nature portfolio

# Peer Review File

## Expanding the CRISPR base editing toolbox in Drosophila melanogaster

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

General comments:

Clark et al. applied Cytosine Base Editors (CBEs) to the fruit flies in this manuscript. Although CBE has been applied in cell lines and mammals, and there have been two recent articles on its application in fruit flies, its application in fruit flies is not yet widespread. This manuscript compares three CBEs, using two promoters, one is an actin driver and the other is a nanos driver specific to germ cells, to quantitatively compare the six conditions of the combination. The authors obtained the efficiency under different conditions and observed their impact on different genders of fruit flies, such as male sterility and female lethality phenotypes. This manuscript provides an effective base editing tool for the Drosophila community.

Specific comments:

1. Figures. It's desirable to provide the images of the flies after base editing, either the entire animals or certain tissues.
2. The actin driver is mainly expressed in somatic cells in the reproductive system, with relatively low expression in germ cells. The authors can discuss the impact and explanation of this phenomenon on the results through experiments or citing relevant literatures.
3. There are multiple references with incomplete titles, and some words such as "Drosophila" have been omitted (such as references 15, 23, 24). All literature needs to be carefully checked for accuracy.
4. Some pioneering papers on the application of CRISPR in fruit flies (2013) have not been cited.
5. Reference 25 cites a corrigendum and it should cite the original paper.

Reviewer #2 (Remarks to the Author):

In this manuscript Clark, Maselko, Del Amo and colleagues describe new transgenic Drosophila strains expressing three base editors either ubiquitously or in the germline. They characterize them through base editing at two target sites in one gene and find that they mediate base editing with high efficiency and purity. However, further characterisation of

one editor at four additional sites reveals varying levels of efficiency. The authors also compare base editing when performed in either the male or female germline.

Overall, I am enthusiastic about this work, which significantly expands the base editing toolbox in the important Drosophila model and also provides interesting insights that might be relevant to expand this technology to insect disease vectors. Particular strengths of this study are generation of base editors that so far have not been tested in insects and the finding that product purity is dependent on the sex of the animal. A major limitation is the low number of tested target sites, which limits the generalizability of the findings.

Before the study can be published the authors will need to revise the manuscript to more accurately describe their findings and compare them to previous literature. While providing some additional experimental data would be welcome, I believe the essential revisions can be achieved by changes to text and figures. I provide detailed comments below.

I would like to congratulate the authors on this study, which makes an important contribution to the field.

Major points:

At several points in the manuscript the authors give the impression that the tested base editor strains mediate near complete base editing. This in particular applies to lines 26, 92, 388, but also other instances. This is misleading and needs to be revised. The authors find that at some target sites (e1, e2, tub1) editing rates are indeed very high, but at others they are intermediate (sxl1) or low (tub2, sxl2). An accurate description would be that editing rates were found to be variable and range between 4% and 99%. The number of sgRNAs that have been used in this study is unfortunately too low to draw definitive conclusions about efficiency. This is particularly true for the AID and ancBE4 editors, which have only been tested at the ebony sites. The data from rAPO1 targeting b-tub and sxl clearly shows that the high levels of editing observed here are likely not representative.

The authors suggest that a difference between the study of Doll et al and their system is that the Doll et al base editors are temperature sensitive, while the ones described here are not. But the authors of this study have not tested their base editors at different temperatures, so whether they are temperature sensitive or not remains unknown. Of note, also the CBEs described in Doll et al mediate efficient editing at 24C, with evoCDA1

mediating rates similar to the ones described here. The authors should revise the sections of the manuscript related to temperature sensitivity.

In the meantime there has been a third publication describing CRISPR base editors in Drosophila (Thakkar et al., PMID: 37917075). The authors need to include this paper in their comparison of their results with prior work.

In the experiments targeting ebony the authors focus on base editing in the germline, but it would be useful to know about the level of somatic editing as well. Could the authors comment, and ideally provide images, of the ebony phenotype in the F1 generation?

Please directly indicate the N numbers for crosses and number of flies used in each of the figures.

Some studies have reported toxicity of base editors in cells and animals. Could the authors please comment on the fitness of their CBE expressing strains.

The writing about the results presented in Figure 6c and d is not clear (Lines 360 - 372). The authors suggest that they will test if base editing is less efficient in females that survive than in those that die, but in the end they only sequence DNA from those that survived. In our experience sequencing from dead individuals is problematic, but if DNA is extracted shortly after death is possible. This would have been a useful comparison. It would also be interesting to quantify editing in males, where you don't have the potential selection for individuals with lower editing rates. These experiments would improve the study, although I don't find them essential for the overall conclusion. In case they are not performed this section should be rewritten to make more clear what has been done.

An interesting observation is that product purity is not only influenced by the sex of the animal, but also the base editor used. Could the authors share their view in the discussion on why in particular the two APO1 derived editors might result in different indel rates.

The levels of indels and other undesired edit types should also be reported for the editing of b-tub and sxl with rApo1.

Minor points:

Line 50: Inaccurate phrasing. The deaminase deaminates the DNA base, endogenous DNA repair then converts it into a point mutation.

Line 67: Explain 'Allele sail', as it is not standard terminology.

Line 326: In reference 24 base editing was used to also target several non-pigmentation genes, including essential genes. Please rephrase.

Line 340 and 353: For one genotype you must have used males. Please correct.

Line 370: it's not possible to induce lethality in escapees (either they are dead or they are escapees). Also, I find the word escapees unusual and have not come across it in the literature (escaper seems to be more common), but I'm not a native speaker so I might be wrong.

Line 388: This is not accurate. Editing rates for sex-determination genes were substantially lower.

Line 449: If the expected phenotype is female lethality and there are some female escapers it is not correct to state full penetrance of the phenotype.

Line 529: Indicate where the script will be accessible.

Line 545: Provide the average and minimum number of reads for these experiments.

Reviewer #3 (Remarks to the Author):

Improving genome engineering techniques in Drosophila and other insects is of great

interest by scientists studying gene function as well as genetic biological control of species. The work by Clark et al. is a welcome addition to the growing list of studies adapting new genome engineering tools that were developed in mammalian cells for insects. This work describes new base editor constructs for ubiquitous and germline expression that are remarkably effective at making heritable base edits. The work is convincing and manuscript well written. I appreciate the use of amplicon sequencing to accurately determine editing efficiency, as well as carefully distinguishing editing in female vs male germline. I recommend this manuscript be accepted, with minimal revisions (mentioned next).

Minor concern:

The sgRNA constructs used by the authors to target ebony, β-Tub, and sxl contain two individual sgRNAs, each with different target sequence. In Figure 3, where ebony is being edited, it is unclear how editing efficiencies compare at the two target sites that are shown in Figure 1c. For example, for a given base editor (e.g. nos-ancBE4), there are two blue dots the graphs of Figure 3. Do these two blue dots correspond to the two different sgRNA target sites? The text “Stop Codon at gRNA1 and/or gRNA2” in Figure 3 is confusing. It leaves me wondering how consistent each editor protein is for the two ebony sgRNA target site. For example, one ebony sgRNA target site could have excellent editing whereas the 2nd ebony sgRNA target site could have poor editing. Giving readers a good sense of base editor “consistency” between different sgRNA target sites is just as valuable as editing efficiency at individual sgRNA target sites. The text/figures need to be rewritten to be more clear about this point.

We sincerely appreciate the valuable feedback provided by all reviewers on our manuscript. The reviewers' comments helped us improve the manuscript substantially, and we hope to address all concerns raised and answer the questions satisfactorily. We are providing a revised version of the manuscript, including the suggested revisions and edits (highlighted in blue through the manuscript). Also, please find point-by-point responses written in blue text below.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

General comments:

Clark et al. applied Cytosine Base Editors (CBEs) to the fruit flies in this manuscript. Although CBE has been applied in cell lines and mammals, and there have been two recent articles on its application in fruit flies, its application in fruit flies is not yet widespread. This manuscript compares three CBEs, using two promoters, one is an actin driver and the other is a nanos driver specific to germ cells, to quantitatively compare the six conditions of the combination. The authors obtained the efficiency under different conditions and observed their impact on different genders of fruit flies, such as male sterility and female lethality phenotypes. This manuscript provides an effective base editing tool for the Drosophila community.

Specific comments:

1. Figures. It's desirable to provide the images of the flies after base editing, either the entire animals or certain tissues.

We have added a new figure (**Supplementary Fig. 1**) showing phenotypes for all F1 and F2 individuals from the conditions tested.

We have modified our results section accordingly as well:

"*Lastly, we provided images of all F1 and F2 individuals within all conditions tested. Interestingly, flies expressing base editors from the actin promoter displayed an ebony phenotype while those expressing from the nos promoter displayed a wildtype phenotype. This indicates the higher expression of the actin promoter in somatic tissues compared to nos, which is more germline specific[46]. Instead, all F2 individuals displayed ebony phenotype for both promoters, as expected (**Supplementary File - Supplementary Fig.1**)*".

2. The actin driver is mainly expressed in somatic cells in the reproductive system, with relatively low expression in germ cells. The authors can discuss the impact and explanation of this phenomenon on the results through experiments or citing relevant literatures.

We have added new text and citations to clarify this point:

"*The overall phenotyping data indicated close to complete base editing efficiency for all actin expressed CBEs, though the nos promoter seemed to be less efficient (**Fig. 2b-e**). While the nos promoter is germline-specific, the act promoter has also shown high expression in both male and female germline cells[40]. In fact, the act promoter has produced higher editing rates in various CRISPR-based technologies, such as gene drives[32], compared to nos, suggesting its overall higher performance*".

3. There are multiple references with incomplete titles, and some words such as "Drosophila" have been omitted (such as references 15, 23, 24). All literature needs to be carefully checked for accuracy.

We have fixed this. It seems our citation software was introducing those inaccuracies.

4. Some pioneering papers on the application of CRISPR in fruit flies (2013) have not been cited.

We have added this work.

5. Reference 25 cites a corrigendum and it should cite the original paper.

Thanks. This has been fixed. This work is ref #26 in the new version.

Reviewer #2 (Remarks to the Author):

In this manuscript Clark, Maselko, Del Amo and colleagues describe new transgenic Drosophila strains expressing three base editors either ubiquitously or in the germline. They characterize them through base editing at two target sites in one gene and find that they mediate base editing with high efficiency and purity. However, further characterisation of one editor at four additional sites reveals varying levels of efficiency. The authors also compare base editing when performed in either the male or female germline.

Overall, I am enthusiastic about this work, which significantly expands the base editing toolbox in the important Drosophila model and also provides interesting insights that might be relevant to expand this technology to insect disease vectors. Particular strengths of this study are generation of base editors that so far have not been tested in insects and the finding that product purity is dependent on the sex of the animal. A major limitation is the low number of tested target sites, which limits the generalizability of the findings.

Before the study can be published the authors will need to revise the manuscript to more accurately describe their findings and compare them to previous literature. While providing some additional experimental data would be welcome, I believe the essential revisions can be achieved by changes to text and figures. I provide detailed comments below.

I would like to congratulate the authors on this study, which makes an important contribution to the field.

We thank Reviewer #2 for these words.

Major points:

1. At several points in the manuscript the authors give the impression that the tested base editor strains mediate near complete base editing. This in particular applies to lines 26, 92, 388, but also other instances. This is misleading and needs to be revised. The authors find that at some target sites (e1, e2, tub1) editing rates are indeed very high, but at others they are intermediate (sxl1) or low (tub2, sxl2). An accurate description would be that editing rates were found to be variable and range between 4% and 99%. The number of sgRNAs that have been used in this study is unfortunately too low to draw definitive conclusions about efficiency. This is particularly true for the AID and ancBE4 editors, which have only been tested at the ebony sites. The data from rAPO1 targeting b-tub and sxl clearly shows that the high levels of editing observed here are likely not representative.

While we agree gRNA efficiencies can vary between genes, it is important to note that these editing rates differences could also be due to the difference between tissues analyzed in both cases (ebony-germline (F2 individuals) vs. b-tub/sxl-somatic tissues (F1 individuals)).

We have added a comment to clarify this point in the discussion:

*"While the ebony gRNAs showed high germline base editing efficiencies under almost any condition when analyzing F2 individuals, gRNAs targeting the β-Tub and sxl genes displayed lower efficiencies. It is important to highlight that in this case, we are analyzing somatic base editing rates as we analyze F1 individuals. Overall, these results indicate that the selection of gRNAs is crucial for effective base editing".*

2. The authors suggest that a difference between the study of Doll et al and their system is that the Doll et al base editors are temperature sensitive, while the ones described here are not. But the authors of this study have not tested their base editors at different temperatures, so whether they are temperature sensitive or not remains unknown. Of note, also the CBEs described in Doll et al mediate efficient editing at 24C, with evoCDA1 mediating rates similar to the ones described here. The authors should revise the sections of the manuscript related to temperature sensitivity.

We meant to identify base editor domains with high efficiency at 25C rather than investigating the temperature sensitivity of the new base editors tested. We agree with Reviewer #2 that this remains unknown still.

We have revised the manuscript regarding the temperature comments and modify some of our statements to clarify our message in the introduction and discussion.

Introduction

From:

"*However, these efficiencies required the elevated temperature of 29˚C, which is not ideal, as the optimal survival and preferred temperature in Drosophila is 25˚C[27,28]. Therefore, we sought to identify different base editor domains displaying high editing rates without temperature requirements and using two different promoters*"

To:

*"Interestingly, both studies required the elevated temperature of 28-29˚C to achieve higher editing rates in specific situations, which is not ideal, as the optimal survival and preferred temperature in Drosophila is 25˚C[27,28]. Therefore, we sought to identify different base editor domains displaying high editing rates (90-100%) at 25˚C and using two different promoters"*

Discussion

From:

*"Importantly, the extremely high efficiency was maintained across all four possible modes of CBE transmission and our system did not require elevated temperatures, as observed before in flies"[25].*

To:

*"Importantly, the extremely high efficiency was maintained across all four possible modes of CBE transmission at 25˚C".*

In the meantime there has been a third publication describing CRISPR base editors in Drosophila (Thakkar et al., PMID: 37917075). The authors need to include this paper in their comparison of their results with prior work.

We have added this work in our resubmission and modified the introduction and discussion accordingly. The new text regarding this work is highlighted in blue.

Introduction:

*"While preparing this manuscript, two studies have demonstrated transgenic expression of CBEs and ABEs in the model organism D. melanogaster targeting the germline[24,25]. Thakkar and colleagues targeted the white gene using first-generation CBEs and ABEs previously optimized in human cell culture[8,9], and reported an average germline-editing rate of 70%"*

Discussion:

*"Takkar and colleagues employed two CBE domains: i) the same APOBEC1 (rAPO-1) tested in this work, and ii) the CBEevoCDA1 tested by Doll and colleagues[25]. However, their study reported lower base editing rates (~70%) compared to ours and the studies by Doll and colleagues. These differences could be due to the nuclease used: while we and Doll and colleagues employed the regular nickase Cas9 tested in previous human cell culture studies[8,9], Takkar and colleagues tested a modified version, xCas9[59], which could be less active, as previously shown in Drosophila[60]"*

3. In the experiments targeting ebony the authors focus on base editing in the germline, but it would be useful to know about the level of somatic editing as well. Could the authors comment, and ideally provide images, of the ebony phenotype in the F1 generation?

Following the Reviewer #2 suggestion (also requested by Reviewer #1), we have added a new figure showing F1 and F2 phenotypes. This can be found in the new **Supplementatry Fig.1**.

We have also added new text clarifying these observations (results section):

"*Lastly, we provided images of all F1 and F2 individuals within all conditions tested. Interestingly, flies expressing base editors from the actin promoter displayed an ebony phenotype while those expressing from the nos promoter displayed a wildtype phenotype. This indicates the higher expression of the actin promoter in somatic tissues compared to nos, which is more germline specific[46]. Instead, all F2 individuals displayed ebony phenotype for both promoters, as expected (**Supplementary File - Supplementary Fig. 1**)*".

4. Please directly indicate the N numbers for crosses and number of flies used in each of the figures.

We have added this information in **Figure 1** and modified the figure legends of all figures indicating where this information can be found (**Supplementary Tables**).

5. Some studies have reported toxicity of base editors in cells and animals. Could the authors please comment on the fitness of their CBE expressing strains.

We did not quantify any fitness cost imposed by the CBEs transgenes. Yet, our stocks have been maintained for 2 years now as regular wildtype flies without any issues.

6. The writing about the results presented in Figure 6c and d is not clear (Lines 360 - 372). The authors suggest that they will test if base editing is less efficient in females that survive than in those that die, but in the end they only sequence DNA from those that survived. In our experience sequencing from dead individuals is problematic, but if DNA is extracted shortly after death is possible. This would have been a useful comparison. It would also be interesting to quantify editing in males, where you don't have the potential selection for individuals with lower editing rates. These experiments would improve the study, although I don't find them essential for the overall conclusion.

In case they are not performed this section should be rewritten to make more clear what has been done.

As suggested by Reviewer #2, we performed amplicon sequencing on males carrying the Cas12a and sxl transgenes, which are viable and suitable for molecular characterization.

We found that these males had higher editing rates compared to the previously studied "surviving/escapees" females carrying both transgenes at "target site 1" (85% vs. 75%). Additionally, we confirmed that "target site 2" for sxl appears to be almost inactive, as previously observed in the “surviving/escapees females”, suggesting that the female lethality phenotype is likely imposed by gRNA-1. We have addressed this in the revised version submitted.

In summary, we have included the data from males in the main figure (**Fig. 6d**) and retained the data from "surviving/escapees" females as a new **Supplementary Fig. 4**. The text has been adjusted accordingly where necessary.

7. An interesting observation is that product purity is not only influenced by the sex of the animal, but also the base editor used. Could the authors share their view in the discussion on why in particular the two APO1 derived editors might result in different indel rates.

We believe that the ancBE4 domain evolved from the original Rattus norvegicus-derived cytosine deaminase APOBEC1 (rAPO-1 in our work) could be a better option for human purposes, as it was obtained through codon optimization using human cell culture. Since preferred codon usage varies between organisms, it is possible that the original APOBEC1 (without any modification) from rat is better suited for *Drosophila* usage rather than the ancBE4 obtained using human cell culture.

In fact, it appears that the usage of synonymous codons is under selective pressure in some organisms such as *Drosophila*, and this could have a significant impact on the transgenes optimization as well.

We have added this view in the discussion,  as suggested:

*“We did not observe major differences between our r-APO-1 and ancBE4 in terms of editing efficiency, though the r-APO-1 generated less indels compared to the ancBE4. It is assumed that synonymous mutations in mammals are generally considered free from*

*natural selection, imposing little to no impact on fitness[55]. However, the usage of synonymous codons in animals such as Drosophila or worms is under selective pressure, significantly affecting the fitness of these organisms[56,57]. This could be the reason why, while ancBE4 was preferred over r-APO-1 in previous work using mammalian cells[29], our results suggest that r-APO-1 is a better choice for base editing in flies".*

8. The levels of indels and other undesired edit types should also be reported for the editing of b-tub and sxl with rApo1.

We have generated a new figure (**Supplementary Fig.5**) to show this information. As shown with the ebony gRNAs. C to T transitions and no indels (insertions and/or deletions) were the most prevalent events observed when targeting *b-tub* and *sxl* genes too using the *nos*-rAPO-1.

Minor points:

Line 50: Inaccurate phrasing. The deaminase deaminates the DNA base, endogenous DNA repair then converts it into a point mutation.

This has been rephrased.

Line 67: Explain 'Allele sail', as it is not standard terminology.

We have added a few sentences to explain it.

Line 326: In reference 24 base editing was used to also target several non-pigmentation genes, including essential genes. Please rephrase.

This has been rephrased.

Line 340 and 353: For one genotype you must have used males. Please correct.

This has been corrected. Thanks.

Line 370: it's not possible to induce lethality in escapees (either they are dead or they are escapees). Also, I find the word escapees unusual and have not come across it in the literature (escaper seems to be more common), but I'm not a native speaker so I might be wrong.

Both options work. So, we decided to keep escapees.

Line 388: This is not accurate. Editing rates for sex-determination genes were substantially lower.

This has been modified for accuracy.

Line 449: If the expected phenotype is female lethality and there are some female escapers it is not correct to state full penetrance of the phenotype.

This has been addressed.

Line 529: Indicate where the script will be accessible.

We have added the link (can be found in the Methods section):

https://github.com/aidantay/Fly_CRISPResso_analysis

Line 545: Provide the average and minimum number of reads for these experiments.

This has been added:

"*A minimum of 100,000 reads aligned in all our experiments using CRISPResso2.*"

Reviewer #3 (Remarks to the Author):

Improving genome engineering techniques in Drosophila and other insects is of great interest by scientists studying gene function as well as genetic biological control of species. The work by Clark et al. is a welcome addition to the growing list of studies adapting new genome engineering tools that were developed in mammalian cells for insects. This work describes new base editor constructs for ubiquitous and germline expression that are remarkably effective at making heritable base edits. The work is convincing and manuscript well written. I appreciate the use of amplicon sequencing to accurately determine editing efficiency, as well as carefully distinguishing editing in female vs male germline. I recommend this manuscript be accepted, with minimal revisions (mentioned next).

Minor concern:

The sgRNA constructs used by the authors to target ebony, β-Tub, and sxl contain two individual sgRNAs, each with different target sequence. In Figure 3, where ebony is being edited, it is unclear how editing efficiencies compare at the two target sites that are shown in Figure 1c. For example, for a given base editor (e.g. nos-ancBE4), there are two blue dots the graphs of Figure 3. Do these two blue dots correspond to the two different sgRNA target sites? The text “Stop Codon at gRNA1 and/or gRNA2” in Figure 3 is confusing. It leaves me wondering how consistent each editor protein is for the two ebony sgRNA target site. For example, one ebony sgRNA target site could have excellent editing whereas the 2nd ebony sgRNA target site could have poor editing. Giving readers a good sense of base editor “consistency” between different sgRNA target sites is just as valuable as editing efficiency at individual sgRNA target sites. The text/figures need to be rewritten to be more clear about this point.

We have added a new **Supplementary Fig.2** to show the contribution of each gRNA to the stop codon generation and modified the text accordingly:

“*Lastly, we examined the contribution of each gRNA separately to extend the stop codon analysis. Indeed, both gRNAs introduced stop codons at the target nucleotide. However, gRNA2 was more active for every base editor and condition tested, suggesting its major contribution to the observed phenotypes (**Supplementary Fig. 2**)*”.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have addressed most of this reviewer's concerns.

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns and I now recommend publication of the manuscript.

Reviewer #3 (Remarks to the Author):

The authors addressed my concerns and I recommend the manuscript be accepted.