FINAL SCRIPT: APPROVED FOR FILMING

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Title: Generation of Maternal Mutants Using *zpc*:*cas9* Knock-in Zebrafish

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

2.4.1, 3.1.2, 4.2.3, 4.5.1

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **7/26/2025**

When you are ready to submit your video files, please contact our China Location Producer, Yuan Yue.

Current Protocol Length

Number of Steps: 20
Number of Shots: 45

Introduction

NOTE: The interview statements have been edited

1.1. **Ming Shao:** In this video, we present an improved oocyte-specific conditional knockout method in zebrafish, offering a versatile platform for studying maternal factors whose zygotic mutations result in lethality or sterility.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 1*

What are the current experimental challenges?

1.2. **Yizhuang Zhang:** Current maternal knockout methods are either technically challenging or time-consuming, whereas previously reported zpc:Cas9 transgene is transcriptionally silenced over generations.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What research questions will your laboratory focus on in the future?

1.3. **Ziping Fu:** Using a zpc:cas9 Knockin line, we have established a robust platform for generating conditional knockouts during oogenesis, enabling the rapid and highly efficient production of maternal mutants.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 7*

Ethics Title Card

This research has been approved by the Institutional Animal Care Committee at Shandong University

Protocol

2. Microinjection and Prescreening of Transgenic Founders

Demonstrator: Yizhuang Zhang

2.1. To begin, collect embryos that have been spawned by homozygous rbm24a-RFP KIzpc:cas9 *(R-B-M-Twenty-four-A-R-F-P-K-I-Z-P-C-Cas-Nine)* fish **[1]**.

2.1.1. WIDE: Talent using a transfer pipette to collect freshly spawned embryos from the breeding tank labeled rbm24a-RFP KIzpc:cas9.

2.2. Combine 1 microliter of pGGDestEB-rbm24a-4sgRNA (*P-G-G-Dest-E-B-R-B-M-Twenty-four-A-Four-S-G-R-N-A)* with 1 microliter of Tol2 *(Tol-Two)* transposase messenger RNA to prepare the injection mixture **[1-TXT]**. Dilute both the plasmid and the Tol2 messenger RNA directly in pure water **[2]**.

2.2.1. Talent pipetting pGGDestEB-rbm24a-4sgRNA and Tol2 transposase messenger RNA into a single tube. **TXT: Purify plasmid via phenol-chloroform to remove RNase**

2.2.2. Talent using a pipette to dilute the reagents in sterile pure water.

2.3. Now, place a glass coverslip in a 90-millimetre dish **[1]**. Align the embryos along the edge of the coverslip **[2]** and gently use a pipette to remove any excess water **[3]**.

2.3.1. Talent placing a coverslip inside a large Petri dish.

2.3.2. Talent using a pipette tip to line up the embryos along the edge of the coverslip.

2.3.3. Talent removing excess water from the embryos using a pipette.

2.4. Then, inject 2 nanoliters of the prepared mixture into the blastodisc of each 1-cell stage embryo **[1]**. Gently rinse the injected embryos with blue water **[2]** and transfer them to another dish **[3]**. Place the dish in an incubator set to 28 degrees Celsius for cultivation **[4]**.

2.4.1. SCOPE: 2.4.1.mp4: 00:04-00:12

2.4.2. Talent gently rinsing the embryos using methylene blue working solution.

2.4.3. Talent transferring rinsed embryos into a clean Petri dish using a pipette.

2.4.4. Talent placing the dish in a labeled 28 degrees Celsius incubator.

2.5. At 24 hours post-fertilization, select embryos that show robust and ubiquitous blue fluorescent protein expression **[1]**. At 4 days post-fertilization, raise only the embryos that maintain strong transgenic fluorescent signals **[2]**.

2.5.1. LAB MEDIA: Figure 9: *Video editor: Highlight the upper panel labelled 24hpf*

2.5.2. LAB MEDIA: Figure 9: *Video editor: Highlight the upper panel labelled 96hpf*

3. Characterization of Double Transgenic Embryo

Demonstrator: Ziping Fu

3.1. Collect embryos produced from mating mosaic transgenic founder females with wild-type males **[1]**. Using a fluorescence stereomicroscope, pick up the blue fluorescent protein-positive embryos at the one-cell stage **[2-TXT]**.

3.1.1. Talent using a pipette to collect embryos from a mating setup labeled with mosaic transgenic female and wild-type male identifiers.

3.1.2. ~~SCOPE:~~ Show fluorescence view of embryos with blue fluorescence at one-cell stage being selected using a micropipette. **TXT: Identify developmental defects seen only in BFP$^+$ embryos** NOTE: This is not a SCOPE shot, use the provided image

3.2. Then, fix the embryos at appropriate stages in 4 percent paraformaldehyde **[1]** at 4 degrees Celsius overnight **[2-TXT]**.

3.2.1. Talent placing embryos into vials with paraformaldehyde.

3.2.2. Talent placing the embryos in a 4 degrees Celsius refrigerator. **TXT: Perform hybridization/IF staining/histological analysis when required**

4. Genotyping Maternal Mutant Embryos

Demonstrator: Ming Shao

4.1. To prepare a 1.5 percent agar solution, weigh and combine agar powder in one-third strength Ringer's buffer **[1]** and boil until fully dissolved **[2]**. Pour the melted agar solution into a 90-millimeter Petri dish and insert a z-mold into the molten agarose **[3]**. Once the agarose has solidified, gently remove the mold to create a ready-to-use plate **[4]**.

4.1.1. Talent adding agar powder to a beaker containing one-third strength Ringer's buffer.

4.1.2. Talent boiling the mixture until it becomes clear.

4.1.3. Talent pouring molten agarose into a 90 millimeter Petri dish and inserting a z-mold carefully.

4.1.4. Talent removing the z-mold from the solidified agar to reveal multiple embryo wells.

4.2. Next, adjust the settings on the needle puller to use two light weights and select the Step 1 procedure **[1-TXT]**. Load the glass capillaries into the puller to make melt-sealed needles **[2]**. Using pointed tweezers, trim the capillary tips to a 30 to 40-micrometer diameter **[3]**. Then, use a microforge to form a spike at the tip, which will aid in embryo penetration and reduce tissue damage **[4]**. NOTE: The VO is edited as per the moved shots

4.2.2 Close-up of puller settings configured to two light weights, Level 1: 60, and Step 1 selected. **TXT: Heating power: Lv1: 60** NOTE: This shot is moved here as per the author's request

4.2.1. Talent loading a glass capillary into the puller.

~~4.2.2. Close-up of puller settings configured to two light weights, Level 1: 60, and Step 1 selected. **TXT: Heating power: Lv1: 60.**~~ NOTE: This shot is moved before 4.2.1

4.2.3. Talent trimming the needle tip using pointed tweezers under a stereomicroscope.

4.2.4. Talent forming a spike at the needle tip using a microforge.

4.3. Mate the putative female founders with wild-type zebrafish and collect embryos **[1]**. At the one-cell stage, isolate embryos that are positive for blue fluorescent protein using a fluorescence stereomicroscope **[2]**.

4.3.1. Talent collecting embryos from a breeding tank containing labeled female founder and wild-type male fish.

4.3.2. ~~SCOPE:~~ Fluorescent view showing selection of blue fluorescent protein-positive embryos at one-cell stage using a pipette. NOTE: This is not a SCOPE shot, use the provided image

4.4. At 3 hours post-fertilization, incubate the embryos in Pronase dissolved in one-third strength Ringer's buffer for 10 minutes with gentle pipetting to dechorionate them **[1-TXT]**. Carefully transfer the dechorionated embryos onto the prepared agarose plate flooded with 1/3 Ringer's buffer supplemented with penicillin-streptomycin **[2]**. Reorient the embryos so that the blastomere is facing toward the capillary tip for cell aspiration **[3]**.

4.4.1. Talent placing embryos into a Pronase solution and gently pipetting to remove the chorions. **TXT: 1 mg/mL Pronase**

4.4.2. Talent transferring dechorionated embryos onto the agarose plate pre-flooded with buffer containing penicillin-streptomycin.

4.4.3. Talent using a pipette or fine tool to rotate embryos so the blastomeres face the needle tip.

4.5. Now, use a microinjector to aspirate 20 to 40 cells from each embryo by gently reducing the equilibrium pressure to counter the capillary aspiration force **[1]**. Increase the equilibrium pressure to release the aspirated cells into 2 microliters of deionized water at the edge of 1.5-millilitre eppendorf tubes **[2]**.

4.5.1. SCOPE: 4.5.1.mp4: 00:08-00:23

4.5.2. Talent expelling aspirated cells into a droplet of deionized water at the edge of an open 1.5 ml eppendorf.

4.6. Then, add 200 microliters of RNA extraction reagent to the tube to wash down the aspirated cells and place the tube on ice for temporary storage **[1-TXT]**.

4.6.1. Talent pipetting RNA extraction reagent into the PCR tube and placing the tube on an ice rack. **TXT: Analyse cell's genotype with or without developmental defects**

4.7. Keep the embryos after cell aspiration on the agarose plate until aberrant phenotypes are visualized **[1]**.

4.7.1. Show the embryos after cell aspiration on the agarose plate.

4.8. Next, add 40 microliters of chloroform to the cells from selected embryos in the extraction reagent and mix gently **[1]**. Then, centrifuge the mixture at 12,000 *g* for 1 minute at room temperature **[2]**.

4.8.1. Talent adding chloroform to the extraction tube and gently mixing by inversion.

4.8.2. Talent placing the tube in a centrifuge and setting the parameters to 12,000 g for 1 minute at room temperature.

4.9. After collecting the supernatant, add 1 microliter of glycogen solution **[1]**. Then, add an equal volume of isopropanol, based on the supernatant volume, and mix thoroughly **[2]**. Incubate the tube at minus 20 degrees Celsius for 30 minutes to allow RNA precipitation **[3]**.

4.9.1. Talent pipetting glycogen solution into the tube with supernatant.

4.9.2. Talent adding isopropanol in equal volume and mixing by tapping.

4.9.3. Talent placing the tube in minus 20 degrees Celsius freezer.

4.10. Now, centrifuge the sample at 12,000 *g* for 10 minutes at 4 degrees Celsius **[1]** and discard the supernatant **[2]**.

4.10.1. Talent places the sample in the centrifuge.

4.10.2. Talent carefully pouring off the supernatant after the spin.

4.11. Then, to wash the RNA pellet twice, add 500 microliters of 70 percent ethanol **[1]** and centrifuge at 12,000 *g* for 1 minute at 4 degrees Celsius **[2]**.

4.11.1. Talent pipetting 70 percent ethanol into the sample tube.

4.11.2. Talent places the tube in the centrifuge it for 1 minute.

4.12. After discarding the supernatant, open the tube lid to let the pellet dry at room temperature for 5 minutes **[1]**.

4.12.1. Talent leaving the tube open on a bench for drying.

4.13. Add 7 microliters of water to dissolve the RNA pellet **[1]**. Perform reverse transcription using a first-strand cDNA synthesis kit, then conduct PCR and Sanger sequencing to analyze mutations in maternal mutant embryos **[2-TXT].**

4.13.1. Talent pipetting 7 microliters of water into the tube to dissolve the pellet.

4.13.2. Talent preparing a reaction using the first-strand complementary DNA synthesis kit and following the instruction manual. **TXT: Amplify and clone the coding region; Sequence 30 clones/embryo for mutation analysis**

Results

5. Results

5.1. Maternal rbm24a mutants were identified by the absence of Rbm24a-RFP, while other maternal mutants were detected by specific phenotypes or cell-aspiration-based genotyping **[1].** Among BFP *(B-F-P)*-positive embryos, those lacking RFP *(R-F-P)* signal were identified as maternal rbm24a mutants **[2]**.

5.1.1. LAB MEDIA: Figure 11.

5.1.2. LAB MEDIA: Figure 11A–D. *Video editor: Highlight cells with blue fluorescence but no red fluorescence (asterisk-marked) in the merged panel.*

5.2. In situ hybridization using nanos3 *(Nanos-Three)* probe revealed that Mrbm24a embryos failed to recruit germ plasm mRNAs to germ granules at the 4-cell stage **[1]** and lacked primordial germ cells at 24 hours post-fertilization **[2]**.

5.2.1. LAB MEDIA: Figure 11E,F.

5.2.2. LAB MEDIA: Figure 11G,H.

5.3. All adult Mrbm24a males failed to fertilize eggs spawned by wild-type females **[1]**, showing anatomical abnormalities with fatty deposits replacing normal testes **[2]**. Histological analysis confirmed the complete absence of germ cells and spermatozoa in Mrbm24a testes **[1]**.

5.3.1. LAB MEDIA: Figure 11I,J.

5.3.2. LAB MEDIA: Figure 11K,L.

5.3.3. LAB MEDIA: Figure 11M,N.

5.4. Western blot analysis confirmed nearly undetectable levels of Rbm24a protein in BFP-positive, RFP-negative embryos **[1]**. RT-qPCR results showed significantly lower rbm24a transcript levels in RFP-negative BFP-positive embryos relative to controls **[2]**.

5.4.1. LAB MEDIA: Figure 12A. *Video editor: Highlight the faint or absent band in the RFP–BFP+ lane compared to strong bands in RFP+ lanes.*

5.4.2. LAB MEDIA: Figure 12B.

5.5. RT-PCR and Sanger sequencing revealed large deletions and indels in both RFP negative BFP positive and RFP positive BFP positive embryos **[1]**, with wild-type transcripts detectable only in RFP positive embryos **[2]**.

5.5.1. LAB MEDIA: Figure 12D.

5.5.2. LAB MEDIA: Figure 12E.

5.6. A maternal GFP marker and nanog sgRNAs *(S-G-R-N-A)* were introduced to generate maternal nanog mutants, and GFP-positive embryos showed a range of dorsalized phenotypes **[1]**. Germline transmission rates ranged from 12% to 32% among GFP-positive transgenic lines **[2]**.

5.6.1. LAB MEDIA: Figure 13B. *Video editor: Highlight merged fluorescence image highlighting with asterisk mark*

5.6.2. LAB MEDIA: Figure 13C.

5.7. A dorsalized phenotype consistent with maternal nanog mutants was observed in 22% to 60% of GFP-positive embryos **[1]**.

5.7.1. LAB MEDIA: Figure 13D.

1. **rbm24a**
   Pronunciation link: No confirmed link found
   IPA: /ɑːr-biː-ɛm-twɛnti-fɔːr-eɪ/
   Phonetic Spelling: ar-bee-em-twenty-four-ay
2. **RFP**
   Pronunciation link: No confirmed link found
   IPA: /ɑːr-ɛf-piː/
   Phonetic Spelling: ar-ef-pee
3. **KIzpc:cas9**
   Pronunciation link: No confirmed link found
   IPA: /keɪ-aɪ-ziː-piː-siː kæz-naɪn/
   Phonetic Spelling: kay-eye-zee-pee-see caz-nine
4. **pGGDestEB**
   Pronunciation link: No confirmed link found
   IPA: /piː-dʒiː-dɛst-iː-biː/
   Phonetic Spelling: pee-jee-dest-ee-bee
5. **Tol2**
   Pronunciation link: https://www.merriam-webster.com/dictionary/toll
   IPA: /toʊl-tuː/
   Phonetic Spelling: tohl-two
6. **transposase**
   Pronunciation link: https://www.merriam-webster.com/dictionary/transposase
   IPA: /trænˈspoʊˌseɪz/
   Phonetic Spelling: tran-spoh-sayz
7. **blastodisc**
   Pronunciation link: https://www.merriam-webster.com/dictionary/blastodisc

IPA: /ˈblæstoʊˌdɪsk/
Phonetic Spelling: blas-toh-disk

8. **nanos3**
Pronunciation link: No confirmed link found
IPA: /ˈnænoʊs-θriː/
Phonetic Spelling: nan-ohs-three
9. **germ plasm**
Pronunciation link: https://www.merriam-webster.com/dictionary/germ%20plasm
IPA: /ˈdʒɝːm ˌplæzəm/
Phonetic Spelling: jerm-plaz-uhm
10. **paraformaldehyde**
Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde
IPA: /ˌpærəfɔːrˈmældəˌhaɪd/
Phonetic Spelling: par-uh-for-mal-duh-hahyd
11. **histological**
Pronunciation link: https://www.merriam-webster.com/dictionary/histological
IPA: /ˌhɪstəˈlɑːdʒɪkəl/
Phonetic Spelling: his-tuh-loj-i-kuhl
12. **agarose**
Pronunciation link: https://www.merriam-webster.com/dictionary/agarose
IPA: /ˈægəˌroʊs/
Phonetic Spelling: ag-uh-rohs
13. **capillary**
Pronunciation link: https://www.merriam-webster.com/dictionary/capillary
IPA: /ˈkæpəˌlɛri/
Phonetic Spelling: kap-uh-leh-ree
14. **microforge**
Pronunciation link: No confirmed link found
IPA: /ˈmaɪkroʊ-fɔːrdʒ/
Phonetic Spelling: my-kroh-forj
15. **dechorionate**
Pronunciation link: https://www.merriam-webster.com/dictionary/dechorionate
IPA: /diːˈkɔːriəˌneɪt/
Phonetic Spelling: dee-kor-ee-uh-nayt
16. **blastomere**
Pronunciation link: https://www.merriam-webster.com/dictionary/blastomere
IPA: /ˈblæstəˌmɪr/
Phonetic Spelling: blas-tuh-meer
17. **Pronase**
Pronunciation link: https://www.merriam-webster.com/dictionary/Pronase
IPA: /ˈproʊˌneɪs/
Phonetic Spelling: proh-nays
18. **penicillin**
Pronunciation link: https://www.merriam-webster.com/dictionary/penicillin
IPA: /ˌpɛnəˈsɪlɪn/
Phonetic Spelling: pen-uh-sill-in

19. **isopropanol**
    Pronunciation link: https://www.merriam-webster.com/dictionary/isopropanol
    IPA: /ˌaɪsəˈproʊpəˌnɔːl/
    Phonetic Spelling: eye-suh-proh-puh-nawl
20. **ethanol**
    Pronunciation link: https://www.merriam-webster.com/dictionary/ethanol
    IPA: /ˈɛθəˌnɔːl/
    Phonetic Spelling: eth-uh-nawl
21. **complementary DNA**
    Pronunciation link: https://www.merriam-webster.com/dictionary/complementary%20DNA
    IPA: /ˌkɑːmpləˈmɛntri ˌdiː-ɛnˈeɪ/
    Phonetic Spelling: com-pluh-men-tree dee-en-ay
22. **Sanger sequencing**
    Pronunciation link: https://www.merriam-webster.com/dictionary/Sanger%20sequencing
    IPA: /ˈsæŋgər ˈsiːkwənsɪŋ/
    Phonetic Spelling: sang-er see-kwen-sing
23. **indel**
    Pronunciation link: https://www.merriam-webster.com/dictionary/indel
    IPA: /ˈɪnˌdɛl/
    Phonetic Spelling: in-del
24. **nanog**
    Pronunciation link: No confirmed link found
    IPA: /ˈnænɒg/
    Phonetic Spelling: nan-og