency and survival.
Steps 40–45, evaluation of editing efficiency: 1–3 d. Evaluation of efficiency could be performed on a subset of injected eggs, while the remaining eggs are left to develop.

Steps 46–52, hatching: 5–7 weeks. This step requires 3–4 d hands-on. However, it also includes a period of 14–20 d for the embryos to develop (d.p.f.) and be ready for hatching, and 3–4 weeks post hatching (d.p.h.) period, for the animals to reach sexual maturity (**Fig. 1a**).
Steps 53–59, germ-line transmission and backcrosses: 1–3 d hands-on and 5–7 weeks for each generation (life cycle from 'egg to egg').
Box 1, Tol2-based transgenesis: 2–3 months
Box 2, disinfecting embryos by bleaching: 1 h

ANTICIPATED RESULTS

This protocol provides detailed guidelines for the successful husbandry and efficient genome engineering in the short-lived turquoise killifish. Although there are many similarities with other fish model systems, the turquoise killifish has several characteristics—a tough chorion, rapid growth and short lifespan—that are probably adaptation to its harsh environment and translate to key differences from protocols for other fish models. Although stable lines can be generated as quickly as 2–3 months, establishing the experimental setup (including a breeding colony) will take ~6 months or more. The generation of NHEJ-based knockout alleles can reach 90% efficiency, and depending mostly on the specific gRNA target and injection skill 2–10 founders are usually sufficient for obtaining a stable line (**Fig. 2**). However, error-free HDR-based knock-in requires an in-frame insertion of a short sequence; therefore, 10–30 founders are usually required, as many events will result in only partial editing[2] (**Figs. 2d** and **6**). When the Tol2-based transgenic approach is used with a fluorescence reporter, screening for germ-line transmission becomes easier, as visual screening can replace genotyping. Initially, during earlier phases of colony setup and until the user is comfortable with all the husbandry steps, it is advised to collect higher numbers (e.g., 200–300) of fertilized eggs for propagation. Similarly, survival and targeting efficiency after microinjection will improve with practice. Even if injection/targeting efficiency is low, these experiments can still be successful by simply increasing the number of injected eggs and number of F0 founders.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS I.H. and A.B. conceived the project, and I.H. performed the experiments. I.H. and A.B. wrote the manuscript, with assistance from D.R.V.

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