# A transgenic system for targeted ablation of reproductive and maternal-effect genes

Sylvain Bertho, Odelya Kaufman, KathyAnn Lee, Adrian Santos-Ledo, Daniel Dellal and Florence L Marlow

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**Editor**: Stephen Wilson

**Review timeline**

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**Original submission**

First decision letter

MS ID#: DEVELOP/2020/198010

MS TITLE: A Transgenic System for Targeted Ablation of Reproductive and Maternal-Effect genes

AUTHORS: Sylvain Bertho, Odelya Kaufman, KathyAnn Lee, Adrian Santos-Ledo, Daniel Dellal, and Florence L Marlow

Apologies for the delay in obtaining reviews on your manuscript. However I have now received all the referees' reports and made 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. 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*

Bertho, Kaufman et al describe effective and efficient tools for creating mutations in zebrafish germ cells and oocytes. These tools will allow the requirement for potential maternal effect genes with essential zygotic function to be determined with much greater efficiency than current methods such as germ line replacement.
They describe multiple proof of principle experiments that clearly demonstrate the efficiency of the method.
This report also highlights that Cas9 can be expressed in the zebrafish germline. In all it is a well supported description of an innovative method and tools that are likely to be used widely within the zebrafish community.

*Comments for the author*

Some minor issues with the manuscript should be addressed to improve the clarity of the presentation. rbpms2 is provided as a proof of principle target gene however no data on targeting efficiency or mutant phenotype is provided. The only reference is to the fluorescence of embryos from F0 females. Was this gene mutated using this method? If not this should be mentioned. If this work will be presented elsewhere this should be indicated.

In the Methods > Genotyping, I assume each usage of the phrase “The genomic region surrounding (gene of interest) was amplified using the primers” should be something like “The genomic region surrounding the target sequence in (gene of interest) was amplified using the primers” since the whole gene is not being amplified

In the Methods > Western blot, I assume “pulled together” should be “pooled together”

In the Results > Phenocopy of Mkif5Ba with mutagenesis vectors:

It appears that the individual fish are given number identifiers in figure 3C which would be useful to indicate in the text. Especially relating to text that identifies a specific individual fish, such as “However one female was heterozygous, indicating de novo mutations in kif5Ba occurred in her father’s sperm”.

The figure for the nanos3 in situ may be hard for a naïve reader to identify the difference among embryos.

An inset with high magnification would be useful. The diagram in Fig3A may be sufficient but it should be closer to the in situs since it isn’t relevant to the genotyping that follows in Fig 3BandC.
The text refers to “we examined the phenotype of embryos from Tg:GMS:kif5Ba F1 females at d1” in FigF-J but the figure indicates that these embryos are from OMS females.

A reference for the dorsalized phenotype classification system V1-V5 should be provided. Do the duplicated axis embryos have a heart to score as gfp+? If not the text should be clarified to indicate that.

In the Results > Phenocopy of buc, the last sentence seems to be a general conclusion and would be more appropriate in the “Overall conclusions” section. Furthermore, the phrase “ranging from lower than to greater than the penetrance of a typical recessive mutation,” could use more elaboration. Does this refer to variable penetrance or expressivity found in some maternal effect mutations? Or does it refer to generating the maternal mutants themselves? In this case, the frequency would be dependent on the transgenesis rate as well as the mutagenesis rate if one is assaying F1 embryos for phenotype or the mutagenesis rate if assaying F2 embryos.

Figure 3:
I think that the legend reading “frequencies in C) F1 and D) F2 progeny”should be “frequencies in B) F1 and C) F2 progeny”

The yellow + and - in C are not described I assume that 2x2 and 3x3 refer to intercrosses of progeny from female 2 and female 3?

Reviewer 2

*Advance summary and potential significance to field*

In this paper, Bertho et al establish a method designed to mutate genes and investigate their role in germline development. The method allows studying the function of proteins that play roles both in germline and somatic cells by directing the Cas9 protein to the germline at different stages of development. The approach reported here involves a transgenic system that is more efficient than the currently used germ-cell replacement method. The mutations are generated by Cas9 expressed in the germline under the control of the bucky ball or ziwi promoters and specific guide RNAs under the control of a ubiquitous promoter. The two promoters differ in their activation time ; bucky ball promotor is activated during early oogenesis and ziwi promoter earlier, in mitotic primordial germ cells. Two maternal-effect genes were mutated using this method, bucky ball and kif5Ba. The expected phenotypes were observed.
In principle the method would be useful for studying the function of genes whose mRNAs are produced in the ovary and play a role in oogenesis and in early embryogenesis.

*Comments for the author*

The main issue the authors should determine rigorously concerns the germline specificity of the method. Namely, they should examine leakage of Cas9 activity by including guide RNAs targeting several genes whose function is important for early embryonic development (e.g. half baked (eCad), one eye pinhead, tyrosinase etc.). Such experiments would prove the main claim of the authors that the method can knockout genes specifically in the germline without affecting the function of the genes in the soma. The authors consider the time of mutagenesis based on the published expression pattern of the buc and ziwi. The authors should confirm that the expression of Cas9 by the construct indeed replicates the expression pattern of those genes (e.g. by RT PCR to exclude early expression in the larvae and in situ hybridization experiments in the developing ovary). Similarly, the ubiquitous expression of the gRNA under the U6 promoter throughout development should be demonstrated e.g., by RT PCR. In case phenotypes are not observed, it would be formally possible that gRNA expression is not present at early stages.

Specific comments in order of appearance in the text-

1. The text includes many grammatical mistakes and should be thoroughly revised- e.g. – "… plasmid-based system for germline specific mutagenesis based on (Ablain et al., 2015).", "…(Ablain et al., 2015; Auer et al., 2014a; Auer et al., 2014b; Barrangou, 2013; Blackburn et al., 2013; Hruscha et al., 2013) et al., 2013; (Hwang et al., 2013a; Hwang et al., 2013b)." and more.

2. The specific advantages of System I vs. II should be discussed. If one of the systems is sufficient, could the other be omitted?

3. "However, the effectiveness of CRISPR/Cas9 in meiotic cells, when there are four copies of each chromosome and distinct checkpoints and repair pathways has not been determined." – Do the author know that the mutagenesis does not occur prior to meiosis?

4. "Here we report cassettes and recovered transgenic (Tg+) founders targeting three genes, rbpms2a, kif5Ba, and buc ". ; only 2 genes were introduced in the intro. Introduce rbpms2a, kif5Ba. Abbreviations like d1 (day 1) should be introduced.

5. Figure 2a. It would be helpful to have the brightfield image of the embryos in addition to the GFP channel. Also, expand the description of this panel. Similarly the legend of figures 3 and 4 should provide more details.

6. Improve the quality of the gel in Figure 2B.

7. Figure 2C-2E are not mentioned in the text.
8. Sup fig 2 is not mentioned in the text.

9. In Figure 3A, present a real result rather than a scheme.

10. " indicating that expressing Cas9 from the ziwi promoter in the presence of guide RNA targeting buc induced germline mutations in founder males." – if the guides are more efficient that those of buc, one might only get sterile males. Should be mentioned.

11. Figure 3C is not clear the way it is presented.

12. It would be helpful to specify what the abbreviation in sup 3A stand for eg GH, pGG.

13. Fig 4B panel is not marked and it is not clear what is presented in the non-marked part of fig 4. It is also not clear what is presented in fig 4B',C'

Reviewer 3

*Advance summary and potential significance to field*

The manuscript by Bertho et al presents a system of CRISPR/Cas9 gene knock out using a transgene carrying a guide RNA and Cas9. In this system, the generation of mutations is driven by a transgenic copy harboring both Cas9 and guide RNAs for the target genes. The system targets function during germ line and oogenesis using promoters specific for those stages.
Transcription of both components during germ cell development of the initial F0 (injected to generate the transgene) individual allows to generate mutations de novo at the targeted endogenous locus, leading to a mosaic germ line. Lesions at the endogenous locus can be recovered in F1 fish and propagated in non-mosaic individuals. Additionally, the transgene itself can be propagated, through males if it has a maternal-effect phenotype, to generate more lesions even when crossed to wild type fish.

The authors show that the system is able to generate lesions in two previously known genes, kif5Ba and Bucky Ball. The study presents a significant advance in the ability to generate mutations in targeted maternal genes, as the system allows in principle targeting lesions specifically in the germ line, thus allowing to assess the germ line function of genes that may be somatic lethal. Additionally, the system allows to generate biallelic mutations in the F0 fish themselves, allowing to test the function of genes in a single generation. This expedites the ability to test the function of target maternal-effect genes through reverse genetics.

*Comments for the author*

Data for Figure 2 has no n numbers, hence it is difficult to assess how representative the images are.

There are no non-transgenic controls in Figure 3, in particular to show lack of background defects and/or technical issues with the labeling (e.g. if a fraction of embryos are not labeling with the nanos probe due to sensitivity reasons)

The abstract and introduction highlight the usefulness of the method to assess the maternal contribution for genes that have a zygotic function (and whose maternal function could not be analyzed in a traditional mutant because of inviability). However, the proof of principle genes chosen are maternal-effect genes and do not directly test the method for somatic-lethal mutations. Given this, the idea that the method is useful to generate germ line mutant chimeras in a wild type background, while reasonable, remains formally untested, and the concept be should toned down in particular in the abstract.

Minor concerns
The "Validation of OMS and.." (page 11) states tests on three genes, but rbpms2a is not mentioned in the manuscript any further

In the statement “GFP should be a proxy for Cas9 because both proteins are produced from the same transcript” (p 11). From the constructs, it appears that system I has two different transcripts (same transgene), only in system II it is the same transcript

For the proof-of-principle genes, it would be helpful if more background on previous studies was presented, in order to better make a comparison between previous approaches and the new one presented in this work. For example, more details on what “Global loss of maternal kif5Ba” (p 12) means.

Does the terminology of oms and gms apply only to System II, or also to System I? From the text, it seems that it depends on the promoter driving Cas9 (so that it would apply to both System I and II) but in Figure 1 only System II is specifically labeled as oms or gms

Related to the terminology issue, it is unclear in Figure 2 which system is represented in the images (I or II)

It is unclear whether the “Egg activation” phenotype in Figure 3J is part of the expected phenotype

Generally it is unclear why the authors use the term “Progeny of" instead of the generation itself (e.g. "progeny of F1” rather than “F2”). Simply stating F0, F1, F2 would be a more direct way to convey the information, in a format that is standard and which would directly match Figure 1C

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**First revision**

Author response to reviewers' comments

*We are grateful to the reviewers for their time and thoughtful comments. We have carefully reviewed the reviewer’s comments, and we appreciate their constructive feedback and are delighted that they found the method to be innovative and a significant advance in the ability to generate mutations in targeted maternal genes and the germline. We have carefully considered each of their comments and have addressed all of them with either experiments or revisions to the text as appropriate based on their feedback. We hope that the reviewers agree that these new data provide support for our conclusions and strengthen our manuscript. Our point by point responses to each of the reviewer concerns are detailed below.*

Reviewer 1 Advance summary and potential significance to field
Bertho, Kaufman et al describe effective and efficient tools for creating mutations in zebrafish germ cells and oocytes. These tools will allow the requirement for potential maternal effect genes with essential zygotic function to be determined with much greater efficiency than current methods such as germ line replacement. They describe multiple proof of principle experiments that clearly demonstrate the efficiency of the method.This report also highlights that Cas9 can be expressed in the zebrafish germline. In all it is a well-supported description of an innovative method and tools that are likely to be used widely within the zebrafish community.

Reviewer 1 Comments for the author

Some minor issues with the manuscript should be addressed to improve the clarity of the presentation.

rbpms2 is provided as a proof of principle target gene however no data on targeting efficiency or mutant phenotype is provided. The only reference is to the fluorescence of embryos from F0 females. Was this gene mutated using this method? If not this should be mentioned. If this work will be presented elsewhere this should be indicated.

*rbpms2 was mutated with system I, but we removed system I per comments from reviewer 2.*

In the Methods > Genotyping, I assume each usage of the phrase “The genomic region surrounding (gene of interest) was amplified using the primers” should be something like “The genomic region surrounding the target sequence in (gene of interest) was amplified using the primers” since the whole gene is not being amplified

*Yes, that is correct. This has been clarified as suggested.*

In the Methods > Western blot, I assume “pulled together” should be “pooled together”

*Yes, your assumption is correct. Thank you for pointing this out. This has been corrected.*

In the Results > Phenocopy of Mkif5Ba with mutagenesis vectors:
It appears that the individual fish are given number identifiers in figure 3C which would be useful to indicate in the text. Especially relating to text that identifies a specific individual fish, such as “However, one female was heterozygous, indicating de novo mutations in kif5Ba occurred in her father’s sperm”.

*This has been indicated in the main text as recommended.*

The figure for the nanos3 in situ may be hard for a naïve reader to identify the difference among embryos. An inset with high magnification would be useful. The diagram in Fig3A may be sufficient but it should be closer to the in situs since it isn’t relevant to the genotyping that follows in Fig 3BandC.

*This has been addressed in the main figure in two ways: 1) The schematic has been enlarged - we could not bring it closer without disrupting the symmetry of the figure. 2) Larger images showing fewer examples have been added to panel E and the original images were moved to supplemental Figure 4.*

The text refers to “we examined the phenotype of embryos from Tg:GMS:kif5Ba F1 females at d1” in FigF-J but the figure indicates that these embryos are from OMS females.

*Thank you for pointing this out - the figure was mislabeled. This has been corrected.*

A reference for the dorsalized phenotype classification system V1-V5 should be provided.

*Kishimoto 1997 has been added as recommended.*

Do the duplicated axis embryos have a heart to score as gfp+? If not the text should be clarified to indicate that.

*The reviewer is correct, the heart was not scoreable in embryos with duplicated axes. The text was revised to clarify this important point.*
*“….among the green heart+ progeny -the heart was not scoreable in embryos with duplicated axes..”*

In the Results > Phenocopy of buc, the last sentence seems to be a general conclusion and would be more appropriate in the “Overall conclusions” section.

*This has been moved as suggested.*

Furthermore, the phrase “ranging from lower than to greater than the penetrance of a typical recessive mutation,” could use more elaboration. Does this refer to variable penetrance or expressivity found in some maternal effect mutations? Or does it refer to generating the maternal mutants themselves? In this case, the frequency would be dependent on the transgenesis rate as well as the mutagenesis rate if one is assaying F1 embryos for phenotype or the mutagenesis rate if assaying F2 embryos.

*This has been revised to clarify. "Although the frequency of phenotype detection varies for both gms and oms mutagenesis, ranging from lower than to greater than the penetrance of a typical recessive mutation, because it depends on the transgenesis and mutagenesis rate, this approach represents a significant advance over germline replacement methods, which are tedious, time intensive, and inefficient."*

Figure 3:
I think that the legend reading "frequencies in C) F1 and D) F2 progeny"should be "frequencies in B) F1 and C) F2 progeny"
*Yes, this is correct. Thank you for pointing this out. This has been corrected in the revised manuscript.*

The yellow + and - in C are not described

*Additional labels have been added to the panel as well as a description in the legend.*

*"Plus indicates digested; minus indicates undigested."*

I assume that 2x2 and 3x3 refer to intercrosses of progeny from female 2 and female 3?
*Yes that is correct. The legend has been revised to clarify.*

*"# x # indicates the individuals intercrossed."*

Reviewer 2 Advance summary and potential significance to field
In this paper, Bertho et al establish a method designed to mutate genes and investigate their role in germline development. The method allows studying the function of proteins that play roles both in germline and somatic cells by directing the Cas9 protein to the germline at different stages of development. The approach reported here involves a transgenic system that is more efficient than the currently used germ-cell replacement method. The mutations are generated by Cas9 expressed in the germline under the control of the bucky ball or ziwi promoters and specific guide RNAs under the control of a ubiquitous promoter. The two promoters differ in their activation time ; bucky ball promotor is activated during early oogenesis and ziwi promoter earlier, in mitotic primordial germ cells. Two maternal-effect genes were mutated using this method, bucky ball and kif5Ba. The expected phenotypes were observed.

In principle the method would be useful for studying the function of genes whose mRNAs are produced in the ovary and play a role in oogenesis and in early embryogenesis.

Reviewer 2 Comments for the author
The main issue the authors should determine rigorously concerns the germline specificity of the method. Namely, they should examine leakage of Cas9 activity by including guide RNAs targeting several genes whose function is important for early embryonic development (e.g. half baked (eCad), one eye pinhead, tyrosinase etc.). Such experiments would prove the main claim of the authors that the method can knockout genes specifically in the germline without affecting the function of the genes in the soma.

*Although it was not clear, in the original submission we provided evidence that mutations are induced in the germline and not the soma in Figures 3B (kif5Ba) and 4D (buc) parents. All potential founders were genotyped and the targeted gene (either buc or kif5Ba) was amplified from and sequenced from somatic tissues (Fin). By comparing the sequences of these genes from the parents and their offspring, we show that mutations are present in the progeny that were not present in the somatic tissues of the parents. We hope that we have clarified this in the revised manuscript.*

*In addition, to further address the reviewers concern about leakage and to prove that knockout is in the germline we performed the following experiments:*

*1) We injected kif5Ba guide RNA into kif5Bb homozygous mutants to determine if the resulting "crispants" phenocopied the zygotic craniofacial defects observed in kif5Ba;kif5Bb double mutants (Santos-Ledo et al., 2017). As expected, this caused defects in jaw extension. These results are in revised Fig. 3L and N.*

*2) We injected gms:kif5Ba into kif5bb homozygous mutants to determine if jaw defects were observed, which would be expected if there were leakage or somatic cell mutations. We observed no jaw defects in green heart positive (those with the transgene) or in their transgene negative siblings. These data are provided in Fig. 3M and N. These transgenic animals were raised to adulthood, further indicating that somatic mutations are not induced because double mutant fish and compound mutant fish are not viable (Santos-Ledo et al., 2017).*

*Taken together these results strongly support our claim that the method can knockout genes specifically in the germline without affecting the function of the genes in the soma.*

The authors consider the time of mutagenesis based on the published expression pattern of the buc and ziwi. The authors should confirm that the expression of Cas9 by the construct indeed replicates the expression pattern of those genes (e.g. by RT PCR to exclude early expression in the larvae and in situ hybridization experiments in the developing ovary).
Similarly, the ubiquitous expression of the gRNA under the U6 promoter throughout development should be demonstrated e.g., by RT PCR. In case phenotypes are not observed, it would be formally possible that gRNA expression is not present at early stages.

*As recommended by the reviewer we have performed RTPCR experiments to compare expression of the constructs to the endogenous genes (Revised Figure 2A). We examined eggs from transgenic mothers to look at the maternal deposition, d10 larvae as this is when ziwi promoter is activated, d36 when both buc and ziwi promoters are active. We examined the follow ziwi, buc, cas9, guide RNA. As expected the guide RNA was detected in transgenic positive animals at all stages examined, ziwi, but not buc was active at d10 and both ziwi, and buc were active by day 31 and cas9/gfp expression was as expected for the respective promoter controlling its expression.*

Specific comments in order of appearance in the text-

1. The text includes many grammatical mistakes and should be thoroughly revised-
e.g. – "… plasmid-based system for germline specific mutagenesis based on (Ablain et al., 2015).", "…(Ablain et al., 2015; Auer et al., 2014a; Auer et al., 2014b; Barrangou, 2013; Blackburn et al., 2013; Hruscha et al., 2013) et al., 2013; (Hwang et al., 2013a; Hwang et al., 2013b)." and more.

*We have corrected these endnote formatting issues and checked the grammar.*

2. The specific advantages of System I vs. II should be discussed. If one of the systems is sufficient, could the other be omitted?

*The main difference is that one marks the expressing germ cells. Because we feel that marking the expressing cells is more advantageous we have omitted I as suggested by the reviewer.*

3. "However, the effectiveness of CRISPR/Cas9 in meiotic cells, when there are four copies of each chromosome and distinct checkpoints and repair pathways has not been determined." – Do the author know that the mutagenesis does not occur prior to meiosis?

*Buc is expressed in meiotic cells; so, for oms mutations would only be made after activation of the buc promoter. For gms, ziwi promoter, mutations are likely made in mitotic cells but could be made at any time after the ziwi promoter is activated.*

4. "Here we report cassettes and recovered transgenic (Tg+) founders targeting three genes, rbpms2a, kif5Ba, and buc ". ; only 2 genes were introduced in the intro. Introduce rbpms2a, kif5Ba. Abbreviations like d1 (day 1) should be introduced.

*These have been introduced/revised as suggested.*

5. Figure 2a. It would be helpful to have the brightfield image of the embryos in addition to the GFP channel. Also, expand the description of this panel. Similarly the legend of figures 3 and 4 should provide more details.

*This figure has been reorganized and brightfield examples are shown in the other revised main figures. Additional details have been added to the legends as requested.*

6. Improve the quality of the gel in Figure 2B.
*We have repeated the Western blot several times and replaced it with a new blot using eggs from a gms:buc mother because system I, which was shown before was removed as recommended. Unfortunately, the abundant yolk proteins in eggs do not run beautifully on gels. Nonetheless, the result is clear.*

7. Figure 2C-2E are not mentioned in the text.

*This has been addressed.*

8. Sup fig 2 is not mentioned in the text.
*We checked and confirmed that it is cited. "Briefly, we generated mutagenesis cassettes that include selectable markers (tissue specific expression of fluorescent proteins (FP)) and that express target guide RNAs ubiquitously (U6 promoter) and Cas9 from germline specific promoters (bucky ball (female meiotic cells)(Heim et al., 2014) and ziwi (all germ cells)(Leu and Draper, 2010); Figure 1A,B, Supplemental Figure 1, 2)."*

9. In Figure 3A, present a real result rather than a scheme.

*3A is a schematic to orient readers that depicts a summary of a previously published result and the "real results" are shown in panel 3E and supplemental Figure 4.*

10. " indicating that expressing Cas9 from the ziwi promoter in the presence of guide RNA targeting buc induced germline mutations in founder males." – if the guides are more efficient that those of buc, one might only get sterile males. Should be mentioned.

*Presumably the reviewer means that one might only get sterile males if the gene being targeted is required for fertility because buc is not required in males. We have mentioned this in the overall conclusions section.*

*"Nonetheless, this system may be suitable for genes transcribed in later oocytes, or for genes of interest that result in sterile male phenotypes using the GMS system."*

11. Figure 3C is not clear the way it is presented.

*This figure has been reorganized.*

12. It would be helpful to specify what the abbreviation in sup 3A stand for eg GH, pGG.

*These abbreviations have been added as recommended.*

13. Fig 4B panel is not marked and it is not clear what is presented in the non-marked part of fig 4. It is also not clear what is presented in fig 4B',C'

*We apologize, the letters inadvertently was not included in the converted image file. We have also added additional labels and outlined the embryos. Hopefully, it is clear now.*

Reviewer 3 Advance summary and potential significance to field
The manuscript by Bertho et al presents a system of CRISPR/Cas9 gene knock out using a transgene carrying a guide RNA and Cas9. In this system, the generation of mutations is driven by a transgenic copy harboring both Cas9 and guide RNAs for the target genes. The system targets function during germ line and oogenesis using promoters specific for those stages.
Transcription of both components during germ cell development of the initial F0 (injected to generate the transgene) individual allows to generate mutations de novo at the targeted endogenous locus, leading to a mosaic germ line. Lesions at the endogenous locus can be recovered in F1 fish and propagated in non-mosaic individuals. Additionally, the transgene itself can be

propagated, through males if it has a maternal-effect phenotype, to generate more lesions even when crossed to wild type fish.

The authors show that the system is able to generate lesions in two previously known genes, kif5Ba and Bucky Ball. The study presents a significant advance in the ability to generate mutations in targeted maternal genes, as the system allows in principle targeting lesions specifically in the germ line, thus allowing to assess the germ line function of genes that may be somatic lethal. Additionally, the system allows to generate biallelic mutations in the F0 fish themselves, allowing to test the function of genes in a single generation. This expedites the ability to test the function of target maternal-effect genes through reverse genetics.

Reviewer 3 Comments for the author
Data for Figure 2 has no n numbers, hence it is difficult to assess how representative the images are.

*These numbers have been added to the figure legend.*

*"4 ovaries and 4 testis were dissected and examined for each transgene."*

There are no non-transgenic controls in Figure 3, in particular to show lack of background defects and/or technical issues with the labeling (e.g. if a fraction of embryos are not labeling with the nanos probe due to sensitivity reasons)

*Non-transgenic controls have been added as supplemental figure 4.*

The abstract and introduction highlight the usefulness of the method to assess the maternal contribution for genes that have a zygotic function (and whose maternal function could not be analyzed in a traditional mutant because of inviability). However, the proof of principle genes chosen are maternal-effect genes and do not directly test the method for somatic-lethal mutations. Given this, the idea that the method is useful to generate germ line mutant chimeras in a wild type background, while reasonable, remains formally untested, and the concept be should toned down in particular in the abstract.

*The experiments that we performed to address R2's concern about leakage and to prove that knockout is in the germline and not somatic cells also address this issue because zygotic kif5Ba and kif5Bb function redundantly to promote craniofacial morphogenesis (Santos-Ledo et al., 2017). Compound zygotic mutants or double mutants lacking both proteins fail to undergo proper jaw morphogenesis and are inviable (Santos-Ledo et al., 2017). In the revised manuscript, we show that injecting kif5Ba guide RNA into kif5bb homozygous mutants "crispants" phenocopies the zygotic craniofacial defects observed in kif5Ba;kif5Bb double mutants (Santos-Ledo et al., 2017). These results are in revised Fig. 3L and N. In addition, we show that injecting gms:kif5Ba into kif5bb homozygous mutants did not cause jaw defects in green heart positive (those with the transgene) or in their transgene negative siblings. These data are provided in Fig. 3M and N. Moreover, these transgenic animals were viable to adulthood, further indicating that somatic mutations are not induced and demonstrating that the system can be used to bypass somatic-lethal mutations.*

Minor concerns

The "Validation of OMS and.." (page 11) states tests on three genes, but rbpms2a is not mentioned in the manuscript any further

*This was for system I, which was removed per R2's suggestion. The text has been revised accordingly.*

In the statement "GFP should be a proxy for Cas9 because both proteins are produced from the same transcript" (p 11). From the constructs, it appears that system I has two different transcripts (same transgene), only in system II it is the same transcript

*System I was removed per R2's suggestion. The text has been revised accordingly.*

For the proof-of-principle genes, it would be helpful if more background on previous studies was presented, in order to better make a comparison between previous approaches and the new one presented in this work. For example, more details on what “Global loss of maternal kif5Ba” (p 12) means.

*Additional details have been added to the revised text.*

Does the terminology of oms and gms apply only to System II, or also to System I? From the text, it seems that it depends on the promoter driving Cas9 (so that it would apply to both System I and II) but in Figure 1 only System II is specifically labeled as oms or gms
System I was removed per R2’s suggestion.

Related to the terminology issue, it is unclear in Figure 2 which system is represented in the images (I or II)
*System I was removed per R2’s suggestion.*

It is unclear whether the “Egg activation” phenotype in Figure 3J is part of the expected phenotype

*Yes, maternal kif5Ba mutants showed variable cortical granule exocytosis impairment (Campbell et al., 2015). This has also been addressed in response to the reviewer’s suggestion to add additional background.*

Generally it is unclear why the authors use the term “Progeny of" instead of the generation itself (e.g. "progeny of F1” rather than “F2”). Simply stating F0, F1, F2 would be a more direct way to convey the information, in a format that is standard and which would directly match Figure 1C

*This has been revised.*

Campbell, P.D., Heim, A.E., Smith, M.Z., Marlow, F.L., 2015. Kinesin-1 interacts with Bucky ball to form germ cells and is required to pattern the zebrafish body axis. Development 142, 2996-3008.
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Second decision letter

MS ID#: DEVELOP/2020/198010

MS TITLE: A Transgenic System for Targeted Ablation of Reproductive and Maternal-Effect genes

AUTHORS: Sylvain Bertho, Odelya Kaufman, KathyAnn Lee, Adrian Santos-Ledo, Daniel Dellal, and Florence L Marlow

I have now received all the referees reports on the above manuscript. 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.

The referees are mostly happy with your revisions but still have some questions, minor comments and suggestions for improving the manuscript before we publish. Referee 2 suggests an experiment for you to consider - this might indeed strengthen the paper but I will leave it in your hands

whether to do any further experimental work. 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*

Bertho, Kaufman et al describe effective and efficient tools for creating mutations in zebrafish germ cells and oocytes. These tools will allow the requirement for potential maternal effect genes with essential zygotic function to be determined with much greater efficiency than current methods such as germ line replacement.
They describe multiple proof of principle experiments that clearly demonstrate the efficiency of the method.This report also highlights that Cas9 can be expressed in the zebrafish germline. In all it is a well-supported description of an innovative method and tools that are likely to be used widely within the zebrafish community.

*Comments for the author*

The authors have addressed my previous comments to my satisfaction. The inclusion of the kif5Ba/kif5Bb genetic interaction certainly strengthens the work. A more accurate comparison would be a ubiquitous Cas9 transgene to replicate the mosaic nature of somatic mutation from the transgene but it's clear that for at least this example there is little somatic mutation and not enough to cause a phenotype and further proof is unnecessary. The manuscript is much improved. However, there are still a few outstanding issues.

In the sentence "we injected kif5Ba guide RNA into kif5Bbe6/e6 homozygous mutants" I assume the authors mean they injected kif5Ba guide RNA and Cas9 mRNA or protein as indicated in the figure. Supplementary figure 3 is confusing to me. The authors reference it for mutation rates but the figure is labeled as Germline Transgene Transmission. The y axes in B and C are labeled "% of positive green heart" which is consistent with it representing germ line transmission. The title of the graphs are "F2…" Are these F1 intercrosses? The transmission rates are pretty high so either they have multiple insertions in the F1's or they are actually mutation rates in green heart embryos? I'm confused...

Similarly, I'm not sure what figure 3 K is supposed to show. All embryos have green hearts? All with green hearts at 1 dpf are dead 2 dpf? It seems unnecessary in any case. Getting rid of it would allow the one extra panel in Supplementary Figure 4 to be included in this figure since most of Supplementary figure 4 is just repeated from Figure 3 anyway. Also, the legend for figure 3 E references black dashed circles but there are arrows on the figure and I see no dashed circles.

Reviewer 2

*Advance summary and potential significance to field*

As stated in theoriginal review, the method theauthors present would be used by researcher who want to induce mutations specifcally in the germline and study maternal-effect mutations.

*Comments for the author*

In their revised version of the manuscript Bertho et al addressed very well most of the points raised.
One issue that would have been easy to check and was not, concerns the proof that the system is useful for inducing mutations specifically in the germline in genes whose function is required in somatic cells during early development. Considering the possibility of cell competition, the lack of mutated cells in the fin etc could theoretically be caused by loss of / strong reduction in the number of mutated cells at late stages, or inability of mutated cells to contribute to specific structures when wild-type cells can take their place.

Injecting 1-cell embryos with guide RNAs directed against genes whose function is essential for early embryonic development (e.g aginst e-cadherin) would have proven the point the authors make beyond doubt and would have encouraged others to employ the method.

Reviewer 3

*Advance summary and potential significance to field*

The manuscript by Bertho et al presents a system of CRISPR/Cas9 gene knock out using a transgene carrying a guide RNA and Cas9. In this system, the generation of mutations is driven by a transgenic copy harboring both Cas9 and guide RNAs for the target genes. The system targets function during germ line and oogenesis using promoters specific for those stages.
Transcription of both components during germ cell development of the initial F0 (injected to generate the transgene) individual allows to generate mutations de novo at the targeted endogenous locus, leading to a mosaic germ line. Lesions at the endogenous locus can be recovered in F1 fish and propagated in non-mosaic individuals. Additionally, the transgene itself can be propagated, through males if it has a maternal-effect phenotype, to generate more lesions even when crossed to wild type fish.

The authors show that the system is able to generate lesions in two previously known genes, kif5Ba and Bucky Ball. The study presents a significant advance in the ability to generate mutations in targeted maternal genes, as the system allows in principle targeting lesions specifically in the germ line, thus allowing to assess the germ line function of genes that may be somatic lethal. Additionally, the system allows to generate biallelic mutations in the F0 fish themselves, allowing to test the function of genes in a single generation. This expedites the ability to test the function of target maternal-effect genes through reverse genetics.
This revision is an improved version of the previous submission, including new data addressing specificity of action of Cas9 in the germ line (as opposed to the soma) and revisions that improve the presentation and clarity of the data

*Comments for the author*

Minor comments
Abstract: there is a typo in the abstrat: "proof of principle" (currently "principal")

Is it known when exactly the transgenes (piwi in the gms construct and buc in the oms construct) are expressed relative to meiosis? A brief description directly addressing this would be valuable to better understand the relationship of Cas9 expression and the number of copies that need to be mutated to produce a maternal-effect phenotype

It would help in the Methods section if there was a section specific for the sequencing of new alleles, in particular given the difficulty to sequence new alleles if the wild type allele is also present.

In Results, in the “Phenocopy of Buc” section, the description and references to screens are not accurate: the screens are “diploidized haploids”, not “haploids”, and the 2004 reference for the relevant research article is "Pelegri et al (Dev Dyn) 2004", not “Pelegri and Mullins, 2004”

It is unclear why the authors state that the oms system is expected to be less efficient than the gms system. Is this because of the number of copies that need to be mutated at the time that Cas9 is active in the oms system (4 in the developing oocyte vs 2 in the germ line)? Here it would be helpful to have more clear discussion, as mentioned above, on how the timing of promoters relates to oogenesis in general and DNA replication and the meiotic divisions and the number of copies that need to be mutated.

The statement that germline replacement methods are “tedious, time intensive, and inefficient” is unnecessary and relatively subjective (since under the right conditions the germ line replacement method could be made more efficient). Ultimately the methodology presented in this study is another tool in the arsenal of tools that can be used to study maternal-effect genes, and all tools available are valuable.

**Second revision**

Author response to reviewers' comments

*We are delighted that the reviewers were enthusiastic about our revisions and appreciate their additional constructive feedback. We have addressed each of the points raised by the reviewers and made the suggested revisions to the manuscript text. Please find our point by point responses below. We hope that you will find our responses and the revised manuscript acceptable.*

Reviewer 1 Advance summary and potential significance to field
Bertho, Kaufman et al describe effective and efficient tools for creating mutations in zebrafish germ cells and oocytes. These tools will allow the requirement for potential maternal effect genes with essential zygotic function to be determined with much greater efficiency than current methods such as germ line replacement. They describe multiple proof of principle experiments that clearly demonstrate the efficiency of the method. This report also highlights that Cas9 can be expressed in the zebrafish germline. In all it is a well-supported description of an innovative method and tools that are likely to be used widely within the zebrafish community.

Reviewer 1 Comments for the author
The authors have addressed my previous comments to my satisfaction. The inclusion of the kif5Ba/kif5Bb genetic interaction certainly strengthens the work. A more accurate comparison would be a ubiquitous Cas9 transgene to replicate the mosaic nature of somatic mutation from the transgene but it's clear that for at least this example there is little somatic mutation and not enough to cause a phenotype and further proof is unnecessary. The manuscript is much improved. However, there are still a few outstanding issues.

In the sentence "we injected kif5Ba guide RNA into kif5Bbe6/e6 homozygous mutants" I assume the authors mean they injected kif5Ba guide RNA and Cas9 mRNA or protein as indicated in the figure.

*Thank you for pointing this out. The sentence has been revised to clarify.*

*"we injected kif5Ba guide RNA and Cas9 protein.."*

Supplementary figure 3 is confusing to me. The authors reference it for mutation rates but the figure is labeled as Germline Transgene Transmission. The y axes in B and C are labeled "% of positive green heart" which is consistent with it representing germ line transmission. The title of the graphs are "F2…" Are these F1 intercrosses? The transmission rates are pretty high so either they have multiple insertions in the F1's or they are actually mutation rates in green heart embryos? I'm confused...

*Supplemental figure 3 shows germline transmission. The embryos in B and C are a F2 generation coming from crosses of F1 transgenic fish. The green heart is used as a secondary marker for the transgene. This allows us to easily find embryos who express the transgene. The transgene germline transmission is independent of the mutation rate. We have revised the figure labels to hopefully clarify this point.*

Similarly, I'm not sure what figure 3 K is supposed to show. All embryos have green hearts? All with green hearts at 1 dpf are dead 2 dpf? It seems unnecessary in any case. Getting rid of it would allow the one extra panel in Supplementary Figure 4 to be included in this figure since most of Supplementary figure 4 is just repeated from Figure 3 anyway.

*3K has been removed as recommended by the reviewer. The revised Figure 3 (panels E) are enlarged versions of the "overview images" provided in Supplementary Figure 4, which we would like to keep because the additional panels do not fit nicely into the main figure.*

Also, the legend for figure 3 E references black dashed circles but there are arrows on the figure and I see no dashed circles.

*The correct version of the figure, with dashed circles, has been uploaded.*

Reviewer 2 Advance summary and potential significance to field
As stated in theoriginal review, the method theauthors present would be used by researcher who want to induce mutations specifcally in the germline and study maternal-effect mutations.

Reviewer 2 Comments for the author

In their revised version of the manuscript Bertho et al addressed very well most of the points raised.

One issue that would have been easy to check and was not, concerns the proof that the system is useful for inducing mutations specifically in the germline in genes whose function is required in somatic cells during early development. Considering the possibility of cell competition, the lack of mutated cells in the fin etc could theoretically be caused by loss of / strong reduction in the number of mutated cells at late stages, or inability of mutated cells to contribute to specific structures when wild-type cells can take their place.

Injecting 1-cell embryos with guide RNAs directed against genes whose function is essential for early embryonic development (e.g aginst e-cadherin) would have proven the point the authors make beyond doubt and would have encouraged others to employ the method.

*We appreciate the reviewer's opinion and suggestion; however, we respectfully disagree that examining e-cadherin "would have been easy to check" in the timeline of a revision or is necessary given that we already provide an example of a genetic interaction (kif5ba;kif5bb) that is required in the early embryo. To include e-Cadherin as another example as the reviewer requests, we would need to generate e-Cadherin guides, test and validate those guides, then generate the mutagenesis vectors with the e-Cadherin guides and inject them into embryos to establish 2-4 transgenic lines. Because we already provide other evidence and examples, we feel that the time, financial investment, and vertebrate animals involved to test additional specific examples that fall outside of the areas of research in the lab are not justified. Moreover, this is a short report and introducing the relevant biology in the space allocated would not be feasible.*

Reviewer 3 Advance summary and potential significance to field

The manuscript by Bertho et al presents a system of CRISPR/Cas9 gene knock out using a transgene carrying a guide RNA and Cas9. In this system, the generation of mutations is driven by a transgenic copy harboring both Cas9 and guide RNAs for the target genes. The system targets function during germ line and oogenesis using promoters specific for those stages.

Transcription of both components during germ cell development of the initial F0 (injected to generate the transgene) individual allows to generate mutations de novo at the targeted endogenous locus, leading to a mosaic germ line. Lesions at the endogenous locus can be recovered in F1 fish and propagated in non-mosaic individuals. Additionally, the transgene itself can be propagated, through males if it has a maternal-effect phenotype, to generate more lesions even when crossed to wild type fish.

The authors show that the system is able to generate lesions in two previously known genes, kif5Ba and Bucky Ball. The study presents a significant advance in the ability to generate mutations in targeted maternal genes, as the system allows in principle targeting lesions specifically in the germ line, thus allowing to assess the germ line function of genes that may be somatic lethal. Additionally, the system allows to generate biallelic mutations in the F0 fish themselves, allowing to test the function of genes in a single generation. This expedites the ability to test the function of target maternal-effect genes through reverse genetics.

This revision is an improved version of the previous submission, including new data addressing specificity of action of Cas9 in the germ line (as opposed to the soma) and revisions that improve the presentation and clarity of the data

Reviewer 3 Comments for the author

Minor comments

Abstract: there is a typo in the abstrat: "proof of principle" (currently "principal")
Thank you for pointing this out – this has been corrected.

Is it known when exactly the transgenes (piwi in the gms construct and buc in the oms construct) are expressed relative to meiosis? A brief description directly addressing this would be valuable to better understand the relationship of Cas9 expression and the number of copies that need to be mutated to produce a maternal-effect phenotype.

*We completely agree. In fact, this was in a previous version and cut out in response to reviewer comments and journal space limits. Trying to find the middle ground, we have briefly included this information in this revision as indicated below.*

*"As expected, the oms system, which is expressed in meiotic cells when there are four copies of each chromosome that must be mutated, was less efficient than the gms system, which is expressed earlier in mitotic germ cells which have only two copies of each chromosome."*

It would help in the Methods section if there was a section specific for the sequencing of new alleles, in particular given the difficulty to sequence new alleles if the wild type allele is also present.

*The methods section has been expanded as recommended.*

*Mutation detection and genotyping*
*Genomic DNA was extracted from adult fins using standard procedures (Meeker et al., 2007). The genomic region surrounding the kif5Ba target sequence was amplified using the primers 5'-GGAGTGCACCATTAAAGTCATGTG -3' and 5'- GTCGGTGTCAAATATTGAGGTC-3'. The genomic region surrounding the buc target sequence was amplified using the primers 5'-TGCAGTATCCTGGCTATGTGAT-3' and 5'- ACCACATCAGGGGTAGAAGAGA-3' (Supplemental Table 2). Products were then digested with T7 endonuclease and visualized on a gel to identify restriction patterns indicative of induced mutations.*

*Sequencing new alleles*

*Genomic DNA was extracted from adult fins using standard procedures (Meeker et al., 2007). The genomic region surrounding the kif5Ba target sequence was amplified using the primers 5'-GGAGTGCACCATTAAAGTCATGTG -3' and 5'- GTCGGTGTCAAATATTGAGGTC-3'. The genomic region surrounding the buc target sequence was amplified using the primers 5'-TGCAGTATCCTGGCTATGTGAT-3' and 5'- ACCACATCAGGGGTAGAAGAGA-3' (Supplemental Table 2). After 35 cycles of PCR at 59°C annealing for kif5Ba and 57°C for buc, PCR fragments were directly TA cloned into pCR4-TOPO vector (K457502, Invitrogen). After transformation, mini-prep DNA was prepared using Qiagen kits, and each plasmid was sequenced using universal primers on the vector. In this way, both the wild-type and any mutant alleles were detected.*

In Results, in the "Phenocopy of Buc" section, the description and references to screens are not accurate: the screens are "diploidized haploids", not "haploids", and the 2004 reference for the relevant research article is "Pelegri et al (Dev Dyn) 2004", not "Pelegri and Mullins, 2004"
Thank you for this correction, this has been revised and the appropriate reference included.

It is unclear why the authors state that the oms system is expected to be less efficient than the gms system. Is this because of the number of copies that need to be mutated at the time that Cas9 is

active in the oms system (4 in the developing oocyte vs 2 in the germ line)? Here it would be helpful to have more clear discussion, as mentioned above, on how the timing of promoters relates to oogenesis in general and DNA replication and the meiotic divisions and the number of copies that need to be mutated.

*Please see response above.*

The statement that germline replacement methods are "tedious, time intensive, and inefficient" is unnecessary and relatively subjective (since under the right conditions the germ line replacement method could be made more efficient). Ultimately the methodology presented in this study is another tool in the arsenal of tools that can be used to study maternal-effect genes, and all tools available are valuable.

*Yes, you are right, and we fully agree that all tools are valuable and having options to choose from is good for science. The sentence has been revised. Thank you!*

*"Although the frequency of phenotype detection varies for both gms and oms mutagenesis, this approach represents a significant advance in the tools available to study maternal-effect genes."*

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Third decision letter

MS ID#: DEVELOP/2020/198010

MS TITLE: A Transgenic System for Targeted Ablation of Reproductive and Maternal-Effect genes

AUTHORS: Sylvain Bertho, Odelya Kaufman, KathyAnn Lee, Adrian Santos-Ledo, Daniel Dellal, and Florence L Marlow
ARTICLE TYPE: Techniques and Resources Report

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