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Functional Studies of Transcriptional Cofactors via Microinjection-Mediated Gene Editing in *Xenopus*

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Abstract

The anuran *Xenopus laevis* has been studied for decades as a model for vertebrate cell and developmental biology. More recently, the highly related species *Xenopus tropicalis* has offered the opportunity to carry out genetic studies due to its diploid genome as compared to the pseudo-tetraploid *Xenopus laevis*. Amphibians undergo a biphasic development: embryogenesis to produce a free-living tadpoles and subsequent metamorphosis to transform the tadpole to a frog. This second phase mimics the so-called postembryonic development in mammals when many organs/tissues mature into their adult form in the presence of high levels of plasma thyroid hormone (T3). The total dependence of amphibian metamorphosis on T3 offers a unique opportunity to study postembryonic development in vertebrates, especially with the recent development gene editing technologies that function in amphibians. Here, we first review the basic molecular understanding of the regulation of *Xenopus* metamorphosis by T3 and T3 receptors (TRs), and then describe a detailed method to use CRISPR to knock out the TR-coactivator SRC3 (steroid receptor coactivator 3), a histone acetyltransferase, in order to study its involvement in gene regulation by T3 in vivo and *Xenopus* development.

Keywords

Xenopus laevis; *Xenopus tropicalis*; Thyroid hormone receptor; Metamorphosis; Postembryonic development; Coactivator; Histone acetyltransferase; Histone modification; CRISPR

1 Introduction

The anuran amphibian *Xenopus laevis* has long been used as a model for vertebrate development. For decades, its external development and the ability to manipulate gene function in vivo through microinjection of mRNAs, proteins, and antisense oligonucleotides, etc., has made possible many seminal discoveries on early embryogenesis in vertebrates. In

addition, after a finite period, the free-living tadpole formed at the end of embryogenesis undergoes a second phase of development: metamorphosis, during which essentially every organ/tissue changes drastically into its adult form (Fig. 1) [1]. This process is controlled by thyroid hormone (T3) and resembled postembryonic development in mammals, a period around birth when many organs/tissues mature into the adult form [1, 2]. Amphibian metamorphosis offers a number of advantages for studying postembryonic development in vertebrates. First, it occurs externally, independent of the maternal influence as in mammals. Second, it is totally dependent on T3 and can be easily manipulated by altering T3 levels available to the tadpoles. Blocking the synthesis of endogenous T3 prevents metamorphosis, thus allowing animals remaining as tadpoles for at least a few years (for comparison, normal development leads to the completion of metamorphosis at about 2 months after fertilization in *Xenopus laevis*). On the other hand, tadpoles as young as the onset of feeding (stage 45, about 1 week old for *Xenopus laevis*) can be induced to undergo precocious metamorphosis with physiological concentrations of exogenous T3 in rearing water. Third, most, if not all, tissue transformations are mostly, if not all, organ-autonomous, enabling studies of this process in organ or primary cell cultures. Finally, while embryonic lethality due to gene alternations makes it impossible to study gene function during postembryonic development in mammals, the ability to micro-inject mRNAs/proteins into fertilized eggs coupled with the external development can rescue the embryonic defect due to gene alteration, leading to the formation of free-feeding tadpoles that allows functional studies of the gene alteration during tadpole development and metamorphosis. Taking advantage of these properties, we and others have been studying the molecular mechanisms by which T3 regulates *Xenopus* development. Here, we focus on epigenetic enzymes that are involved in gene regulation by T3.

1.1 Gene Regulation by Thyroid Hormone Receptors (TRs)

T3 can regulate target gene transcription through its nuclear receptors, TRs. T3 can both activate and repress gene expression. While the mechanism for gene repression by T3 is less clear, for genes induced by T3, TRs generally bind to thyroid hormone response elements (TREs) present in the target genes as heterodimers with *9-cis* retinoic acid receptors (RXRs). TR regulates gene regulation by recruiting corepressor or coactivator complexes. In the absence of T3, unliganded TR binds to corepressors. Two best-studied corepressors are the highly related proteins N-CoR (nuclear corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors), both of which can form large complexes containing histone deacetylase 3 (HDAC3) [3–13]. Thus, gene repression by unliganded TR is often accompanied by deacetylation of the histones around the TRE. When T3 is present, TR releases corepressors and recruits coactivators. Many TR-coactivators have been identified. Among them include ATP-dependent chromatin remodeling proteins, histone acetyltransferases such as steroid receptor coactivators (SRC1,2, 3) and p300, and histone methyltransferases PRMT1 (protein arginine methyltransferase 1) and CARM1 (coactivator-associated arginine methyltransferase 1, or PRMT4) [12,14–24]. Consistently, gene activation by T3 is associated with increased local acetylation and methylation.

1.2 TR Function During *Xenopus laevis* Development

Based on the molecular properties of TRs above and expression profiles of TRs during *Xenopus laevis* development, we have previously proposed a dual function model for TRs during *Xenopus* development [25,26]. That is, during embryogenesis, there is little T3 or TRs and T3-inducible genes are expressed at basal level for embryonic development. At the end of embryogenesis, a free-living tadpole is formed and the animal begins to feed (stage 45). By this stage, high levels of expression of TR α and RXR α lead to the binding of TR/RXR heterodimers to the target genes (Fig. 1) [27–29]. As there is little T3 present [30], the unliganded TR/RXR recruits corepressor complexes to repress these genes. The repression of these genes prevents precocious metamorphosis and ensures proper tadpole growth and development. After stage 54, the rising concentration of endogenous T3 enables T3-binding to TR, thus releasing the corepressor complexes and recruiting coactivator complexes. This results in the activation of the target genes and metamorphosis.

Over the years, several laboratories have provided strong molecular and transgenic evidence to support the dual functions of TRs during *Xenopus* development [31–38]. More recently, with the development of TALEN and CRISPR-nuclease-mediated gene editing technologies for both the pseudo-tetraploid *Xenopus laevis* and diploid *Xenopus tropicalis*, one can study endogenous genes during *Xenopus* development [39–45]. Using the TALEN technology, two groups independently knocked down the endogenous TR α during *Xenopus tropicalis* development and observed that the TR α knockdown animals developed faster and initiate metamorphosis at younger age than the wild-type siblings, accompanied by the upregulation (derepression) of T3-target genes [46–49]. Similar findings were made in total knockout animals [50, 51]. These results indicate that TR α is important for metamorphic timing and the rate of metamorphosis progression, further supporting the dual function model for TR in *Xenopus* development.

1.3 Roles of Cofactors in TR Action and *Xenopus* Development

As indicated above, TRs repress or activated T3-inducible gene by recruiting corepressors or coactivators in the absence or presence of T3, respectively. The dependence of amphibian metamorphosis on T3 and the ability to manipulate this process make it possible to study the involvement of such cofactors in gene regulation by T3 during vertebrate development in vivo. Early experiments by using chromatin immunoprecipitation (ChIP) assays on tadpole tissues have shown that TRs indeed recruit corepressors or coactivators to endogenous T3-inducible genes in premetamorphic tadpoles when there is little endogenous T3, or during metamorphosis when T3 is present (Fig. 1) [19, 36, 52–58]. Furthermore, transgenic studies have revealed that interferes with the interaction of endogenous corepressor complexes with unliganded TR leads to premature derepression of T3-inducible genes and precocious metamorphosis [59]. On the other hand, disrupting coactivator function with TR inhibits gene activation by T3 and metamorphosis (Fig. 2) [53, 54] while transgenic overexpression of the wild-type coactivator PRMT1 (protein arginine methyltransferase 1) enhances gene activation induced by T3 and accelerates metamorphic progression [19].

TR-interacting corepressor complexes are known to contain histone deacetylases while coactivator complexes contain histone acetyltransferases and/or methyltransferases and/or

chromatin remodeling activity. Consistently, ChIP assays with antibodies against core histones H2B and H3 have recently shown that T3 induces the removal of core histones, equivalent to about two nucleosomes, from the TRE regions of T3 target genes during metamorphosis in *Xenopus tropicalis* [60], in agreement with what was observed in the reconstituted frog oocyte transcription system [28, 61, 62]. They have further revealed T3-dependent histone modifications at T3 target genes [60,63,64]. While direct evidence for a role of such epigenetic modifications in gene regulation by T3 is still lacking, treating premetamorphic tadpoles with a histone deacetylase inhibitor increases the expression of T3 target genes [65, 66], suggesting that epigenetic modifications may be important for gene regulation by TR.

These studies summarized above indicate that corepressor and coactivator complexes are both involved in coordinating amphibian development by altering chromatin structure and histone modifications to affect TR function in the absence and presence of T3, respectively. However, so far, there have been no reported analyses on the roles of endogenous cofactors in gene regulation by TR and metamorphosis. With the newly developed TALEN and CRISPR gene editing technologies [39–45], we can now begin to investigate the function of endogenous genes such as SRC3 (Fig. 3) during *Xenopus* development, particularly during metamorphosis. Many transcription cofactors, such as the histone methyltransferases Dot1L and PRMT1, are known to be essential for mouse embryogenesis. This makes it impossible to study the roles of such genes during postembryonic development (around birth) in mammals. Anuran metamorphosis offers a unique opportunity to address this gap since metamorphosis can be induced with exogenous T3 as early as the onset of tadpole feeding (around stage 45 or 3 days old in *Xenopus tropicalis*). This is possible because if the knockout of a transcription cofactor leads to embryonic lethality, one can rescue this development by injecting mRNA encoding wild-type cofactor into fertilized egg. Since the mRNA and the resulting translated protein can persist for a few days in the embryos, this should rescue the knockout effect to allow animal development into feeding stage tadpoles (3 days or older) for studying the role of the cofactor during T3-induced metamorphosis. Thus, by combining the advantage of the anuran development and the gene-editing technologies, it will be possible to investigate cofactor function during postembryonic development regardless of its role during embryogenesis. Furthermore, if a CRISPR is highly efficient (e.g., >80% mutation rate) in causing mutations after injecting the corresponding RNAs into fertilized eggs, it is even possible to study the function of the cofactor in the resulting F0 generation animals, allowing one to know the outcome within days after micro-injecting of the RNAs.

2 Materials

2.1 Design and Synthesize a CRISPR Guide RNA for the Target Site

Gene knockdown/knockout methods by using CRISPR/Cas9 system in frog were as described by [67].

1. RNA transcription kit, Ambion mMessage® SP6 mMachine.
2. MEGAscript® T7 Transcription Kit.
3. pCS2 + hSpCas9 (Cat: Plasmid #51815, Addgene, MA).

4. Zero Blunt TOPO + mCherry (a gift from Dr. Rudolf Jaenisch (Addgene plasmid # 59995).
5. QIAGEN QIAquick® PCR Purification Kit (250).
6. Takara PrimeSTAR Max.
7. Primers: The primers used for the target gene [67]:
Forward;
5'-TAATACGACTCACTATAG(N)16-19GTTTTAGAGCTA-GAAATAGCAAG-3',
Reverse;
5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGAT AACGGACTAGCCT TATTTAACTTGCTATTTCTAG CTCTAAAAC-3'.
8. Online tools for CRISPR guide RNA selection
CHOPCHOP (<https://chopchop.cbu.uio.no>) [68].
CRISPR/Cas9 target predictor (<http://crispr.cos.uni-heidelberg.de>) [69].
CRISPR direct (<http://crispr.dbcls.jp/>) [70].

2.2 Generate Knockdown/Knockout Animals Through mRNA Injection into Fertilized Eggs

Adults of the West African clawed frog *Xenopus tropicalis* were obtained from Nasco (Wisconsin). Embryos were prepared by in vitro fertilization as described [31].

Developmental stages were determined according to [71].

1. Mark's Modified Ringer's (MMR): 0.1 M NaCl, 2.0 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM HEPES (pH 7.8), 0.1 mM EDTA.
2. Merck Human Chorionic Gonadotropin (HCG).
3. Adult *Xenopus tropicalis*.
4. Sutter Instruments P-97 Needle puller (*see Note 1*).
5. Drummond Microcaps Needles 1-000-0300 (*see Note 1*).
6. World Precision Instruments PV820 Injector (*see Note 1*).

2.3 Identify Out-Frame Mutants via a Color Assay

1. QIAGEN DNeasy Blood and Tissue kit.
2. QIAGEN PCR Purification Kit.
3. QIAGEN Gel Extraction Kit.
4. NEB Quick Ligation kit.
5. Invitrogen *E. coli* TOP10 competent cells.

6. SalI-HF.
7. EcoRI-HF.
8. NEB CutSmart Buffer.
9. IPTG solution: 10 mM IPTG.
10. Xgal solution: 20 mg/mL Xgal in DMSO.
11. Takara PrimeSTAR GXL DNA Polymerase.
12. Primers for PCR-amplification of the SRC3 gRNA target region:
SRC3 Forward: 5'-GGCTGCATCTTTGTCGCTAA-3'.
SRC3 Reverse: 5'-GAGAGCCAATCACAGCCTTG-3'.
SRC3 NestedF: 5'-CCGCATCGTCGACAATGCGCCTT
CAGCTGCCTTTTTG-3' (SalI site in underline).
SRC3 NestedR: 5'-GCCAGTTGAATTCCTACTGTTTGC CATGCCTC-3'
(EcoRI site in underline).
SRC3 gRNA forward: 5'-TAATACGACTCACTATAGGA
AAACTCCTTGGATCCGCGTTT-3'.
5'-TAGAGCTAGAAATAGCAAG-3' [67].
SRC3 gRNA reverse: 5'-AAAAGCACCGACTCGGTGCCAC
TTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA
CTTGCTATTTCTAGCTCTAAAAC-3' [67].
13. pLacZα-GFP [72] (Note that it is available through Addgene distribution at https://www.addgene.org/Yun-Bo_Shi/).
14. Nano Drop 2000.
15. Fluorescent dissection microscope.
16. MicroPublisher 5.0 digital camera attached to the microscope.

3 Methods

3.1 Design and Synthesize a CRISPR Guide RNA for the Target Site

3.1.1 Design CRISPR Guide RNA—The first step to generate gene knockout animals is to design a CRISPR guide RNA that contains a sequence 16–20 bp of the target site. The target site should have a G residue at the +1 position and should be followed by a PAM sequence in the target gene [*S. pyogenes* Cas9 (SpCas9): 3 bp NGG], which is recognized by Cas9 to make a double strand break (Fig. 3) (*see* Note 2). One can use online tools to search the optimal target site and putative off-target site based on the *Xenopus tropicalis* genome for designing CRISPR guide RNA [68–70]. We designed some CRISPR guide RNAs for SRC3 (Fig. 3) as described [67] (*see* Note 3).

3.1.2 In Vitro Transcription for the CRISPR Guide RNA

1. The 5' Primer, 5'-TAATACGACTCACTATAG(N)₁₆₋₁₉ GTTTTAGAGCTAGAAATAGCAAG-3', consists of T7 promoter sequence, under lined and the target specific sequence, G(N)₁₆₋₁₉ which is designed as describe above. On the other hands, the 3' primer is used for all CRISPR guide RNA and as shown below 5'-AAAAGCACCGACTCGGT GCCACTTTTT-CAAGTTGATAACGGACTAGCCTTATTTTAACTTGC-TATTCTAGCTCTAAAAC-3' (see Note 4).
2. The primeSTAR Max DNA polymerase was used for template assembly by PCR. Assembly reactions were performed in a final volume of 100µl and carried out with the PCR program: 98 °C for 10 s, 60 °C for 5 s, 72 °C for 15 s repeat 15 cycles.
3. After electrophoresis to identify the specific band, the PCR product was purified by QIAGEN PCR purification kit.
4. One µg of the DNA was transcribed in vitro with T7 RNA polymerase (see Note 5).
5. Remove template DNA, purify RNA and quantitate it (see Note 6).
RNA can be aliquoted and stored at -80°C for months.

3.1.3 Prepare Cas9 and mCherry mRNAs by In Vitro Transcription

1. Linearize the plasmid DNA with an appropriate restriction enzyme such that it can be used for in vitro transcription to produce sense mRNA. Prepare enough linearized DNA (e.g., digest about 20 µg) for at least several transcription reactions at 1–1.5 µg per reaction.
2. Purify and quantitate the DNA, and confirm its linearization, purity, and quantification by running a small aliquot on an agarose gel (see Note 7).
3. Transcribe RNA by following the directions of Ambion's mMessage mMachine kit (see Note 5). In our studies, pCS2+hSpCas9 plasmid and Zero Blunt TOPO + mCherry were linearized with Not I, twice phenol/chloroform-extracted, and the ethanol-precipitated. One µg of the DNA was transcribed in vitro with SP6 RNA polymerase.
4. Remove template DNA, purify RNA, and quantitate it (see Note 6).
RNA can be aliquoted and stored at -80 °C for months.

3.2 Generate Knockdown/Knockout Animals Through mRNA Injection into Fertilized Eggs

3.2.1 Fertilize Eggs In Vitro

1. *X. tropicalis* females are primed with 20 U of human chorionic gonadotropin (HCG) 1 day before ovulation.
2. The primed frogs are injected with 200 U of HCG next morning.

3. A *X. tropicalis* male frog is sacrificed. The testes are removed for in vitro fertilization (see Note 8) and if needed, the remainder can be stored in $1 \times \text{MMR}$ at 4°C for up to several days.
4. With gentle squeezing, eggs are expelled from ovulating females onto petri dishes containing 1 mL $1 \times \text{MMR}$ into which a small fragment of testes has recently been macerated.
5. After gently splaying out of eggs into the sperm solution and 2 min incubation, the dishes are filled with $0.1 \times \text{MMR}$ and incubated for an additional 15 min.
6. To remove the jelly coat, the fertilized eggs are washed with $0.1 \times \text{MMR}$ -3.5% cysteine, pH 8.0, until they started to touch each other (about 3–5 min).
7. Then wash the fertilized eggs/embryos 4–6 times with $0.1 \times \text{MMR}$ before being transferred into fresh $0.1 \times \text{MMR}$ (see Note 8).
8. Embryos are immediately transferred to $0.1 \times \text{MMR}$ -6% Ficoll.

3.2.2 Inject and Culture the Embryos

1. The embryos above are injected with RNAs (guide RNA for SRC3, mRNAs for Cas9 and mCherry at 400 pg, 400 pg, 200 pg per embryo, respectively) in a 2 nL total volume per embryo (see Note 9).
2. Control and injected embryos are kept in $0.1 \times \text{MMR}$ -6% Ficoll for 4–6 h after injection and then transferred to $0.1 \times \text{MMR}$ for rearing.
3. Embryos are incubated for 3 days at 25°C in incubator. The culture medium is changed daily.
4. At 48, 72 h after fertilization, the embryos are phenotypically examined and sorted based on mCherry signal under the microscope.
5. Five embryos are randomly selected for mutation analysis, and the rest of the F0 generation animals are kept in a 3-L plastic container for further rearing.

3.3 Identify Out-of-Frame Mutants via a Color Assay

3.3.1 Extract and Purify Genomic DNA

1. Collect five embryos 3–5 days after fertilization and isolate genomic DNA by using DNeasy Blood and Tissue kit.
2. Design two pairs of primers for detection and screening of CRISPR/Cas9-induced out-of-frame mutations in SRC3 gene: F (GGCTGCATCTTTGTCGCTAA) and R (GAGAGC-CAATCACAGCCTTG), and NestedF (CCGCATCGTCGACAATGCGCCTTCAGCTGCCTTTTTG) and NestedR (GCCAGTT GAATTCCTACTGTTTGCCATGCCTC), respectively (see Note 10).
3. Perform the first round PCR amplification by using primers F and R for 15–20 cycles on the genomic DNA (see Note 11).

4. Dilute the PCR products at 1:100 to 1:1000, respectively, to serve as templates in the second round PCR amplification with the nested primers (Nested F and Nested R) for 25–30 cycles (*see* Note 11).
5. Check the PCR specificity by analyzing the PCR products on an agarose gel.
6. Proceed to purify the nested PCR products by using PCR Purification Kit.
7. Double digest the purified PCR products with restriction enzymes Sall-HF and EcoRI-HF, in CutSmart Buffer, at 37 °C for at least 2 h (*see* Note 12).
8. Add calf intestinal alkaline phosphatase (CIP) to the reactions (1 µL CIP per 100 µL reactions) and incubate for 10 min to dephosphorylate the DNA ends (*see* Note 13).
9. Add 10 µL 5 M NaCl and 80 µL isopropanol to 100 µL reaction, centrifuge at $15,000 \times g$ at 4 °C for 10 min.
10. Carefully remove the supernatant by using a pipette. Air dry the DNA and add 10–20 µL TE buffer to dissolve the DNA.
11. Use agarose gel electrophoresis to resolve the dephosphorylated PCR product bearing an EcoRI and a Sall end and excise out the agarose gel slice containing the DNA band.
12. Proceed to purify the DNA fragment by using Gel Extraction Kit.
13. Measure the concentration of the DNA fragments by using Nano Drop 2000 and store it at –20 °C.

3.3.2 Prepare the Color Assay Vector

1. Double-digest the assay vector pLacZα-GFP with the same restriction enzymes used for digesting the PCR product above (*see* Subheading 3.3.1, **step 7**), and perform single digestions with each enzyme separately as controls to make sure that both enzymes digest the vector completely (*see* Note 14).
2. Check the efficiency of the enzyme digestions by using agarose-gel electrophoresis. Note that the digested vector in all three reactions would be seen as a single band in the agarose gel because the small fragment released by double digestion from the multiple cloning sites within the vector is too small to be visible in the gel.
3. Add 10 µL 5 M NaCl and 70 µL isopropanol per 100 µL reaction, centrifuge at $15,000 \times g$ for 10 min at 4 °C.
4. Carefully remove the supernatant by using a pipette. Air dry and add 10–20 µL TE buffer to dissolve the linear pLacZα-GFP.
5. Purify the linear plasmid by using agarose gel electrophoresis and Gel Extraction Kit.
6. Measure the concentration of the linear pLacZα-GFP, and store it at –20 °C.

3.3.3 Clone the Genomic DNA and Screen for Out-of-Frame Mutations

1. Ligate the double-digested PCRproducts (get from Subheading 3.3.1) into pLacZ α -GFP predigested with EcoRI and SalI (get from Subheading 3.3.2) by using Quick Ligation kit for 5–10 min at room temperature (*see* Note 15).
2. Prepare IPTG/Xgal/Amp plates by spreading evenly 50 μ L Xgal and 100 μ L IPTG each on the surface of ampicillin plates.
3. Transform the ligated DNA above into *E. coli* TOP10 competent cells and use 300 μ L SOC medium to activate the transformed bacteria.
4. Add 30–300 μ L transformed *E. coli* TOP10 competent cells per IPTG/Xgal/Amp plates, and then spread the solution evenly (*see* Note 16).
5. Invert the plates and incubate them at 37 °C overnight.
6. Observe the plates under a fluorescent dissection microscope (Model MZ10F) and photograph with a MicroPublisher 5.0 digital camera attached to the microscope under bright or fluorescent field (*see* Note 17).
7. Process the images with Adobe PhotoShop.
8. Count GFP-negative colonies under florescent conditions and the total number of blue or greenish blue colonies on the same plate. GFP-negative blue colonies contain out-of-frame mutations in the SRC3 fragment.
9. Determine the out-of-frame mutation rate by dividing the numbers of GFP-negative colonies by the total number of blue/ greenish blue colonies on the same plate.

4 Notes

1. A variety of instruments and approaches can be used to pull needles and inject RNA or DNA samples. They include simple hand pulled needles using a Bunsen Burner, and forced air injectors using “house air” found in most laboratories. The crucial criteria are choosing a needle and a method that allow for a small needle diameter (about 10 μ m) to be pulled and a way to calibrate or control injection volumes (about 2–5 nL per injection).
2. When designing the CRISPR guide RNA, it is preferable to choose sequences near the 5'-end encoding a region important for the function of the target protein. If there are multiple in frame initiation codons, the guide RNA should target the last one. If there are multiple spliced forms, the guide RNA should target a common region.
3. The online tools often provide a list of potential CRISPR guide RNA sequences and putative off targets. We choose the highly ranked guide RNA with fewer off targets. To control for specificity, we would recommend analyzing possible mutations at 3–5 off target sites in the list, especially if one uses F0 or F1 generation animals for phenotypic studies.

4. According to [67], two types of templates can be used for in vitro transcription to prepare CRISPR guide RNA. One is plasmid-based and the other PCR-based. We used the PCR-based strategy for our CRISPR guide RNA because it allows prompt synthesis of CRISPR guide RNA without cloning. Alternative strategy may also be used to produce gRNA through CSY4 nuclease processing which generates gRNA starts from any nucleotides at its 5'-end and expands the choices of CRISPR targets on the gene of interest [73].
5. Though we used a commercial RNA transcription kit, in vitro transcription can be performed with transcription components being purchased individually at cheaper costs.
6. Once the transcription reaction is complete (following the removal of the DNA template with DNase), the RNA must be purified prior to injection. LiCl precipitation is a good and quick method of removing unincorporated nucleotides and proteins. Phenol/chloroform extraction followed by ethanol precipitation is a more stringent method of purification, though care must be taken to remove all traces of phenol and chloroform prior to injection.
7. Use gel electrophoresis to confirm that the DNA is pure and completely linearized. Circular plasmid templates will generate extremely long, heterogeneous RNA transcripts because RNA polymerases are very processive.
8. In vitro fertilization for *Xenopus tropicalis* is essentially the same as that for *Xenopus laevis*. For details, see *Xenopus* fertilization protocols [74].
9. It would be better to complete the injection before the first cell division, i.e., within about 90 min after fertilization.
10. pLacZ α -GFP vector [72] is used for the color assay here. Primers F and R are designed for amplifying an extended region encompassing the CRISPR/Cas target (the PCR product should be around 500–1000 bp). The nested PCR primers (NestedF and NestedR) should each contain a unique restriction enzyme site present in the multi-cloning sites (MCS) of the pLacZ α -GFP vector but absent in the rest of the resulting PCR product. The 6–8 extra bp at the 5'-end beyond the restrict site is necessary to ensure that the nested PCR product can be digested efficiently by the restriction enzymes. It is preferable that the two chosen enzymes can digest their substrate DNA efficiently in a single reaction buffer at the same incubation temperature. Restriction enzyme star activity may lead to false outcomes from the assay, thus high fidelity enzymes are preferred.
11. To ensure accuracy during PCR amplification, high fidelity DNA polymerases such as Takara PrimeSTAR GXL DNA Polymerase are preferred.
12. Set up a 100 μ L double enzyme digestion reaction as below: DNA 1 μ g; 10 $\times$ CutSmart Buffer 10 μ L; SalI-HF 1 μ L; EcoRI-HF 1 μ L; plus water to 100 μ L.
13. Dephosphorylation of the double restriction enzyme-digested PCR products is necessary to ensure that only a single insert is ligated into the color assay vector. Multiple inserts would produce false positive results, thus should be avoided.

14. Another color assay vector, pmCherry-GFP, can also be used for determining the out-of-frame mutations by using two fluorescent proteins, i.e., mCherry and GFP [72]. Complete digestion of the vector DNA by the chosen two restriction enzymes is critical for accurate evaluation of the out-of-frame mutation rate from CRISPR/Cas-mediated mutagenesis (linear DNA from single-enzyme digestion would be self-ligated very efficiently, leading to false negative colonies).
15. For ligation, the insert to vector molar ratio should be between 2 and 6 to obtain high ligation efficiency.
16. When plating transformed *E. coli* TOP10 competent cells, it is better to plate two different volumes of *E. coli* per plate for each transformation sample in order to obtain optimal numbers of colonies per plate for quantification purpose, such as 50 and 150 μ L.
17. The double positive pLacZ α -GFP colonies look greenish in the bright field and bright green under fluorescent condition for GFP. This may be due to relatively weaker blue color (in frame GFP expression may reduce the color from LacZ α expression) of the X-gal staining plus weak green color due to the GFP under visible light [72]. Restriction enzyme star activity may lead to false results from the assay, and low quality of the nested PCR primers, i.e., having wrong nucleotides inserted during DNA synthesis, can also contribute to false positive results. If a high background mutation rate is observed, please check if the primer sequences and the sequences at the cloning sites are correct.

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Fig. 1.

A dual function model for TR during frog development. Top: The embryos hatch around stage 35 and tadpole feeding begins around stage 45 [71]. Subsequently, the tadpole enters the premetamorphic phase (till stage 54) when it undergoes mostly growth with little morphological changes as the plasma concentration of T3 remains very low [30]. Metamorphosis begins after stage 54 (prometamorphosis: stage 54–58) and most dramatic changes occur at climax stages 58–66 and end at stage 66 when the tail is completely resorbed. Bottom: A molecular mechanism for gene regulation by TR during development. T3-inducible genes are expressed at the basal levels during embryogenesis when there are little T3 and TRs. After the onset of tadpole feeding (stage 45), the expression of TR and RXR leads to the formation of TR/RXR heterodimers that bind to the T3 response elements (TREs) in the target genes and recruit corepressor complexes to the promoter region. This represses the expression of the genes prior to the synthesis of endogenous T3 around stage 54. Subsequently, T3 binds to TR and causes the release of the corepressor complexes and recruitment of coactivator complexes to the genes, leading to the activation of T3-inducible genes and metamorphosis. Note that exemplary cofactor complexes are shown here

Fig. 2.

Transgenic overexpressing a Flag-tagged dominant negative (F-dn) p300 inhibits T3-induced and natural metamorphosis. (a) Schematic representation of the double promoter construct used for generation of transgenic animals. The constitutively active cytomegalovirus (CMV) promoter drives the expression of the transgene, F-dnp300, and is followed by the SV40 polyadenylation signal. The construct also harbors GFP under the control of the eye-specific γ -crystallin promoter as a marker to identify transgenic animals. (b) F-dnp300 inhibits T3-induced metamorphic changes. Wild-type (WT) and transgenic (Tg) tadpoles at premetamorphic stage 54 were treated with 5 nM T3 for 3 days in the same container. At the end of the treatment, transgenic tadpoles were identified by the green fluorescence in the eye lens. The wild-type tadpoles which were treated with T3 underwent the typical T3-induced changes such as resorption of gills (bracketed) and development of the Meckel's cartilage leading to protrusion of the jaw (marked as J). Furthermore, the anterior end of the olfactory lobe (marked as O in the wild-type animal without T3 treatment) is closer to the nostrils (marked as N) in wild-type animals treated with T3 (WT + T3) as compared to untreated wild-type siblings (WT). These changes were not observed or greatly reduced in F-dnp300 transgenic animals treated with T3 (Tg + T3). (c) The overexpression of F-dnp300 arrests or delays natural metamorphosis. The progression of natural metamorphosis of F-dnp300

transgenic animals (Tg) and their wild-type siblings (WT) was monitored. Note that a tadpole which displayed arrested metamorphosis, remained at stage 61 for more than a month and then died, possibly due to partial metamorphosis, compared to a wild-type sibling which completed metamorphosis at about 2 months of age. For more details, *see ref. 54*

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Fig. 3. Schematic diagram for targeting SRC3 with CRISPR/Cas9 in *Xenopus tropicalis*. The protein coding exons are shown as numbered, colored boxes. The CRISPR/Cas9-recognized sequence is shown as red boxes (top) or red letters and blue line (below). The PAM sequences that are required for gRNA guided target-recognition and cleavage by Cas9 protein (light blue shape) are shown in green letters