Linda Z. Holland[1] and Guang Li[2]

Laboratory Culture and Mutagenesis of Amphioxus (*Branchiostoma floridae*)

[1] Co-corresponding author: Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093-0202

Email: lzholland@ucsd.edu

[2] Co-corresponding author: State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen, Fujian 361102, China.

Email: guangli@xmu.edu.cn

Running head: Laboratory culture and mutagenesis of amphioxus

Key words: Cephalochordate, amphioxus, *Branchiostoma*, aquaculture, germ-line mutagenesis, Tol2, TALEN, transgenics

Linda Z. Holland and Guang Li

Laboratory Culture and Mutagenesis of Amphioxus (*Branchiostoma floridae*)

Abstract

Cephalochordates (amphioxus) are invertebrate chordates closely related to vertebrates. As they are evolving very slowly, they are proving to be very appropriate for developmental genetics studies aimed at understanding how vertebrates evolved from their invertebrate ancestors. To date techniques for gene knockdown and overexpression have been developed, but methods for continuous breeding cultures and generating germline mutants have been developed only recently. Here we describe methods for continuous laboratory breeding cultures of the cephalochordate *Branchiostoma floridae* and the TALEN and Tol2 methods for mutagenesis. Included are strategies for analyzing the mutants and raising successive generations to obtain homozygotes. These methods should be applicable to any warm water species of cephalochordates with a relatively short generation time of 3-4 months and a life span of 3 years or more.

Key Words

1. Introduction

There are three genera of cephalochordates (amphioxus): *Brachiostoma* with 33 named species, *Asymmetron* with 7 named species and *Epigonichthys* with two named species. The last two genera likely have additional cryptic species. Once thought to be the nearest invertebrate relative of vertebrates, molecular phylogenetic studies with nuclear genes have shown they are the sister group of vertebrates plus the fast-evolving tunicates ***(1)***.

Because of its resemblance to vertebrates, amphioxus species have long been the focus of embryological and evolutionary studies designed to give insights into how vertebrates evolved from their invertebrate ancestors. All three genera are exclusively marine, generally inhabiting near-shore waters. In nature, adults live burrowed in sand with just their extreme anterior ends

exposed to the seawater, only emerging after sunset when they spawn. For many years, research on developing embryos of *Branchiostoma* was hampered by inability to control spawning in the laboratory (Fig. 1). Female gametes are stored in the gonad as primary oocytes; on spawning days, which occur every ~10-14 days in summer, meiotic divisions commence in the early afternoon. Meiosis arrests at second metaphase and spawning occurs about 30 min after sundown. On the same day, the sperm acquire the ability to become motile on dilution into seawater. After fertilization, the zygote completes the meiotic divisions. Semi-controlled laboratory spawning of *Branchiostoma floridae* during the breeding season was developed in 1988 ***(2)***. It was found that spawning could be delayed if animals that would normally spawn that day were collected in the afternoon and kept in the light; spawning could then be induced by a mild electric shock or by putting the animals in the dark. This allowed the development of techniques for manipulating embryos such as microinjection of antisense morpholino oligonucleotides for gene knockdowns, DNA constructs for analyzing gene regulatory sequences and mRNAs for gene overexpression ***(3-5)***. The *B. floridae* genome is sequenced and assembled ***(6, 7)***, and a cDNA library of 210,000 clones has been end-sequenced (ESTs) ***(8)***; individual clones are available from L. Z. H. In the laboratory, the life span of *B. floridae* is at least three years.

Until recently, however, one essential technique for understanding how the genotype makes a phenotype--the generation of germline mutants--was missing. The first roadblock to obtaining germline mutants was that while several species of *Branchiostoma* (*B. belcheri, B. japonicum, B. lanceolatum, B. floridae*) as well as *Asymmetron lucayanum* (*see* **Note 1**) had been bred in the laboratory ***(9-12)***, most of the *Branchiostoma* species studied are cool-water species with a larval life of 3 months or more and relatively low survival to metamorphosis; *A. lucayanum* has not been raised to metamorphosis. Only *B. floridae*, a warm-water species, proved easy to raise through metamorphosis in the laboratory with nearly 100% survival (Figs. 1,4). *B. floridae* embryos and larvae develop normally from 21^{o}C-30^{o}C. This is a major advantage as microinjection and manipulations of eggs and embryos can all be done at room temperature. Moreover, at the higher temperature, the life cycle of *B. floridae* from egg to ripe adult is only 3-4 months. A given individual will spawn repeatedly throughout its lifetime of at least 3 years. This means that the multiple generations necessary to obtain homozygous mutants require only 18-24 months. This time frame is similar to that of the zebrafish. Because of these advantages, L.

Holland established continuous breeding cultures of *B. floridae* in in the laboratory, and G. Li has adopted this species in developing techniques for transgenesis.

Methods for generating germline transgenics include Tol2 transposons, TALEN and CRISPR/Cas9 ***(13)***. Tol2 cannot be directed to mutate a specific gene, but has been used to insert exogenous DNA fragments at 10-30% ratios in amphioxus ***(14, 15)]***. To date, however, only TALEN has been adapted for genome editing in amphioxus ***(16)***. Transcription activator-like (TAL) effectors were initially isolated from the bacterium *Xanthomonas*, a plant pathogen. The principle is that two peptides are constructed. Each consists of a TALE backbone including a central region with 12 to 31 nearly identical amino acid repeats, each composed of 34 amino acids differing from one another by only two amino acids at the 12^{th} and 13^{th} positions. These repeat-variable di-residues (RVD) determine the single nucleotides recognized by the TAL effector. Thus, NI binds to adenine, NH or NN to guanine, HD to cytosine, and NG to thymine. Two of these TALE proteins are engineered to opposite strands of DNA. At the C-terminal, each peptide has a spacer and the FokI restriction enzyme. Since FokI works as a dimer (it cleaves between any two bases to create double-stranded breaks), two arms containing assembled modules and a FokI domain are required to introduce double strand breakage at the target locus (Fig. 5). The only constraint is that the 5' bases to which the TALENs bind must be T. In most genomes, target sites for TALENs occur about every 35 bp. The repeat modules can be assembled in a specific order upstream of the FokI nuclease in a pre-constructed vector, and the assembled modules can then bring a FokI nuclease to the target site. The TALEN method has been shown to be highly efficient in introducing indel mutations through non-homologous end joining (NHEJ) pathway at the target loci in amphioxus. However, in amphioxus, it is currently almost impossible to insert exogenous DNA fragments at the target site with the TALEN method (< 1% efficacy) [13], which might reflect a very low activity of homologous recombination (HR)-mediated DNA repair system. For introducing deletions into the Cerberus gene, we used the Golden Gate TALEN and TAL Effector kit 2.0 (#1000000024) from Addgene (www.addgene.org) together with the RCIscript-Goldy TALEN backbone (Addgene plasmid #38142) to construct the two TALEN plasmids. These plasmids are used as templates to synthesize mRNA and the two mRNAs were injected into unfertilized amphioxus eggs. The present chapter describes methods for continuous culture and mutagenesis of *B. floridae.*

2. Materials

2.1 Materials for aquaculture of amphioxus

1. 50-150 larval or adult individuals of *B. floridae*
2. 3, 5, and 10-l translucent plastic bins with lids
3. Aquarium air pumps, air stones, tubing
4. Fish nets –fine and medium mesh
5. Natural or artificial sea water (e. g. Instant Ocean® or Crystal Sea® Marinemix)
6. fluorescent lights
7. timers for lights
8. jars for culturing phytoplankton (4-l)
9. constant temperature rooms or chambers at 17°C and 18-19°C
10. Penicillin, Na or K salt
11. Petri dishes, 9 x 2 cm, 6 cm
12. Clear plastic cups (cocktail glasses)
13. dissecting microscopes
14. pasteur pipettes with rubber bulbs
15. Phytoplankton cultures (*Isochrysis, Tisochrysis, Pavlova, Thallasiosiera*). Starter cultures can be obtained from several suppliers: see reference ***(4)***.
16. Algal culture medium such as F/2 (stock solutions can be made from individual components or purchased, see https://utex.org/products/f-2-medium) or GPM: see reference ***(4)***.
17. Table top centrifuge
18. Autoclave

2.2 General Materials for Transgenesis

1. 50 mg/ml ampicillin stock. Dissolve 1 g of ampicillin in 20 ml of ddH2O. Filter-sterilize, aliquot into microcentrifuge tubes, and store at −20 °C.
2. LB liquid medium: 5 g/l yeast extract, 10 g/l tryptone, 10 g/l sodium chloride. Autoclave.
3. Autoclaved LB liquid medium with 10 mg/l tetracycline, 50 mg/l spectinomycin, or 100 mg/l ampicillin.
4. LB agar petri plates (1.5% agar) with 10 mg/l tetracycline, 50 mg/l spectinomycin, or 100

mg/l ampicillin. Autoclave medium and agar, let cool to 60^{o}C, add antibiotics, pour plates.

5. DH5α chemically competent cells (custom-made or commercial).
6. Plasmid Mini Kit I, Gel Extraction Kit, and Cycle Pure Kit (OMEGA Co. China or Omega Bio-tek, Inc., Norcross, GA 30071).
7. Phenol and chloroform.
8. mMESSAGE mMACHINE T3 Transcription Kit (Thermo Fisher Scientific, AM1348).
9. Animal Tissue Direct PCR Kit (Foregene Co., China) or Phire Animal Tissue Direct PCR Kit (Termo-Fisher, USA).
10. Razor blades and fine tweezers
11. Taq DNA polymerase, buffer and dNTPs (Takara, R007B) or New England Biolabs, Massachusetts, USA.)

2.2.1 Materials for TALEN construct assembly

1. Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene, Kit #1000000024). These kits have 86 different vectors (http://www.addgene.org/taleeffector/goldengatev2/#kit-contents)
2. RCIscript-GoldyTALEN backbone (Addgene: Plasmid #38142).
3. Restriction enzymes *Bsa*I (NEB, R0535S) and *Esp*3I (ThermoFisher, ER0451—supplied with Tango buffer or NEB R0734S--supplied with CutSmart buffer). (*see* Note 2).
4. T4 DNA ligase and 10× T4 DNA ligase buffer (Promega, M1804).
5. 100 mM DTT and 25 mM ATP.
6. Plasmid-Safe nuclease (Epicentre Biotechnologies, E3101K).
7. 10 mg/ml tetracycline stock. Dissolve 200 mg of tetracycline in 20 ml of 95 % ethanol. Aliquot into microcentrifuge tubes and store at −20 °C.
8. 50 mg/ml spectinomycin stock. Dissolve 1 g of spectinomycin in 20 ml of ddH 2 O. Filter-sterilize, aliquot into microcentrifuge tubes, and store at −20 °C.
9. Primers for module amplification and correct clone identification and sequencing:
   Talen-Modual-PCR-F1: 5’-GACTATCGTCGCCGCACTTA-3’
   Talen-Modual-PCR-R1: 5’-CCGCTTACCGGATACCTGTC-3’

pCR8_F1: 5'-TTGATGCCTGGCAGTTCCCT-3'

pCR8_R1: 5'-CGAACCGAACAGGCTTATGT-3'

TAL_F1: 5'-TTGGCGTCGGCAAACAGTGG-3'

TAL_R2: 5'-GGCGACGAGGTGGTCGTTGG-3'

2.2.2 Materials for TALEN mRNA synthesis and egg injection

1. Restriction enzymes *Sac*I (or *Pst*I, *Sal*I, *Bam*HI, *Sma*I).
2. Phenol and chloroform.
3. mMESSAGE mMACHINE T3 Transcription Kit (Thermo Fisher Scientific, AM1348).
4. Glycerol (SIGMA, 49767).
5. 5 cm petri dishes
6. Poly-lysine, 30,000-70,000 mol. Wt., 0.25 mg/ml in distilled water
7. Micromanipulator
8. Horizontal capillary puller
9. Dissecting and compound microscopes
10. Glycerol (SIGMA, 49767).
11. Dextran, Texas Red®, 3000 MW, Neutral (Thermo Fisher Scientific, D-3329).
12. Glass Capillaries (World Precision Instruments, 1702328 or Frederick Haar & Co., 30-30-0

2.2.3 Materials for Tol2 mutagenesis

1. pMiniTol2 (Addgene: Plasmid #31829) and pT3TS-Tol2 (Addgene: Plasmid #31831).
2. Restriction enzymes used for vector reconstruction and plasmid DNA linearization.
3. T4 DNA ligase and 10× T4 DNA ligase buffer (Promega, M1804).
4. Taq DNA polymerase, buffer and dNTPs (Takara, R007B or other suppliers).
5.

3. Methods

3.1 Method for amphioxus aquaculture (Figs. 2-4)

3.1.1 Obtaining adult amphioxus and establishing a culture.

B. floridae lives in shallow water on the west coast of Florida, USA and can be collected with a

shovel and sieve as previously described ***(4)*** .Alternatively, they can be purchased from Gulf Specimens (http://www.gulfspecimen.org/specimen/chordata/amphioxus/), but are relatively expensive. Animals collected in the wild or from laboratory cultures can be shipped by overnight express. Approximately 25-50 adults in about 800 ml of seawater in a 1-liter container with 50-100µg/ml penicillin G can be shipped. Jars are wrapped in a layer of insulating material (e.g. newspaper, bubble wrap, Styrofoam) and shipped in a Styrofoam container. Blue ice packs are included if the weather is very warm. Larvae and newly-metamorphosed adults are transported or shipped 25-100 individuals in ~40 ml seawater with 50-100µg/ml penicillin G in a 50 ml bottle or centrifuge tube. To ship them alive or fixed out of the USA, clearance from the U.S. Department of Fish and Wildlife is required. Obtaining the "Declaration For importation or exportation of Fish and Wildlife" is straight-forward as *B. floridae* is not an endangered species. Some countries require permits to import marine invertebrates.

3.1.2. **Salinity**

If animals are collected in the wild, the salinity of the seawater should be determined with a refractometer before the animals are placed in seawater from a different location. Natural seawater is typically 33-34 ppm. However, in shallow bays with limited water circulation, the salinity can be lower or occasionally a little higher. Individuals of *B. floridae* can acclimate over 7-10 days to sea water with salinity ranging from a low of about 25 ppm to a high of 40 ppm. Instant Ocean® has a salinity of 30 ppm if a standard 15 pound bag is diluted into 50 gallons. If the salinity of the water in which the animals are shipped differs from that in which they will be cultured by 2 ppm or more, or if an artificial seawater is used for culture, then 20% of the water should be replaced every two days with the sea water in which they will be cultured until the salinity of the culture water is achieved.

3.1.3. **Parasites and diseases.**

In the wild, *B. floridae* can harbor larvae of a tape worm *Acanthobothrium brevissime*, whose adults live in the gut of sting rays ***(17)***. Laboratory-raised animals generally do not harbor this tapeworm for lack of the intermediate host. A more serious problem is the "red disease" – probably a *Rhodobacter* --that is endemic in amphioxus populations world-wide. If amphioxus is stressed, it can become infected and eventually die. Animals kept under the culture conditions

described here rarely become infected. Culturing in artificial sea water rather than natural sea water should largely eliminate the red disease. The use of penicillin after spawning (see below) and for early larvae helps stave off bacterial infection.

3.1.4 Laboratory Breeding Culture (*see* Note 2)

B. floridae can tolerate a wide range of temperature. This is highly advantageous not only for shipping animals but for breeding them. Animals cooled to 11°C during shipping can be returned over a few hours to room temperature (23°C) with no ill effects. Adults of *B. floridae* can be maintained in the laboratory at 23-28°C until their gonads ripen. Then they can be transferred to a constant-temperature room set to 17-18°C, mimicking winter conditions. If left at 17-18°C for two weeks or more, and then transferred to 25-28°C, 20-80% will spawn the next day when the lights go out. The only caveat is that if ripe females are maintained at 17-18°C for an extended period, the percentage of embryos developing normally decreases. For microinjection, select egg batches that do not have partially elevated fertilization envelopes, as they make microinjection difficult. Methods for microinjection of unfertilized eggs are in ***(3)***.

B. floridae has been in continuous breeding culture in the Holland laboratory since 2013. Cultures can be established relatively inexpensively; the greatest expense aside from artificial seawater is the labor involved in cleaning tanks and raising phytoplankton for food. For *B. floridae* animals are housed in 10-12 liter, translucent or clear plastic bins with translucent or clear lids. Each bin contains 8 liters of seawater with up to 50 small adults < 3 cm long or 20 large ones, 3-5 cm long (Fig. 2). Sand is not necessary. The water is aerated with an airstone connected to a small aquarium air pump or, if there is one, to the building's airline. The air temperature of the culture facility is maintained at 27-28°C. If that is not possible, aquarium heaters can be used. To achieve growth and sexual maturity of the animals, each bin is fed twice daily about 250 ml of phytoplankton cultures at the end of log phase, with the two feedings are as far apart as possible during a normal work day. For faster growth, animals can be feed three times/day at 8 hour intervals. If the animals are to be bred, they are kept on a 14 hr day/10 hr night cycle. A reversed day/night cycle can be maintained with 'sunset' occurring in the late morning or at noon. This allows tanks to be cleaned in the morning under adequate white light, while red LED lights can provide sufficient illumination for feeding in the afternoon without disrupting the animals' photoperiod; their photoreceptors perceive blue light, but are relatively

insensitive to red. To create dark for the animals during the day, double sheets of black plastic from the ceiling. An alternative is keeping the animals in a room lacking windows with a light trap at the door. Moonlight has proven not to be a major factor in the timing of breeding in *Branchiostoma floridae* ***(18)***.

When adults are maintained without sand, the feces and about half the water should be siphoned out daily. Each tank should be scrubbed out with fresh water once a week to remove the bacterial film.

3.1.5 Feeding adults and algal culture (Figs. 2, 3)

Adults and larvae of *B. floridae* can be fed the same diet. Pilot experiments with 10 animals in 2 l seawater kept at 28oC showed that gonad growth is best promoted by a diet of brown diatoms such as *Pavlova lutheri*, *Isochrysis sp.* (CCMP 1244) and *Tisochrysis lutea* (CCMP463) (Fig. 3). The last used to be considered an *Isochrysis* species. The amount fed is critical. The more that is fed, the faster gonads build up; however, the amount should never exceed that which the animals can clear from the seawater in about 5 hrs. Adults can be fed green algae such as *Tetraselmis*, but it is less digestible than the brown algae; larvae can overeat green algae to the point where their guts will burst. This is not a problem with brown phytoplankton. Algae can be cultured under constant light at 18-23^{o}C (Fig. 2B). Appropriate media are F/2 or GPM. When fed to the animals, cultures should be just at the stationary phase (typically after about 1 week of culture at 18-20^{o}C depending on the amount of culture used for seeding new cultures). Details for phytoplankton cultures are in ***(4)***. Twenty large adults in 8 liters should be fed about 250 ml of the cultures twice a day at intervals of 8 hrs or more. If possible, a 3rd feeding can be done. Since the algal medium can be somewhat toxic, especially for larvae, if small adults are raised in less water (e.g. 20 animals in 2 liters) or if larvae are being fed, it is desirable to centrifuge the algae at 1500xg for 5 min, pour off the medium and resuspend the algal cells in sea water before feeding. The adult diet can be supplemented with commercial diets such as EZ larva (10-50 um) from Zeigler. If feeding artificial food, the aeration should be doubled and tanks cleaned at least twice a week as the commercial diet will settle faster than live phytoplankton and serve as a substratum for bacteria.

3.1.6 Breeding

For breeding, ripe animals are placed for at least 2 weeks at 17-18^{o}C under the same light and feeding conditions. Before the onset of the dark period on the day before spawning is desired, males and females, about 6 of each sex, are separated, placed in separate 4-l containers of 17-18^{o}C seawater and moved to the 27-28^{o} C facility. The next day, before the onset of the dark period, each animal is placed in a plastic cocktail cup with about 50 ml of seawater containing 50 µg/ml penicillin. Between 20-80% of the animals typically spawn the next day within 45 min of the onset of the dark cycle. After spawning, adults are separated from the eggs or sperm and placed in 2-l containers of sea water with 50 µg/ml penicillin. Before the end of the work day, they are returned to the 4-1 containers with 50 µg/ml penicillin. The next morning, they are returned to the 17-18^{o}C facility. Penicillin is usually no longer necessary but can be added if the animals show signs of the "red disease". The spawned animals will eventually refill their gonads and can be spawned again. Leaving the animals at 27-28^{o}C after spawning allows the gonads to ripen faster, but the animals are more susceptible to the "red disease." Spawned sperm can be kept for 24 hr on ice or in the refrigerator at 4^{o}C to allow fertilization later. Eggs can be kept unfertilized for 2-4 hours after spawning to allow for microinjection and other treatments, although the percentage of fertilization does decrease with time.

3.1.7 Raising larvae of *B. floridae* (Fig. 4)

The rate of development is directly proportional to temperature. Below 20^{o}C, development is abnormal. Optimal is 28-30^{o}C. A schedule of development is in ***(4)***. The eggs are transferred from the spawning cups into 2 x 9 cm-deep petri dishes. Maternal feces and/or other debris should be removed, and the eggs fertilized with a few drops of the water in which males spawned. The fertilized eggs should be diluted so that after elevation of the fertilization envelope, which requires up to 20 min for full elevation, the embryos form a monolayer on the bottom of the dish. For the first 24 hrs, it is advisable to keep the embryos in 25-50 µg/ml penicillin to inhibit bacterial growth facilitated by dying sperm. Embryos will not develop normally if excessively crowded. If fertilized at 1300-1400h and incubated at 30^{o}, they will be hatched neurulae by the next morning. The neurulae have a single photoreceptor in the nerve cord and swim up towards the light. By shining a light on one side of the dish at the top of the water, the embryos can be easily collected with a Pasteur pipette and transferred into a dish of fresh seawater. Once embryos hatch, antibiotics are no longer necessary. The mouth opens by

28-30 hours after fertilization. Like adults, larvae feed continuously. Larvae should be fed twice a day as soon as their mouths open. They must be fed within 1 day of the mouth opening or they will cease feeding and never start again even if food is present. Before the second feeding of the day, it is useful to pour the larvae into a clean 2 cm x 9 cm petri dish to remove any algae that have settled to the bottom and might provide a substratum for bacteria. Larvae should be changed to fresh seawater daily. For a 2 cm x 9 cm dish with about 50 ml sea-water, about 40-45 ml algal culture should be centrifuged at 1500xg for 5 min and the pellet resuspended in seawater before adding to the dish of larvae as the algal medium is somewhat toxic. At 30^{o}C, metamorphosis will commence in about 2.5 weeks when animals have about 7 gill slits (Fig. 4). Metamorphosis can be synchronized by the application of T3 thyroid hormone at 1x10^{-}8 M or 1x10^{-}7M TRIAC ***(19)***. Metamorphosis will be complete in 1-2 weeks after its onset.

3.2. Generation of amphioxus mutants with TALEN method (Figs. 5-7).

3.2 1. TALEN target site design

Figure 5 shows the TALEN site used for targeting the amphioxus *Cerberus* gene ***(20)***. This site is in the coding region (bases 202 -252). In selecting the site, care must be taken to ensure that introns do not interrupt the target site. Lengths of the arms and spacer are chosen to be 14-17 bp in amphioxus, although lengths over 20 bp have been used in other species. A nucleotide T located immediately upstream of both arms is required for efficient binding of TALEN proteins to the target site. It is useful if the target site includes a restriction endonuclease site near the center of the spacer for convenient mutation detection. Making sure that this site is conserved across species of *Branchiostoma* will help avoid problems caused by polymorphism. Genome sequences of three amphioxus from genus *Brachiostoma* (*B. floridae*, *B. belcheri* and *B. lanceolatum*), which are available now, can be accessed at https://genome.ucsc.edu/ (for *B. floridae* and *B. lanceolatum*) and http://genome.bucm.edu.cn/lancelet/index.php (for *B. belcheri*).

3.2.2 TALEN construct assembly (Figs. 5, 6)

RVD repeat modules are assembled using the Golden Gate assembly method, essentially as described in ***(21)*** but with minor modifications for more efficient assembly. The Golden Gate kit (Addgene.org) provides a set of 86 plasmid vectors, 50 of which code for RVD modules. First, plasmids containing the requisite RVD modules are selected and then the RVD modules are

amplified by PCR and used for assembling the arms. The intermediate vector originally used to host the first ten RVD modules has been modified to generate pFUS_A1 and pFUS_A2, which can respectively adapt 1-5 and 6-10 RVD modules. Since this modification removes part of *LacZ* coding sequence, the resultant colonies of FUS_A1 and pFUS_A2 cannot be screened with blue-white selection. Second, pFUS_A1, pFUS_A2, pFUS_B and RCIscript-GoldyTALEN vectors are pre-cut with *Bsa*I or *Esp*3I, and purified before use, to remove most of the colonies with empty vectors (*see* Note 5).

3.2.3 Before Golden Gate assembly

1. PCR-amplify RVD modules. Since the arm-length of TALENs for amphioxus genome editing is less than 17 base pairs, and since the pFUS_A1 and pFUS_A2 vectors are used for cloning the amplified RVD modules, only 24 of these modules (NI 1-6, HD1-6, NI 1-6 and NN 1-6 need to be amplified. The PCR program is 95°C for 3 min, 38 × (95°C for 30 sec, 58 °C for 30 sec, 72 °C for 20 sec), 72 °C for 5 min, and hold at 4 °C.

10 × Buffer	10 µl
dNTP Mix (2.5 mM each)	8 µl
ddH2O	69 µl
Talen-Modual-PCR-F1 primer (10 mM)	4 µl
Talen-Modual-PCR-R1 primer (10mM)	4 µl
Taq HS polymerase (5 U/µl)	1 µl
RVD module plasmid DNA (1 ng/µl)	4 µl
ddH2O to final volume of	100 µl

2. Purify the above PCR products with the Cycle Pure Kit and adjust each RVD module to 50 ng/µl with ddH2O. Store at -20 ° C.
3. Cut the intermediate vectors FUS_A1, pFUS_A2 and pFUS_B5 with *Bsa*I (100 µl each) for overnight at 37 °C, purify them with the Gel Extraction Kit, and adjust each vector to 50 ng/µl. Store at -20° C. There are ten pFUS_B plasmids (numbered pFUS_B, 1-10) for cloning arms of different lengths. For amphioxus, as our arms are usually <17 bp, we use only pFUS_B, 1-6 vectors.

10 × CutSmart Buffer	10 µl
BsaI (10 U/µl)	2 µl
Plasmid DNA	2 µg

ddH2O	to 100 µl

4. Cut the final vectors RCIscript-GoldyTALEN with *Esp*3I overnight at 37 °C, purify them with the Gel Extraction Kit, and adjust each vector to 50 ng/µl. Store at -20°C.

10 × Tango buffer	10 µl
Esp3I (10 U/µl)	2 µl
Plasmid DNA	2.5-5 µg
DTT (20 mM)	5 µl
ddH2O	to 100 µl

3.2.4 Golden Gate assembly: Day 1

1. Thaw PCR products for each of the RVD modules for positions 1 to $N - 1$ (N is the total length of TALEN = number of RVDs). Thaw the pre-cut intermediate plasmid DNA for pFUS_A1, pFUS_A2 and pFUS_B ($N - 11$). To assemble the example TALEN in Figure 5, the PCR or plasmid DNAs needed are NG1, NG2, HD3, NI4, NN5, and pFUS_A1 for positions 1–5 and NI1, HD2, NG3, HD4, NG5, and pFUS_A2 for positions 6–10, HD1, NN2, NN3, NN4, NG5, and pFUS_B5 for positions 11 to 15 (the last RVD will be added in the second Golden Gate reaction).
2. Prepare the Golden Gate reaction #1 for positions 1 to 5.

PCR products for RVDs 1-5 (0.5 µl each)	2.5 µl
Pre-cut pFUS_A1	0.5 µl
10 × T4 Ligase Buffer	1 µl
T4 Ligase	0.5 µl
*Bsa*I	0.5 µl
ddH2O	to 10 µl

3. Prepare Golden Gate reaction #2 for positions 6 to 10.

PCR products for RVDs 1-5 (0.5 µl each)	2.5 µl
Pre-cut pFUS_A2	0.5 µl

10 × T4 Ligase Buffer	1 µl
T4 Ligase	0.5 µl
*Bsa*I	0.5 µl
ddH2O	to 10 µl

4. Prepare Golden Gate reaction #3 for postions $N - 10$ to $N - 1$.

PCR products for RVDs 1- ($N - 11$) (0.5 µl each)	X µl
Pre-cut pFUS_B ($N - 11$)	0.5 µl
10 × T4 Ligase Buffer	1 µl
T4 Ligase	0.5 µl
*Bsa*I	0.5 µl
ddH2O	to 10 µl

5. Place the above Golden Gate reactions in a PCR machine and run the following cycle: 30× (37 °C/10 min + 16 °C/10 min) + 50 °C/5 min + 80 °C/5 min. Hold at 4°C.
6. Add 0.5 µl Plasmid-Safe nuclease and 0.5 µl 25 mM ATP to each of above Golden Gate reactions, and incubate them at 37 °C for 1-2 hours to destroy unligated linear dsDNA fragments, including incomplete ligation products with less than the desired number of repeats, and linearized, unligated vector backbones.
7. Transform 5 µl of each Golden Gate reaction into 20-50 µl of *E. coli* (DH5α) competent cells.
8. Spread half volume of the transformation mixture on LB agar plates with 50 mg/l spectinomycin. Incubate overnight at 37 °C

3.2.5 Golden Gate assembly: Day 2 (Figure 6)

1. Pick 3-5 colonies from each plate and screen for correct array assembly by colony PCR using primers pCR8_F1 and pCR8_R1. The PCR program is 95 °C for 60 sec, 30× (95 °C for 20 sec, 55 °C for 30 sec, 68 °C for 60 sec), 68 °C for 5 min.
2. Check PCR products on a 1.5 % agarose gel. The products from correct clones will form a ladder of bands due to the repetitive nature of the RVD array. The full length product of the expected size will be the strongest band of 743 bp in pFUS_A1 and pFUS_A2 vectors, and 1,271 − [(10 − N) × 102] bp (N is the number of the pFUS_B vector used) in pFUS_B

vectors.

3. Inoculate 2-3 positive clones for each transformation into 5 ml of LB with 50 mg/l spectinomycin. Incubate and shake overnight at 220 rpm, 37 °C.

3.2.6 Golden Gate assembly: Day 3

1. Purify plasmid DNA for each culture with the Plasmid Mini Kit I, adjust them to 150 ng/µl, and confirm the insert sequence by DNA sequencing with primer pCR8_F1.
2. Prepare the last Golden Gate reaction as follows.

pFUS_A1 vector with correct insert	0.5 µl
pFUS_A2 vector with correct insert	0.5 µl
pFUS_B vector with correct insert	0.5 µl
pre-cut RCIscript-GoldyTALEN vector	0.5 µl
10 × T4 Ligase Buffer	1 µl
T4 Ligase	0.5 µl
*Esp*3I	0.5 µl
ddH2O	to 10 µl

3. Place above Golden Gate reaction in a PCR machine and run the following cycle: 30× (37 °C/10 min + 16 °C/10 min) + 50 °C/5 min + 80 °C/5 min.
4. Add 0.5 µl Plasmid-Safe nuclease and 0.5 µl 25 mM ATP to the reaction, and incubate it at 37 °C for 1-2 hours to destroy unligated linear dsDNA fragments.
5. Transform 5 µl of above Golden Gate reaction into 20-50 µl of *E. coli* (DH5α) competent cells.
6. Spread half volume of the transformation mixture on LB agar plates with 100 mg/l ampicillin, supplemented with X-gal and IPTG. Incubate overnight at 37 °C.

3.2.7 Golden Gate assembly: Day 4

1. Pick 3-5 colonies from the plate and screen for correct array assembly by colony PCR using primers TAL_F1 and TAL_R2. The PCR program is 95 °C for 60 sec, 30× (95 °C for 20 sec, 55 °C for 30 sec, 68 °C for 2 min), 68 °C for 5 min.
2. Check PCR products on a 1.0 % agarose gel. The products from correct clones will also form a ladder of bands with the strongest of around 2000 bp in size.
3. Inoculate one positive clone for each transformation into 5 ml of LB with 100 mg/l

ampicillin. Incubate and shake them overnight at 220 rpm, 37 °C.

3.2.8 Golden Gate assembly: Day 5

1. Purify plasmid DNA for each culture with the Plasmid Mini Kit I and confirm the insert sequence by sequencing.
2. After sequence confirmation, store the TALEN constructs at 4°C or -20 °C for use.

3.2.9 TALEN mRNA synthesis, microinjection and analysis of somatic mutations (Figures 6,7)

1. Linearize 4-8 μg TALEN vectors with *Sac*I (or *Pst*I, *Sal*I, *Bam*HI, *Sma*I).

2. Purify the linearized vectors with phenol/chloroform, and re-suspend them in RNase-free water (5-10μl) to a final concentration of ~200 ng/μl. For the plasmid purification method see https://www.addgene.org/protocols/purify-plasmid-dna/.

3. Prepare a 5-20 μl mRNA transcription reaction for each TALEN vector in 200 μl RNase-free PCR tubes, following the T3 mMESSAGE, mMACHINE T3 kit manual (Ambion/Invitrogen, available from Thermo-Fisher). Mix thoroughly, centrifuge briefly, cap the tubes and incubate them in 37 ^{o}C water bath for 2-3 hours.

4. Mix 0.5 μl above reaction solution with 9.5 μl RNase-free water and electrophorese 1-2 μl on a 1% agarose gel to analyze the quality and quantity of the synthesized mRNA. One or two sharp bands (of size equal to 1-2 Kb double stranded DNA bands) of 300-600 ng/μl in total is the normal yield for a successful reaction. If most mRNA is degraded or its concentration is less than 100 ng/μl, re-prepare the linearized vector DNA and resynthesize the mRNA with the newly purified vector template. The synthesized mRNA can be either used directly for injection or if a higher concentration is required, concentrated with LiCl according to the manual for the mMACHINE T3 kit.

5. Prepare the injection solution by mixing equal amounts of mRNA encoding forward and reverse TALEN sites (for final concentrations of 150-300 ng/μl of each mRNA) with 20% glycerol and 5 mg/ml Texas Red dextran. If necessary, more mRNA can be injected. Inject amphioxus unfertilized eggs with the injection solution (1-2 pl per egg) as previously described ***(22-24)***. (*see* Notes 3 & 4).

6. 500-1000 injected eggs for each TALEN are required to ensure a successful cultivation. The injected eggs are fertilized and the resultant embryos are cultured essentially as described above.

7. When the embryos injected with the TALEN mRNAs reach mid-neurula stage, lyse 20-30 of them as well as 20-30 of the uninjected control embryos with the Animal Tissue Direct PCR Kit. Use the lysates as templates to amplify the DNA region spanning the target site. Mutation efficiency is then determined with restriction digestion. The amplified DNA fragments are cut with the appropriate restriction enzyme and analyzed on a 1-2% agarose gel. The DNA fragment from wild-type embryos should be cut into two pieces of the expected sizes, while that from the injected embryos should not be cut completely. Mutation efficiency is calculated by dividing the total DNA amount (cut and uncut) by that of the uncut DNA. The uncut DNA is then purified with a gel purification kit and cloned into the pGEM-T vector. To determine the type of mutation, 3-5 positive colonies are analyzed. If mutations are detected at the target locus, culture the remaining embryos as described in ***(20)*** or above.

3.2.10 Detection of mutations in founder and F1 amphioxus (Figure 7).

1. When the animals injected with the TALEN mRNA develop fairly large gonads, induce them to spawn as described in ***(12)*** or above, and cross them with wild type (WT) animals to get F1 embryos. Mutations in the gametes of the founders are determined by analyzing their F1 offspring embryos as in section 3.2.9 above.

2. For F1 embryos of founders carrying mutations, 10-20 individuals (depending on the mutation ratios) are randomly selected and lysed individually at mid-neurula or later stages with 10 μl lysing solution from the Animal Tissue Direct PCR Kit. Their genotypes are then determined by digestion with restriction enzymes. PCR amplicons from 3-5 heterozygous embryos are then sequenced to determine the mutation types. If about half of them carry frameshift mutations, embryos from these batches are kept and cultured as described above; if not, screen more founders until one is found with a high ratio of frameshift mutations in its gametes. To get one such animal, it is normally necessary to screen fewer than five founders.

3. When the F1 animals grow to 2-3 centimeters, cut a tiny piece of tissue from the end of their

tails with a razor blade and lyse the tissues with 15-20 μl lysing solution. Their genotypes are then determined as described above. Animals carrying frameshift mutations are kept and those carrying the same mutation are raised together in 5-l plastic tanks (>5 individuals) or in 1-l plastic beakers (< 5 individuals). Normally, more than 10 animals carrying one type of frameshift mutation can be acquired by screening fewer than 100 individuals. If fewer than four heterozygotes are acquired or the identified heterozygotes are all male or female, screen more F1 animals, or cross the F1 individual with WT animals and then screen the F2 animals to obtain enough heterozygotes.

3.2.11 Obtaining and analyzing homozygous mutant embryos (Figure 7).

1. Obtaining homozygous mutants. When the F1 or F2 heterozygotes are ready to spawn, cross them to get homozygous mutant embryos, as well as heterozygous and WT embryos, the ratios of which should be one to two to one. Before hatching, divide the embryos into several petri dishes to avoid biased ratios of different genotypes in subsequent analyses. The number of dishes required depends mainly on how many stages of embryos you intend to fix, but at least one or two dishes with >100 embryos are required for phenotypic analysis.

2. Phenotypic analysis. After fertilization, the embryos should be observed carefully at different stages to determine the phenotypes potentially caused by gene loss-of-function. In our experience, homozygous mutants normally develop well before gastrulation. Therefore, abnormalities in embryos before gastrulation are almost certainly due to a low quality of gametes or excessive agitation of cleavage stages causing cells to separate, but not to loss of gene function. When a uniform phenotype is first detected in ~25% of the embryos, 5-10 embryos with the phenotype plus 5-10 normal embryos should be photographed and then genotyped as described above. Another 50 embryos should also be fixed for paraffin or plastic sections or in situ hybridization ***(25)***. If all embryos showing the phenotype are homozygous mutants and all normal embryos are WT or heterozygotes, this means the phenotype is caused by loss-of-function of the target gene. For confirming the phenotype, similar analyses on later stage embryos are required. It should be noted that occasionally, mutation at one allele of the target gene also leads to a phenotype which is milder than those caused by mutations at both alleles. In addition, some homozygous

mutants may show no obvious phenotype or abnormality before 3-gill slit stage but may die before metamorphosis, while a few may survive to the juvenile stage albeit at a lower or normal expected ratio. For those genes, especially ones expressed during embryonic development that have no closely related paralogues, lines carrying mutations in other exons of the gene are needed to confirm that homozygous mutants are relatively normal.

3. Functional analysis of the mutants. Currently, there are only few custom-made and commercial antibodies available for amphioxus proteins. This limits analysis of gene function in amphioxus development largely to the level of gene transcription. One method for analyzing mRNA expression is whole-mount *in situ* hybridization. Generally, 20-30 embryos are hybridized with tissue-specific markers and/or potential downstream targets genes of the mutant gene. Other analyses include immunostaining, scanning and transmission electron microscopy and RNA-seq.

4. Genotyping embryos after functional analysis. In analyses with homozygous embryos before they exhibit a phenotype, genotyping is required to distinguish the homozygotes from WT/heterozygotes. For embryos older than 32-cell stage fixed with 4% MOPS-PFA or prepared for scanning electronic microscopy, and for gastrula-stage or older embryos analyzed by WISH, genotyping can be done with the above protocol if the fixative is removed with a 30-min wash in 500 ml filtered sea water or PBS before lysis. However, for *in situ* hybridized embryos younger than late blastula, it is generally difficult to amplify the DNA fragment by PCR.

3.3 Generation of amphioxus transgenic line with Tol2 transposon system

The transposable element Tol2 from the medaka fish is the first functional transposon identified in vertebrates ***(26)***. It has been used for transferring exogenous DNA fragments into genomes of zebrafish, frog and mammals through a "cut-and-paste" mechanism ***(27)***. Two vectors are normally required to generate transgenic organisms using the Tol2 system, with one containing the minimal transposable element and the other containing a sequence encoding Tol2 transposase. We used the Tol2 transposon system to insert exogenous DNA fragments at 10-30% ratios in amphioxus ***(14)***. In our protocol, the pMiniTol2 and pT3TS-Tol2 vectors generated by Ekker and colleagues ***(28)*** are used, although other vectors may also work.

3.3.1 Constructs

1. For promoter activity analysis. To test whether a DNA fragment upstream of a gene has promoter activity, an intermediate vector containing a reporter encoding sequence and a polyA signal should first be prepared by integrating these sequences into the multiple cloning sites of the pMiniTol2 vector. mCherry or LacZ reporter sequences are normally chosen since amphioxus eggs and embryos have endogenous green fluorescent protein ***(29, 30)***. After the intermediate vector is constructed, the sequence of potential the potential promotor (normally locating 1-3 kb upstream of the transcript initiation site) is then inserted upstream of the reporter.

2. Enhancer activity analysis. The construct used for enhancer activity analysis is generated in a similar way as described above, except that a minimal promoter (e.g. the one from amphioxus cytoplasmic *Actin* ***(3)***) should be inserted between the enhancer and the reporter.

3. For overexpression of genes. The gene can be an amphioxus gene or one from another species. A promoter/enhancer that directs temporal or spatial expression and the coding sequence of the examined gene can be inserted to the pMiniTol2 vector as described above. However, there are only few sequences of regulatory activity which have been characterized in amphioxus ***(7, 31-33)***.

3.3.2 Preparation of the injection reagent and embryonic injection

1. Linearize 4-8 μg pT3TS-Tol2 vector with *Sac*I (or *Sma*I, *Xma*I, *Bam*HI, *XbaI*, *Sph*I) enzyme, purify it with phenol/chloroform, and re-suspend it in RNase-free water (5-10μl) to a final concentration >200 ng/μl.

2. Prepare a 20 μl mRNA transcription reaction in 200 μl RNase-free PCR tubes according to the manual for the T3 mMESSAGE, mMACHINE kit (Ambion/Invitrogen, available from Thermo-Fisher) and incubate in a 37 ^{o}C water bath for 2-3 hours to synthesize the Tol2 transposase mRNA.

3. To analyze the quality and quantity of the synthesized mRNA, mix 0.5 μl of the mRNA with 9.5 μl RNase-free water and electrophorese 1-2 μl on a 1% agarose gel. Concentrate

the Tol2 transposase mRNA with LiCl precipitation to 2 μg/μl, divide into several RNase-free PCR tubes (1.0 μl/tube) and store at -80 ^{o}C.

4. Purify the pMiniTol2-derived construct with phenol/chloroform to remove the RNase and re-suspend it in RNase-free water to 2-3 μg/μl.

5. Prepare injection solution by mixing 0.85 μl purified pMiniTol2-derived construct, 0.9 μl concentrated Tol2 transposase mRNA, 0.5 μl glycerol and 0.25 μl 50mg/ml Texas Red dextran.

6. Inject amphioxus unfertilized eggs with above injection solution (1-2 pl per egg), fertilize them and culture the embryos as described by ***(22)*** or above. In other species like zebrafish and frogs, a PCR-based assay for generation of a transposon-excision footprint is normally conducted to determine whether integration of the target fragment occurs in the genome of the injected embryos. Several sets of primers have been tried to amplify the excised fragment in amphioxus, but all of them gave strong non-specific bands, excluding the possibility of using this method to identify gene integration in amphioxus. In our experience, to ensure successful transfer of the target sequence from the vector to the genome, injecting as high concentration of plasmid DNA and transposase mRNA as possible is recommended.

7. Examine the injected embryos under a fluorescence microscope (compound or dissecting). Normally there is very strong mosaic and ectopic red fluorescent signals in most of the injected embryos. Although the fluorescent signal cannot show the precise spatiotemporal expression pattern of the transgene, it can reveal if the construct has been correctly made.

3.3.3 Detection of germline transmission in F0 founders

Methods for detecting transgenes in F0 founders and the F1 generation vary depending on the construct used. The following two sections present a protocol for identifying transgenic amphioxus carrying the mCherry reporter construct.

1. When the animals injected with the DNA construct and transposase mRNA develop fairly large gonads, induce them to spawn as previously described ***(12)***, and mate them with wild type (WT) animals to generate F1 embryos.
2. Examine the F1 embryos at the stage when the construct is expected to, express under a

fluorescence microscope. A red fluorescent signal in some embryos indicates that the exogenous DNA fragment was successfully integrated into the genome of germ cells of the founders and transmitted to the F1 embryos. Raise the red fluorescent F1 (RSF) embryos to adults and maintain the corresponding F0 founders. When the F0 founders have refilled their gonads, cross them with WT animals to obtain enough embryos for other analyses.

3. To control for the possibility that some transgenic amphioxus lines carrying constructs of low or no promoter/enhancer activity may not have red-fluorescent embryos, a PCR-based detection strategy is recommended. Randomly select 15 or more F1 embyos, lyse them individually as in section 3.2.10 above and perform PCR with primers corresponding to the mCherry gene. The program is 95^{o}C for 60 sec, 35x (95^{o}C for 20 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec), 72^{o}C for 5 min and hold at 4^{o}C. If PCR of some embryos yields a band of 371 bp, some germ cells of the F0 founders have the transgene. Raise more of the F1 embryos. Maintain the corresponding F0 founders and when ripe, cross them with WT animals to obtain embryos for other experiments.
4. For RFS-negative but PCR-positive F1 embryos, whole-mount *in situ* hybridization with an antisense mCherry probe should be done on sibling embryos to determine if no RFS is due to low or no activity of the promoter/enhancer. If some embryos do show a tissue-specific stain, then the promoter/enhancer is of low activity; if none has a tissue-specific stain, then the promoter/enhancer probably cannot activate transcription.
5. Because Tol2 insertion is not specific to a single site in a genome, the copy number of the insert and the insertion site may differ among F0 individuals or among cells of the same F0 individual. As a result, the expression patterns or intensity of the transgene may vary among embryos of the F1 generation. Therefore, it is best to analyze the expression pattern of the reporter gene in F1 embryos derived from two or more F0 founders.

3.3.4 Identification of F1 transgenic amphioxus

1. Transgenic embryos or larvae of the F1 generation that emit red fluorescence can be separated from non-transgenic ones under the fluorescence dissecting microscope. However, for transgenic embryos and larvae that show no RFS signal, PCR must be done at the juvenile stage when they are 2-3 cm long. The procedure is as in section 3.2.9, but the primers are specific to mCherry. Indidviduals for which PCR gives a 371 bp fragment

are kept. They can regenerate the tips of their tails.

2. Insertion sites and coy number of the insertions in each individual can be determined with inverse PCR and sequencing as described in ***(14)***. Since only the tip of the tail is used for PCR, most, if not all of the animals will survive for further analysis.

Acknowledgements: Particular thanks are due to technicians and many students who helped establish and maintain the amphioxus cultures. These include Ms Patti Brewer, Ms Christine Steinke, Mr. Dan Hartsook, Ms. Esmeralda Alcantar, Mr. Joel Lashomb, Ms Taylor Crabtree, Mr. Zaren Youngblood, Ms. Denisse Larin Enriquez, Mr. Pablo Perez and a host of volunteers. Special thanks are given to Prof. Yiquan Wang and student Jun Feng and Chenggand Shi for their controbutions to the development of genome-editing and transgenic methods in amphioxus. Culture of *B. floridae* was supported by NSF IOS 1160880 to L.Z.H. Development of genome-editing and transgenesis of amphioxus was supported by NSFC 31672246 to G.L.

References

1. Delsuc F, Brinkmann H, Chourrout D et al (2066) Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439**:**965-968
2. Holland ND, Holland LZ (1989) Fine structural study of the cortical reaction and formation of the egg coats in a lancelet (= amphioxus), Branchiostoma floridae (phylum Chordata: supphylum Cephalochordata = Acrania). Biol Bull 176:111-122
3. Holland LZ, Onai T (2011) Analyses of gene function in amphioxus embryos by microinjection of mRNAs and morpholino oligonucleotides. Methods Mol Biol 770:423-438
4. Holland LZ, Yu JK (2004) Cephalochordate (amphioxus) embryos: procurement, culture, and basic methods. Methods Cell Biol 74:195-215
5. Yu JK, Holland LZ (2009) Cephalochordates (amphioxus or lancelets): a model for understanding the evolution of chordate characters. Cold Spring Harb Protoc Sept (9):pdb.emo130
6. Roberts RG, Bobrow M (1998) Dystrophins in vertebrates and invertebrates. Hum Mol

Genet 7:589-595

7. Holland LZ, Albalat R, Azumi K et al (2008)The amphioxus genome illuminates vertebrate origins and cephalochordate biology. Genome Research 18:1100-1111
8. Yu J-K, Wang M-C, Shin-I T et al (2008) A cDNA resource for the cephalochordate amphioxus *Branchiostoma floridae*. Dev Genes Evol 218:723-727
9. Zhang QJ, Sun Y, Zhong J et al (2007) Continuous culture of two lancelets and production of the second filial generations in the laboratory. J Exp Zool 308B:464-472.
10. Yasui K, Urata M, Yamaguchi M, et al (2007) Laboratory culture of the orental lancelet *Branchiostoma belcheri*. Zool Sci 24:514-520
11. Benito-Gutiérrez È, Weber, H, Bryant, DV et al (2013). Methods for generating year-round access to amphioxus in the laboratory. PLoS ONE 8. http://dx.doi.org/10.1371/journal.pone.0071599
12. Li G, Yang X, Shu ZH et al (2012) Consecutive spawnings of Chinese amphioxus, *Branchiostoma belcheri*, in captivity. PLoS 1 7:e50838.
13. Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32:347-355
14. Shi C, Huang J, Chen S et al (2018) Generation of two transgenic amphioxus lines using the Tol2 transposon system. J Genet Genomics 45:513-516
15. Kozmikova I, Kozmik Z (2015) Gene regulation in amphioxus: An insight from transgenic studies in amphioxus and vertebrates. Marine Genomics 24:159-166
16. Li G, Feng J, Lei Y et al (2014) Mutagenesis at specific genomic loci of amphioxus *Branchiostoma belcheri* using TALEN method. J Genet Genomics 41:215-219
17. Holland ND, Campbell TG, Garey JR et al (2009) The Florida amphioxus (Cephalochordata) hosts larvae of the tapeworm *Acanthobothrium brevissime*: natural history, anatomy and taxonomic identification of the parasite. Acta Zoologica 90:75-86
18. Stokes MD, Holland ND (1996) Reproduction of the Florida lancelet (*Branchiostoma floridae*): spawning patterns and fluctuations in gonad indexes and nutritional reserves. Invert Biol 115:349-359
19. Paris M, Escriva H, Schubert M et al (2008) Amphioxus postembryonic development reveals the homology of chordate metamorphosis. Curr Biol 18:825-830
20. Li G, Liu X, Xing C, et al (2017) Cerberus–Nodal–Lefty–Pitx signaling cascade controls

left–right asymmetry in amphioxus. Proc Natl Acad Sci USA 114:3684-3689

21. Cermak T, Doyle EL, Christian M F (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 39:e82
22. Liu X, Li G, Feng J et al (2013) An efficient microinjection method for unfertilized eggs of Asian amphioxus *Branchiostoma belcheri*. Dev Genes Evol 223:269-278
23. Holland LZ, Onai T (2011) Analyses of genefFunction in amphioxus embryos by microinjection of mRNAs and morpholino oligonucleotides. In: Pelegri FJ (ed) Vertebrate Embryogenesis: Embryological, Cellular, and Genetic Methods. Totowa, NJ: Humana Press 423-438
24. Holland LZ, Yu JK (2004) Cephalochordate (amphioxus) embryos: procurement, culture, basic methods. Meth Cell Biol 74:195-215
25. Yu JKS, Holland LZ (2009) Amphioxus whole-mount In Situ hybridization. Cold Spring Harbor Protocols 2009:pdb.prot5286
26. Koga A, Suzuki M, Inagaki H et al (1996) Transposable element in fish. Nature 383:30.
27. Kawakami K (2007) Tol2: a versatile gene transfer vector in vertebrates. Genome Biol 8 Suppl 1:S7
28. Balciunas D, Wangensteen KJ, Wilber A et al (2006) Harnessing a high cargo-capacity transposon for genetic applications in vertebrates. PLoS Genet 2:e169
29. Deheyn DD, Kubokawa K, McCarthy JK et al (2007) Endogenous green fluorescent protein (GFP) in amphioxus. Biol Bull 213:95-100
30. Feng J, Li G, Liu X et al (2014) Functional analysis of the promoter region of amphioxus beta-actin gene: a useful tool for driving gene expression in vivo. Mol Biol Rep 41:6817-6826
31. Beaster-Jones L (2112) Cis-regulation and conserved non-coding elements in amphioxus. Brief Funct Genomics 11:118-130
32. Yue J-X, Kozmikova I, Ono H (2016) Conserved Noncoding Elements in the Most distant genera of cephalochordates: The Goldilocks principle. Genome Biol Evol 8:2387-2405
33. Xing, C, Li, G, Liu, X et al (2017). Characterization of an amphioxus heat-shock protein gene promoter and its application *in vivo*. Int J Dev Biol 61, 10-12.

34. Kon T, Nohara M, Nishida H et al (2006) Hidden ancient diversification in the circumtropical lancelet *Asymmetron lucayanum* complex. Mar Biol 149:875-883
35. Holland ND (2011) Spawning periodicity of the lancelet, *Asymmetron lucayanum* (Cephalochordata), in Bimini, Bahamas. Ital J Zool 78:478-486
36. Holland ND, Holland LZ (2010) Laboratory spawning and development of the Bahama lancelet, *Asymmetron lucayanum* (Cephalochordata): fertilization through feeding larvae. Biol Bull 219:132-141

4. **Notes**

Note 1: *Asymmetron lucayaum* can be collected in shallow water off the northeastern coast of the Yucatan peninsula; Bimini, Bahamas, the Pacific coast of Costa Rica, Bermuda, Ryukyu islands, Maldives, and Great Barrier Reef. Phylogenetic analysis indicated that *Asymmetron* falls into three clades, suggesting that they may not all be the same species ***(31)***. Like *Branchiostoma, A. lucayanum* burrows in the sand. Because *A. lucayanum* is much smaller (maximum 1.5-2 cm long) than *Branchiostoma*, it is collected in sieves with a smaller mesh size than the window-screening used for sifting *B. floridae* from the sand. *A. lucayanum,* unlike *Branchiostoma*, spawns in synchrony with the cycles of the moon. Specifically, it spawns over 2-3 days just before each new moon ***(35,36)***. This allows spawning to be controlled in the laboratory.

Culture conditions for *A. lucayanum* are similar to those for *B. floridae.* Typically ~30 adults are maintained at 28^{o}C in about 2 l of seawater in a shallow dish without aeration. Because they are in a relatively small volume, it is best to centrifuge algae for feeding twice a day. For breeding, they are maintained on a reversed day/night cycle under artificial moonlight (e.g. from Sera, a German company). Although this moonlight controller comes with a moonlightit is best to substitute a strip or two of blue LEDs. A separate ballast must be purchased with the moonlight controller. Each day, the moonrise and moonset times are manually set according to a moonlight schedule (we use the one for Bimini available at Time and date.com). This moonlight controller has a linear increase and decrease during the moon cycle, but this can be adjusted to be more of a sine wave. More expensive moonlight controllers such as those available from

Neptune systems, Morgan Hill, CA can be programmed to mimic the moon cycle more accurately.

Note 2: restriction enzymes, ligases and polymerases are sold by a variety of companies in Asia, USA and Europe. The names of the buffers that accompany a given enzyme vary from company to company. In general, it is safe to use the buffer supplied with the enzyme, ligase or polymerase even if different one is listed in the methods.

Note 3: Injection needles must be pulled on a horizontal puller with a 2-2.5 mm filament. The taper should exceed 1 cm. After filling and before injection, the tip is broken off with fine tweezers.

Note 4: Only unfertilized eggs can be injected. This limits the volume that can be injected to 1-2 pl as the meiotic divisions and migration of the sperm nucleus in the egg cytoplasm occur after fertilization. Although there is a cortical reaction, complete elevation of the fertilization envelope takes 20-25 min during which the zygote continues to secrete material. The envelope can be removed mechanically, but the surface of the zygotes remains very sticky. Attempts to inject through the elevated fertilization envelope fail because the zygote simply rotates inside the fertilization envelope underneath the needle.

Note 5: The original pFUS_A vector can be used instead of the modified pFUS_A1 and pFUS_A2 vectors, but it generally gives few or no colonies with the right insert. pFUS_A1, pFUS_A2, pFUS_B and RCLscript-GoldyTALEN vectors can also be used without precutting, but most such colonies will have empty vectors.

Note 6: Thirty or more cycles of enzyme digestion and ligation are suggested, but fewer can also be done if adequate for obtaining enough colonies with the right insert.

Figure captions:

Figure 1. Ripe female and ripe male specimens of *Branchiostoma floridae.* Anterior to the right. As the gonads are transparent, their color is that of the gametes. Each animal has 26 gonads per

side. In the wild, most females have yellow oocytes, although a few have rather white ones. In the laboratory, the oocytes are relatively white. The difference in color between gonads from animals in the field versus the laboratory is presumably due to differences in the food. The males all have white to slightly grey gonads. Maximum length of adults is about 5 cm.

Figure 2. Simplified culture system for *Branchiostoma floridae.* A. Tanks of animals (T) without sand are on the table to the right. They are illuminated by overhead fluorescent lights (FL). Timers control the lights for a 14 hr day, 10 hr night cycle. Night falls at noon to allow animals to spawn if needed in the early afternoon. Arrow shows the airline for aeration. Carboys of seawater (S) are equilibrating to the room temperature, which is 28oC. Black curtains (B) can be drawn to maintain the dark cycle when the lights are off but room lights are kept on. R-LED is a strip of red LED lights. Amphioxus photoreceptors have maximal sensitivity in the blue. Therefore, they do not perceive red light. This allows afternoon feedings or spawnings to be carried out without disturbing the animals' photoperiod.

Figure 3. The amount of food is critical for ripening gonads. Ten animals in 2 liters were fed 2x/day either 1.6 ml or 0.8 ml of suspension of Isochrysis and Pavlova at ~$4x10^6$ cells per ml. Dark grey bars = females, light grey bars = males. The animals were maintained at 28^oC. AT constant warm temperature, spawning is not synchronized. The gonad index was measured as the maximum height of the gonads divided by the length of the animal x 100. Asterisks indicate animals that spawn before the next measurement. The question marks indicate an animal that had a small gonads one date and no apparent gonad at the next measurement. Most of the animals fed twice a day on the larger amount of phytoplankton spawned within 6 weeks of initiation of the experiment. Then they refilled their gonads. In contrast, a few of the animals fed half as much spawned near the beginning of the experiment and a few spawned later; however, none of the animals ever made very large gonads.

Figure . 4 A series of late larvae of *Branchiostoma floridae* showing the metamorphosis of the pharynx. Whole mounts. Anterior to the right. A. whole mount of late larva with 7 gill slits. Scale bar = 1 mm. B. Higher magnification of the anterior end of the larva in A. The first row of ill slits is numbered. The mouth (m) on the left is barely visible through the transparent larva. The club-shaped gland (csg) is immediately posterior to the chevron-shaped endostyle (e). C. A slightly later stage than B. The second row of gill slits has begun to develop above the first row,

which is migrating to the left. The clubshaped gland has undergone apoptosis. The endostyle (e) has not yet begun to migrate. The mouth (m) is still on the left. D. At a later stage, the first row of gill slits has largely moved to the left. The tongue bars are growing down from the dorsal border of the second gill slits to divide each into two. The mouth has begun to move anteriorly. The endostyle has moved ventrally and is no longer visible on the right. The atrial cavity (a) has grown over the gill slits. E. A larva that has nearly completed metamorphosis. Scale bar = 1 mm. The atrial cavity (a) has completely formed. Both right and left gill slits are divided by tongue bars and the mouth (m) has almost completed moving anteriorly. G. A still later stage showing the cirri (c) forming around the mouth. Atrium = a. G. a very advanced stage of metamorphosis with the digestive diverticuclum (dd) growing anteriorly, the tongue bars (tb) elongating and the cirri well formed. H. A fully metamorphosed juvenile. The digestive diverticulum (dd) will continue to grow forward. a = atrium, c= oral cirri.

Figure 5. The design of Talen peptides for generating deletions in the amphioxus *Cerberus* gene. Forward and reverse peptides consist of a TALE backbone with an insertion of 16 modules each of 34 amino acids with RVD sequences designed to bind to the relevant bases. At the C-terminal each peptide has the FokI enzyme. The binding sites of the two peptides are separated by 14-17 base pairs on the target DNA.

Figure 6. Assembly of RVD modules and synthesis of mRNA of the forward arm of *B. floridae Cerberus* TALEN. Five RVD modules amplified by PCR are cut with BsaI and ligated in the relevant order into a different pFUS plasmid. The resultant pFUS plasmids ad the pLR vector containing a half NG module are cut with Esp31, and the inserts ligated together into the RCL-Script-Goldy TALEN vector containing sequences coding for the Fok-I enzyme. These plasmids are then replicated in bacteria, linearized and used as templates for mRNA synthesis.

Figure 7. Scheme for injection, breeding, genotyping and crossing amphioxus to obtain homozygous mutants. Unfertilized eggs are injected. Co-injection with a fluorescent dye enables injected eggs to be distinguished from uninjected ones. Restriction enzyme digests determine the percentage of mutations at the target locus (in this instance for hedgehog, Hh). Injected animals (F0) are raised to sexual maturity, spawned, and their gametes are assayed by restriction enzyme digest and DNA sequencing to determine if they carry frameshift mutations. F0 animals carrying frameshift mutations are crossed with wild type. Offspring (F1) are genotyped by restriction

enzyme digests, raised to sexual maturity and crossed with one another to obtain homozygous mutants and with wild type to obtain more heterozygous animals. The phenotype of the homozygous F2 individuals is then analyzed.