Rapid reverse genetics systems for *Nothobranchius furzeri*, a suitable model organism to study vertebrate aging

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The African turquoise killifish *Nothobranchius furzeri* (*N. furzeri*) is a useful model organism for studying aging, age-related diseases, and embryonic diapause. CRISPR/Cas9-mediated gene knockout and Tol2 transposon-mediated transgenesis in *N. furzeri* have been reported previously. However, these methods take time to generate knockout and transgenic fish. In addition, knock-in technology that inserts large DNA fragments as fluorescent reporter constructs into the target gene in *N. furzeri* has not yet been established. Here, we show that triple-target CRISPR-mediated single gene disruption efficiently produces whole-body biallelic knockout and enables the examination of gene function in the F0 generation. In addition, we developed a method for creating the knock-in reporter *N. furzeri* without crossing by optimizing the CRISPR/Cas9 system. These methods drastically reduce the duration of experiments, and we think that these advances will accelerate aging and developmental studies using *N. furzeri*.

African turquoise killifish (*N. furzeri*) is an emerging model vertebrate¹-². It can be maintained in the laboratory at a low cost, similar to other small laboratory fish, like zebrafish and medaka. Some inbred lines of *N. furzeri* have also been established. Among these, the GRZ strain is the most frequently used line that originates from a sample collected in the Gona-Re-Zhou National Park of Zimbabwe³. GRZ larvae rapidly grow and mature sexually by 4–5 weeks, with a maximum lifespan of only 3–4 months. This is the shortest life span among vertebrate species and is beneficial for studying aging and age-associated diseases⁴. Furthermore, the developmental process of *N. furzeri* embryo is also quite different from that of zebrafish and medaka embryos and has three unique features; the first one is a slow cell cycle during early cleavage, the second one is the cell dispersion and reaggregation before starting gastrulation, and the third one is the ability to enter diapause, a state of facultative developmental arrest. Diapause allows the organism to survive long-term in extreme environments⁵-⁶.

To explore gene function, knockout animals and reporter-transgenic animals that enable visualization of gene expression patterns are powerful tools. Methods for CRISPR/Cas9-mediated gene knockout and Tol2 transposon-mediated reporter transgenesis in *N. furzeri* have been previously reported⁷-⁸. However, these methods require a long time and large breeding areas to produce knockout and transgenic fish. For example, generating the whole-body biallelic knockout of *N. furzeri* by the CRISPR/Cas9 system required at least two rounds of mating that take a further six months, because the body of the first generation-transgenic fish consists of wild-type, heterozygous, and a small percentage of biallelic mutant cells. If the mutant cells contribute to the germline, the next-generation offspring contain hetero-knockout fish, and further crossing can produce homo-knockout fish at a Mendelian rate. Tol2 system-mediated production of fluorescent reporter fish also requires at least one crossing, and it is difficult to judge whether the reporter can reflect endogenous gene expression patterns in the reporter-containing F0 generation. Apart from the Tol2 system, gene knock-in is also useful for generating a reporter *N. furzeri*. Although a gene knock-in technology that inserts 30–50 bp DNA fragments into the *N. furzeri* genome for exchanging a few amino acid sequences has been previously reported⁹, methods for introducing large DNA fragments as fluorescent reporter genes (around 700 bp) have not been established.

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In this study, we improved the CRISPR/Cas9 method by introducing three short-guide RNAs (sgRNAs) targeting a single gene to produce a whole-body biallelic knockout in *N. furzeri* in F0 generation with an almost 100% success rate. Furthermore, we created a highly efficient method for generating a fluorescent reporter that reflects endogenous gene expression patterns in F0 generation by injecting Cas9 mRNA with sgRNAs to digest a specific genomic region and donor plasmid containing a heat-shock promoter and fluorescent reporter gene. These methods drastically reduce the time and space required for experiments and we hope that these will accelerate developmental and aging studies using *N. furzeri*.

### Results

The triple-target CRISPR-mediated efficient production of whole-body biallelic knockout. A multiple-target CRISPR strategy, in which multiple sgRNAs are used to target a single gene, has efficiently produced biallelic KO animals in the F0 generation in several model organisms. First, to examine whether a multiple-target CRISPR strategy would also work efficiently in *N. furzeri*, we compared the single- and triple-target CRISPR for the rate of generating the whole-body biallelic knockout of the tyrosinase gene. The *tyrosinase* gene encodes an enzyme which converts tyrosine into melanin, and loss of *tyrosinase* function results in pigmentation defects. We selected three specific sgRNA target sites on the *tyrosinase* gene that did not overlap with other genomic sequences, were close to their translational initiation sequences (Fig. 1a) and injected them in the same *N. furzeri* embryos. These results indicate that the triple-target CRISPR can efficiently produce whole-body biallelic knockout in *N. furzeri* embryos. These results indicate that the triple-target CRISPR can efficiently produce whole-body biallelic knockout in *N. furzeri* in the F0 generation.

Next, to further confirm the efficacy of triple-target CRISPR in *N. furzeri*, we targeted the *tcf7l1* and *tbx16* genes. The phenotypes of these gene knockouts have been well-characterized in zebrafish. The *tcf7l1* gene encodes a transcriptional repressor in the Wnt/β-catenin signaling pathway and zebrafish embryos with *tcf7l1* loss-of-function mutation exhibit a posteriorized phenotype lacking the eye and a part of the forebrain due to excessive activation of Wnt/β-catenin signaling. The *tbx16* gene is expressed in paraxial mesoderm precursors, and loss-of-function of *tbx16* disrupted the paraxial mesoderm tissue from which somites are derived, causing a drastic reduction in the tail region in zebrafish. We designed three specific sgRNAs targeting *tcf7l1* or *tbx16* genomic loci in *N. furzeri* that are close to their translational initiation sequences (Fig. 2a) and injected them in the same way as described above. Remarkably, 95.2% of embryos injected with triple sgRNAs targeting the *tcf7l1b* gene showed a posteriorized phenotype (loss of eyes and reduction of anterior brain regions (Fig. 2b,c), which was similar to that of zebrafish mutants lacking *tcf7l1* gene. Furthermore, 100% of embryos injected with triple sgRNAs targeting the *tbx16* gene exhibited tailless phenotype (Fig. 2b,c), which is the same phenotype observed in zebrafish lacking *tbx16* gene. These results indicate that triple-target CRISPR can be used for several genes, suggesting that this strategy could be applied to systematic functional analysis of genes in *N. furzeri* development and aging models.

Application of the triple-target CRISPR for mosaic analysis. Genetic mosaic analysis can be used to gain insights into the cell specificity of gene function. Because the triple-target CRISPR strategy can efficiently and quickly introduce biallelic knockout in the cell receiving the injection and its descendant cells, we believe that
this strategy can also be used to stably generate genetic mosaics in *N. furzeri* by injecting gene-targeting triple sgRNAs into a single cell at the multi-cell stage. To test this idea, we established a DsRed2-transgenic *N. furzeri* line, which expresses the red fluorescent protein DsRed2 ubiquitously. Triple sgRNAs targeting the DsRed2 gene were injected into the DsRed2-transgenic eggs (Fig. 3a). In the uninjected control embryos, DsRed2 expression was observed in all cells throughout the body (Fig. 3b). However, the embryos injected with DsRed2-targeting triple sgRNAs into the cell body of 1-cell stage fertilized eggs eliminated DsRed2 expression completely in all the cells, confirming that the triple-target CRISPR produces whole-body knockout (Fig. 3b). Importantly, injection of the triple sgRNAs into the single-cell bodies of the 4-cell stage embryo eliminated DsRed2 expression only in some cells (Fig. 3b). These results indicate that triple-target CRISPR is applicable for generating the genotypically mosaic *N. furzeri*.

### Efficient production of knock-in reporter fish using CRISPR/Cas9

CRISPR/Cas9 was effective in modifying the genome of *N. furzeri*, and we believe that this method is also useful for generating reporter gene knock-in *N. furzeri* at F0 generation. To test this possibility, we applied the protocol for generating the previously reported CRISPR/Cas9-mediated reporter knock-in system in zebrafish or medaka. As described in Fig. 4a, this protocol requires two sgRNAs for digesting the target gene on the genome and donor plasmid. One sgRNA targets the 5’-UTR region, approximately 100–200 bp upstream of the translational initiation site of the genomic target gene. The second sgRNA targets the Tbox sequence, upstream of the hsp70 basal promoter fused with the GFP gene on the donor plasmid. Concurrent digestion of the genome and plasmid DNA by the Cas9 protein results in the integration of the donor plasmid into the genome via a homology-independent repair system. Then, cis-regulatory sequences controlling the spatiotemporal expression pattern of the target gene drive the GFP expression in target gene expressing cells through the hsp70 promoter (Fig. 4a). We chose *noto* (notochord homeobox) and *tbx16*, the transcripts of which are specifically expressed in the notochord and paraxial meso-
derm lineage, respectively (Fig. 4b, right), as target genes. Surprisingly, almost 20–30% of embryos strongly and specifically expressed GFP in the area expressing endogenous noto or tbx16 mRNAs (Fig. 4b, left). Using this method, we also succeeded in visualizing the expression of hemoglobin embryonic subunit alpha (hba) and ecto-
nucleoside triphosphate diphosphohydrolase 5a (entpd5a) genes, which are well-established markers for erythrocytes and skeletal tissues, respectively, in zebrafish18,19 in the F0 generation (Fig. 4c,d, Supplemental Video 1). These results indicate that CRISPR/Cas9-mediated knock-in systems allow for rapid identification of gene expression patterns in *N. furzeri*.

**Discussion**

Genetic engineering tools, such as gene knockout and reporter animal models, facilitate the analysis of gene functions and regulation. Many mutant and reporter-transgenic zebrafish lines have been generated and have promoted zebrafish studies, consequently making zebrafish one of the most popular animal models. *N. furzeri* has emerged as a new fish model that allows the study of important biological processes such as aging, age-associated diseases, and embryonic diapause. It is difficult to study these processes using other well-established
model organisms, such as mice and zebrafish. Although methods for generating knockout and transgenic lines in N. furzeri have been established, the number of studies using mutant and transgenic N. furzeri is still very limited. These previously established methods require a long time and large breeding areas to produce knockout and transgenic fish. Therefore, to accelerate N. furzeri research, the development of rapid and efficient methods for N. furzeri reverse genetics is required. In this study, we successfully developed a method to effectively generate whole-body biallelic knockout N. furzeri without crossing. We also established a method for monitoring endogenous gene expression patterns by creating a knock-in reporter gene without crossing. These methods for N. furzeri without the need for crossing would facilitate not only the identification of genes involved in vertebrate aging and diapause but also an understanding of their expression patterns. Furthermore, we established a method to generate genetically mosaic organisms. This method enables us to understand the molecular mechanism in more detail, allowing us to examine the non-autonomous functions of the genes related to aging and diapause, such as cell–cell and/or organ-organ interaction processes. These sequential analyses in N. furzeri may facilitate rapid identification of the genes and pathways that are critical for aging and diapause throwing light on the unexplored biological phenomena.

Methods

Ethical approval. All experimental animal care was performed in accordance with institutional and national guidelines and regulations. The study protocol was approved by the Institutional Animal Care and Use Committee of the respective universities (Gunma University Permit# 17–051; Osaka University, RIMD Permit# R02-04). The study is reported in accordance with ARRIVE guidelines.

Maintenance of fish lines. The GRZ (GRZ-AD) strain of N. furzeri was gifted by Prof. Adam Antebi. Fish were maintained at 26.5 °C, 0.7 conductivity on 12/12 h light/dark cycle in the fish breeding system (METO, Nagoya, Japan) at a density of 1 fish per 1.4 L tank for adult fish. Fish were fed freshly hatched brine shrimp twice a day from Monday to Saturday, and once a day on Sunday, fish that were over 2 weeks old were also fed bloodworms (Kyorin, Himeji, Japan). For mating, one adult male and 3–4 female fish were kept in 4 L tanks, and females spawned on a sand substrate in the plastic cases. Embryos were collected and incubated in the egg water (0.03% sea salt containing methylene blue). Ten days later, the embryos were plated on sterile dry peat moss until they were ready to hatch. Usually, one month after egg collection, embryos were ready to hatch and incubated in the 0.07% ice-cold humic acid solution (53680, Sigma-Aldrich, St. Louis, MO, USA) for 30–60 min, and transferred into the 4 L hatching tank with air supply. Hatched fry were kept in a hatching tank, and half of the solution was changed with the fish breeding system water every day. After 2 weeks, by which time they had grown to be around 1–1.5 cm, they were transferred to 1.4 L tanks similar to the adult fish.

Triple-target CRISPR. The sgRNA targeting sites are listed in Supplementary table 1 and the gene sequences were retrieved using NCBI Gene IDs (tyrosinase:107380455, tcf7l1:107393717, tbx16:107393822). We selected three target sites on the protein coding sequences for each gene that did not overlap with other genomic sequences and were close to the translational initiation sequence, aiming to induce frameshift mutation, using the chop-chop program. The protocol for sgRNA synthesis was based on a previously reported method. The oligonucleotides containing a T7 promoter sequence, target sequence, and the sgRNA templates were PCR-amplified from pDR274 vectors using the oligonucleotides and primer sgRNA-RV with PrimeSTAR Max Cas9 nuclease was synthesized using the pCS2 + hSpCas9 vector as a template using the mMessage mMachine database (Gene ID of from the 5' UTR region using the chop-chop program, and genes sequences were retrieved from the NCBI Gene SP6 Kit (Thermo Fisher Scientific). The solution was injected into the cell body of 1-cell stage fertilized eggs or 4-cells stage embryos to generate genetically mosaic organisms. This method enables us to understand the molecular mechanism in more detail, allowing us to examine the non-autonomous functions of the genes related to aging and diapause, such as cell–cell and/or organ-organ interaction processes. These sequential analyses in N. furzeri may facilitate rapid identification of the genes and pathways that are critical for aging and diapause throwing light on the unexplored biological phenomena.

Microinjection. The injection plates were generated by melting 2% agarose with egg water in a 100 mm petri-dish, and the injection mold was floated with 0.9 mm width 1.0 mm height (AM6540-0904-1, IPN-Factory, Hekinan, Japan). For injection needle preparation, a glass capillary (G-1, Narishige, Tokyo, Japan) was pulled by one-step pulling using a PC-10 puller (Narishige), and the tip of the capillary was slightly broken off. RNA and DNA solutions were introduced into the needle using a microloader (5242956003, Eppendorf) and injected using an IM 300 microinjector (Narishige).
In situ hybridization. Embryos were fixed with 4% paraformaldehyde. Whole-mount in situ hybridization was performed according to a standard protocol. Probes for the noto and lbx16 genes were generated from *N. furzeri* embryonic cDNA by PCR using the sequences retrieved using the gene IDs from the NCBI Gene database (nto:107391787, lbx16:107393822).

Imaging. Bright-field images of embryos and fry were captured using a stereomicroscope (Leica, Wetzlar, Germany). Fluorescent images of the embryos were captured using FV3000 (Olympus, Tokyo, Japan).

Data availability
The datasets analyzed during the current study are available in the NCBI repository, [tyrosinase:107380455, tcf7l1:107393717, lbx16:107393822, noto:107391787, hba:107378369, entpd5a:107392375].

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Author contributions
Conception and design: M.O. and T.I. wrote the main manuscript text and T.O-A., H.M., and K.A. modified it and M.O., M.N., T.O-A., K.A., S.O, C.M., and T. I. analyzed data and M.O., T.O-A., and T. I. prepared Figures. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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