

A Microinjection Protocol for the Generation of Transgenic Killifish (Species: *Nothobranchius furzeri*)

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Background: A challenge in age research is the absence of short-lived vertebrate model organisms. The turquoise killifish *Nothobranchius furzeri* has an exceptionally short lifespan of 4–10 months depending on the strain. Thus, it possesses the shortest known maximum lifespan of a vertebrate species that can be bred in captivity. **Results:** Here we show the successful introduction of DNA and RNA molecules into the one-cell embryo of *N. furzeri*. For this purpose, we adapted existing microinjection protocols to inject through the remarkably thick and robust chorion of *N. furzeri*'s eggs. The injected DNA transgene was integrated into the genome and transmitted to subsequent generations as indicated by the expression of the fluorophore enhanced green fluorescent protein (EGFP). Furthermore, we could confirm a special phase during embryonic development in which embryogenesis occurs within a re-aggregated mass of previously dispersed cells as it has been described for other related cyprinodont fish species. **Conclusions:** The transgenesis protocol described here provides a basis for a variety of genetic manipulations including overexpression of genes and determining their effects on lifespan and longevity. The feasibility to perform transgenesis is an important step to establish *N. furzeri* as a new model in age research. *Developmental Dynamics* 241:1133–1141, 2012. © 2012 Wiley Periodicals, Inc.

Key words: aging; transgenesis; *tol2*; short-lived teleost fish

Key findings:

- A transgenesis protocol is presented for the short-lived fish *Nothobranchius furzeri*.
- Successful germline transmission of the transgene is shown.
- *N. furzeri* possesses unique phases of embryonic development.

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INTRODUCTION

Small laboratory fish such as zebrafish (*Danio rerio*) or medaka (*Oryzias latipes*) are very popular as vertebrate models in developmental biology and genetics, because they can be maintained in large numbers at relatively low costs. In the past years, particularly zebrafish has been used as a model for various human diseases ranging from cancer (Etchin et al., 2011) to

neurodegenerative diseases (Paquet et al., 2010; Linder et al., 2011). Of interest, zebrafish has also turned out to be an excellent model for regeneration (Jopling et al., 2010; Kikuchi et al., 2010; Diep et al., 2011). It would be desirable to use these species in aging and longevity studies, however, their maximum lifespan of up to five years (Egami, 1971; Gerhard et al., 2002) prevents them from being used as a routine model in age research.

The turquoise killifish (*Nothobranchius furzeri*) is a small laboratory fish that has the shortest known maximum lifespan of a vertebrate species which can be kept in captivity (Valdesalici and Cellerino, 2003). Depending on the strain, maximum lifespan ranges from 4 to 10 months, which makes *N. furzeri* an ideal model for aging studies (Terzibasi et al., 2008). The short lifespan is considered to be the consequence of its natural habitat

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characterized by alternating rainy and dry seasons.

The fish live in temporary bodies of fresh water located in South-Eastern Africa and are absent in permanent streams or ponds. The population survives the dry season in the form of embryos encased in the mud. During the subsequent rainy season, the embryos hatch and the larvae rapidly grow to maturity. Sexual reproduction occurs as early as 4–5 weeks after hatching. Survival of the population in the embryo form has been correlated with unique developmental characteristics (Peters, 1963). Embryos can enter a facultative developmental arrest or diapause at three distinct stages of development, which allow them to survive the variable durations of the dry seasons. Little is known about the mechanisms that trigger the onset and duration of each diapause, except that in *Nothobranchius guentheri* low temperature and low oxygen concentration increase the proportion of embryos entering diapauses (Peters, 1963; Markofsky and Matias, 1977).

Another characteristic of *N. furzeri* is that the short lifespan has been associated with rapid aging as shown by an early onset of aging biomarkers, a decline in learning and behavioral capabilities, age-related telomere shortening and an age-related impairment of mitochondrial function (Terzibasi et al., 2008; Hartmann et al., 2009, 2011). Further genetic studies in *N. furzeri* will benefit from completion of the genome and transcriptome project, which are under way (Reichwald et al., 2009; Valenzano et al., 2009). A very important aspect in establishing *N. furzeri* as a model organism in age research is the feasibility to manipulate gene expression and gene function by insertion of foreign DNA into the genome and its transmission to subsequent generations.

Several different approaches including electroporation and particle bombardment have been tested for germline transgenesis in fish, but up to now microinjection has been shown to be the method of choice (Rembold et al., 2006). This approach requires the injection of DNA constructs directly into the embryo at the one-cell stage. To facilitate early integration of foreign DNA into the host genome, one technique involves the co-injection of

meganuclease *I-Sce*, whereas other approaches use different transposon systems (Grabher and Wittbrodt, 2007). Recently, the *Tol2* transposon system, originally isolated from the medaka fish, has been successfully used in zebrafish and other vertebrates (Kawakami, 2007; Mosimann and Zon, 2011). *Tol2* belongs to the *hAT* (*hobo*/*Activator*/*Tam3*) transposable element family, which is flanked by inverted repeats and encodes their own transposase (Koga et al., 2002).

Here we used the *Tol2* system to analyze the feasibility of injecting DNA molecules into *N. furzeri* embryos and generating transgenic animals. We describe optimized injection conditions and show that the introduced transgene is integrated into the germline and transmitted to the F2 generation.

RESULTS AND DISCUSSION

Establishing Microinjection Conditions for *N. furzeri*

To establish transgenesis in *N. furzeri*, we tested several conditions how to optimally inject RNA or DNA molecules into the one-cell embryo. One factor influencing the microinjection procedure is the thickness of the outer envelope, or chorion, of the fish egg. Whereas zebrafish embryos have a rather thin chorion and are hence relatively easy to inject, other fish species such as medaka or stickleback have a thicker and more robust chorion. Because *N. furzeri* embryos survive in a dry environment, their chorion is exceptionally thick and robust (Fig. 1A). First experiments to remove the chorion manually with forceps or enzymatically with pronase or trypsin were not successful. Instead, we generated glass needles that were stable enough to penetrate through the chorion and thin enough to allow injections into the cytoplasm of the blastodisc (one-cell stage; Fig. 1A). Another step that improved the microinjection procedure (Fig. 1C–E) was the fixation of the embryos while injecting. Although microinjection was possible when holding embryos in agarose trenches as described for other fish species (Rembold et al., 2006), we experienced a higher efficiency and survival rate when the complete

embryo was fixed in 1% low melting agarose (Fig. 1B). The injected embryos were removed from the agarose the next day and cultivated in 0.3× Danieau's medium. There was no detectable difference in survival rate of agarose-embedded and nonembedded embryos.

Injection of EGFP-mRNA Visualizes Developmental Stages of *N. furzeri*

To monitor embryonic development we injected *EGFP*-mRNA (60 ng/μl; EGFP, enhanced green fluorescent protein) into the blastodisc of *N. furzeri* embryos. This one-cell stage occurred at approximately 2 hr post fertilization (hpf) and was clearly visible (Fig. 1A). Expression of *EGFP* was first observed after 12 hpf at the 16- to 32-cell stage (Fig. 2A), and cleavage during the first day after fertilization produced a typical teleost blastula (Fig. 2B; Iwamatsu, 2004). The gastrula stage began at day 2 with the expansion of the blastoderm over the surface of the yolk sphere (Fig. 2C) and epiboly was completed at day 3 (Fig. 2D). Blastomeres dispersed during epiboly and appeared to be arranged in a striking pattern of near-uniform distribution (Supp. Fig. S1A, which is available online). It has been shown in *Nothobranchius* species and other closely related cyprinodont fishes that there is no embryonic shield or axial organization during the dispersed phase (Fig. 2D; Wourms, 1972; van Haarlem, 1983; Carter and Wourms, 1991). This is in contrast to other teleost fishes, such as *Fundulus heteroclitus* (Betchaku and Trinkaus, 1978), *Oryzias latipes* (Iwamatsu, 2004), and *Danio rerio* (Kimmel et al., 1995) in which blastomeres aggregate before and during epiboly to form the germ ring and embryonic shield. Embryogenesis in these fishes takes place in the shield and is usually well advanced before the completion of epiboly.

In *N. furzeri*, the stage of complete dispersion lasted for several days and embryos might enter the first developmental arrest in this phase (diapauses I). A localized region of high cell density usually appeared at around day 6 and marked the future

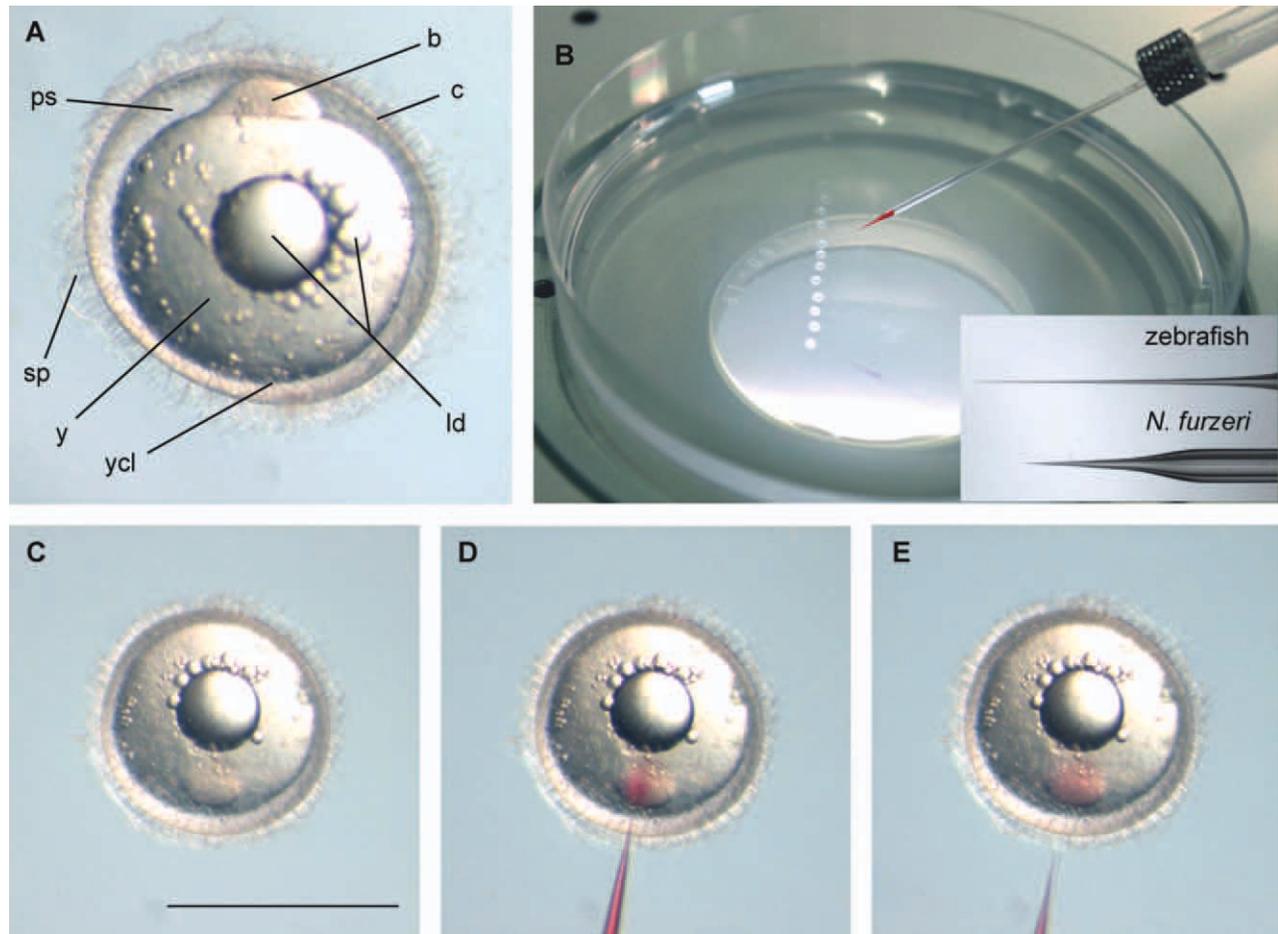


Fig. 1. Microinjection procedure in *N. furzeri*. **A:** Fertilized eggs are allowed to develop for 2 hr. During this time, the blastodisc or one-cell stage develops. **B:** Embryos at the one-cell stage are embedded in 1% low melting agarose for fixation and are oriented with the blastodisc facing the injection needle. Glass needles for zebrafish have a longer and thinner tip, whereas needles for *N. furzeri* have a shorter and more robust tip to penetrate through the hard chorion of the eggs (inset). **C–E:** Correct microinjection into the cytoplasm of the blastodisc is visualized by injecting a solution containing phenol red dye. Scale bar = 1 mm. b, blastodisc; c, chorion; ld, lipid droplets; ps, perivitelline space; sp, surface projections of the chorion; y, yolk; ycl, yolk cytoplasmic layer (adapted from Carter and Wourms, 1991).

site of re-aggregation (Fig. 2E, Supp. Fig. S1B). During the following day the aggregate increased in area and cell number until the embryonic axis appeared which is marked by linear ordering of cells within the aggregate. The aggregate was further increasing and resulted in the pre-somite embryo that usually appeared at day 8 (Supp. Fig. S1C). There was some variation among *N. furzeri* embryos, despite embryos were always kept in 0.3× Danieau's medium at 23°C (Supp. Fig. S1D). For instance, half of the embryos entered the 12-somite stage at day 9 (8 of 16 analyzed embryos), but the overall range varied from day 8 to 11.

The somite phase lasted for several days (here from day 10 to day 24) and

diapause II might occur at this stage. Surprisingly, *EGFP* expression was still detectable at day 10 and 17 which is most likely due to the high stability of the *EGFP* protein. It is worth noting that the *N. furzeri* somite stage at 17 days post fertilization (dpf; Fig. 2G) roughly corresponds to the zebrafish somite stage at 20 hpf in which *EGFP* expression from injected mRNA can be easily detected. Organogenesis occurred before day 31 (Fig. 2I) and growth proceeded until day 36 (Fig. 2J). This is the prehatching state and diapause III might occur at this stage. Overall, this description of embryonic stages in *N. furzeri* is in accordance with studies based on other *Nothobranchius* species and on other annual fish species such as *Austrofundulus myersi* and *Cynolebias spec.*

(Wourms, 1972; van Haarlem, 1983; Carter and Wourms, 1991).

Generation of Transgenic *N. furzeri*

We constructed a *Tol2* vector carrying the *enhanced green fluorescent protein (EGFP)* expression cassette flanked by *Tol2* elements (Fig. 3A). The vector additionally contained the SV40 late polyadenylation (polyA) signal sequence and a 5.3 kb promoter element from the zebrafish β -*actin2* gene (Kwan et al., 2007). This promoter element has been shown to drive broad expression throughout the zebrafish embryo (Higashijima et al., 1997). We co-injected the DNA construct (25 ng/ μ l) with the *Tol2* transposase mRNA (25 ng/ μ l) directly

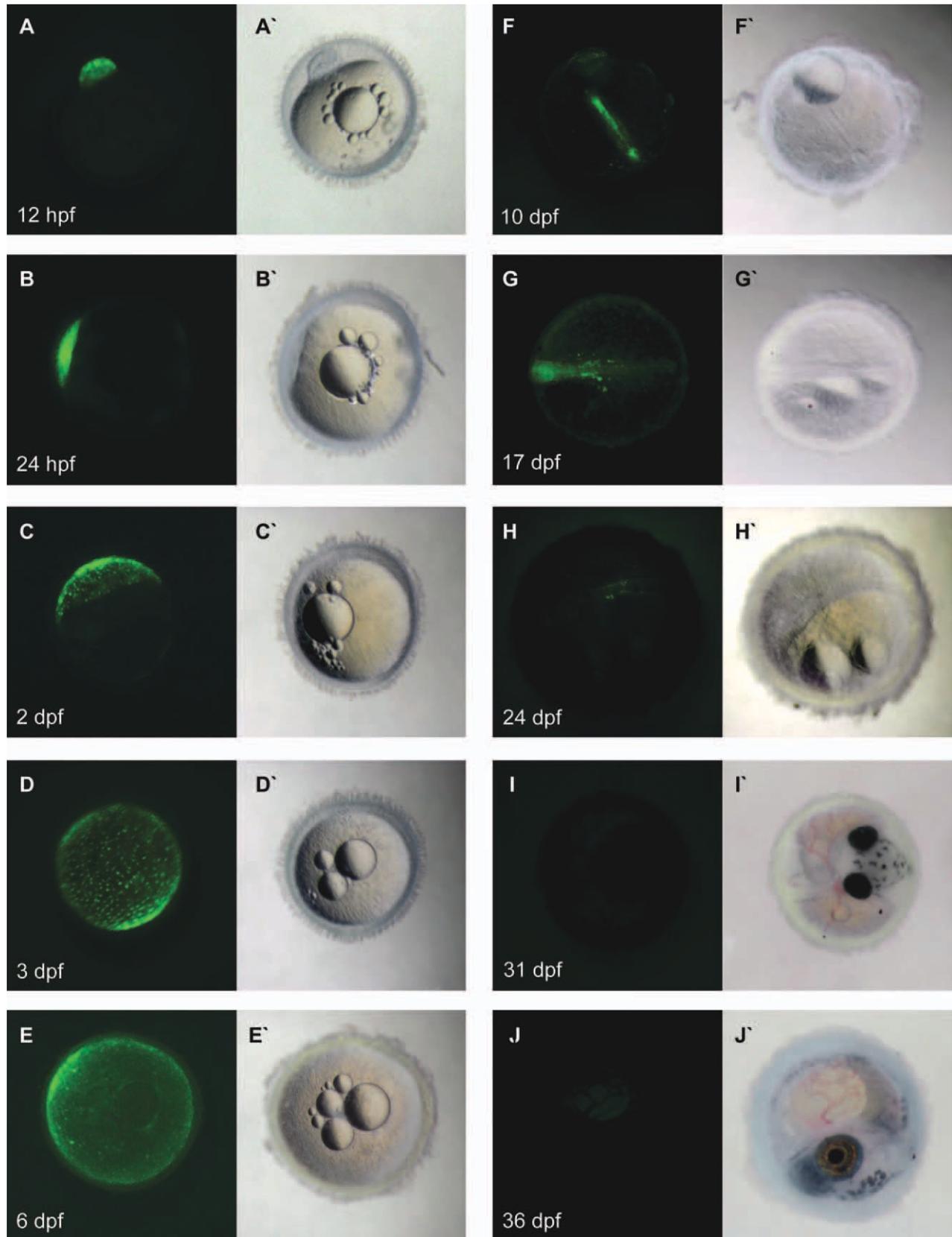


Fig. 2. Embryonic development of *N. furzeri*. *EGFP*-mRNA was injected into the cytoplasm of the blastodisc and *EGFP* expression was monitored while the embryo was cultivated in $0.3\times$ Danieau's medium at $22\text{--}23^\circ\text{C}$. Embryos are always shown using fluorescence (left) and phase contrast (right) optics. **A,A'**: 16- to 32-cell stage, **B,B'**: Blastula stage, **C,C'**: Early epiboly, **D,D'**: Completion of epiboly and dispersed phase (dispause I may occur at this stage), **E,E'**: Beginning of re-aggregation, **F,F'**: Early somite embryo, **G,G'**: Mid somite stage (dispause II may occur at this stage), **H,H'**: Late somite embryo, **I,I'**: Organogenesis is nearly completed, **J,J'**: Prehatching (dispause III may occur at this stage); hpf, hours post fertilization; dpf, days post fertilization. Embryonic staging has been adapted from Wourms, 1972. EGFP, enhanced green fluorescent protein.

into the blastodisc of *N. furzeri* at the one-cell stage. We screened for *EGFP* expression 24 hr after injection and from 350 injected embryos we found 70 (20%) surviving embryos expressing *EGFP* (Fig. 3B). Embryos were cultivated in 0.3× Danieau's medium for 1 week until they reached the somite stage and were then kept on peat moss until hatching. As in the un-injected embryos, a considerable amount of embryos underwent extended diapauses or died during development. From seven hatched embryos, four larvae reached sexual maturity (5 weeks after hatching). Analysis of *EGFP* expression revealed that all four larvae showed a mosaic expression pattern of *EGFP* as it has been frequently described for the founder generation of transgenic animals (Fig. 3C,D; Winkler et al., 1991; Hosemann et al., 2004; Cho et al., 2011). *EGFP* expression continued throughout adulthood as determined at the age of 7 months (not shown). This is in agreement with recent studies in zebrafish and medaka showing *EGFP* expression under the control of different β -*actin* promoters to be active in numerous tissues of adult fish (Burket et al., 2008; Cho et al., 2011). In this particular experiment we obtained four potential founders from 350 injected oocytes indicating a very low efficiency of around 1%. However, it has to be considered that only 10% of the routinely collected embryos survive and reach sexual maturity. The rest of the embryos does not survive the various phases of development. Thus, by further improving husbandry conditions we would also significantly improve efficiency of transgenesis.

Germline Transmission

To analyze whether the injected transgene was transmitted to the next generation, all four founder fish expressing *EGFP* were mated with wild-type fish of the same strain. One out of four founder fish, a female, transmitted the transgene to its offspring as observed by *EGFP* expression in the F1 embryos (Fig. 4). This F0 female transmitted the transgene at a high frequency of 70.2% (33 of 47 embryos), suggesting more than one independent integration site. The

EGFP expression was strong and could be observed as early as at the one-cell stage (~2 hpf), suggesting maternal deposition of *EGFP* protein and/or mRNA (Fig. 4A; Burket et al., 2008). In all analyzed F1 embryos, *EGFP* expression continued to be strong during embryonic development (Fig. 4).

To characterize the integration of the transgene we performed Southern blotting with genomic DNA of four transgenic F1 animals. We restricted genomic DNA with either *SacI* or *HindIII* and used the *EGFP* gene as a probe (Fig. 5A). Two animals showed one band, suggesting one integration site per animal, whereas the other two animals showed two bands indicating two integration sites. The number of bands per animal was consistent with *SacI* or *HindIII* restriction. In total, we detected four bands that differed in size, suggesting four different integration sites in these analyzed F1 animals. Thus, we assume that integration of the transgene occurred at least four times in the founder fish. We also performed an inverse polymerase chain reaction (PCR) approach and restricted genomic DNA of transgenic animals with several restriction enzymes that cut outside the transgene. After ligation of the fragments to form circular molecules we performed a long-range PCR. In case of the restriction of genomic DNA with *MscI* and *SpeI*, we obtained two PCR products that included the border between transgene and host DNA (Fig. 5B). Sequence analysis of these integrations sites revealed that eight nucleotides of host DNA had been duplicated as it is typical for the integration mechanism of the *tol2* transposase (Koga et al., 2002; Kondrychyn et al., 2009). This demonstrates that transgene integration was indeed mediated by the transposase and not by other means of recombination.

To avoid any maternal effects we crossed one male of the F1 progeny animals to non-transgenic females. The resulting F2 embryos only expressed *EGFP* as early as at the blastula stage (24 hpf) as expected when the transgene is transmitted paternally. Fluorescence intensity of the F2 embryos was lower compared with the F1 embryos (Fig. 6). The transmitted frequency was 48.1% (25

of 52 embryos), which is close to the expected Mendelian ratio of 50% indicating the transmission of the transgene that had integrated into one locus.

The feasibility to manipulate gene expression is a prerequisite to establish *N. furzeri* as a new model organism for age research. While this manuscript has been prepared, a successful transgenesis protocol for *Nothobranchius furzeri* has also been presented by others (Valenzano et al., 2011). The authors also used the *tol2* transposon system with slight variations compared with our injection protocol. The ability to perform transgenesis will be useful for introducing a variety of molecules including morpholinos, RNAs or DNA transgenes into *N. furzeri*. This should be particularly useful when overexpressing candidate genes and determining their effect on longevity and lifespan. Together with the analysis of genome-wide linkage mapping of the chromosome regions that effect lifespan, this method should also help to identify new genes involved in the aging process.

EXPERIMENTAL PROCEDURES

Fish Husbandry

The *Nothobranchius furzeri* strain MZCS-08/122 originates from Southern Mozambique (Dorn et al., 2011) and was used for all experiments described herein. Fish were kept at $26 \pm 1^\circ\text{C}$ on a 12 hr: 12 hr light: dark cycle. Adult fish were fed with red bloodworms (Chironomidae) once a day, while fry until the age of five weeks was fed with brine shrimp (*Artemia*) twice a day. Animals were either kept in 40-liter breeding tanks consisting of one male and several females or individually, that means two separated fish per 5-L tank. All animals were maintained in accordance with the current version of the German Law on the Protection of Animals.

Construction of Vector and mRNA Preparation

The MultiSite Gateway system (Invitrogen) and vectors from the *Tol2* kit were used to generate the pDest-Tol2A_*bactin2:EGFP* vector (#1091).

This vector was created by combining the *Tol2* kit vectors #299 (p5E-*bactin2*), #383 (pME-*EGFP*), #302 (p3E-polyA), and #394 (pDestTol2pA2) using LR Clonase II Plus (Invitrogen) according to standard protocols. *Transposase* mRNA was generated using the Tol2kit vector #396 (pCS2FA-*transposase*) as a template. The plasmid was linearized with *NotI* (Roche) and capped mRNA synthesis was carried out using the mMessage mMachine SP6 kit (Ambion). RNA was purified using phenol:chloroform extraction and isopropanol precipitation. The *EGFP*-mRNA was generated in the same way using the pCSII vector #300 as a template (kindly provided by Jochen Wittbrodt) and using *Asp718* (Roche) for linearization.

Injections

Five to 10 breeding tanks were equipped with sand boxes that were subsequently checked for newly laid eggs. Embryos with a clearly visible blastodisc were chosen for embedding in 1% low melting agarose (Biozym). Embryos were lined up, so that the blastodisc was facing toward the injection needle. After hardening of the agarose the embryos were fixed and the tip of the needle could penetrate

through the chorion directly into the blastodisc. This was carried out with glass capillary needles using a pres-

sure injector (World Precision Instruments), micromanipulator (Saur) and stereomicroscope (Olympus). The

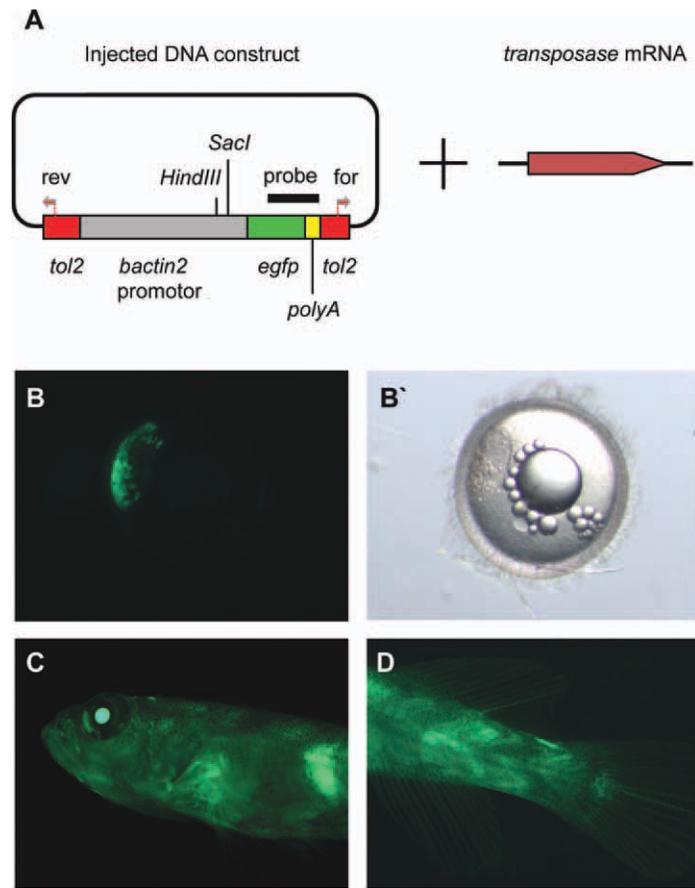


Fig. 3.

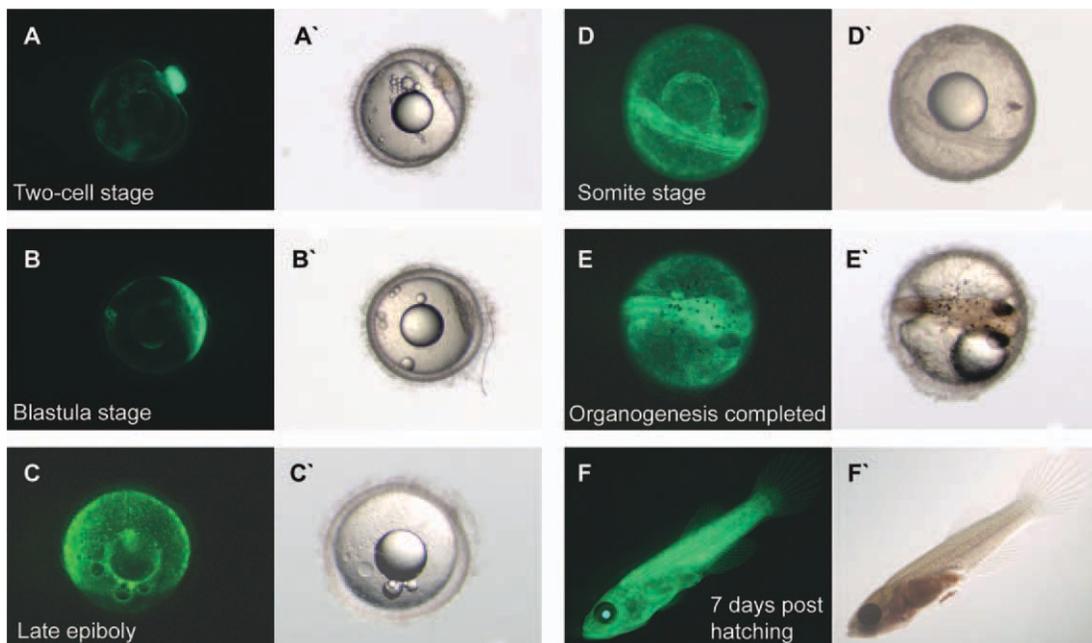


Fig. 4.

injection solution contained either the DNA-construct (25 ng/ μ l) and the *transposase* mRNA (25 ng/ μ l) in 1 \times Yamamoto's medium or the *EGFP*-mRNA (60 ng/ μ l) in 1 \times Yamamoto's medium.

Monitoring and Upbringing of Transgenic Animals

Embryos injected with *EGFP*-mRNA were cultivated in 0.3 \times Danieau's medium at 22–23°C. Pictures were taken with a Zeiss Discovery V8 stereomicroscope. Embryos injected with the DNA-construct and expressing *EGFP* were first cultivated in 0.3 \times Danieau's medium for one week and then kept on peat moss for 2 to 3 weeks until embryos were ready to hatch. After hatching, fish reached sexual maturity within 5 weeks and were crossed to wild-type fish. *EGFP*-positive F1 embryos were raised to adulthood and crossed to wild-type fish to establish a transgenic line.

Southern Blotting and Inverse PCR

Genomic DNA was extracted from entire F1 hatchlings (~10 days old) using the QIAamp tissue kit (Qiagen, Hilden, Germany). Approximately 15 μ g of genomic DNA was digested with either *Hind*III or *Sac*I (NEB, Frankfurt, Germany) at 37°C overnight. DNA samples were ethanol precipitated and 10 μ g of DNA were loaded on a 0.7% agarose gel (1 \times TBE). The gel was treated for 10 min with 0.25 M HCl for depurination, then for 20 min with denaturing buffer (1.5 M NaCl, 0.4 M NaOH) and finally for 20 min with renaturing buffer (1.5 M NaCl, 0.1 M Tris-HCl [pH 7.5]). DNA was blotted onto a Hybond XL membrane (GE Healthcare, Munich,

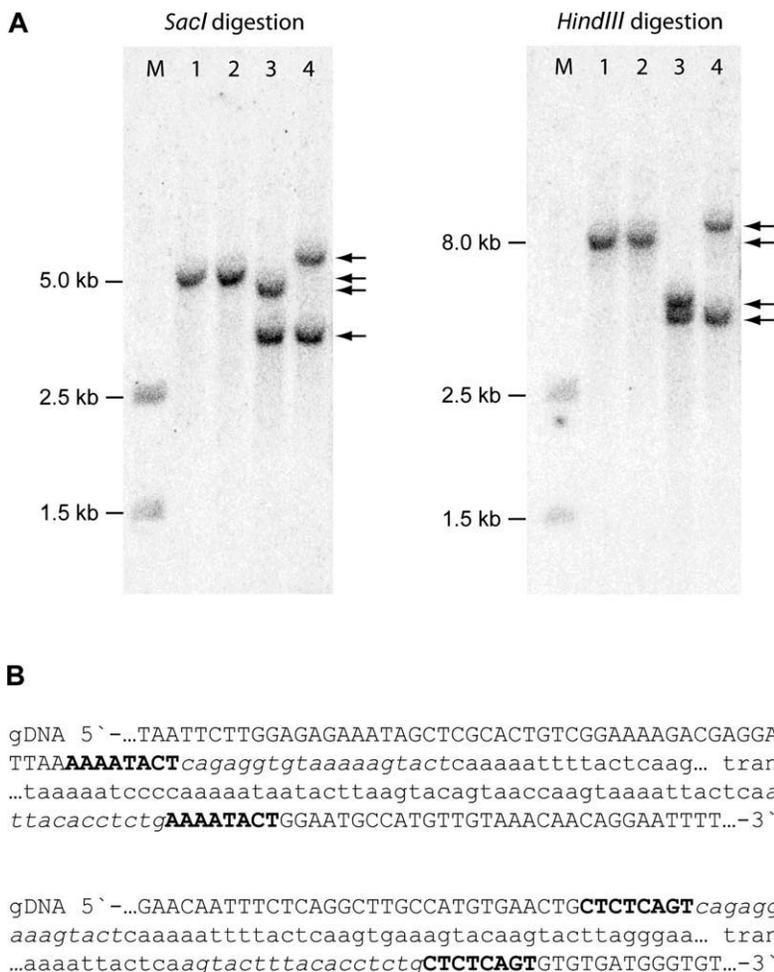


Fig. 5. Characterization of *tol2* integration sites. **A:** Southern blot analysis of genomic DNA from four transgenic F1 animals, which were either restricted with *Sac*I or *Hind*III. Positions of the restriction enzymes in the transgene and position of the used probe are shown in Fig. 3A. Animal #1 and #2 display one band indicating the presence of only one integration site, whereas animal #3 and #4 show two bands indicating two integrations. The total number of bands that differ in size adds up to four (arrows) with both restriction enzymes, respectively. **B:** The inverse polymerase chain reaction (PCR) approach revealed two PCR products that include genomic host DNA (capital letters) and transgene DNA (lower case). The terminal inverted repeats of *tol2* consist of 17 and 19 nucleotides and are marked in italic. The inverted repeats are flanked by eight-nucleotide target site duplications of host DNA (bold) as it has been described for the *tol2* integration mechanism. M, marker.

Germany) by capillary transfer overnight and cross linked. A PCR product (625 bp) that included part of the

EGFP gene was radioactively labeled with [α -³²P]-dCTP using the NEBlot kit (NEB, Frankfurt, Germany) and subsequently purified using Probe-Quant G-50 columns (GE Healthcare, Munich, Germany). Hybridization was performed in 10 ml Rapid-hyb buffer (GE Healthcare) with a probe concentration of about 1,000,000 cpm/ml at 65°C for 3 hr. Stringency washing was performed once in 3 \times standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) and twice in 0.5 \times SSC/0.1% SDS at 65°C. Signal analysis was performed using a PhosphoImager FLA-7000 (Fujifilm, Düsseldorf, Germany).

Fig. 3. Generation of transgenic *N. furzeri*. **A:** Schematic of the injected DNA construct containing *EGFP*, polyA site and the zebrafish β -*actin2* promoter flanked by two inverted *tol2* sites. The *Hind*III and *Sac*I restriction sites were used for subsequent analysis of the integration by Southern Blot, whereas the primers were used for the inverse polymerase chain reaction (PCR) approach. The DNA construct was co-injected with the *transposase* mRNA to facilitate integration into the genome. **B, B':** Successful injection can be observed by *EGFP* expression after 24 hr. **C, D:** Young fish that hatched from injected eggs (14 days after hatching) show a mosaic expression pattern that is typical for the F0 generation. *EGFP*, enhanced green fluorescent protein.

Fig. 4. Developmental time course of *EGFP* expression in transgenic F1 embryos. The same embryo is always shown using fluorescence (left) and phase contrast (right) optics. **A–E:** Ubiquitous and strong *EGFP* expression was present throughout embryonic development and (**F**) remained present after hatching in the F1 generation. *EGFP*, enhanced green fluorescent protein.

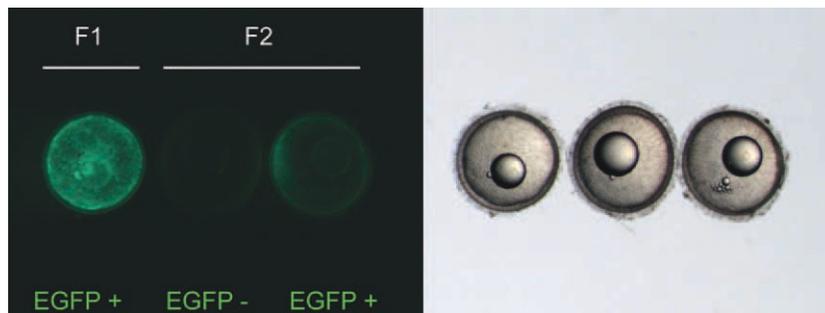


Fig. 6. Different levels of *EGFP* expression in F1 and F2 embryos. After crossing an *EGFP*-positive F1 male to wild-type females, *EGFP* fluorescence is reduced in the F2 generation and can be found in approximately 50% of all F2 embryos. Embryos were in the dispersed phase (3–6 days post fertilization [dpf]). The middle embryo (F2) exhibits no *EGFP* expression. Fluorescence (left) and phase contrast (right) photomicrograph. *EGFP*, enhanced green fluorescent protein.

For the inverse PCR approach, aliquots of 3 μ g of genomic DNA of F1 animals were digested with 1 of 12 different restriction enzymes that do not cut within the transgene. A total of 500 ng of restricted DNA was ligated with the T4 DNA ligase (Fermentas, St. Leon-Rot, Germany) in a 100- μ l volume at 12°C overnight. The 100 ng of ligated DNA was used as template for a long-range PCR using the inverse primers indicated in Figure 3A (*for*: *TGGGCAT CAGCGCAATTCAA*; *rev*: *TTGAGTAG CGTGTACTGGCATT*) and the expanded long template PCR system (Roche Applied Science, Mannheim, Germany). PCR products were cloned and sequenced.

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