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TITLE:

Determining the Role of Maternally-Expressed Genes in Early Development with Maternal Crispants

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early development, CRISPR-Cas9, maternal-effect, zebrafish, genome editing, reverse genetics,

SUMMARY:

Early development is dependent on maternally-inherited products, and the role of many of these products is currently unknown. Herein, we described a protocol that uses CRISPR-Cas9 to identify maternal-effect phenotypes in a single generation.

ABSTRACT:

Early development depends on a pool of maternal factors incorporated into the mature oocyte during oogenesis that perform all cellular functions necessary for development until zygotic genome activation. Typically, genetic targeting of these maternal factors requires an additional generation to identify maternal-effect phenotypes, hindering the ability to determine the role of maternally-expressed genes during development. The discovery of the biallelic editing capabilities of CRISPR-Cas9 has allowed screening of embryonic phenotypes in somatic tissues of injected embryos or "crispants," augmenting the understanding of the role zygotically-expressed genes play in developmental programs. This article describes a protocol that is an extension of the crispant method. In this method, the biallelic editing of germ cells allows for the isolation of a maternal-effect phenotype in a single generation, or "maternal crispants." Multiplexing guide RNAs to a single target promotes the efficient production of maternal crispants, while sequence analysis of maternal crispant haploids provides a simple method to corroborate genetic lesions that produce a maternal-effect phenotype. The use of maternal crispants supports the rapid identification of essential maternally-expressed genes, thus facilitating the understanding of early development.

INTRODUCTION:

A pool of maternally deposited products (e.g., RNAs, proteins, and other biomolecules) is necessary for all early cellular processes until the embryo's zygotic genome is activated[1]. The

premature depletion of these products from the oocyte is typically embryonic lethal. Despite the importance of these genes in development, the role of many maternally-expressed genes is currently unknown. Advancement in gene-editing technology in zebrafish, such as CRISPR-Cas9, enables the targeting of maternally-expressed genes[2–4]. However, the identification of a maternal-effect phenotype requires an extra generation when compared to a zygotic phenotype, thus requiring more resources. Recently, the biallelic editing capability of CRISPR-Cas9 has been used to screen for embryonic phenotypes in somatic tissues of injected (F0) embryos, known as "crispants"[5–10]. The crispant technique permits resource-efficient screening of candidate genes in somatic cells, facilitating understanding of specific aspects in development. The protocol described in this paper allows for the identification of maternal-effect phenotypes, or "maternal crispants," in a single generation[11]. This scheme is attainable by multiplexing guide RNAs to a single gene and promoting biallelic editing events in the germline. These maternal crispant embryos can be identified by gross morphological phenotypes and undergo primary characterization, such as labeling for cell boundaries and DNA patterning[11]. Combined analysis of the observable phenotype and basic molecular characterization of the induced INDELs allows for the prediction of the targeted gene's role in early development.

In zebrafish, during the first 24 h post-fertilization (hpf), a small group of cells develops into the primordial germ cells, a precursor to the germline[12–15]. In clutches laid by F0 females, the proportion of maternal crispant embryos recovered depends on how many germ cells contain a biallelic editing event in the targeted gene. In general, the earlier the editing event occurs in the embryo, the higher the probability of CRISPR-Cas9 mutations being observed in the germline. In most cases, the phenotypes of maternal crispant embryos come from the loss of function in the two maternal alleles present in the developing oocyte. As the oocyte finishes meiosis, one of the maternal alleles is extruded from the embryo via the polar body, while the other allele becomes incorporated into the maternal pronucleus. The sequencing of multiple maternal crispant haploids will represent a mixture of the mutations (insertions and/or deletions (INDELs)) present in the germline that contribute to the phenotype[11].

The following protocol describes the necessary steps to create CRISPR-Cas9 mutations in maternal-effect genes and identify the corresponding phenotype using a maternal crispant approach (**Figure 1**). Section one will explain how to effectively design and create guide RNAs, while sections two and three contain critical steps for creating maternal crispants by microinjection. After injecting the CRISPR-Cas9 mixture, injected embryos are screened for somatic edits via PCR (section four). Once the injected F0 embryos develop and reach sexual maturity, the F0 females are crossed to wild-type males, and their offspring are screened for maternal-effect phenotypes (section five). Section six includes instructions on making maternal crispant haploids that can be combined with Sanger sequencing to identify the CRISPR-Cas9-induced INDELs. In addition, the Discussion contains modifications that can be made to the protocol to increase the sensitivity and power of this method.

PROTOCOL:

In studies leading to the development of this protocol, all zebrafish housing and experiments were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee (IACUC-M005268-R2).

1. Synthesis of Guide RNAs

NOTE: Zygotic crispants have been created using a single guide RNA or multiplexing multiple guide RNAs to a single target[5–10]. The multiplexing of guide RNAs increases the percentage of embryos showing a zygotic crispant phenotype[10]. Due to this increased frequency of embryos exhibiting a phenotype, maternal crispants are created by multiplexing four guide RNAs to a single gene. A more detailed protocol on using CHOPCHOP to design guide RNAs and an annealing method to synthesize guide RNAs for zebrafish can be found elsewhere[16–20].

1.1. To identify a maternally-expressed gene to target, ascertain the mRNA transcript levels during development via an RNA-sequence database that provides transcriptome information from zygote to 5 days[21]. In general, maternal-specific genes are highly expressed in the early embryo and are degraded after the zygotic genome is activated[22].

1.2. Once a maternally-expressed target gene has been identified, determine the first predicted protein domain using the "domains and features" section available on the Ensembl genome browser[23]. Use this domain as the target region for the four guide RNAs.

1.3. Use the guide RNA selection program CHOPCHOP to identify four guide RNA target sites in the first active domain. Design gene-specific oligonucleotides, as shown below for each target site. In the gene-specific oligonucleotide, the N^{20} section corresponds to the target sequence minus the PAM site (NGG) from CHOPCHOP. Order these gene-specific oligonucleotides and the constant oligonucleotide using standard desalt purification (see **Table of Materials**).

Gene-specific oligonucleotide
5' TAATACGACTCACTATA- N^{20} -GTTTTAGAGCTAGAAATAGCAAG 3'

1.4. To create a guide RNA template for each gene-specific oligonucleotide, anneal it to the constant oligonucleotide and fill in the overhangs with T4-DNA polymerase as previously described[16]. After the four guide RNA templates are assembled, purify and concentrate them together using a DNA clean-up and concentrator kit according to the manufacturer's instructions (see **Table of Materials**).

1.5. Synthesize the sgRNA mixture from the pooled guide RNA template using an *in-vitro* T7 transcription kit (see **Table of Materials**). Perform the *in-vitro* transcription according to the manufacturer's instructions. Using half-reactions of the T7 Transcription kit can decrease the cost per reaction.

1.6. After RNA synthesis, purify the resulting pool of sgRNAs using an ethanol/ammonium acetate protocol as previously described[16,20,24]. After the RNA has been isolated, resuspend it in

20 µL of nuclease-free water. If half-reactions of the T7 Transcription kit were used to transcribe the pool of sgRNAs, resuspend the purified RNA into 10–15 µL of nuclease-free water.

1.7. Quantify the amount of pooled sgRNAs that were created using a spectrometer. Dilute the pool of sgRNAs in nuclease-free water to a dilution of 1500 ng/µL ± 500 ng/µL. Typically, the final volume of the working dilution ranges from 30–50 µL.

1.8. After determining the concentration of the pool of sgRNAs, verify the integrity of the sgRNAs on a 1% agarose gel.

1.8.1. Cast a 1% agarose/0.5 µg/mL ethidium bromide/TBE gel. Once the gel has solidified, place it in TBE running buffer.

1.8.2. Mix 1 µL of the sgRNA mixture and 1 µL of RNA gel loading buffer (see **Table of Materials**). Load this sample in the gel and run the gel at 100 V for 5 min.

1.9. Visualize the bands using ultraviolet (UV) light. The pool of sgRNAs should appear as a single band. If a smear is observed, RNA degradation has likely occurred.

1.10. Store the pool of sgRNAs in single-use 1 µL aliquots in nuclease-free PCR strip tubes in the -80 °C freezer. For large volumes of sgRNAs mixture (30 µL or more), aliquot half of the volume into the nuclease-free PCR strip tubes and store the other half as a larger volume in a nuclease-free microcentrifuge tube. Thaw out and aliquot when needed.

1.11. To prevent RNA degradation, ensure that the samples in the microcentrifuge tube undergo no more than two freeze-thaw cycles.

2. Preparing reagents and materials for microinjection

NOTE: In zebrafish, the injection of Cas9 mRNA can create zygotic crispants. However, studies have shown that Cas9 protein is more efficient in creating INDELs in injected embryos[16,25]. This protocol uses Cas9 protein to generate maternal crispants because this protein does not experience the same lag in activity as injected Cas9 mRNA. In theory, this should increase the probability of a biallelic mutation early in development resulting in an increased chance of a more extensive section of the germline being affected. Other protocols and resources detailing how to prepare for microinjections can be found elsewhere[24,26].

2.1 Purchase or generate Cas9 protein (see **Table of Materials**). Resuspend the Cas9 protein in nuclease-free water to make a 2 mg/mL solution and aliquot 1 µL into RNase-free polypropylene microcentrifuge tubes. Store these as single-use tubes at -80 °C.

2.2 The afternoon before the injection, use a micropipette puller to pull a glass capillary and create an injection needle. Store the unbroken needle in an enclosed needle holder until the morning of microinjections.

2.3 To create an injection plate, pour 20 mL of 1.5% agarose/sterile H_2O to fill half of a 100 mm X 15 mm Petri dish and wait for it to solidify. Once the agarose solution is set, add 20 mL of 1.5% agarose/sterile H_2O to the Petri dish and place the plastic mold (see **Table of Materials**) into the liquid agarose and allow it to harden.

2.4 After the agarose has hardened, remove the plastic mold and store the injection plate upside down in a refrigerator until the morning of injections. A single plate can be used for multiple injections as long as the wells maintain their integrity.

3. Microinjection of CRISPR-Cas9 cocktail into a one-cell zebrafish embryo to generate maternal crispants

NOTE: More resources for microinjection into zebrafish embryos can be found elsewhere[24,26,27]. Injecting the CRISPR-Cas9 mixture into the developing blastodisc of one-cell embryos may increase the probability of creating maternal crispants. The mixture can also be injected into the yolk sac up to the 2-cell stage. However, mixtures injected into the yolk depend on ooplasmic streaming to reach the blastodisc, so CRISPR-Cas9 injected into the yolk could decrease the cutting efficiency of the CRISPR-Cas9[28].

3.1 The afternoon before microinjections, set up wild-type crosses in zebrafish mating boxes. Keep both the male and female fish in the same tank but separate them with a mating box divider or place the female inside an egg-laying insert.

3.2 On the morning of the experiment, take out one 2 mg/mL aliquot of Cas9 protein and one aliquot of the pool of sgRNAs. In the RNase-free polypropylene microcentrifuge tube that contains the Cas9 protein, assemble a 5 µL injection mixture that includes the pool of sgRNAs, 1 µL of 0.5% phenol red solution, and nuclease-free water. Aim for a final concentration of 400 ng/µL Cas9 protein and 200 ng/µL of the pooled sgRNAs in RNase-free water or a 2:1 ratio of Cas9 protein to sgRNAs in the injected embryo. This injection mixture can be stored on ice for the morning of the injection.

3.3 Remove an injection plate from the refrigerator and let it warm up to room temperature (RT) for at least 20 min.

3.4 After the injection cocktail is assembled, allow the male and female to mate, e.g., by removing the mating box divider or by placing the male in the same egg-laying insert as the female, as appropriate.

3.5 After the fish have laid but before the embryos have been collected, cut the tip of an unbroken needle using a new razor blade or fine forceps to create a needle that has a bore small enough to avoid embryo damage but is wide enough so that it will not become clogged with injection mixture. After the needle has been cut, load the needle with the injection mixture using a microloader pipette tip inserted into the back end of the capillary (see **Tables of Materials**).

3.6 After the needle is filled, incubate the needle for 5 min at RT to assemble Cas9-sgRNA complexes.

3.7 Turn the microinjector on and insert the needle into the micromanipulator. Place a drop of mineral oil onto a micrometer slide and calibrate the needle by adjusting the injection pressure until the needle ejects a 1 nL bolus into the mineral oil.

NOTE: When injecting into the mineral oil, a 1 nL bolus will have a diameter of approximately 0.125 mm (or radius of 0.062 mm) as measured with the micrometer slide.

3.8 To synchronize the embryos, collect them after 10 min using a plastic strainer and rinse them into a Petri dish using 1x E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and add 20 µL of. 0.03 M Methylene Blue per 1 L of 1x E3). Remove 10–15 embryos and place them into a separate Petri dish to be kept as uninjected controls

3.9 Transfer the rest of the developing embryos into the wells of the injection plate.

3.10 Inject 1 nL of solution (a total of 400 pg of Cas9 protein and 200 pg of sgRNAs) into the developing blastodisc of a one-cell embryo. If the tip of the needle becomes clogged, use forceps to break the tip back and recalibrate the needle to eject a 1 nL bolus. Ensure to inject all embryos during the first 40 min of development after fertilization.

3.11 After the injection is completed, return the injected embryos into a labeled Petri dish that contains 1x E3 media and allow them to develop throughout the day. Remove any embryos that are unfertilized or are not developing normally according to the zebrafish staging series[29].

3. Screening for somatic INDELs in F0 injected embryos

NOTE: Other methods for identifying INDELs, such as T7endonuclease I assay or high-resolution melting analysis, can be used when determining if the embryos contain somatic INDELs [30].

4.1 The next day after injections, remove defective and lysed embryos from the Petri dish and replace the 1x E3 media to maintain embryo health.

4.2 After cleaning out the dish, collect six healthy injected embryos and two control embryos from the uninjected plate. Place each embryo individually into a single well of a PCR strip tube and label the top of the tubes.

4.3 To extract the genomic DNA of individual embryos, remove the excess E3 media from the wells of the strip tube and add 100 µL of 50 mM NaOH per well.

4.4 Incubate the embryos at 95 ˚C for 20 min. Then cool the samples down to 4 ˚C, add 10 µL of 1 M Tris HCL (pH 7.5), and vortex them for 5 to 10 s. This extracted DNA can be stored at -20 ˚C for at least 6 months without significant DNA degradation.

4.5 Design unique screening primers for each guide site to amplify a 100–110 bp DNA fragment that includes the CRISPR-Cas9 target site. If possible, place the target site in the middle of the amplified fragment, allowing for the identifications of larger deletions.

4.6 For each of the four guide target sites, set up eight 25 µL PCR reactions using 5 µL of the prepared single-embryo genomic DNA, PCR mix, and the guide-specific screening primers to identify somatic mutations in the target site (**Table 1**).

4.7 Cast a 2.5% agarose/0.5 µg/mL ethidium bromide/TBE gel using combs that create approximately 0.625 cm wide wells. This wide comb allows for better resolution when detecting size changes to the genomic sequence. After the gel has solidified, place it into an electrophoresis chamber that contains TBE running buffer.

4.8 Add 5 µL of 6x loading dye to the PCR product and load 25 µL of this mixture into the gel. Make sure that the injected and control samples are run on the same row of the gel. After all the samples are loaded, add 5 µL of ethidium bromide per 1 L of TBE running buffer to the positive end of the gel box.

4.9 Run the gel at 120 V until the DNA bands resolve or the DNA has approached the end of the lane. If the Cas9 created INDELs in the target site, a smear is typically observed in injected samples but not the controls.

4.10 If smears are observed in a minimum of three out of the four guide sites in embryos injected with four guide RNAs, grow up the sibling injected embryos.

4.11 Whenever the injected samples do not contain smears in the required number of guide sites, design new guide RNAs to replace those that did not work and create a new pool of guide RNAs that includes the ones that worked and the newly designed ones. Inject and test the new pool for somatic INDELs as described above.

5. Identification of maternal-effect phenotypes in maternal crispant embryos

NOTE: Once the injected F0 females have reached sexual maturity, their germline cells have the potential to generate a mixture of maternal crispant and wild-type embryos. Even though this mixture allows for internal controls for fertilization and developmental timing, it is still beneficial to set up a wild-type incross as an external control in case a clutch from F0 female contains only maternal crispant embryos.

5.1 The afternoon before the experiment, set up the F0 injected females against wild-type males and control wild-type crosses in standard zebrafish mating boxes. Place both the male and

female fish in the same tank but separate them with a mating box divider or place the female inside an egg-laying insert.

5.2 On the morning of the experiment, allow the male and female to start mating, e.g., by removing the mating box divider or placing the male in the same egg-laying insert as the female.

5.3 Collect the embryos every 10 min by moving the egg-laying insert into a new mating tank bottom that contains fresh system water and label the tank with a tag identifying the individual F0 female. Take the old mating tank and pour the water through a tea strainer to collect the embryos from one individual 10-min clutch.

5.4 Once the embryos from a single 10-min clutch have been collected in the strainer, transfer them to a Petri dish containing 1x E3 media. Label the Petri dish with the time of collection and the fish information.

5.5 Under a dissecting microscope with a transmitted light source, observe the embryos undergoing development every hour for the first 6–8 h and daily for the next 5 days.

5.6 Identify potential maternal crispant embryos by gross morphological changes in their development compared to time-matched wild-type controls[29].

5.7 Move the potential maternal crispant embryos to a Petri dish that contains 1x E3 media and assay for morphological phenotype at 24 hpf and viability (e.g., swim bladder inflation) at 5 days post fertilization

6. Sequencing alleles in maternal crispant haploids

NOTE: Maternal crispant haploids contain a single allele in the targeted locus, allowing for the identification of INDELs in the target gene via Sanger sequencing. Maternal crispant haploids embryos can also be analyzed using next-generation sequencing assays. Embryos that show a maternal crispant phenotype are expected to carry a lesion in at least one of the four target sites (See Discussion).

6.1 The afternoon before the experiment, set up mating pairs of F0 females known to produce maternal crispant embryos crossed to wild-type males. Keep the wild-type males physically separated from the females using a mating box divider or place the female in the egg-laying insert.

6.2 On the morning of the experiment, remove the physical partition or place both the males and females within the egg-laying insert to initiate mating. At the first sign of egg-laying, interrupt breeding by separating the male and F0 females. Keep each separated F0 female in individual mating boxes.

6.3 Prepare UV-treated sperm solution using testes from one wild-type male for every 100 µL of Hank's solution (**Table 2**), sufficient to fertilize extruded eggs from one female, as previously described[31].

6.4 Manually extrude mature eggs from the pre-selected F0 females and perform *in vitro* fertilization (IVF) using the UV-treated sperm[31].

6.5 After *in vitro* fertilization, allow the haploid embryos to develop until the maternal crispant phenotype is observed and place those embryos into a different Petri dish.

6.6 Once the maternal crispant haploid embryos have been identified, allow them to develop for at least 6 h post-fertilization.

6.7 To extract the genomic DNA from at least ten maternal crispant haploid embryos, place a single haploid embryo into an individual well of a PCR strip tube, remove excess E3 media from the well and add 50 µL of 50 mM NaOH.

6.8 Incubate the embryos at 95 ˚C for 20 min. Then cool the samples down to 4 ˚C, add 5 µL of 1 M Tris HCL (pH 7.5), and vortex for 5–10 s. The extracted DNA can be stored at -20 ˚C for up to 6 months.

6.9 To identify which guide sites contain INDELs, design sequencing primers to amplify a DNA fragment that includes all four CRISPR-Cas9 target sites. These sequencing primers allow for the identification of INDELs that span multiple guide sites.

6.8 Set up two 25 µL PCR reactions per embryo using 5 µL of the prepared genomic DNA and the sequencing primers.

6.9 After the PCR is finished, purify and concentrate the two samples using a DNA clean-up and concentrator kit (see **Table of Materials**). Then submit the DNA fragment to Sanger sequencing using both the forward and reverse sequencing primers.

6.10 After the haploid maternal crispant fragment has been sequenced, align it to the wild-type sequence and identify INDELs in the target sites using a sequence alignment program.

REPRESENTATIVE RESULTS:

The experimental approach described in this protocol allows for the identification of maternal effect phenotypes in a rapid, resource-efficient manner (**Figure 1**).

Generating maternal crispants:

When designing the four guide RNAs to target a single candidate maternal-effect gene, special consideration should be given to where the guide RNAs will bind to DNA. In general, they should all be clustered together with minimal to no overlapping regions between guide RNAs at the start of the first predicted protein domain (**Figure 2A**). Targeting the guide RNAs to this domain

increases the chance that both in-frame and out-of-frame INDELs will affect the protein's function. Other variables that should be considered when designing guide RNAs are cutting efficiency and the number of off-target sites in the genome.

After injecting the CRISPR-Cas9 solution, the somatic activity of the Cas9 can be determined by running a small PCR fragment, approximately 100 bp, on an agarose gel. If INDELs were created in the injected embryo, a smear should be observed in the injected samples but not the uninjected control (**Figure 2B**). Each guide site should be tested independently for Cas9 activity in somatic cells. If smears are seen in at least three guide sites, the sibling injected embryos should be grown up and screened for maternal crispant phenotypes.

Identification of maternal crispants:

To determine if maternal crispants are created in natural crosses, the embryos from an F0 female fish can be compared to time-matched wild-type controls to observe any changes in early development. F0 clutches should also be scored at 24 hpf and 5 days post-fertilization to examine the development of the basic body plan and viability, respectively, to identify maternal factors that could regulate later stages of embryonic development. Identifying a shared phenotype in clutches from different F0 females facilitates distinguishing effects caused by the loss of function of a target gene from off-target or non-specific effects.

Additionally, clutches containing maternal crispants are typically mosaic (i.e., they include both phenotypical wild-type and maternal crispant embryos), allowing wild-type embryos to act as an internal control for variables such as fertilization timing and developmental rate. On average, clutches from F0 females will contain approximately 69% maternal crispant embryos, with clutches containing up to 100% maternal crispant embryos observed[11].

After identifying maternal crispant embryos, they can be used for primary molecular characterization, i.e., immunolabeling for cell boundaries or staining of DNA with DAPI, which can provide insight into the cellular nature of the affected developmental process[11]. The maternal crispant method can also be used to phenocopy known maternal-effect mutations, such as *motley*, *tmi*, and *aura* (**Figure 3**)[11].

Sequencing of maternal crispant haploids:

To identify the genetic lesion(s) that contribute to the maternal crispant phenotype, UV-treated sperm and IVF are combined to create maternal crispant haploids (**Figure 4**). UV-treated sperm provides a centriole but does not contribute paternal DNA, thus permitting cellular division to occur with only maternal genomic material. The creation of a haploid allows Sanger sequencing of the maternal allele and identification of maternal crispant INDELs (**Figure 4**). On average, two alleles per clutch of maternal crispant haploid were observed. The INDELs identified via Sanger sequencing include edits both in single guide sites and deletions spanning multiple guide sites (**Figure 4C**, **Table 3**). A survey of maternal crispant haploid INDELs from different F0 females shows that the same guide sites are edited in multiple embryos, and most of the recovered mutations are premature stop codons (**Table 3**)[11].

FIGURE AND TABLE LEGENDS:

Figure 1: Maternal crispant workflow. To create a maternal crispant, begin by designing four gRNAs that target the first active domain of the gene. 1) Then synthesize the four gRNAs in a single reaction. 2) After synthesizing and purifying the gRNAs, create a CRISPR-Cas9 cocktail and inject it into the blastodisc of a single-cell embryo. 3) Next, screen the injected embryos for somatic mutations using PCR and gel electrophoresis. If INDELs were created in injected samples, a smear would appear in the injected embryos, in contrast to the tight band of the wild-type control. 4) Allow the siblings of the injected embryos to grow for 3-6 months to reach sexual maturity. After sexual maturity is reached, cross an F0 injected female against a wild-type male. The resulting progeny can be a mixture of wild-type and maternal crispant embryos. Identify an F0 injected female whose embryos display the maternal-effect phenotype. 5) To identify the lesions that contribute to the phenotype, IVF is performed using UV-treated sperm to create maternal crispant haploids for Sanger sequencing.

Figure 2: Generation of INDELs in targeted genes. (**A**) Gene structure diagram showing hypothetical protein domains (light and dark purple blocks), location of gRNAs (red lines), and PAM sites (red stars). The gRNAs are targeted to the first active domain. Exons are shown as blocks, and introns are shown as lines. (**B**) Smears in a 2.5% agarose gel are indicative of INDELs in somatic cells in injected embryos.

Figure 3: Maternal crispants recapitulate the phenotype of known maternal-effect mutations. Representative comparison of live, time-matched wild-type (left column), known maternal mutants (middle column), and maternal crispant embryos (right column). (**A**) *motley*/*birc5b*, (**B**) *tmi*/*prc1l*, (**C**) *aura*/*mid1ipIl* mutants/maternal crispants show defects in cytokinesis in early embryonic divisions, leading to fully syncytial blastula (**A**, **B**), or partially acellular embryos (**C**, white box).

Figure 4: Using maternal crispant haploids to sequence CRISPR-Cas9-induced mutations. (**A**) An F0 injected female crossed against a wild-type male results in a diploid embryo with a maternal effect phenotype. (**B**) IVF is performed using UV-treated sperm to create maternal crispant haploids, allowing for the sequencing and analysis of induced INDELs in the maternal allele. (**C**) Representative sequencing of *birc5b* maternal crispant haploid showing a large deletion between guide sites 3 and 4 (boxed).

Table 1: PCR mix.

Table 2: Hank's solution.

Table 3: The location and type of INDELs found in two different sets of maternal crispant haploids*: birc5b* and *prc1l*.

DISCUSSION:

The protocol presented in this manuscript allows for the identification and primary molecular characterization of a maternal-effect phenotype in a single generation instead of the multiple

generations required for both forward and reverse genetic techniques. Currently, the role of many maternally expressed genes is unknown. This lack of knowledge is partly due to the extra generation required to visualize a phenotype when identifying maternal-effect genes. In the past, the rapid identification of maternal-effect genes in zebrafish could be achieved by injecting translation-blocking morpholino oligonucleotides into cultured oocytes[32]. This method was proven successful by phenocopying multiple known maternal-effect genes, but manipulating an immature oocyte can be a delicate, time-consuming experiment. Maternal RNAs can also be targeted for degradation using CRISPR-RfxCas13d complexes, but the injection of these complexes into the one-cell embryo cannot target maternally provided protein[33]. More recently, it has been discovered that CRISPR-Cas9 can induce biallelic mutations in the germline, allowing for the rapid identification of novel maternal-effect genes in a single generation[11].

This protocol includes several critical steps that contribute to the recovery of maternal crispant embryos. In theory, because germ cells are specified in early embryonic development, the earlier a DNA lesion is created in a target gene, the higher the probability that a cell containing mutations will become part of the germline. This method uses Cas9 protein injected into the developing blastodisc of a one-cell embryo to increase the probability of edits in the germline. Another critical factor that affects the percentage of recovered maternal crispant embryos is the efficiency of the guide RNAs in creating genetic edits in target sites. This procedure includes a section on determining the ability of guide RNAs to create somatic INDELs at 24 hpf by PCR. If a guide RNA fails to make somatic edits, it has a low probability of producing edits in the germline at a high enough rate to generate a maternal crispant. This protocol directs the user to test somatic edits, which should be visible in three or more guide sites.

After observing the phenotype of maternal crispant embryos, the genetic lesion(s) contributing to the phenotype can be analyzed at the sequence level via IVF with UV-treated sperm. To acquire enough starting material for PCR, maternal crispant haploid embryos should develop for at least six to eight hours, allowing multiple cycles of DNA replication to occur. The genomic DNA should also be extracted in 50 µL of 50mM NaOH to concentrate the DNA. If the maternal crispant haploid embryos cannot survive for 6–8 h, collect the embryos at an earlier time point. To account for the embryo undergoing fewer cycles of DNA replication, extract the DNA in a smaller volume of 50 mM NaOH while maintaining the same proportion of NaOH and Tris-HCL. Another option is to concentrate the extracted DNA by using a DNA clean-up and concentrator kit. After the DNA has been extracted, the PCR fragment used for sequencing should include all four target sites, if possible. This fragment will allow for the identification of large deletions that span multiple guide sites in maternal crispant embryos.

When collecting maternal crispant haploids for Sanger sequencing, collect all the haploids that show the phenotype and send a minimum of 10 unique haploid embryo samples to Sanger sequencing. The sequencing of multiple haploid embryos per clutch will allow for the identification of multiple INDELs found in the germline. Past sequencing data of maternal crispant haploids have shown that multiple alleles can be identified in a set of maternal crispant haploids[11]. However, these alleles are not recovered in the expected 1 to 1 ratio[11]. The sequencing of multiple embryos will also help support the idea that the phenotype is caused by

a CRISPR-Cas9 INDEL in the target gene. Any wild-type sequence observed in sequenced haploid embryos that show a specific phenotype will suggest that the phenotype is not associated with the targeted gene. Any novel maternal-effect phenotypes identified through targeting previously uncharacterized genes should also be confirmed by establishing a stable line using the sibling F0 males[11].

In some cases, it may be challenging to identify INDELs via haploid analysis where the identified maternal crispant phenotype has a certain phenotypic characteristic. For example, maternal crispant haploid embryos that appear to be unfertilized or lysis during the cleavage stage may be impossible to select maternal crispant haploids. Maternal crispant embryos with axis extension defects similar to those corresponding to the haploid syndrome may also not be distinguishable for analysis when generating maternal haploids[34]. In such cases, the researcher is advised to conduct the analysis directly using stable lines for gene-phenotype confirmation.

One limitation of the current maternal crispant protocol is that it only identifies maternal-effect genes by gross morphological defects. To increase the method's sensitivity and make it more specific for certain aspects of early development, transgenic reporters could be used to highlight specific structures or cell types, as has been done for other screens[9,10,35]. For example, the CRISPR-Cas9 mixture could be injected into the Buc-GFP transgenic line to identify non-lethal genes that regulate the formation and development of primordial germ cells[36]. Another limitation of the maternal crispant protocol is that, though useful for genes solely expressed during early development, it may not be effective for genes with both maternal and zygotic function since the CRISPR-Cas9 targeting of the gene could be lethal to the developing embryo. To study the maternal function of genes expressed throughout development, Cas9 activity could be targeted to the germline[37], thus, leaving the somatic function of the gene unaffected and permitting the survival of the F0 females to adulthood.

Maternal crispants are an effective tool to identify novel maternal-effect genes necessary for early development. The combination of multiplexing guide RNAs to a single target and the biallelic editing ability of Cas9 allows the maternal-effect phenotype to be observed in a single generation, avoiding multi-generation breeding schemes typically needed when performing targeted gene editing. The bypassing of multiple generations decreases the amount of space and resources necessary to identify maternal-effect genes.

In addition to identifying new maternal-effect genes, this protocol permits any zebrafish laboratory to phenocopy and study any known maternal-effect mutant without establishing and maintaining stable lines, facilitating detailed analysis of genetic pathways. In principle, this maternal crispant approach can also determine the function of maternal products in non-genetic model species.

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DISCLOSURES:

The authors declare no competing financial interests.

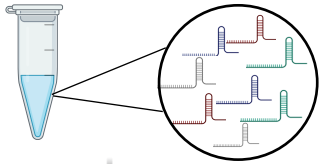
REFERENCES:

1. Pelegri, F. Maternal factors in zebrafish development. *Developmental Dynamics*. **228** (3), 535–554 (2003).
2. Campbell, P. D., Heim, A. E., Smith, M. Z., Marlow, F. L. Kinesin-1 interacts with Bucky ball to form germ cells and is required to pattern the zebrafish body axis. *Development (Cambridge, England)*. **142** (17), 2996–3008 (2015).
3. Eno, C., Solanki, B., Pelegri, F. aura (mid1ip1l) regulates the cytoskeleton at the zebrafish egg-to-embryo transition. *Development (Cambridge, England)*. **143** (9), 1585–1599 (2016).
4. He, W. -X. et al. Oocyte-specific maternal Slbp2 is required for replication-dependent histone storage and early nuclear cleavage in zebrafish oogenesis and embryogenesis. *RNA (New York, N.Y.)*. **24** (12), 1738–1748 (2018).
5. Burger, A. et al. Maximizing mutagenesis with solubilized CRISPR-Cas9 ribonucleoprotein complexes. *Development (Cambridge, England)*. **143** (11), 2025–2037 (2016).
6. Jao, L. -E., Wente, S. R., Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences of the United States of America*. **110** (34), 13904–13909 (2013).
7. Shah, A. N., Davey, C. F., Whitebirch, A. C., Miller, A. C., Moens, C. B. Rapid reverse genetic screening using CRISPR in zebrafish. *Nature Methods*. **12** (6), 535–540 (2015).
8. Shankaran, S. S., Dahlem, T. J., Bisgrove, B. W., Yost, H. J., Tristani-Firouzi, M. CRISPR/Cas9-directed gene editing for the generation of loss-of-function mutants in high-throughput zebrafish F0 screens. *Current Protocols in Molecular Biology*. **119** (1), 31.9.1–31.9.22 (2017).
9. Trubiroha, A. et al. A Rapid CRISPR/Cas-based mutagenesis assay in zebrafish for identification of genes involved in thyroid morphogenesis and function. *Scientific Reports*. **8** (1), 5647 (2018).
10. Wu, R. S., Lam, I. I., Clay, H., Duong, D. N., Deo, R. C., Coughlin, S. R. A Rapid method for directed gene knockout for screening in G0 zebrafish. *Developmental Cell*. **46** (1), 112–125.e4 (2018).
11. Moravec, C. E., Voit, G. C., Otterlee, J., Pelegri, F. Identification of maternal-effect genes in zebrafish using maternal crispants. *Development (Cambridge, England)*. **148** (19), dev199536 (2021).
12. Braat, A. K., Zandbergen, T., van de Water, S., Goos, H. J., Zivkovic, D. Characterization of zebrafish primordial germ cells: morphology and early distribution of vasa RNA. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*. **216** (2), 153–167 (1999).
13. Eno, C., Hansen, C. L., Pelegri, F. Aggregation, segregation, and dispersal of homotypic germ plasm RNPs in the early zebrafish embryo. *Developmental Dynamics*. **248** (4), 306–318 (2019).
14. Knaut, H., Steinbeisser, H., Schwarz, H., Nüsslein-Volhard, C. An evolutionary conserved region in the vasa 3'UTR targets RNA translation to the germ cells in the zebrafish. *Current biology: CB*. **12** (6), 454–466 (2002).

15. Yoon, C., Kawakami, K., Hopkins, N. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development (Cambridge, England)*. **124** (16), 3157–3165 (1997).
16. Gagnon, J. A. et al. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLoS ONE*. **9** (5), e98186 (2014).
17. Labun, K., Montague, T. G., Gagnon, J. A., Thyme, S. B., Valen, E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Research*. **44** (W1), W272–W276 (2016).
18. Labun, K., Montague, T. G., Krause, M., Torres Cleuren, Y. N., Tjeldnes, H., Valen, E. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research*. **47** (W1), W171–W174 (2019).
19. Montague, T. G., Cruz, J. M., Gagnon, J. A., Church, G. M., Valen, E. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Research*. **42** (W1), W401–W407 (2014).
20. Moravec, C. E., Pelegri, F. J. An accessible protocol for the generation of CRISPR-Cas9 knockouts using INDELs in zebrafish. *Methods in Molecular Biology (Clifton, N.J.)*. **1920**, 377–392 (2019).
21. White, R. J. et al. A high-resolution mRNA expression time course of embryonic development in zebrafish. *eLife*. **6**, eLife.30860 (2017).
22. Aanes, H. et al. Zebrafish mRNA sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition. *Genome Research*. **21** (8), 1328–1338 (2011).
23. Aken, B. L. et al. The Ensembl gene annotation system. *Database: The Journal of Biological Databases and Curation*. **2016**, baw093 (2016).
24. Sorlien, E. L., Witucki, M. A., Ogas, J. Efficient production and identification of CRISPR/Cas9-generated gene knockouts in the model system Danio rerio. *Journal of Visualized Experiments: JoVE*. **138**, 56969 (2018).
25. Kotani, H., Taimatsu, K., Ohga, R., Ota, S., Kawahara, A. efficient multiple genome modifications induced by the crRNAs, tracrRNA and Cas9 protein complex in zebrafish. *PloS One*. **10** (5), e0128319 (2015).
26. Rosen, J. N., Sweeney, M. F., Mably, J. D. Microinjection of zebrafish embryos to analyze gene function. *Journal of Visualized Experiments: JoVE*. **25**, 1115 (2009).
27. Xu, Q. Microinjection into zebrafish embryos. *Methods in Molecular Biology (Clifton, N.J.)*. **127**, 125–132 (1999).
28. JoVE Science Education Database. *Biology II: Mouse, Zebrafish, and Chick*. *Zebrafish Microinjection Techniques*. JoVE, Cambridge, MA, (2021).
29. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F. Stages of embryonic development of the zebrafish. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*. **203** (3), 253–310 (1995).
30. D'Agostino, Y. et al. A rapid and cheap methodology for CRISPR/Cas9 zebrafish mutant screening. *Molecular Biotechnology*. **58** (1), 73–78 (2016).
31. Baars, D. L., Takle, K. A., Heier, J., Pelegri, F. Ploidy manipulation of zebrafish embryos with Heat Shock 2 treatment. *Journal of Visualized Experiments: JoVE*. **118**, 54492 (2016).
32. Nair, S., Lindeman, R. E., Pelegri, F. In vitro oocyte culture-based manipulation of zebrafish maternal genes. *Developmental Dynamics: An Official Publication of the American Association of*

Anatomists. **242** (1), 44–52 (2013).
33. Kushawah, G. et al. CRISPR-Cas13d induces efficient mRNA knockdown in animal embryos. *Developmental Cell*. **54** (6), 805-817.e7 (2020).
34. Kroeger, P. T., Poureetezadi, S. J., McKee, R., Jou, J., Miceli, R., Wingert, R. A. Production of haploid zebrafish embryos by in vitro fertilization. *Journal of Visualized Experiments: JoVE*. **89**, 51708 (2014).
35. Xiao, T., Roeser, T., Staub, W., Baier, H. A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. *Development (Cambridge, England)*. **132** (13), 2955–2967 (2005).
36. Riemer, S., Bontems, F., Krishnakumar, P., Gömann, J., Dosch, R. A functional Bucky ball-GFP transgene visualizes germ plasm in living zebrafish. *Gene Expression Patterns: GEP*. **18** (1–2), 44–52 (2015).
37. Moreno-Mateos, M. A. et al. CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nature Methods*. **12** (10), 982–988 (2015).

1. Synthesis of gRNA (1 day)



2. Microinject CRISPR-Cas9 Cocktail into One-Cell Embryo (2h)

3. Screen for INDELs (5h)

4. Grow Injected Embryos to Sexual Maturity (3-6 months)

Identify Maternal Crispant Phenotypes (1 day)

Wild Type

Maternal Crispant

5. Creation and Sequencing of Maternal Crispant Haploids (2 days)

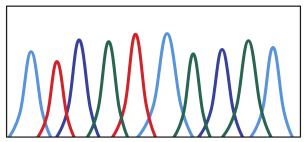
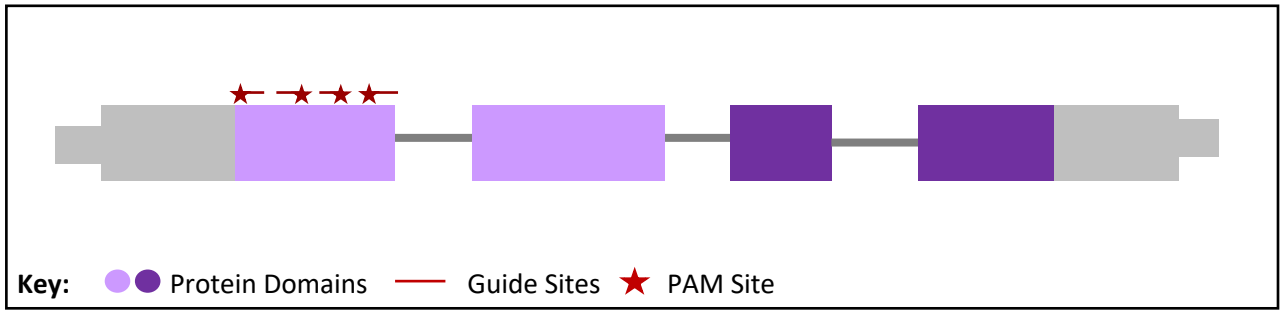


Figure 1

Figure 2

A Structure of Targeted Gene



B INDEL Identification via Gel Electrophoresis

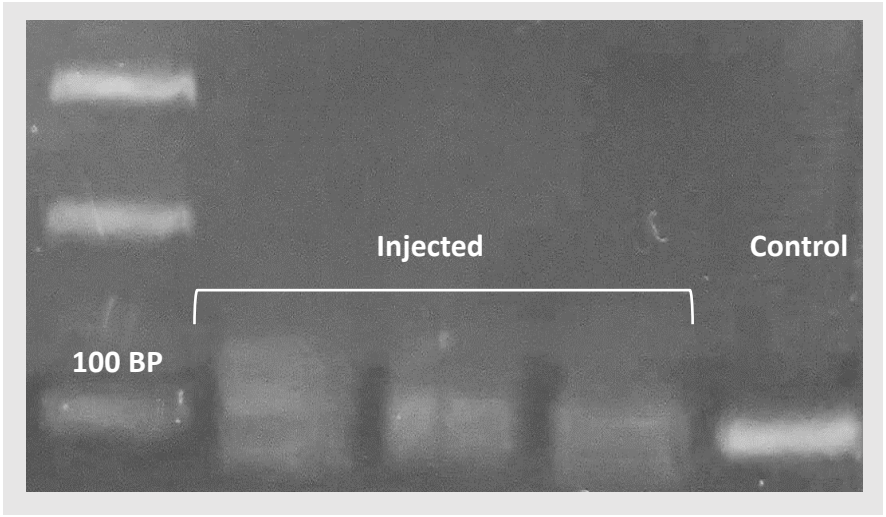


Figure 2

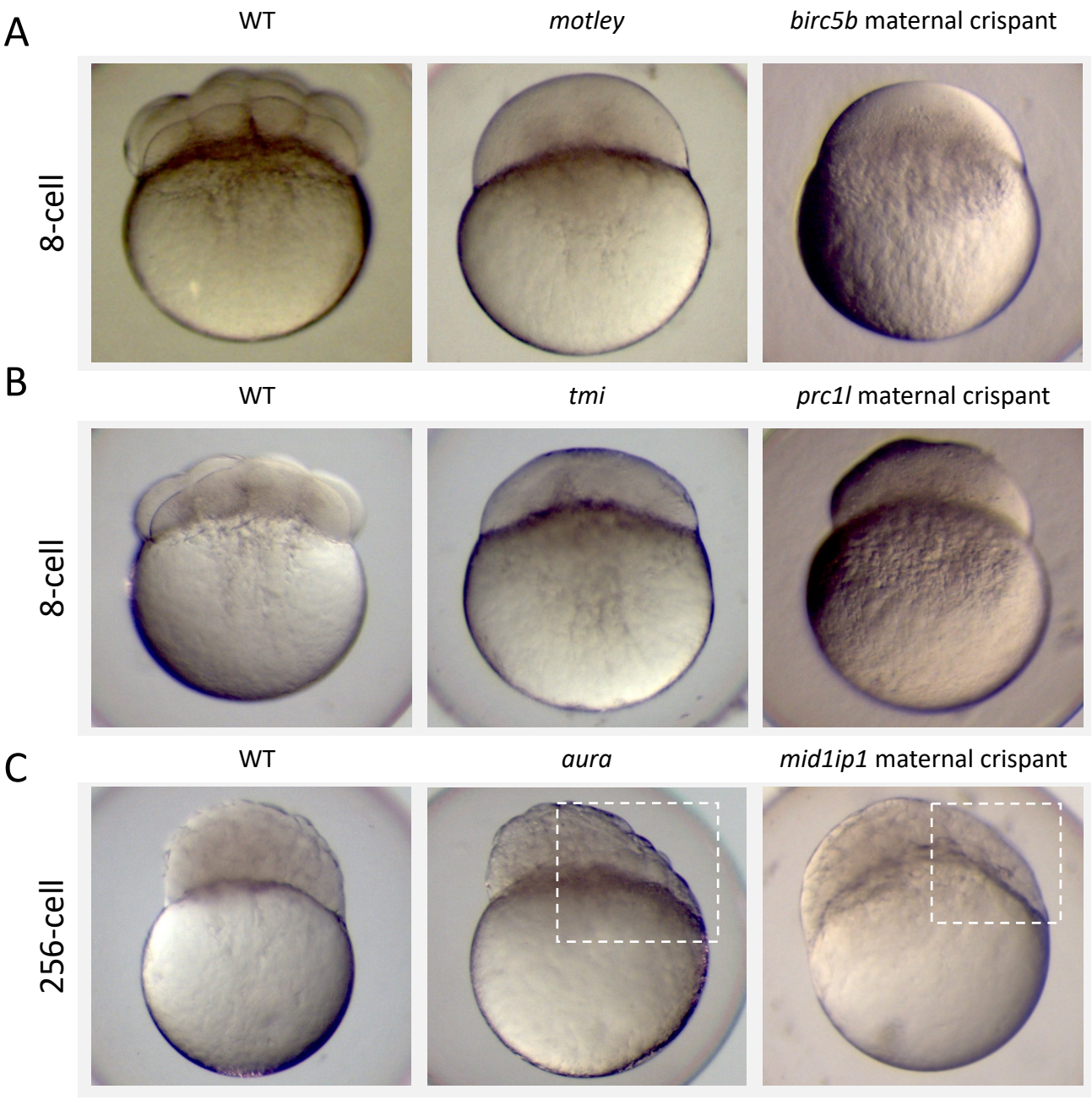


Figure 3

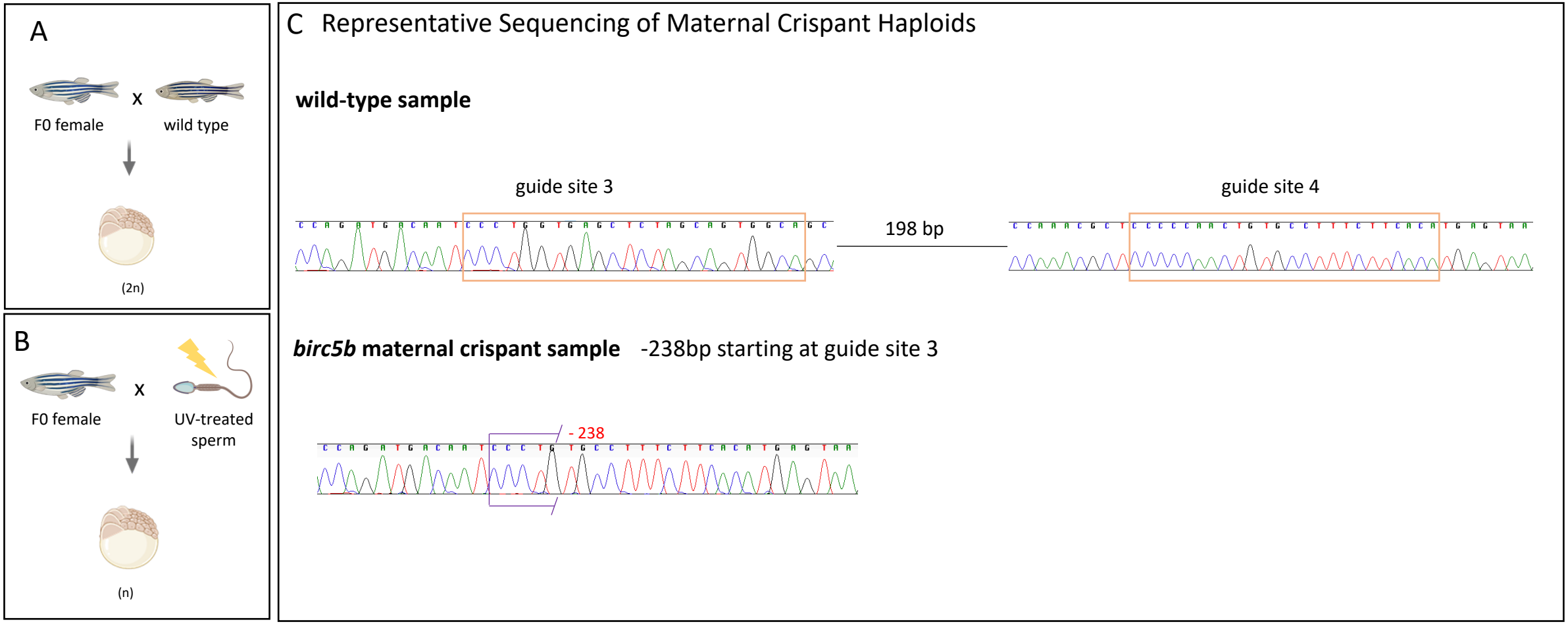


Figure 4

Table 1

Click here to access/download;Table;Table 1- PCR mix updated.xlsx

PCR Mix

Add		
	sterile H_2O	171.12 mL
	$MgCl_2$ (1 M)	0.393 mL
	Tris-HCl (1 M, pH 8.4)	2.618 mL
	KCl (1 M)	13.092 mL
Autoclave for 20 min, then chill the solotion on ice. Next add		
	BSA (100 mg/mL)	3.468 mL
	dATP (100 mM)	0.262 mL
	dCTP (100 mM)	0.262mL
	dGTP (100 mM)	0.262 mL
	dTTP (100 mM)	0.262 mL
Alliquot into sterile microcentrifuge tubes		
PCR Recipe		per sample
	PCR Mix	17.9 µL
	F+ R Primer (10 µM)	0.2 µL
	RoH2O	1.8 µL
	Taq DNA Polymerase	0.1 µL
	DNA	5 µL

Table 2

Hank's Premix
Hank's Stock Solution #1
Hank's Stock Solution #2
Hank's Stock Solution #4
Hank's Stock Solution #5
Hank's Stock Solution #6
Hank's Final Working Solution

Hank's Solution
Combine the following in order: (1) 10.0 mL of HS #1, (2) 1.0 mL of HS#2, (3) 1.0 mL of HS#4, (4) 86 mL of ddH2O, (5) 1.0 mL of HS#5. Store all HS Solotions at 4 °C
8.0 g of NaCl, 0.4 g of KCl in 100 mL of ddH_2O
0.358 g of Na_2HPO_4 anhydrous; 0.60 g of $K_2H_2PO_4$ in 100 mL of ddH_2O
0.72 g of $CaCl_2$ in 50 mL of ddH_2O
1.23 g of $MgSO_4 \cdot 7H_2O$ in 50 mL of ddH_20
0.35 g of $NaHCO_3$ in 10.0 mL of ddH_20; make fresh on the day of use
Combine 9.9 mL of Hank's Premix with 0.1 mL of HS Stock #6

Table 3 Click here to access/download;Table;Table 3- INDELs.xlsx

	birc5b #1	*birc5b* #2	*birc5b* #3
Total number of embryos sequenced	3	9	12
Total number of embryos with INDELs	3	9	12
Mutation in one site	0	0	0
Mutations in multiple sites	3	9	12
Location of INDELs			
Guide site 1	0	0	0
Guide site 2	0	0	0
Guide site 3	3	9	12
Guide site 4	3	9	12
Types of INDELs			
In-frame mutation	1	2	2
Frame shift mutation	4	7	10

prc1l #1	*prc1l* #2
8	10
8	10
0	0
8	10
0	0
8	10
8	10
0	10
7	10
9	10

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript to ensure there is no spelling or grammar errors.

2. Please provide an institutional email address for each author.

This information has been added

3. Please revise the following lines to avoid previously published work:

We have revised the following lines: 131-132 ,179-180, 202-204,304-307,344-346.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the text to avoid personal pronouns

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: DNA Clean & Concentrator kit, MEGAshortscript T7 Transcription kit, NanoDrop, etc.

We have removed commercial language from the manuscript

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

We have adjusted the numbering system and revised to avoid bullets and dashes

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
The text has been revised to use the imperative tense

8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

We have moved the discussion about the protocol to the discussion section.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step (e.g., Section 1(step 8, 9), Section 2 (step 3), Section 3 (step 2,4,5,6,7,8), Section 4 (step 3, 7,8), Section 5 (step 3,4), etc.)

We have simplified the steps as requested.

10. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm.

We have changed the text to use standard abbreviations

11. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

We have changed the text to use abbreviations for durations as requested

12. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have revised the manuscript to make sure that all steps will be suitable for generating a script.

13. Line128-128: Is the purification and concentration based on the manufacturer’s protocol? Please specify if any changes were made.

The purification and concentration steps are according to the manufacturer's instructions. We have added this information to the step

14. Line 253: How are the embryos confirmed as inviable?
Inviable embryos are defective or lysed. We have revised the text and additionally added to remove the remove defective and lysed embryos from the petri dish.

15. The highlighting in this version of the manuscript exceeds the 3-page limit (please limit it to <3 pages). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences)? Please ensure that the highlighted part of the step includes at least one action that is written in the imperative tense.

We have reduced the highlighted text and make sure it has a cohesive narrative with a logical flow.

16. Figure 1: Please revise "hours" to "h".

As suggested, we have changed hours to h In figure one.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This interesting manuscript by Moravec et al. describes an alternative strategy to obtain maternal mutants in a rapid and straightforward crispant way. Although unable to obtain maternal mutants of zygotic lethal genes, this method can be used for functional screenings of maternal genes that specifically control early embryonic development in zebrafish. So it will have broad interest to the zebrafish community. In general, the method is well presented. However, before publication, the authors should carefully address several important issues.

Major Concerns:

1. The haploid genotyping method is insufficient "to present INDELs in the germline that contribute to the phenotype." Because the maternal products are generated in the diplotene stage of primary oocytes. At this stage the homologous chromosomes have not separated, thus, the mutant products contributing to the phenotype are supplied by diploid genome. The author states that only one of the two alleles passes to the embryo and can be identified by sequencing in maternal crispant haploids. That means the information

obtained by haploid sequencing is not adequate to reveal all the mutation events that occurred in a maternal mutant embryo.

We have edited the text to specify to test multiple (10) haploid embryos to gain statistical confidence that the (diploid) germ line producing the phenotypically mutant haploid embryos only contains mutated alleles and no wild-type ones. We also clarify that this method is most effective to recreate an already known phenotype and for preliminary gene-phenotype association and that, in the case of potential new phenotypes, the phenotype must be confirmed using CRSIPR-derived stable lines.

2. The authors used the haploid genotyping method to examine genomic lesions in embryos with apparent defective phenotypes as the proof of principle. Suppose mutation of a maternal gene does not produce a distinguishable morphological phenotype (PGCs defect, for example). In that case, this genotyping strategy seems risky if applied to identify maternal mutant embryos because heterozygous mutant oocyte can pass its mutant allele to the embryo. This embryo is, however, not the maternal mutant.

We agree with this assessment. Indeed, the strategy as presented depends on the identification of a mutant maternal-effect phenotype in the embryo. Such a phenotype could be in the embryo's somatic tissue or (if assayed with appropriate markers) in the germline (PGCs). If there is a maternal-effect phenotype in PGCs, the embryos could be analyzed in the same way as with mutations affecting somatic tissue, using PGC markers such as a Buc-GFP transgenic line, in situ hybridization, or other methods, and again with full confirmation of the gene-function relationship requiring the analysis of a stable, germ line-transmitted mutation. To address the concern that a mutant allele could be found in individuals from heterozygous oocytes, we have edited the text to specify to test multiple (10) haploid embryos to gain statistical confidence that the (diploid) germ line producing the phenotypically mutant haploid embryos only contains mutated alleles and no wild-type ones.

3. There is a concern about the efficiency in inactivating sperm by UV treatment. In Figure 4C, sample 2, double peaks appear in the chromatogram data, indicating the existence of a wild-type allele. Theoretically, as only one of the maternal mutant alleles transmitted to the haploid embryo, no wild-type genome should be detected. All chromatogram data should be single-peaked. It is thus possible that the wild-type allele is from the sperm not inactivated adequately by the UV treatment.

In Figure 4c, there are some background peeks most apparent at the end of the sequenced fragment. We believe that the lower peaks in figure 4c (birc5b maternal crispant sample 2) correspond to the background found at the sequencing reaction's end. We do not believe these smaller peaks correspond to the wild-type allele because the sequence resulting from

these smaller peaks does not match the wild-type reference genome. To prevent confusion, we have removed the *birc5b* maternal crispant sample 2 from the 4c panel of the figure.

Minor Concerns:
1. Haploid embryos exhibit systematic developmental defects in zebrafish (so-called haploid syndrome). Is it easy to distinguish the specific maternal mutant phenotype from the defects of haploid syndrome?

The maternal crispant phenotype is first identified in diploid embryos and only later for DNA analysis in haploid embryos. Additionally, many if not most maternal-effect phenotypes appear prior to somitogenesis, prior to the time when the haploid syndrome becomes apparent. We have added a clarification in the discussion to state that any maternal crispant phenotypes observed in diploid embryos similar to those corresponding to the "haploid syndrome" may be difficult to identify for DNA analysis in a haploid background. In such cases, the user can generate a stable line directly for gene-phenotype confirmation.

2. There is an error in line 233, where 0.125 mm should be 0.062 mm.

We have verified the sentence so that it presents the correct diameter and volume: 0.125 mm diameter (0.062 mm radius also included) corresponding to roughly 1 nl volume.

3. In the discussion section, the authors should compare their crispant method with other strategies to generate maternal mutants.
We expanded the discussion to compare to other forward and reverse genetics approaches.

Reviewer #2:
Manuscript Summary:
Not exactly groundbreaking - similar results regarding roles of maternally expressed genes can be obtained with previous methods but the manuscript does provide a method that will speed up studies.

Major Concerns:
None really.

Minor Concerns:
For a protocol of this kind it would be useful with references to alternative common methods for similar tasks. For example, haploids and sanger sequencing can't be the only ways to identify indels -(e.g. paralell sequencing or FLA). Alternative methods doesn't have to be described in detail of course, just as references for readers.

We have added a note to step 6- sequencing alleles in maternal crispant haploids - that these haploid embryos can also be sequenced by next generation sequencing assays.

Row/Figure Comment

90 Ethical permit number?

We have added our Wisconsin-Madison Institutional Animal Care and Use Committee Permit number to the start of the protocol

132, 134 Add TM (MEGAshortcriptTM....)

To our understanding, Jove policy states not to include the TM, although we are happy to modify the text according to editorial guidelines

179-181 I would not recommend to store a needle in the needle holder over a longer period.

We have modified the text to state to pull the needle the afternoon before the injection.

261 100 ul NaOH to lyse embryos seems a bit too much. 30 ul are usually used for clipped fins and we use 15ul for embryos.

This protocol uses a higher amount of NaOH. because the reader will have to do all four sets of PCR reactions to look at the somatic activity at each target site in the same embryo. This higher volume provides plenty of backup DNA in case of PCRs failures in the event that the sample needs to be verified by sequencing.

In the discussion it says in row 498 that genomic DNA of embryos can also be extracted in 50 ul to concentrate the DNA. But as said above I would recommend ever less volume.

Resuspending the maternal crispant haploid embryos in 50 ul of NaOH allows for multiple PCRs to be run without worrying about running out of sample. As IVF is hard on the females, we aim

to create a protocol that wouldn’t require multiple IVF procedures to get a high number of samples.

266 Shouldn't primers have been designed earlier in the protocol?

Oligos for gRNA production prior to the production of maternal crispants are indeed described early in the text, in section one. This section (4.4.5) corresponds to analysis after the production of maternal crispants, to generate primers to produce a PCR fragment for the analysis of INDELS. Each set of primers has been placed in the protocol in a location that corresponds to the step in which they are used. However, we will be happy to revise this position with further editorial guidance

397 Why not FLA but gel?

This protocol uses a gel to screen for somatic mutations because it is a cost-efficient way to visualize somatic activity, and a majority of molecular labs will have the supplies on hand. We have added a note and reference stating that other methods can be used to screen for somatic INDELs.

Figure 3 Labels are not really symmetrically centered

The labels in Figure 3 have been symmetrically centered.

Figure 4 In both panel A and B there is a lot of space. Would be good to enlarge the images and use the free space.

We have adjusted the spacing of Figure 4.