Identification of maternal-effect genes in zebrafish using maternal crispants

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MS TITLE: Identification of Maternal-Effect Genes in Zebrafish using Maternal Crispants

AUTHORS: Cara E Moravec, Gabriella C Voit, Jarred Otterlee, and Francisco Pelegri

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some criticisms and suggestions for improvements to your manuscript. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Moravec et al present a clear and accessible description of a clever method to efficiently assay the essential function of essential maternal effect genes by CRISPR mutation. They describe a method to identify likely candidate genes for analysis by examining gene expression patterns. They demonstrate that these genes can be mutated and consistent mutant phenotypes recovered efficiently enough for analysis of the resulting phenotype without a 3 generation inbreeding strategy. They demonstrate proof of principle by testing genes with known mutant phenotypes as well as new genes that yield novel phenotypes demonstrating that the method is likely to yield novel insights. Furthermore, they generate haploids to investigate the preferential use of some guide RNAs over others and see a allelic transmission distortion which is interesting and important to document even if the mechanism is not clear yet.

Comments for the author

I have only a couple of minor concerns intended to improve accessibility and impact.
One addition to support this as a technique paper would be a statement of generalizability. With current transcript data sets, can the authors provide an estimate of how many genes fit the expression criteria of genes with primarily maternal expression? Just a ball park figure would go a long way. Is it 10 or is it 1000?
Would other labs be interested or will the Pelegri lab have them all mutated by the time this paper comes out?
Although it is intended to be a technique paper, the data presentation should be accessible. The frames of the supplemental movies of kpna7 and control should be placed side by side in a single movie so the differences can be appreciated. It's challenging to run them side by side. Further annotations can be added to show the cells missing nuclei. I suggest make the movie 2 loops one without cells missing nuclei indicated and one with.

Reviewer 2

Advance summary and potential significance to field

In this work, the authors validate a reverse genetic technique to identify and characterize maternal-effect genes using adult zebrafish females who have had their genomes directly edited during embryogenesis. In the process of validating this forward genetic pipeline, the authors identified two maternal-effect genes, kpna7 and fhcd3. Using this three-part approach, a researcher can identify prospective maternal-effect genes and the edited sequence in a single generation, saving both time and resources. This makes identifying maternal-effect genes more accessible to researchers and would thus move the field further along. Maternal-effect genes are important for developmental competence and embryogenesis, which makes this manuscript of broad interest to the developmental biology community and of interest to Development readers.

The work presented would make an important contribution to identifying maternal-effect genes that are critical early in development. The research presented was clear and easy to follow. However, there are some concerns that weaken the strength of the manuscript. Satisfactory efforts to resolve these concerns would make the manuscript suitable for publication in Development.

Comments for the author

Major:
1) In the Material and Methods section, the concentration of guide RNA and Cas9 protein are a lot lower than what other groups have published using. It is also lower than what is reported in Moravec and Pelegri, 2019. Assuming a 1 nl injection, the authors would have injected only 0.4 pg of guide RNA and 0.2 pg of Cas9 protein. Did the authors mean to use the units pg/nl instead of pg/µl?

2) In the text in referring to Fig 2I, the authors state "mid1ipil maternal crispants ……with B-catenin present in unreleased cortical granules and unavailable for recruitment to the furrow (Figure 2I)." How do the authors know that the vesicle-like staining is from cortical granules or that the lack of their release (most appear to have been released) causes the loss of B-Catenin at the furrow? The

figure legend suggests that a previous publication showed that. If so, please reference it. If not, please provide more information that substantiates it.

3) The authors use the phrase “data not shown” several times. It would be best to include the data especially when it is in reference to the description of a phenotype, as in the characterization of the fhdc3 maternal crispants.

4) The authors suggest that fhdc3 may regulate actin dynamics, but they do not look at actin in the fhdc3 crispants. Given the maternal-effect phenotypes associated with the edited fhdc3 alleles, the authors should assess whether there are any actin cytoskeletal irregularities in fixed fhdc3 crispants or stable lines by using an actin stain.

5) Did the authors observe any developmental delays in the fhdc3 crispants? The images for dome stage embryos and the 1 dpf embryos in Figure 6A and B suggest there might be a delay. Also, a picture of the full 1 dpf embryo should be included in Figure 6 since there appears to be a difference between WT and fhdc3 maternal crispants that might extend into the tail.

6) The authors state on p 14 “DNA lesions observed in single haploid maternal crispants represent a mixture of induced alleles, potentially including unique lesions from different germ cell precursors in the mosaic F0 female germ line.” This is a bit confusing and requires clarification. A single haploid embryo should not have a mixture of alleles from different germ cell precursors. A single haploid is derived from one oocyte and as a haploid will have only one mutant allele, although that allele may be caused by multiple different CRISPR-induced mutations due to the multiplexing, correct? Since it was diploid, it could of course have multiple different alleles at the RNA level, derived from the 2nd allele. But at the DNA level, it should not. I expect that the authors would agree, it is just a bit confusing as written.

7) The authors confirmed the kpna7 crispant phenotypes with the stable kpna7uw107 line, but they do not mention the phenotype of any other stable line from the other kpna7 alleles. Was the maternal-effect phenotype the same for the stable line of other kpna7 allele(s)?

8) Since the germ line is derived from multiple cells at 4 distinct locations in the embryo, the observation of non-equal proportions of two alleles through the germline may be related to the frequency of particular mutations induced and the proportion of the germ line overall that a given germ cell gives rise to. Additionally when a lesion is made in a germ cell, the number of other germline precursors exist at that time. If a particular allele is only generated at the point of 8 germ cells, then the lesion may only be present in 1 of 16 haploid progeny, for example. Identical CRISPR lesions may also be made in independent germ cells, which could contribute also to unequal frequencies in progeny. The Discussion points made on page 17-18 about ratios of alleles found in haploids is not clear to this reviewer.

Minor:
1) Misspelled crispant in Figure 5D.
2) Misspelled transcript on the y-axis title in Figure 3A.
3) Nair et al, submitted, cannot be used as a reference 4) Table 4, since protein size is not directly tested, it is better to state “Average predicted protein length”

Reviewer 3

Advance summary and potential significance to field

The manuscript “Identification of Maternal-Effect Genes in Zebrafish using Maternal Crispants” reports on the use of CRISPR-Cas9 for the generation of fish that reveal maternal-effect phenotypes in Zebrafish in Go.
The results presented in the paper are an extension of a known method for rapid generation of zygotic mutations where the same technology is employed. Being aware of this possibility is of interest for readers of the Journal, and would facilitate following new research paths. Analysis of maternal-effect mutations in zebrafish is a somewhat neglected field due the extensive fish breeding involved, a point the presented findings solve to a certain degree.

Comments for the author

In general, the results are convincing and the documentation level is very high.
My general suggestion would be to provide readers with a very concise presentation of the method- How the authors suggest choosing genes for analysis using their approach, the practical details of how to perform the procedure and how to check the results. Mutants generated in this work can be presented as a proof of principle, but that should be limited to the molecular analysis and a very brief presentation of some of the phenotypes. As is, the description of so many rather unrelated phenotypes "masks" the method, making it more difficult to follow. There is no real take-home message concerning the Biology and the mutants should be analysed more deeply and presented in future papers.
If such a revision path is followed, I believe a short paper presenting the option the authors suggest would fit the Journal as a "Resource" paper.
Specific points in order of appearance in the manuscript-
1. The first paragraph of the introduction should better be removed. The paper would primarily be read by people who already work with zebrafish and others that are highly likely to be familiar with the "rapid transparent development" etc. . One does not include such "motivation paragraphs" for other mainstream vertebrate or invertebrate models, and it would be good if such a paragraph is not included here as well.
2. "Knockdowns have been achieved with the use of morpholino-conjugate oligonucleotides, although there is an uncertain correlation between morpholino and knockout phenotypes (Kok et al., 2015).". There is no need to "attack" the morpholinos. For example, these have been shown in some cases to be a more reliable tool for revealing gene function as compared with genetic mutations (Rossi et al Nature 2015). Same for mentioning the other tools for inducing mutations. It would be sufficient to indicate that CRISPR is good and that it is used here.
3. "In animal species, mutations in maternally-expressed genes disrupt early cellular processes, such as egg polarity and activation, pronuclear fusion, cytokinesis, cell adhesion, and axis induction (Abrams and Mullins, 2009; Dosch, 2015) ". An important relevant process that the authors surprisingly do not clearly mention here is germ-cell specification.
4. If a resource-paper path is chosen, the "general methods" such as the fact that the work was approved by the University institutional Animal Care and use committee, the antibody staining procedure, the fact that a Leica MZ FLIII microscope was used etc should be included as supplemental material. This way, readers can focus on the actual new procedure, which should useful for users of other microscopes as well.
5. Fig 1 would be clearer if both sexes are presented in the same colour and the male/female symbol appears above the fish. In 1A, if one uses the */+ and */* , the WT should be +/+. The * should be replaced by - . In 1A at the F2, the authors should indicate as much as possible what is crossed with what, as they do in the F1 and F3. Otherwise, this point might not be clear for those not familiar with F3 screens.
6. Would be good to provide readers with data concerning the effect of the injection mix on fish viability.
7. Fig 1B should be cited in the sentence before.
8. "Characterization of the maternal crispants confirmed cellular defects identical to those caused by loss of function of the corresponding target genes." – I believe that showing the brightfield photos would be sufficient. The information in 2 G and H is not very clear, and in my opinion not needed.
9. Assign the same colour for the DNA in Fig 2, 4 and 6.
10. In Figure 2 (and other figures with similar information) provide the reader with information explaining what the symbols mean – e.g. at the bottom left, put * - PAM sequence.
11. " In nearly all cases (9 of 10 F0 females), F1 clutches contained embryos with defects indistinguishable" – the authors could include examples of that in Suppl. Material.
12. At the beginning of the "Identification of novel maternal-effect genes via maternal crispants" section, would be good to include a few sentences that introduce the problem of generating females that carry homozygous mutant germline. The issue with a lethal embryonic phenotypes due to roles in the soma etc. and use this to introduce the gene expression criteria the authors suggest to employ when deciding on which genes to knock out.
13. "..in spite of the production of multiple INDELS in F0 embryo somatic tissues 24 hours after injection (data not shown)." – the data should be presented in the Suppl. Material.
14. In my opinion, the "Kpna7 is necessary for nuclear stability in the early embryo" and the "Fhdc3 is necessary for the yolk-blastodisc boundary function" sections should be removed from the

paper. The phenotypes are very interesting and deserve being analysed deeper in separate manuscripts. The current description level does not reach that expected from a paper in Development.
15. In Fig 5A not clear why fhdc4 the fhdc1 data is not presented for the pre ZGA stages.
16. “Immunolabeling of fhdc3 maternal crispants with ß-catenin and DAPI shows that at the 8-cell stage, the cellular layout (a 2x4 cell-matrix at this stage) is similar between the fhdc3 maternal crispants and stage-matched wild-type controls” – cite a figure.
17. I do not think panel A in Figure 7 is needed. Simply say that to have a clean sequence one examines a single chromosome of the female. No need to put in a figure what not to do. In general, in all figures the embryos obtained from the mutant females could benefit from better graphics.
18. “For four out of the five targeted genes: birc5b, prc1l, kpna7, and fhdc3, we sequenced single maternal crispant haploid embryos” – why four and not all?
19. “…sequenced single maternal crispant haploid embryos from every female that presented the maternal crispant phenotype (three to thirteen embryos per female)”. Why not testing the same number of embryos from each female? Indicate how many females were used for each genotype in Table 3.
20. The discussion is very long, especially if the “resource paper” path is taken and could probably be omitted. For example, the advantage of the method over the F3 screen approach should not be mentioned again, the re-presentation of the results is not needed (even in the case of discussion in a regular paper) etc. An exception that should probably be included is the discussion concerning the lack of phenotype in the cases of zgc:114123 and kcns3b.
21. The relevance of the tool for analysing maternal-zygotic rather than only maternal phenotypes should be mentioned.

First revision

Author response to reviewers' comments

Response to Reviewers’ comments

We thank all Reviewers for their time and efforts to provide critical comments on the work. We have tried to address all comments and concerns in as much as possible, resulting in what we think is an improved manuscript.

Reviewer 1:
- On providing an estimate of how many genes fit the expression criteria of genes presented in the study to select for candidate maternal-effect genes to target:
We have added information on the number of genes that have high expression levels in the oocyte and are degraded at the time of zygotic genome activation, known from previous studies. Additionally, we have added previous estimates for the number of gene families that have gene duplicates. This allows to estimate the number of genes that conform to the criteria (having maternal-specific expression and being a gene duplicate) to have a maternal-specific function, which can be targeted by the approach presented in this manuscript.

- On placing side-by-side movies (based on optical sections) of kpna7 maternal crispants and wild type fixed embryos, to better show cells missing nuclei
We have edited the movie so that it includes a section where the frames of the wild-type control and the kpna7 maternal crispant are placed side by side, allowing for easier comparison. After this section, the movie has a loop of the wild-type control and then kpna7 maternal crispant frames alone. Additionally, we have also annotated the video with an arrow to highlight missing nuclei in kpna7 maternal crispants.

Reviewer 2:

Major:

1) On the units of Cas9 concentration in the Material and Methods section
We had indeed meant to use the units pg/nl instead of pg/µl, and have corrected the text. Thank you for noticing this error.

2) On the ectopic b-catenin localizing to unreleased cortical granules in mid1ipil maternal crispants and mid1ip1l maternal mutants.
We have more clearly referenced Eno et al., 2016 which shows that aura/mid1ipl1 maternal mutant embryos contain unreleased cortical granules, which were found in that study to contain ectopic b-catenin.

3) On presenting the data referred to as “not shown” in the initial submission.
We have included data (previously referred to as “data not shown”) in Supplementary Figures 4 and 5.

4) On analyzing actin in fhdc3 maternal crispants.
We have added an analysis of the cytokinetic (F-actin) ring at the base of the blastodisc, thought to be normally involved in the contraction and relaxation of the blastodisc, in fhdc3 maternal crispants. We find that the cytokinetic ring is significantly wider in the fhdc3 maternal crispants when compared to wild-type controls, consistent with the exaggerated contractions found in Fhdc3 maternal crispants.

5) On potential developmental delays in fhdc3 maternal crispants.
Although we observe a minor developmental delay in fhdc3 maternal crispant embryos when they start gastrulation, at 1dpf the embryos are indistinguishable from wild-type controls (with the exception of the described yolk inclusions). However, at the end of the gastrulation period fhdc3 maternal crispant embryos do not exhibit defects in yolk closure, a common consequence of severely delayed epiboly. We have modified the text to both clarify that there is a delay early in gastrulation and that in spite of this initial delay gastrulation proceeds within a normal temporal frame, as shown by the absence of yolk closure defects in 24 hour embryos

We have also replaced the images of the 1 dpf fhdc3 maternal crispant and wild type embryos to present the entire embryo, to show that (other than yolk inclusions) there are no gross morphological defects in fhdc3 maternal crispants.

6) On clarifying the description of the analysis of single maternal crispants in mosaic clutches:
We have adjusted the text to clarify that the lesions refer to the collection present in a group of individually analyzed haploid embryos. Collectively, the lesions represent the constitution of alleles in the germ line as described in the text. The key sentence now reads as follows:
“Collectively, DNA lesions observed in the set of individually analyzed haploid maternal crispants, represent the mixture of induced alleles present in the germ line, either in different germ cells or in either of the two different gene copies present in diploid, pre-meiotic germ line cells.”

7) On the phenotype of stable kpna7 alleles, other than kpna7uw107.
We only propagated one stable line carrying an allele for kpna7 (kpna7uw107). The maternal crispant phenotype in F1 embryos and the maternal-effect phenotype in the stable line concur and provide separate independent evidence for the phenotype. This is consistent with the use of at least two independent lines of evidence to confirm gene assignment in studies in various model systems including our previous work in the zebrafish (e.g. Eno and Pelegri 2018)

8) On the possibility that the chimeric germline (e.g. 1 in 8 germ cells) provides an explanation for the unequal proportions of analyzed alleles in maternal crispants
We have revised the Discussion to include the possibility that gene editing at different times in development can generate different ratios of induced alleles in the germline.

Minor:

We have revised the text throughout, including figure legend 5D and Figure 3 for misspellings and typos. The label in the table 3 has been changed to say “Average predicted protein length”.
We provide a BioRxiv reference for the Nair et al submitted manuscript.

Reviewer 3:

- On presenting the work on a more concise manner, focusing on the method alone and not on identified phenotypes.

We agree with the Reviewer that superficially analyzed mutants can detract from what should be a focus on the method. Our intention in this work has been to present proof of principle for the approach to first validate that the method accurately recreates known phenotypes and subsequently a minimal analysis of novel phenotypes to show that the method can indeed reveal previously unknown gene-function relationships. With regards to the latter, we only perform a primary characterization of the newly discovered maternal crispants, similar to previous published work to identify maternal-effect genes in forward genetic screens using ploidy manipulation (Pelegri et al. 2004) or mixed pool strategies (Dosch et al. 2004 and Wagner et al 2004). Previous studies have shown that live phenotypes in early embryos can have very different underlying causes at the cellular level. Additionally, because early embryos lack cell cycle check points, cell division can proceed even in the presence of underlying cellular defects. For these reasons, we routinely couple the analysis of all new phenotypes with the characterization of at minimum adhesive membrane (e.g. b-catenin) and DNA. This allows us to place phenotypes into broad classes related to the associated cellular defect. When warranted by the molecular gene identity and/or phenotype, additional minimal assays are included, such as the analysis of the cytokinetic ring in maternal crispants for the cytoskeletal regulator fhdc3, also included in this revision after being requested by another Reviewer.

Having stated this, we agree with the Reviewer that an abbreviated format can help better highlight the method, and have shortened and condensed the text in multiple sections, in particular in the Discussion.

We appreciate the suggestion to make the manuscript a Resource paper but consider that the word and figure limit of the Resource paper format would not allow inclusion of the validation assays. We also highlight that Reviewers 1 and 2 asked us to add more experimental details to our primary analysis. However, if requested by the Reviewers and Editor we will be happy to consider a Resource format.

Specific points in order of appearance in the manuscript-
1. On removing the initial “motivation” paragraph
We have removed the first paragraph in the introduction.

2.On avoiding direct comparisons between morpholinos and gene editing methods and simply indicate the work uses CRISPR.
We have revised the text to avoid making direct comparisons between the methods.

3. On the list of maternal traits including germ-cell specification.
We have added germ cell specification to the list. It was not meant to be a comprehensive list but we appreciate the encouragement from the Reviewer to include a topic that is close to our own research focus.

4.On placing general methods as supplemental material if a resource-paper path is chosen.
We appreciate the suggestion to use a resource format but feel a Methods format best allows inclusion of key validation experiments (see above). We will be happy to consider a Resource format if requested by the reviewing team and Editor.

5. On clarifying notations and symbols in Fig. 1
We have clarified the notations in this figure. We have added the male/female symbol next to the genotype and used the same color for males and females. We have also changed the WT to +/+, and changed the * to a – (We had used the asterisk as it is standard in forward genetic screens, but we agree this in this reverse genetics approach a minus sign may be more appropriate and thank the Reviewer for the suggestion). We have also rearranged the F2 generation to make it clearer of what fish are crossed together to create the F3 generation. Additionally, we have also reworked the graphics of this figure.

6. On providing on the effect of the injection procedure on viability.
We have added data on day 5 viability in embryos injected with the guide RNA/Cas9 mixture. This is presented in Supplementary Figure 1.

7.On the placement of Fig 1B
We have cited Figure 1 B a sentence earlier in the text as suggested.

8. On showing only the brightfield images (and not the immunolabelings, specifically panels G and H) in Figure 2
Live phenotypes can be similar in spite of different underlying cellular defects, therefore primary immunolabeling of cell adhesive membrane and DNA provides valuable information to place new phenotypes into broad phenotypic classes (e.g. nuclear processes, mitosis, early cytokinesis, late cytokinesis). In this figure, the panels are meant to allows the reader to better determine that the embryos are indeed syncytial. We realize however that the images as presented did not adequately convey this information (and therefore did not clearly justify their presence) and have added higher magnification inserts to better present the information in the images.

9. On assigning the same color for the DNA in Fig 2, 4 and 6.
We have adjusted the color of the DAPI stain to gray in all figures throughout the paper.

10. On explaining symbols in Figure 2, in particular the PAM sequence
We have added to the figure key the PAM sequence in Figures 2, 3,5, and Supplementary Figure 3.

11. On including examples of the phenotypes for mutations used to validate the method.
We have included stage-matched live images of the known maternal mutants (motley, tmi, and aura), which we use to validate the method, in Supplementary Figure 2. We thank the Reviewer for this suggestion.

12. On addressing the use of this method and selection criteria to bypass lethal phenotypes caused by somatic function.

We have added a sentence on the difficulty of studying maternal functions in the context of zygotic functional requirement, leading to our presentation of our criteria for the selection of maternal specific target genes.

13. On presenting the data for INDEL production in F0 embryo somatic tissues after injection.

We have added gel images showing multiple INDELS in 24 hpf injected embryos in Supplementary Figure 4.

14. On the required depth of analysis of Kpna7 and Fhdc3 and whether the analysis should be left for future publications.

We agree with the Reviewer that each of the newly discovered maternal effect crispants deserves to be analyzed in more depth in separate manuscripts and that, on their own the level of their analysis does not reach that expected in Development. In this article we present a primary characterization of the observed phenotypes in order to provide minimal evidence that the method is effective at identifying novel phenotypes (see above). This primary characterization should still allow ample material for a full-fletched analysis of the phenotypes and gene function in future publications. Similar publication paths were taken for genes initially described with minimal detail in early screen publications (e.g. Pelegri et al 2004, Dosch et al 2004) and which were subsequently analyzed in greater depth in follow-up publications (e.g. Yabe et al 2007, Yabe et al 2009, Putiri, 2011; Ge et al, 2014; Eno et al 2018).

15. On why some pre-ZGA stages are not depicted in Fig 5A
This was an unintended presentation of the data. For those stages, the counts were below significance.
We have adjusted the graph in figure 5 to reflect “zero”.

16. On citing a figure for the similar cellular layout in the immunolabeling of fhdc3 maternal crispants with ß-catenin and DAPI at the 8-cell stage
We have cited the figure in the text in the appropriate Results section

17. On removing panel A in Figure 7 (which represented ambiguity in the sequencing of diploid heterozygous embryos) and improving the graphics in Figure 7.
In Figure 7, we have removed panel A and improved the “maternal crispant” embryo graphics. We have replaced the maternal crispant graphics with the improved version in all relevant figures of the manuscript.

18. On why we sequenced single maternal crispant embryos from only four of five targeted genes.

We could not sequence haploids from the mid1ip1l maternal crispant because the eggs and embryos have a fragile cortex (Eno et al 2016) and did not survive the IVF procedure (involving manual extrusion of eggs). We have added this information to the text.

19. On why we sequenced varying numbers of maternal crispant embryos per female, and indicating how many females were used for each genotype in Table 3.

Haploid production through IVF involves manual extrusion of eggs from anesthetized females and often times can have as a side effect that females do not fully recover to become gravid again and contain mature eggs. For this reason, females are manipulated for IVF as little as possible (ideally only once) and all resulting maternal crispant embryos, which varied in fraction within the clutch and number, were analyzed. We have added a clarification of this in the Methods section. We have also added in a row to table 3 to indicate how many females were used for sequencing.

20. On the length of the discussion, re-presentation of the advantage of the method and results, and lack of phenotype in the cases of zgc:114123 and kcns3b.

We have shortened the Discussion, in particular in the re-stating of the advantage of the method and re-stating of the results. As suggested, we have kept in the text a brief explanation addressing the lack of phenotype in the cases of zgc:114123 and kcns3b.

21.On the relevance of the tool for analyzing maternal-zygotic rather than only maternal phenotypes.
In order to target maternal-zygotic genes, the tool should be adapted to specifically target the germ line, in order to avoid inducing subviability in the injected fish due to lesions in the soma. This could be achieved for example using an injected Cas9 targeted to the germ line with a localizing 3’UTR (e.g. nanos3’ UTR). We thank the Reviewer for the suggestion and include this suggestion in the Discussion.

Second decision letter

MS ID#: DEVELOP/2021/199536

MS TITLE: Identification of Maternal-Effect Genes in Zebrafish using Maternal Crispants

AUTHORS: Cara E Moravec, Gabriella C Voit, Jarred Otterlee, and Francisco Pelegri

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, two of the referees are largely happy with your revisions. The third still thinks that your paper is weakened by presenting some of the phenotypic analyses rather than focussing solely

on validation of the methods. I will leave it to you to decide whether or not to further address this criticism in the final version of your manuscript.

Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Moravec et al present a clear and accessible description of a clever method to efficiently assay the essential function of essential maternal effect genes by CRISPR mutation. They describe a method to identify likely candidate genes for analysis by examining gene expression patterns. They demonstrate that these genes can be mutated and consistent mutant phenotypes recovered efficiently enough for analysis of the resulting phenotype without a 3 generation inbreeding strategy. They demonstrate proof of principle by testing genes with known mutant phenotypes as well as new genes that yield novel phenotypes demonstrating that the method is likely to yield novel insights. Furthermore, they generate haploids to investigate the preferential use of some guide RNAs over others and see a allelic transmission distortion which is interesting and important to document even if the mechanism is not clear yet.

Comments for the author

The authors have addressed all of my concerns. I do suggest a change in the text. The sentence ending
"although embryos do not show yolk closure defects characteristic of strong epiboly" should read "although embryos do not show yolk closure defects characteristic of previously described strong epiboly delay mutant phenotypes."

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all my previous concerns and strengthened their manuscript. This is a valuable method, in addition to revealing two new maternal-effect mutants of Formin Fhdc3 and Kpna7.

Comments for the author

In Fig 6A, the blue triangle arrowheads make it unclear what is being pointed to. Use arrows or more asymmetric arrowheads, so it is clear.
In the absence of time-lapse imaging or staining for yolk protein or globules, it is better to refer to the inclusions in Fig 6B fhdc3 crispants as ‘presumptive ectopic yolk inclusions’.
In some of the new text added, there are typos, which should be corrected prior to publication.

Reviewer 3

Advance summary and potential significance to field

The current version of the manuscript is very much improved and could provide the zebrafish community with a nice tool.

Comments for the author

My main suggestion - to focus on the method and avoid presentation of poor analysis of mutants was not followed.

I believe it is a pity, as it would have made the paper more readable and useful for the target audience. The fact that people used to do things like that almost 20 years ago and others followed up on poorly analysed mutants 10 years ago does not make the approach necessarily desirable now.

Second revision

Author response to reviewers' comments

Regarding Reviewer 1 comment: The sentence ending "although embryos do not show yolk closure defects characteristic of strong epiboly" now reads "although embryos do not show yolk closure defects characteristic of previously described strong epiboly delay mutant phenotypes."

Regarding Reviewer 2 comment: In figure 6, all arrowheads are now arrows to increase clarity. We have also revised all other figures through the manuscript for clarity in the placement and visibility of arrowheads and arrows

Regarding Reviewer 2 comment: "Ectopic yolk inclusions" are now referred to as "presumptive ectopic yolk inclusions"

Regarding Reviewer 2 comment: Typos in the new text have been corrected

Regarding Reviewer 3 comment: We understand and appreciate Reviewer 3's suggestion to reduce any phenotypic analysis and only leave the basics of the method. We were torn between the suggestion to reduce the number of details in the analysis and that of other Reviewers who requested additional details. We have re-assessed whether any of the phenotypic analysis is unnecessary and feel that, to the best of our knowledge, it is better to have the minimal characterization presented precisely to better validate the method. The basic primary characterization assay of b-catenin/DAPI labeling for example allows to know that defects in live embryos indeed involve lack of cellular membranes in spite of cell cycling - this is something that may not be appreciated by looking at the wild-type embryo alone, since membranes are not clearly visualized in live embryos and additionally embryos with defects in cellularization could be interpreted as simply being at an earlier developmental stage or simply unfertilized. Other additional assays presented help provide confidence that the method indeed is causing mutations in the targeted genes. In the case of kpna7, an importin, loss of some nuclei but not others as observed in the bcat/DAPI primary analysis may not be easily explained through a function related to nuclear import. This spotty phenotype, however, becomes easier to understand (and the targeting more credible) when identifying aberrant (and likely to be lost) DNA masses forming during chromosome segregation in the subsequent microtubule/DAPI labeling, together with previous studies that show a link between nuclear envelope re-formation during anaphase and the loading of nuclear envelope proteins during the very rapid cell cycling of the early embryonic cell divisions. In the case of fhdc3, a putative actin regulator, it provides confidence in the method to show that the phenotype involves aberrant formation of the cytokinetic ring at the base of the blastodisc (an experiment that was suggested by one of the other Reviewers and which we added in the Revision). Thus, the minimal analysis presented in the manuscript helps provide confidence in the method, in particular that phenotypes obtained are indeed those caused by loss of function of the intended targets. This confidence is what we hope will encourage other investigators to use the method to identify phenotypes in other target genes of their choice. The presentation of minimal analysis also provides a convenient blueprint (primary characterization with membrane and nuclear markers, followed when appropriate by other assays that specifically address expected gene function or any perceived incongruencies) for other investigators to follow while using this method with new targets.

Third decision letter

MS ID#: DEVELOP/2021/199536

MS TITLE: Identification of Maternal-Effect Genes in Zebrafish using Maternal Crispants

AUTHORS: Cara E Moravec, Gabriella C Voit, Jarred Otterlee, and Francisco Pelegri
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.