

Video Article

Primordial Germ Cell Transplantation for CRISPR/Cas9-based Leapfrogging in *Xenopus*

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Abstract

The creation of mutant lines by genome editing is accelerating genetic analysis in many organisms. CRISPR/Cas9 methods have been adapted for use in the African clawed frog, *Xenopus*, a longstanding model organism for biomedical research. Traditional breeding schemes for creating homozygous mutant lines with CRISPR/Cas9-targeted mutagenesis have several time-consuming and laborious steps. To facilitate the creation of mutant embryos, particularly to overcome the obstacles associated with knocking out genes that are essential for embryogenesis, a new method called leapfrogging was developed. This technique leverages the robustness of *Xenopus* embryos to "cut and paste" embryological methods. Leapfrogging utilizes the transfer of primordial germ cells (PGCs) from efficiently-mutagenized donor embryos into PGC-ablated wildtype siblings. This method allows for the efficient mutation of essential genes by creating chimeric animals with wildtype somatic cells that carry a mutant germline. When two F₀ animals carrying "leapfrog transplants" (*i.e.*, mutant germ cells) are intercrossed, they produce homozygous, or compound heterozygous, null F₁ embryos, thus saving a full generation time to obtain phenotypic data. Leapfrogging also provides a new approach for analyzing maternal effect genes, which are refractory to F₀ phenotypic analysis following CRISPR/Cas9 mutagenesis. This manuscript details the method of leapfrogging, with special emphasis on how to successfully perform PGC transplantation.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56035/>

Introduction

How genotype encodes phenotype has been a major question in biology since the rediscovery of Mendel's laws. An understanding of the roles of genes, their regulation and interactions within gene networks, and the functions of encoded products promises to provide tools for uncovering new biology and ameliorating disease states. For over half a century¹, the African clawed frog, *Xenopus*, has been a leading model for studies on a wide variety of topics in basic biology and biomedicine, including the genetic control of development. Historically, most research on *Xenopus* has used the allotetraploid frog, *X. laevis*, but more recently, due to its diploidy, *X. tropicalis* has been developed as an amphibian genetic model. Complete genome sequences have been assembled from both *Xenopus* species^{2,3}. The "frog community" is now at a turning point where basic genome modification technology permits the study of gene function, virtually at will. Programmable CRISPR/Cas9 endonucleases have made the mutagenesis of genes highly efficient, with biallelic mutation possible in most cells of the animal^{4,5,6,7,8,9,10,11}. These studies, underscored by Bhattacharya *et al.*¹² and Shigeta *et al.*¹³, have shown that the function of many genes can be studied by mutagenesis in F₀ mosaic animals. This approach has many advantages; however, Cas9-sgRNA-microinjected embryos often display variable phenotypes due to incomplete loss of function (LOF). More significantly, the generation of mutant lines is highly advantageous for some applications—in particular when studying genes that have a maternal mRNA contribution. Maternal mRNAs and proteins, and their influences on epigenetics, persist for an extended period into embryogenesis^{14,15,16}, rendering the early developmental contributions of many genes refractory to F₀ analyses. Therefore, other LOF approaches are required.

When seeking to create mutant lines, the path to obtaining homozygous LOF embryos has several obstacles. First, efficient mutagenesis to produce biallelic mutations can be disadvantageous because loss of essential gene functions results in failure to survive to sexual maturity, interfering with the production of a viable line. A common solution is the careful titration of the amount of Cas9-sgRNA delivered. Here, the goal is to achieve a balance between reducing lethality while also maximizing germline mutagenesis efficiency. A second problem arises from the standard breeding scheme, where phenotypic analyses are deferred until the F₂ generation. Using the standard approach, sexually mature F₀ animals that transmit mutant alleles through the germline are outcrossed to produce F₁ heterozygous "carriers", which are then grown to sexual maturity. Two F₁ heterozygotes are then intercrossed to produce F₂ mutant embryos at an expected Mendelian frequency of 25%. Thus, two generations of breeding are necessary for analysis of mutant phenotypes. Mutant animals could be either homozygotes or compound heterozygotes (*i.e.*, progeny containing two different mutant alleles, which depends on the genotypes of the parental animals used in the F₁ intercross).

These obstacles can be overcome by confining programmable nuclease-mediated mutagenesis to germ cells, which underlies a new method called leapfrogging¹⁷. Leapfrogging has two main components: (1) the microinjection of Cas mRNA together with sgRNA, or nuclease-sgRNA

complexes (or, in principle, TALENs or zinc finger nucleases) at the single-cell stage to efficiently mutagenize embryonic genomes, followed by (2) the transplantation of PGCs into wildtype sibling embryos, where the endogenous PGCs were removed. When both steps are efficient, complete germline replacement with mutant germ cells can be obtained. Blackler demonstrated in the early 1960s that *Xenopus* PGCs could be transplanted between embryos at the late neurula and early tailbud stages^{18,19}. For leapfrogging, Blackler's approach was modified by performing the transplantations at the blastula stage¹⁷, when PGCs are localized in the vegetal pole of the embryo^{20,21}. Transplantation before gastrulation has two main advantages. First, the engraftment of transplants and the subsequent normal development was found to be more efficient when the transplantation is performed at the blastula stage (unpublished observations). Second, by performing blastula-stage transplantations shortly after zygotic transcription has begun, one can avoid the lethality arising from developmental gene mutations that disrupt gastrulation or that otherwise lead to malformed late neurulae. PGC transplant-bearing ("leapfrogged") embryos are grown to sexual maturity, and intercrosses between these F₀ animals have demonstrated that, in many cases, 100% of the F₁ progeny display the LOF phenotype (most being compound heterozygotes), indicating complete germline replacement with the targeted mutations.

It is expected that leapfrogging will accelerate genetic approaches in *Xenopus*. Leapfrogging also provides an alternative to the "host transfer" method²² for the LOF analysis of maternal-effect genes (unpublished observations). In the current publication, a detailed description of the method, especially focusing on PGC transplantation, is presented in *X. tropicalis* (with minor modifications for *X. laevis*). The transplantation of PGCs is demonstrated here to facilitate a more rapid transfer of this technology to other laboratories working with *Xenopus*. The principles of this method should be successful in other amphibians (e.g., urodeles), and organism-specific modifications in the methodology should allow for application to many other animals in which efficient mutagenesis can be accomplished and PGCs are readily transplantable.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

1. Preparations for PGC Transplantation

1. Prepare dissection tools in advance, as previously described²³.
NOTE: Eyebrow hair knives are used to make incisions with the assistance of a hair loop to stabilize the embryo while performing the surgeries. Eyebrow hairs and hair loops are glued into borosilicate glass Pasteur pipettes that have first been drawn in a flame and broken on the thinned portion (see **Figure 1A**, arrowhead) to create a shorter, narrower tip (**Figure 1B**) for greater dexterity when performing surgeries.
2. Generate sgRNAs using procedures previously described²⁴.
NOTE: Briefly, short DNA templates coding for sgRNAs are created using overlapping deoxyoligonucleotides containing 5' bacteriophage T7 promoters, which are annealed and "filled in" by an error-free, thermostable DNA polymerase. *In vitro* sgRNA synthesis reactions are incubated for several hours to overnight (depending on the kit used). Reactions are DNase-treated to remove the template. The sgRNAs are purified by standard methods of phenol/chloroform extraction and ammonium acetate/isopropanol precipitation, according to the kit manufacturer's instructions²⁴.
3. Shortly before performing microinjections, prepare Cas9-sgRNA complexes by first denaturing ~250 ng of sgRNA in a 3 μ L total volume of RNase-free (diethylpyrocarbonate-treated) H₂O at 60–65 °C for 5 min, followed by quick-cooling on ice for 5 min. Centrifuge the sgRNA for a few seconds, add 1 μ L of 1 μ g/ μ L Cas9 protein, and incubate for 10 min at 37 °C to promote complex formation.
NOTE: Following this incubation, the tube can be kept on ice while preparing the embryos for microinjection.
4. Obtain *X. tropicalis* embryos by *in vitro* fertilization using standard methods, as previously described²⁵. De-jelly^{25,26} the embryos at 10 min post-fertilization.
NOTE: Fertilization time is considered the time after the sperm suspension is added to the eggs and the dish is flooded with 1/9thX Marc's Modified Ringers (MMR)²⁶. For *X. tropicalis*²⁵, unlike *X. laevis*, perform the de-jellying in 1/9x MMR containing 3% cysteine free base (not the HCl salt), pH adjusted to 7.8–8.0, followed by multiple washes in 1/9x MMR. Transfer the embryos to an agarose (1%)–coated dish containing 1/9x MMR at room temperature.
5. Immediately transfer the embryos to be injected to a 1% agarose-coated dish containing 1x MMR using a Pasteur pipette.
6. **Inject at the 1-cell stage. See references^{26,27} for detailed descriptions of *Xenopus* microinjection methods.**
 1. Inject each embryo at a single site in the animal pole, with 4 nL of Cas9-sgRNA complex (final amount = 1 ng of Cas9 and ~250 pg of sgRNA; see the **Discussion**)²⁴. After 10–20 min of injection site healing, transfer the embryos to an agarose-coated dish containing 1/9x MMR and incubate at 25 °C. Also create a dish of uninjected sibling embryos that will serve as graft recipients.
7. **Prepare Petri plates (60 mm) that contain a ~5 mm-thick layer of 1% agarose made in 0.3x MMR, if doing transplantations in *X. tropicalis*, or 1x MMR for *X. laevis*.**
 1. Create depressions ~3–4 mm deep by inserting a 3- x 4-well mold (created by cutting a 96-well PCR plate) into the molten agarose when pouring the plates (see **Figure 1C** and **D**). Hold the mold several millimeters above the bottom of the Petri dish using a clamp attached to a ring stand until the agarose has hardened.
 2. Additionally, make a 24-well plate to house individual embryos by coating the wells with a thin layer of 1% agarose made in 1/9x MMR.
NOTE: The culture medium added to these wells is 1/9x MMR supplemented with 50 μ g of gentamycin sulfate/mL.

2. Transplantation of PGCs

1. When the embryos just reach Nieuwkoop and Faber²⁸ blastula stage 9 (**Figure 2A**), ~4.5 h post-fertilization (hpf) for *X. tropicalis*, remove them from the 25 °C incubator and allow them to equilibrate to room temperature for an additional 0.5 h.
2. To begin the transplantation at 5 hpf (**Figure 2B**), use a Pasteur pipette to transfer one PGC donor embryo (injected with Cas9-sgRNA) to the 60 mm agarose dish containing depressions (created in step 1.7) in 0.3x MMR (or 1x MMR if using *X. laevis*). Also transfer one uninjected sibling embryo, the graft recipient, to this dish.
NOTE: It is critical that the identities of each of these embryos are not confused.

3. Manually remove the vitelline envelopes from each embryo using forceps and rotate both embryos so that their vegetal poles are in view and accessible for surgery.
NOTE: A description of manual de-vitellination of embryos has previously been described²⁶.
4. Using the sharp tip of an eyebrow hair knife, make four shallow incisions in the shape of a square on the vegetal pole of the recipient embryo, inside the zone where future bottle cells that mark the blastopore will form. Make the incisions by inserting the tip of the eyebrow hair knife into the embryo, just below the surface, and make upward slicing movements while stabilizing the embryo with the hair loop.
NOTE: **Figure 2** shows vegetal views of an embryo at half-hourly intervals from 4.5-7.0 hpf to show the cell size and to provide a guide for estimating where the bottle cells will form (dashed white circle). The aim is to make incisions where the dashed black box is indicated.
5. Once the four sides of the square are delineated by incisions, deepen each incision with the eyebrow hair knife using similar cutting motions to reach a depth of approximately 1/3 to 1/2 of the distance to the blastocoel floor.
6. Free the vegetal tissue explant from the recipient embryo (**Figure 3A and B**) by making a horizontal (parallel to the vegetal surface) cut(s) in the deep vegetal region.
NOTE: The size of the PGC-containing vegetal explant is approximately 0.4-0.45 mm per side and 0.25-0.3 mm in depth. This vegetal explant from the recipient embryo is no longer needed and should therefore be set aside to be discarded.
7. Working quickly, repeat this procedure (steps 2.4-2.6) on the PGC donor embryo to create a similarly sized vegetal tissue fragment for transplantation.
NOTE: It is important to carry out this second dissection with little delay to minimize the time for the recipient embryo to heal its open wound.
8. Once the tissue containing PGCs is removed from the donor embryo, use the eyebrow hair knife and hair loop to move this explant into position in the opening created in the recipient embryo.
NOTE: The interior surface of the donor graft must be facing the interior of the recipient embryo. It is not necessary to match the orientations of the dorsal-ventral and left-right axes of the graft with the recipient embryo.
9. Use the long edge of the shaft of the eyebrow hair knife, held parallel to the surface of the graft, to gently press the graft into the opening in the vegetal surface of the recipient embryo.
10. Once the graft has been placed, use a hairloop or the shaft of an eyebrow hair knife to gently slide the "carcass" of the donor embryo across the agarose surface and into an agarose depression. Make sure that the open wound of the carcass is facing the bulk liquid. If not, use the eyebrow hair knife to rotate the embryo to achieve this orientation.
11. Slide the recipient embryo, with the graft healing in place, into an adjacent depression and likewise make sure that the grafted vegetal pole tissue is facing the bulk liquid.
12. Repeat this procedure (steps 2.1-2.11) using another pair of donor and recipient embryos to create another transplant/carcass pair; transfer these to empty depressions.
NOTE: It is critical that one makes notations to keep track of matched pairs of carcasses and transplant-bearing embryos. The donor carcasses will be used as a proxy for the efficiency of Cas9-sgRNA-induced mutagenesis in the transplanted PGCs, which cannot be easily assayed until the transplant-bearing animals reach sexual maturity (see step 3.3, below, and the **Discussion**).
13. Continue making transplants until the embryos reach approximately early gastrula stage 10, which is approximately 6.5 hpf in *X. tropicalis* (see **Figure 2E**).

3. Post-healing Care of Embryos

NOTE: Grafts heal into place within ~30 min, and it is normal to observe some yolk debris from cell lysis exuding from the embryo (**Figure 3C**).

1. **Once healed, use a Pasteur pipette to very gently transfer embryos from the depressions to individual wells of an agarose-coated 24-well plate. The exudate will be removed (Figure 3D) by fluid mixing during the transfer.**
 1. Rotate the embryos so that they are placed vegetal-pole up, facing the bulk solution. Again, place the donor carcasses and graft recipients in adjacent wells to assist in keeping track of embryo pairs; record this information.
2. Transfer the 24-well plate containing embryos to a 25 °C incubator for overnight culture.
NOTE: The next day, the embryos will have reached tailbud stages.
3. Move the graft recipients to clean agarose-coated 6-well plates containing 1/9x MMR supplemented with 50 µg of gentamycin sulfate/mL, with 1 embryo per well. Maintain a clear record of donor carcasses that match these graft recipients.
4. To assess mutagenesis efficiency by sequencing PCR amplicons^{5,17,24} or using other methods that rely on PCR amplicons (see the **Discussion**), move individual carcasses to 0.2 mL PCR strip tubes. Remove most of the medium and homogenize in 100 µL lysis buffer²⁴ containing proteinase K (PK) by repeated up-and-down pipetting using a P200 pipette.
5. Perform embryo lysis²⁴ at 56 °C for 6 h to overnight to permit PK digestion. Inactivate PK by heating it to 90-95 °C for 10 min, followed by quick cooling to 4 °C. Use lysates without further cleanup steps to seed the PCR reactions to obtain short amplicons for direct Sanger DNA sequencing²⁴. Store the lysates at -20 °C.
NOTE: Within several days, the tadpoles will reach feeding stages (approximately stage 45-46) and can be fed a suspension of planktonic powder (sera Micron).
6. After ~1 week, with daily changes of culture medium to maintain cleanliness and minimize microbial overgrowth, move tadpoles to small tanks in a circulating aquatic system with drip flow. Use the sequencing data to segregate tadpoles with highly efficiently mutagenized PGCs from tadpoles with lower mutagenesis efficiency. Once metamorphosis is complete, move the froglets to the adult aquatic system in the lab.
NOTE: Regimens developed by the National *Xenopus* Resource (Marine Biology Laboratory, Woods Hole, MA) provide a guide for tadpole feeding²⁹ and adult frog maintenance³⁰.

Representative Results

Following transplantation, the qualitative determination of the efficacy of CRISPR/Cas9 mutagenesis should be performed before expending the effort in animal husbandry to grow and maintain the animals to sexual maturity. Because animals carrying leapfrog transplants are somatically wildtype and the germline is difficult to access for direct measurements, saving the carcasses of donor embryos becomes important. DNA analysis from the carcasses serves as a proxy for the extent of mutagenesis that occurs in the transplanted germline¹⁷.

One approach to assess the success of mutagenesis is to directly sequence PCR amplicons spanning the genomic region being targeted. This becomes especially costly and inefficient if one wishes to sequence numerous *individually-cloned* DNA fragments from many animals. Instead, sequencing amplicons heterogeneous at the target site, as they are derived from genetically mosaic embryos, is used to assess mutagenesis efficiency in each embryo¹⁹. Visualization of chromatograms shows the wild type sequence (peaks) upstream of the target sequence and peaks become "scrambled" (*i.e.*, the co-occurrence of peaks corresponding to multiple bases appearing at each nucleotide position) downstream of the site of the Cas9-catalyzed double-strand break. Examples of such profiles can be found in Figure S1 of Blitz *et al.*¹⁷. Accurate quantitation of the extent of mutagenesis represented by these scrambled peaks can be obtained using a sequence decomposition algorithm, TIDE (<https://tide-calculator.nki.nl/>)³¹. Screening individual embryos using this technique gives confidence that each embryo was properly injected and targeted for mutagenesis. A tracer, such as a fluorescently tagged dextran, can be used to confirm successful injection²⁴ and can also aid in establishing that a tadpole is indeed carrying the transplanted tissue (unpublished data). It is important to note that the formal possibility that the coinjection of a fluorescent dextran together with Cas9-sgRNA might alter Cas9 mutagenesis efficiency has not been explored. Any "leapfrogged" embryos containing transplants from poorly or unmutagenized donors can be discarded.

Leapfrogging circumvents the need for F₂ generation analysis in standard genetic breeding schemes, by permitting efficient phenotypic analyses in the F₁ embryos. When using the standard approach to mutate essential genes, careful titration of Cas9-sgRNA dose is required to ensure the viability of founder embryos, resulting in a reduced mutagenesis efficiency. Surviving F₀ animals from this standard scheme are then outcrossed with wildtypes to obtain viable F₁ embryos, which are screened to identify carriers. Finally, these F₁ heterozygotes are grown to sexual maturity and intercrossed to obtain homozygous F₂ individuals displaying mutant phenotypes. In contrast, because leapfrogging produces animals that have the complete replacement of their germlines with mutant alleles, intercrossing these F₀ animals produces mutant phenotypes in the F₁ generation. Blitz *et al.*¹⁷ reported that a majority of F₀ animals targeted at the *tyrosinase (tyr)* locus, which is required for pigmentation of melanocytes and retina, showed 100% germline transmission (measured by outcrossing with *tyr*^{-/-} albino animals) of donor-derived mutant genomes (see **Figure 4A-B** and **Table 1**). Thus, intercrossing two animals produced by leapfrogging would yield homozygous or compound heterozygous mutant F₁ embryos.

Similar results were obtained¹⁷ by intercrossing F₀ leapfrogged animals carrying mutations in the homeobox (encoding the DNA-binding homeodomain) of the *gooseoid (gsc)* gene (see **Figure 4A, C-H**). Knockdowns of Gsc expression using morpholino antisense oligonucleotides in *X. laevis* suggested that the *gsc* gene is necessary for the complete development of the anterior head³², and Cas9-sgRNA injected tadpoles also show embryonic lethal phenotypes¹⁷. However, the leapfrogged F₀ animals, being somatically wild type, are viable and robust, and the F₁ intercross produced embryos with identical LOF phenotypes to those published in *X. laevis* knockdowns¹⁷. These data provided genetic corroboration of the morpholino phenotype, as well as a proof of principle that leapfrogging can be used to overcome embryonic lethal phenotypes.

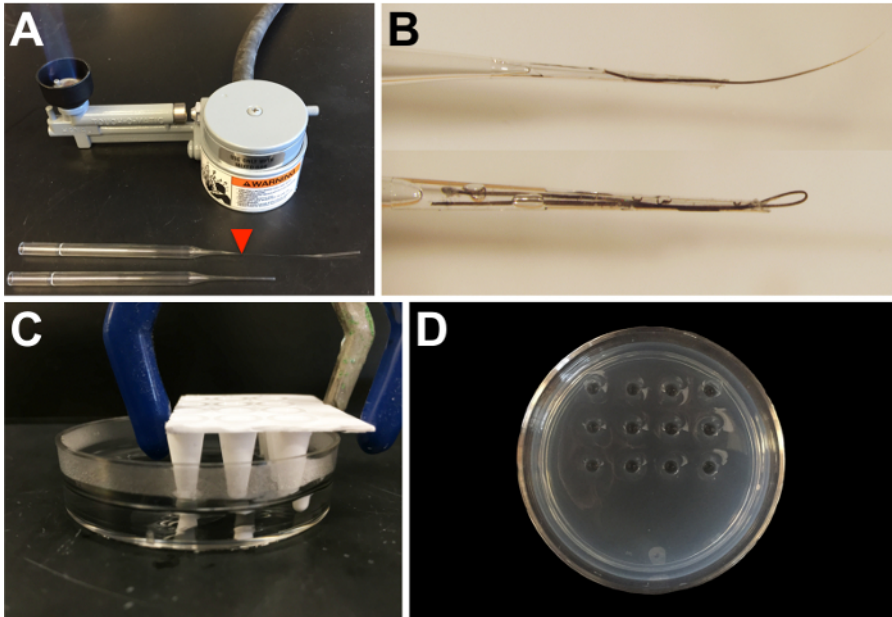


Figure 1: Materials needed for transplantation. (A) Borosilicate glass Pasteur pipettes are used to make eyebrow hair knives and hair loops for microsurgery on early embryos. Drawing out the glass using a Bunsen burner allows for a narrower end for the insertion of the hair. The red arrowhead marks the approximate position at which to break the glass. (B) An example of an eyebrow hair knife (top) and hair loop (bottom), fixed into pipettes with fast-drying glue. (C) A portion of a 96-well PCR plate is used to create depressions in an agarose plate by allowing the 1% agarose to solidify around the mold (agarose is made in 0.3x or 1x MMR, depending on whether the transplantations are being performed in *X. tropicalis* or *X. laevis*, respectively). The thickness of the agarose is ~5 mm, with 3-4 mm-deep wells. (D) An example of an agarose plate for transplantation, with 12 depressions, as shown in panel C. [Please click here to view a larger version of this figure.](#)

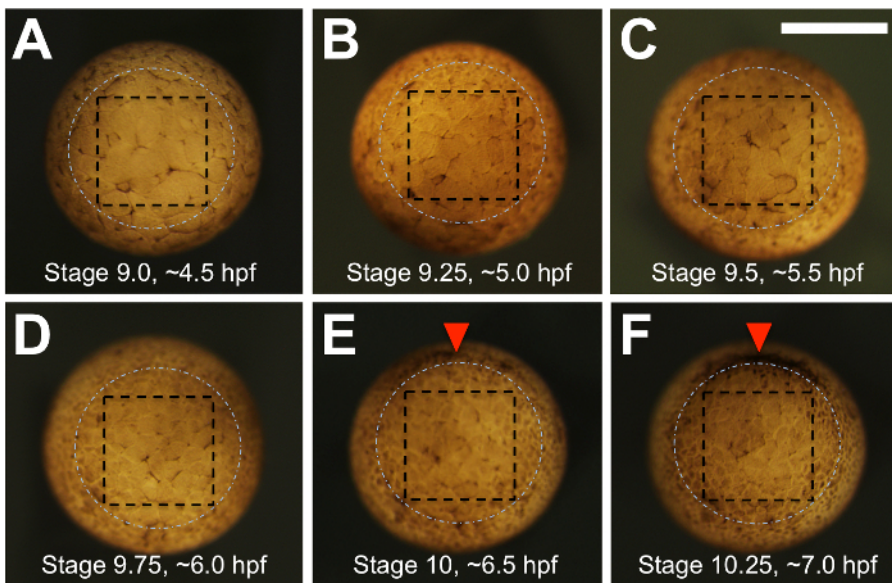


Figure 2: Morphology of the vegetal pole during the late blastula to early gastrula stages and the strategy for dissection. (A-F) *Xenopus tropicalis* embryos are shown in vegetal view at half-hourly time intervals, from the beginning of blastula stage 9 (4.5 hpf) to early gastrula stage ~10.25 (7 hpf). Prior to 5 hpf, blastomeres are large and more easily damaged. The white, dashed circle marks the expected position of bottle cell formation during gastrulation, which can be seen forming beginning on the dorsal side (top) in panels (E and F). For leapfrogging, four incisions are made within a square, depicted by black dashed lines, with a final cut made parallel to the surface of the tissue (not illustrated) to free the vegetal explant. Scale bar = 400 μ m. [Please click here to view a larger version of this figure.](#)

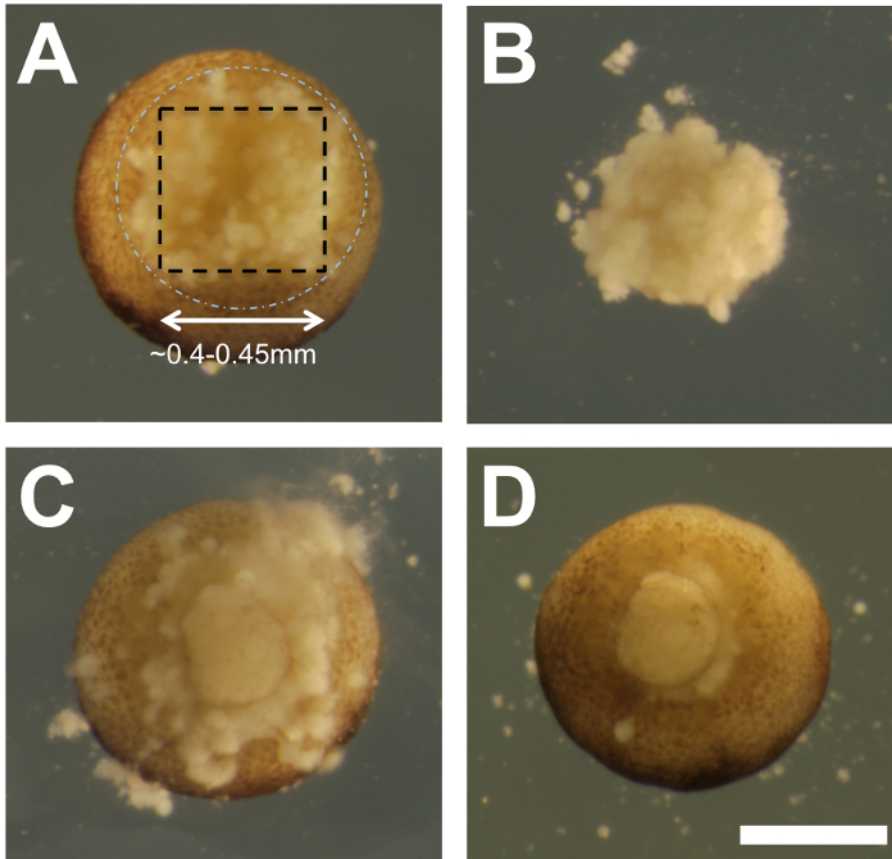


Figure 3: Transplantation. (A) An example of a ~5.5 hpf embryo after the removal of the vegetal PGC-containing tissue. The black dashed box and white dashed circle are the same relative dimensions as those shown in **Figure 2**. (B) The vegetal explant removed from the embryo in panel A, inner surface facing the viewer, is approximately 0.4-0.45 mm per side and 0.25-0.3 mm in depth. (C) An embryo with the transplanted vegetal tissue. The image was acquired ~10 min after transplantation. (D) 30 min after transplantation, the vegetal tissue is seen to have healed into place (gentle swirling with an eyebrow hair knife above the embryo was used to create a vortex that swept away the debris seen in panel C). After transfer to 1/9x MMR and incubation at 25 °C, most embryos complete gastrulation normally. Scale bar = 400 μ m. [Please click here to view a larger version of this figure.](#)

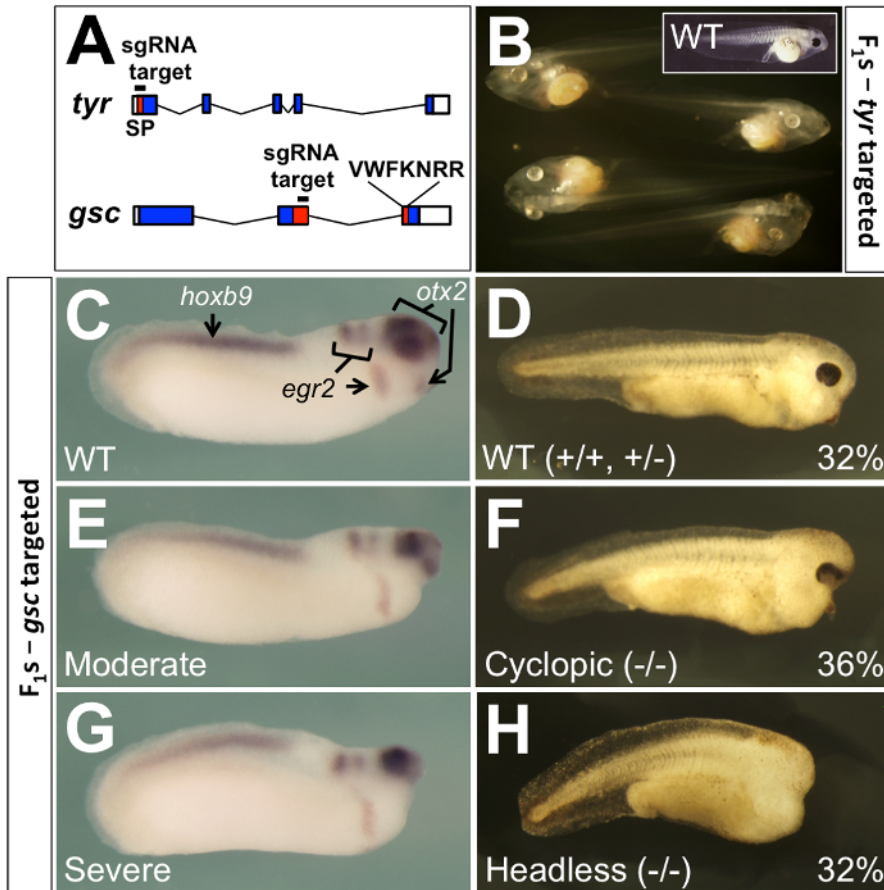


Figure 4: Germline transmission from F₀ animals produced by leapfrogging. (A) For leapfrogging, two genes were targeted by CRISPR/Cas9, *tyrosinase* (top) and *goosecoid* (bottom). The *tyr* gene was targeted^{4,5} within the coding sequencing (blue shading) for an N-terminal signal peptide (SP, red shading), while the *gsc* gene was targeted¹⁷ within coding sequence at the beginning of the homeobox (red shading), which is split between two exons. Untranslated 5' and 3' exonic regions are unshaded. (B) All 160 F₁ tadpoles from (see also **Table 1**) a mating of a leapfrogged F₀ male (LF1) and an albino (*tyr*^{-/-}) female were albino, indicating that the entire germline from the leapfrogged male had been replaced with *tyr* mutant germ cells. The inset shows a wild-type tadpole with normal retinal and melanocyte pigmentation. (C-H) F₁ embryos derived from an intercross between two F₀ leapfrogged *Xenopus* targeting the homeobox gene *gsc*. Approximately 2/3rds of the embryos were phenotypically mutant for *gsc*, with the phenotype being varying degrees of anterior truncation of the head (E-H). Half of the mutants were either cyclopic or had closely-spaced eyes (F), while the other half were eyeless (H). Genotyping showed these to be homozygotes or compound heterozygotes. The 1/3rd of embryos showing wild-type phenotype (C, D) were either homozygous wild type or heterozygotes. *In situ* hybridization for *otx2* (a marker of forebrain, midbrain, and eyes), *egr2* (a marker of hindbrain rhombomeres 3 and 5, and a stream of neural crest), and *hoxb9* (a marker of the spinal cord) shows that loss of *gsc* function affects only the anterior portion of the *otx2* domain. These data are reproduced from Blitz *et al.*¹⁷ with permission from the Company of Biologists. [Please click here to view a larger version of this figure.](#)

Cross	Male	Female	Albino	Wild type	Total	% Albino
1	LF1	<i>tyr</i> ^{-/-}	160	0	160	100
2	LF2	<i>tyr</i> ^{-/-}	148	0	148	100
3	<i>tyr</i> ^{-/-}	LF3	409	0	409	100
4	<i>tyr</i> ^{-/-}	LF4	0	519	519	0
5	<i>tyr</i> ^{-/-}	LF5	223	0	223	100
6	LF6	<i>tyr</i> ^{-/-}	493	703	1196	41.2
7	<i>tyr</i> ^{-/-}	LF7	1	94	95	1.1
8	<i>tyr</i> ^{-/-}	LF8	125	0	125	100
9	<i>tyr</i> ^{-/-}	LF9	0	417	417	0
10	<i>tyr</i> ^{-/-}	LF10	252	0	252	100

Table 1: Efficient germline replacement by leapfrog transplants carrying mutations in tyrosinase. Targeting *tyrosinase*, transplant-bearing animals of both sexes were obtained. These animals were crossed with albinos, which are homozygous *tyr*^{-/-}, to test for the efficiency of the germline transmission of mutant alleles, and the percent of the F₁ embryos that showed albinism was assayed at the tadpole stage. Entries in the columns labeled "Male" and "Female" indicate which parent is either *tyr*^{-/-} or the test animal derived from leapfrogging (LF). The percent of embryos within a cross showing the albino phenotype are indicated under "% Albino." Note that 6 of 10 animals with leapfrog transplants showed complete (100%) replacement with mutant alleles. This table is reproduced from Blitz *et al.*¹⁷, with permission.

Discussion

This report provides a detailed protocol for the transplantation of vegetal tissue containing PGCs. Transplantation of PGCs is used in conjunction with genome-editing technologies (*e.g.*, CRISPR/Cas9) to modify the germline of an animal while maintaining nearly all of its somatic tissues as genetically wild type. For leapfrogging to be successful, there are a number of critical factors to consider prior to performing transplantations.

To ensure the complete replacement of the germline with mutant alleles, it is recommended that one first determines the *in vivo* efficiency of sgRNAs. Experience suggests that most sgRNAs designed by CRISPRDirect (<https://crispr.dbcls.jp/>) or CRISPRScan (<http://www.crisprscan.org/>) are efficient when used at 250 pg/embryo. However, in some cases, it may be necessary to use higher concentrations (*e.g.*, the *gsc* sgRNA published by Blitz *et al.*¹⁷, required ~1 ng/embryo). Assessing the efficiency of mutagenesis of the target site is essential and the use of TIDE permits a quantitative assessment³¹. While the method emphasized here uses the direct Sanger sequencing of PCR amplicons (representing the population of mutant alleles in F₀ embryos), numerous other methods have been used in the genome-editing literature for this purpose. Other low-to-moderate-cost techniques that are commonly used include fragment length polymorphism analysis using either the loss of restriction enzymes³³ or Cas9/TALEN cleavage sites³⁴, the T7 endonuclease¹³⁵ and Surveyor³⁶ assays, the heteroduplex mobility assay³⁷, the high-resolution melt assay³⁸, fragment analysis by capillary electrophoresis^{39,40}, and qPCR-based methods^{41,42}.

A second consideration is the choice of target site location within the target gene. To permit the efficient recovery of mutant phenotypes in the F₁ generation, it is important to maximize the chance for complete LOF alleles. When making mutant lines using classical approaches it is a common strategy to target Cas9 cleavage near the 5' end of coding regions, to elicit premature translation termination near the beginning of the protein coding region. F₁ heterozygotes would be identified with frameshift mutations, and these animals would be selected for further work because they are expected to carry null alleles. Because the leapfrogging strategy uses F₀ intercrosses, the approach of targeting the 5' end of the coding sequence would be highly inefficient. F₀ embryos have mosaic germlines, and intercrossing results in populations of F₁ mutants that are mostly compound heterozygotes¹⁷. Since large-scale analysis verifies that, on average, the expected ~1/3rd of mutations produced by double-strand breaks are in-frame⁴³, most F₁ embryos produced in this fashion would have at least one allele with an in-frame mutation that might retain wild type activity. Therefore, a different strategy is needed to ensure that even these in-frame mutant alleles are likely to be nulls. Targeting double strand breaks within protein-folding domains that are essential for normal protein function is expected to result in a higher chance of complete LOF (null alleles)^{17,44}. Careful consideration to the atomic-level structure(s) of protein domains, when available, can assist in identifying regions of secondary structure that, even when containing a single amino acid in-frame deletion, might be expected to disrupt domain structure (*e.g.*, the Δ3bp mutation in *gsc* published by Blitz *et al.*¹⁷). If CRISPR/Cas9 target sites can be identified within these regions, one has a higher chance of successfully creating null alleles. Small deletions within solvent-exposed loops between protein secondary structural components are less desirable because mutations in more flexible regions might still result in functional proteins.

A third consideration for leapfrogging is the efficiency of PGC removal from the recipient embryo. This step needs to be efficient in order to ensure complete germline replacement. It is currently unclear whether all graft recipient embryos produced by the method described here have complete removal of their PGCs. Whole-mount *in situ* hybridizations show variability in the localization of PGC markers in blastula-stage embryos. In many cases, all or nearly all the signal is found near the vegetal-most end of the embryo and would therefore be removed by the surgery outlined here (Blitz *et al.*¹⁷ and citations therein). However, some embryos show PGC marker expression in a few cells closer to the blastocoel floor. Germ plasm first coalesces at the vegetal pole after fertilization and then is swept along cleavage furrows during early cleavages. Some germ plasm may become distributed in cells in the middle of the vegetal hemisphere and not be effectively removed without using transplants that extend to the blastocoel. However, it is also important to note that these deep cells containing germ cell markers might not contribute to the germline, as microRNAs are known to clear PGC mRNAs from somatic cells^{45,46}. These deep cells staining positive for PGC markers might be somatic cells undergoing such clearance. Methods to ensure complete PGC removal from recipient embryos are currently being developed.

Finally, it is also important to consider how many F₀ animals to generate by leapfrogging. As a variable number of animals will not survive to sexual maturity, it is recommended that more than 20 embryos containing transplants are created. It will be necessary to have enough surviving animals to ensure (1) that sufficient numbers of both sexes will be obtained and (2) that these transmit mutant alleles at a sufficiently high frequency to be suitable for the analyses planned by the researcher.

Using the protocol described here, with some practice, amphibian embryologists should be able to master the PGC transplantations technique with some practice. Leapfrogging should be possible, not only in other amphibians, but also other organisms containing PGCs that are readily transplantable between individuals at early stages of embryogenesis.

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The author has nothing to disclose.

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