

Enhanced RNA-targeting CRISPR-Cas technology in zebrafish

Corresponding Author: Dr Miguel Moreno-Mateos

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors optimized CRISPR-RfxCas13d system to knock down maternal and zygotic mRNA/microRNA in zebrafish embryos. They showed that chemically modified gRNAs, nuclear RNA-targeting, and RNA targeting model predicting could be utilized to enhance CRISPR-RfxCas13d system in zebrafish. The study is well designed, and the optimized CRISPR-RfxCas13d tool may be of broad use for RNA targeting in zebrafish. While the credibility and practicality of this system is not well explored.

1. Previous work (Gopal Kushawah et al (2020)) showed that CRISPR-RfxCas13d works well in zebrafish, with 76% average decrease in transcript levels, as well as undetected collateral activity. The current article does not see higher efficiency improvements. In addition, in the section of the comparison between alternative transient RNA-targeting systems, DjCas13d displayed less collateral activity than RfxCas13d but similar efficiency. So what's the new about this article?
2. On line 148, the maternal and mid-zygotically expressed genes (only one gene) are too little to draw a conclusion that "RNP complexes were much less active when targeting mRNAs from genes whose main expression occurs later during development after 7-8 hpf".
3. On line 182, the authors demonstrate that IVTcd RfxCas13d gRNAs have toxic effects, which is unprecise. Firstly, the purity of RNA between in vitro transcription synthesis and chemically synthesis may be not the same. Secondly, the collateral activity between IVTcd gRNAs and chemically synthesized gRNAs is not evaluated, since collateral activity could also cause embryogenesis delay or lethal. Except for RfxCas13d system, other CRISPR systems (e.g. CRISPR-SpCas9, CRISPR-DjCas13d) should be introduced as controls or supplement to support this conclusion.
4. In Fig. 1E and Extended Data Fig. 1B, whether gRX3 and gRX3-1 are the same gRNA? If it's the same, the combination of RfxCas13d mRNA and cm-gRNA is more efficient than RNP form in rx3, albino and tyr. Why did this happen?
5. Except for nanog, no-tail, rx3 phenotypes, the authors should give more specific phenotype from other knock-down genes, including evaluating other unknown genes by this system. Moreover, for the purpose of RNA-targeting, compared to morpholino, the advantages of RfxCas13d should be explored.
6. How to choose the target sequence? If targeting the same gene on different domains, the phenotype is the same or not?
7. On line 264, is there significant difference between 10 or 25 gRNAs as a set to detect highly efficient gRNAs? Why choose 25 gRNAs as a set to evaluate the KD efficiency?
8. Some detailed information should be added to figures. Such as in Fig. 3B, 3D, mRfxCas13d or pRfxCas13d should be showed as a control.

Reviewer #2

(Remarks to the Author)

The manuscript by Moreno-Sanchez et al. is an excellent example of a comprehensive characterization of a new CRISPR-related tool for research in zebrafish.

The authors performed an impressive amount of high-quality work that will hopefully allow the whole zebrafish field to start using this tool.

Below are some comments to further improve the manuscript.

Major:

- In Summary, please indicate what Cas13d generally does.
 - Line 281: why do you have to classify the whole set? Are there cases when individual guide RNAs are classified well, but the whole set is somehow defective?
- Can you mention what was wrong with the other 3 sets?
- Lines 291-292: The phrase "to moderately classify CRISPR-RfxCas13d activity delivered in vivo as RNP complexes" has a few issues: "moderately" does not have a precise meaning here, the second one is that you are classifying the guide RNAs, not the protein activity.
 - Phenotype-based verification of miR430-targeting guideRNAs needs to be significantly substantiated by molecular measures of targeting.
 - Figure 3A: It is unclear if your guide RNAs actually targeted inside the miRNA encoding parts of the transcript.
 - Figure 3: There should be verification of mature miR430s levels after the knockdown
 - Line 554: The phrase "together with translational inhibitors" is unclear. How would this be done in practice without killing the fish?

Minor:

- Line 46: "RNA-targeting" should be 2 words in this case. Otherwise, "RNA-targeting" is appropriate as an adjective.
- Line 58: Please indicate the origin of RfxCas13d and its history prior to this study, especially its usage in zebrafish.
- Line 125: The phrase "later during development after 7-8 hours post-fertilization" is somewhat confusing. Please make the timing more precise and the sentence grammatically correct.
- Line 132: It is unclear what was combined with RfxCas13d. Please revise.
- Line 170-171: The phrase "was lethal with previous developmental defects even by 2 hpf" is unclear. In what sense defects are previous?
- Line 224: "it" is not needed.
- Line 236-238: need to indicate the type of experiment and that 203 is not a sample number.
- Line 302: do you mean that ribosomes are targeted?
- Line 313: The phrase "KD of 20 or more pg of gfp mRNA" is not suitable for a scientific publication. Please describe things more precisely.
- Line 315-316: Please check if "data not shown" is allowed.
- Line 361: "eliminated" is not the right word, "targeted" may be better.
- Line 371: "a reduced or lack of" can be shortened to "little or no".
- Line 416: down-regulation should be indicated by negative log values or small numbers less than 1.
- Line 445: incorrect usage of "allow", you could write "allow us to ...".
- Line 468: "that" should be "than". "approaches with a constitutive expression" should be "approaches using constitutive expression".
- Line 498-499: check the grammatical usage of "despite" and correct the sentence.
- Line 516: After "chemically synthesized" should be a noun and the phrase itself should be "chemically-synthesized".
- Line 566: "being" is at the wrong position in the sentence.
- Line 567: "to a lower degree" is the correct version.
- Line 568-569: "broadening and enhancing" is the correct usage.
- For all figures, please indicate whether a protein or mRNA RfxCas13d was used such as mRfxCas13d or pRfxCas13d

Reviewer #3

(Remarks to the Author)

This manuscript by Moreno-Sanchez, Hernandez-Huertas, Nahon-Cano and colleagues benchmarks the performance of various RNA-targeting CRISPR systems in zebrafish. Specifically, they improve the performance of the RfxCas13d system via use of chemically-modified gRNAs and optimized placement of nuclear localization signals. They report interesting discrepancies in silencing efficiency of temporally-restricted transcripts when RfxCas13d and its guide are delivered via RNP or mRNA-gRNA formulations. The thorough comparison of on-target efficiency and collateral activity of RfxCas13d, DxCas13d and Cas7-11 is useful for the CRISPR community.

Major point:

In Figure 2, the authors show convincing data that certain IVTed gRNAs induce toxicity in zebrafish embryos, whereas chemically synthesized gRNAs do not. In the Discussion section, the authors state that the difference in toxicity between chemically synthesized and IVTed gRNAs is "remains to be determined".

It is known that T7 in vitro transcription results in the production of RNAs that carry 5'-triphosphate groups. This 5'ppp can trigger a RIG-I-mediated innate immune responses which may lead to cell death, particularly in primary mammalian cells (Wienert et al., 2018, PLOS Biology; Kim et al., 2018, Genome Biology). The extent of the innate immune response is protospacer sequence-specific, which may be why the authors see different toxicities between gRNAs 1-3. Additionally, T7 RNA polymerase is reported to have some RNA-dependent RNA transcription activity, which can lead to the generation of dsRNAs via anti-sense transcription and/or spurious "turn-around" transcription in cis. dsRNA can also activate innate immune responses.

Removal of the 5'ppp via e.g. phosphatase treatment has been shown to prevent such an immune response and thereby rescue loss in cell viability when using IVTed gRNAs. It doesn't appear as though this step is included in the IVT protocol. Did the authors profile the interferon response following administration of the IVTed gRNAs? Does 5'ppp removal rescue this toxic effect, as it does in mammalian cells?

Minor points:

- Recent reports have directly benchmarked RfxCas13d against PspCas13b in mammalian cells, concluding that PspCas13b exhibits comparable on-target activity but superior specificity due to the absence of noticeable collateral activity. (Hu et al., NSMB, 2024). It is obviously not feasible to test all orthologues in every study, but I am curious as to whether authors have tested PspCas13b in the zebrafish system? It would be interesting to see how this compares with RfxCas13d, and particularly with DjCas13d, in terms of both on-target efficiency and collateral activity.
- "One-cell stage zebrafish embryos were injected with 300 pg of RfxCas13d mRNA or 3 ng of RfxCas13d protein together with 1 ng of a mix of three gRNAs (~300 pg from each gRNA)." How were the doses of mRNA vs protein selected? Do these doses lead to approximately equal Cas13 expression level? Does delivering a higher or lower dose of Cas13 affect the targeting window (i.e. early- vs -late transcripts)?
- The authors convincingly show that the RNAtargeting algorithm performs better at predicting gRNAs efficiency vs TIGER or DeepCas13. Why does it perform better for some guide sets (i.e. sets 1-4) but worse for others (e.g. set 5, 7). What happens if you look at individual guide performance rather than pooled guide sets?
- In Figure 6B, injection of Cas7-11 alone shows a decently strong epiboly phenotype (in ~30% of embryos), even in the absence of gRNA. This is not the case for the no-tail phenotype in Figure 6C. Why is Cas7-11 alone affecting the epiboly phenotype?

Stylistic points:

- The phenotype severity for noto, rx3 and tyrosinase are ordered as least-to-most sensitive from left-to-right, but nanog is ordered from most-to-least severe.
- In this reviewer's opinion, it would be beneficial to show all four stages from Figure 1B (maternal, early zygotic, mid-zygotic and late-zygotic) in panels 1D and 1E, for both protein and mRNA. This would make the contrast between delivery modes at each timepoint clearer, and saves the reader flicking between the main and supplementary figures.
- The data showing collateral targeting of the DsRed reporter in the context of high-abundance eGFP targeting is an important control and a relevant finding. I recommend the authors include this data in the main figures, rather than in the supplementary data.
- Please also include the colour code legend (from 6A) for the various proteins in Extended Data Fig 9a.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors did a fantastic job in addressing most of my points, and these revisions have significantly improved the manuscript.

Reviewer #2

(Remarks to the Author)

The authors did a great job in addressing my comments on the previous version of the manuscript and made extensive edits to the manuscript text. These changes should contribute to the manuscript becoming acceptable for publication.

Reviewer #3

(Remarks to the Author)

The authors have addressed my concerns. The new data presented in Figure 2 is a valuable addition to the manuscript.

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Reviewer comments & Author responses

Dear Reviewers,

We are thankful to the reviewers for their thoughtful comments and valuable suggestions. We have modified the manuscript according to the reviewers' comments and the changes were highlighted in red. The detailed point-by-point responses are listed below.

Reviewer #1 (Remarks to the Author):

The authors optimized CRISPR-RfxCas13d system to knock down maternal and zygotic mRNA/microRNA in zebrafish embryos. They showed that chemically modified gRNA, nuclear RNA-targeting, and RNAtargeting model predicting could be utilized to enhance CRISPR-RfxCas13d system in zebrafish. The study is well designed, and the optimized CRISPR-RfxCas13d tool may be of broad use for RNA targeting in zebrafish. While the credibility and practicality of this system is not well explored.

1. Previous work (Gopal Kushawah et al (2020)) showed that CRISPR-RfxCas13d works well in zebrafish, with 76% average decrease in transcript levels, as well as undetected collateral activity. The current article does not see higher efficiency improvements. In addition, in the section of the comparison between alternative transient RNA-targeting systems, DjCas13d displayed less collateral activity than RfxCas13d but similar efficiency. So what's the new about this article?

We thank the reviewer for pointing out our efforts to enhance CRISPR-Cas RNA-targeting in zebrafish and his positive comments on our experimental design. We apologize if the novelty of our work that further enhanced CRISPR-Cas RNA-targeting has not been clearly described. We review below all new improvements developed in this manuscript that are beyond to increase the targeting efficiency and **cover other important aspects of the technology that needed to be tested and enhanced**. Indeed, we acknowledged exactly this point in the introduction of our manuscript, lines 68-70 that it is more detailed in lines 71-91. Beyond this paragraph in the introduction, all these novel CRISPR-Cas enhancements developed in this work are point-by-point further considered and contextualized in the Discussion section that is introduced by a paragraph highlighting our novel improvements of CRISPR-Cas RNA-targeting technology in zebrafish (lines 497-502).

Therefore, while in our previous paper Kushawah et al., 2020, Developmental Cell (hereafter Kushawah et al) we were able to implement CRISPR-RfxCas13d system in zebrafish and other vertebrate embryos, we identified a set of issues from that paper and our continued work from 2021 that could be corrected. This is what we have precisely addressed now:

1. In Kushawah et al we have shown that nuclear targeting was not optimized in zebrafish embryos and much lower efficiency was obtained using a standard NLS formulation (Kushawah et al, Supp. Fig. 1K). We detailed this previously detected

drawback in our current manuscript in the Results section lines 248-252. Here, we have explored other NLS combinations and found that RfxCas13d-2C-NLS significantly improved nuclear targeting in comparison to our original RfxCas13d-NLS version. **This novel optimization will broad the use of the technology** in zebrafish particularly **when nuclear RNA is targeted** circumventing an issue that was not previously solved.

2. We also found that long-term RNA targeting to eliminate mRNA from genes expressed after 7-8 hpf was not as efficient as when depleting maternal mRNAs or genes expressed during first waves after zygotic genome activation during first 5-6 hpf. This was clearly showed in Kushawah et al comparing the lower level of phenotype penetrance targeting *tyr* mRNA a gene expressed at 16-18 hpf (Supp. Fig. 3O in Kushawah et al., 2020) vs targeting *nanog* or *tbxta* mRNA genes that are maternally provided or early expressed (at 4-5 hpf), respectively (Supp. Fig. 3H and Fig. Supp. 1M in Kushawah et al). This issue was mentioned in the Introduction section, lines 71-72 and in Results section, lines 126-127. Now, using chemically modified gRNAs we have been able to enhance the phenotype penetrance and obtain a more robust targeting on genes whose highest level are after 7-8 hpf. **The advantages of this novel improvement of the system** were extensively discussed in lines 502-519.

3. In Kushawah et al, we discussed about CRISPR-RfxCas13d variable efficiency *in vivo* using our transient approaches as it had been observed in other studies in mammalian cell culture employing plasmids or lentiviral systems to express gRNAs and RfxCas13d (Konermann S et al., 2018 Cell, Wessels HH et al., 2020 Nature Biotech.). In that paper, Kushawah et al 2020, we speculated that it would be convenient to have a tool to predict CRISPR-RfxCas13d activity *in vivo* using RNP (see Limitations section from Kushawah et al for details). We, additionally, highlighted this constraint in the introduction of this manuscript, lines 74-76. Now, applying different computational models developed in mammalian cell culture with a constitutive and strong expression of gRNAs and RfxCas13d, we have tested their ability to predict CRISPR-RfxCas13d activity in zebrafish embryos injected with RNPs. We have been able to identify a computational model that outperforms others in predicting CRISPR-RfxCas13d targeting efficacy in zebrafish (RNAtargeting, Wei J et al., 2023 Cell Systems). Therefore, **this new analysis will help to select active gRNAs** when using transient approaches such as RNP complexes in zebrafish and in other *in vivo* systems. However, we also acknowledged RNAtargeting presented limitations, and a computational model specifically designed and developed for transient and *in vivo* applications will likely increase the accuracy of the predictions that we have reported here using RNAtargeting (Discussion: lines 530-553).

4. Most of the gRNAs used in Kushawah et al were *in vitro*-transcribed (IVTed) gRNAs and we showed that they were a bit less efficient than chemically synthesized gRNAs and equally specific. However, from 2021 until now we have generated and used much more IVTed gRNAs, and we noticed that some of them can trigger toxicity in the zebrafish embryo. This was not observed when using an equivalent gRNA but chemically synthesized. To further clarify and optimize the use of CRISPR-RfxCas13d in zebrafish we believed that it was needed to report this observation. In addition, we have characterized this occasional toxicity of some IVTed gRNAs. Importantly, IVTed

gRNAs are less expensive than chemically synthesized gRNAs, and therefore more economically accessible for the scientific community. Consequently, we also tested alternative conditions to generate a useful IVT-ed gRNA, such as using a different RNA polymerase or 5' triphosphate phosphatase treatment of the IVT-ed gRNA (Kim S et al., 2018; Wienert B et al., 2018) that did not eliminate the toxicity. Alternatively, we have developed a protocol to pre-select high-quality IVT-ed gRNAs or demonstrated that IVT-ed gRNAs for other CRISPR-Cas13d systems could be used instead if that particular region of the mRNA and no other needs to be targeted (see below at point 3 and lines 213-235 and 475-487 for details).

Altogether, **our new results from this section of the manuscript will democratize the application of CRISPR-RfxCas13d technology** with a more controlled use of IVT-ed gRNAs without the need of employing chemically synthesized and more expensive gRNAs. We have particularly pointed out this whole subject in the Discussion section, lines 575-603.

5. In 2020, only a report using CRISPR-Cas13a had shown collateral activity in eukaryotic cells (Wang Q et al., 2018 Adv Science) and there was not any evidence of this effect when using CRISPR-RfxCas13d. Indeed, the collateral activity in eukaryotic cells was only initially described and it was not clearly detected. In Kushawah et al, we did not observe differences in RIN or embryonic toxicity in KD compared to control conditions when CRISPR-RfxCas13d was used as mRNA-gRNA or RNP complexes. These results were compatible with the absence of collateral activity. However, later in 2022 and 2023 several reports showed that CRISPR-RfxCas13d could trigger collateral activity in mammalian cells specially when the targets were highly expressed and always under constitutive expression of gRNAs and RfxCas13d. Additionally, these papers showed further molecular effects from the collateral activity that helped to unequivocally identify it. Nevertheless, the collateral activity induced by CRISPR-RfxCas13d was not deeply studied when employing transient approaches frequently used *in vivo* such as mRNA-gRNA or RNP. Besides, in Kushawah et al we did not study this effect when targeting highly abundant reporter or endogenous mRNAs. The issue related to the collateral activity triggered by CRISPR-RfxCas13d and the lack of a comprehensive study *in vivo* is detailed in the manuscript in several sections such as lines 77-91 and lines 345-352 in the Introduction and Results section, respectively. Therefore, **we aimed to update our study on the collateral activity *in vivo* using transient reagents and zebrafish embryos as vertebrate model.** Here, and for first time, we were able to demonstrate that CRISPR-RfxCas13d triggers collateral activity in zebrafish embryos but only when targeting extremely abundant and exogenous mRNAs without any substantial or minimal toxic effect when highly expressed endogenous transcripts were depleted. We analyzed the collateral activity not only measuring RIN, mRNA reporter degradation or developmental defects but also through RNA-seq in conditions where a mild (10 pg of mRNA GFP reporter per embryo) or extreme (50 pg of mRNA GFP reporter per embryo) collateral activity was induced. Moreover, we determined that rRNA fragmentation is a very sensitive and straightforward assay to detect collateral activity *in vivo* even in conditions where no developmental defects were found during zebrafish embryogenesis. **Altogether, our new comprehensive characterization of the CRISPR-RfxCas13d collateral activity *in vivo* not only provides a guidance to detect this effect and allows a safer and a more**

controlled use of the technology through transient reagents such as RNP or mRNA-gRNA but also demonstrates that the system can potentially induce toxic effects under severe conditions (i.e. when extremely abundant RNAs are targeted). To circumvent this, we optimized 3 more CRISPR-Cas RNA-targeting systems that showed absent or lower collateral activity than CRISPR-RfxCas13d in mammalian cells where gRNAs and Cas are expressed from constitutive promoters. Under transient targeting conditions, we demonstrated that high-fidelity RfxCas13d is not a highly efficient system *in vivo* used as either RNP or mRNA-gRNA and **CRISPR-Cas7-11 and specially CRISPR-DjCas13d are suitable novel systems to be employed as RNP *in vivo***. Moreover, we compared the collateral activity of these systems under conditions triggering minor and extreme effects by CRISPR-RfxCas13d and conclude that DjCas13d can also induce collateral activity but with less severity and Cas7-11 did not induce this toxicity but, in an exchange, showed a global less on-target efficacy. With our results, **we now provide new systems for RNA-targeting *in vivo* through transient approaches expanding the CRISPR-Cas toolbox**. The appropriate system *in vivo* can be chosen now depending on the final desired goal including potential biomedical applications where a more safety method is needed. Still, **we confirmed that CRISPR-RfxCas13d is an efficient and specific system used as RNP or mRNA-gRNA** but in those circumstances where a potential collateral may be triggered, we now provide alternative systems that can be used. The optimization and potential of these alternative systems, the comparison with similar and other RNA-targeting approaches implemented in mammalian cells and other aspects regarding this section of the manuscript has been extensively described and discussed through the whole text (Introduction, lines: 111-119, Discussion, lines: 618-651)

In summary, **our new results have circumvented several issues that we anticipated in our previous report (Kushawah et al) and we have provided novel and complementary strategies to enhance CRISPR-Cas RNA-Targeting**. This will help to expand the use of the technology in zebrafish and other *in vivo* systems where transient approaches are used as mRNA-gRNA or RNP complexes.

2. On line 148, the maternal and mid-zygotically expressed genes (only one gene) are too little to draw a conclusion that “RNP complexes were much less active when targeting mRNAs from genes whose main expression occurs later during development after 7-8 hpf”.

We thank the reviewer for this comment. We apologize if the text was not clear enough. In Figure 1 and Extended Data Figure 1, we targeted 7 different mRNAs in total that were classified in two groups: three maternally provided or early-zygotically transcribed mRNAs (whose highest levels are detected between 4-6 hpf) and four mid- and late-zygotically expressed mRNAs whose main expression occurs after 7-8 hpf. Indeed, these mRNAs were grouped in 2 sets because each one has similar features regarding targeting mRNA optimization: maternally provided or early zygotically transcribed vs mid-late zygotically transcribed. Nevertheless, we have included new data with two more examples: one maternally provided mRNA and one mid-zygotically expressed transcript. For the four genes with maternal or early zygotic expression, whose highest mRNA levels are between 0-6 hpf, we combined 3 gRNAs per gene

(*nanog*, *smad5*, *noto* and *tbxta*) and in all cases RNP complexes, with or without chemically modified gRNAs, essentially triggered the most robust and highest phenotype penetrance. We have further clarified this point in Results section lines 152-156 (Fig. 1D-E, Extended Data Fig. 1B-C).

Then, we depleted the expression of five mid- and late-zygotically transcribed genes whose highest mRNA levels are after 7-8 hpf with 8 targeting assays (*rx3* mRNA using 3 individual gRNAs and these three together, and *tbx6*, *tyr*, *albino* and *golden* mRNA combining 3 gRNAs per gene) where in most experiments, mRNA-cm-gRNA was the most efficient formulation outperforming RNP approach. Therefore, after the results with these multiple independent targeting experiments we concluded that for genes whose main expression is after gastrulation, cm-gRNA together with RfxCas13d mRNA is the most robust approach to be used. We have revised the text to clarify that our conclusions are based on data from different genes that were clustered according to their expression pattern, lines 160-164 (Fig. 1F-G, Extended Data Fig. 1B-D). We have also clarified the conclusions in the Results section, lines 164-167 (Fig. 1D-G, Extended Data Fig. 1B-D). To facilitate the classification of the different targets according to their expression pattern, we have also included the color code of the gene names in Fig. 1B through other panels in Fig. 1 and Extended Data Figure 1.

3. On line 182, the authors demonstrate that IVTcd RfxCas13d gRNAs have toxic effects, which is unprecise. Firstly, the purity of RNA between in vitro transcription synthesis and chemically synthesis may be not the same. Secondly, the collateral activity between IVTcd gRNAs and chemically synthesized gRNAs is not evaluated, since collateral activity could also case embryogenesis delay or lethal. Except for RfxCas13d system, other CRISPR systems (e.g. CRISPR-SpCas9, CRISPR-DjCas13d) should be introduced as controls or supplement to support this conclusion.

We appreciate the points raised by the reviewer. We agree that the grade of purity of RNA from *in vitro* transcription and chemical synthesis is not the same. Chemically synthesized gRNAs are much purer while *in vitro* transcription reactions may generate byproducts that are not the *in silico* designed gRNAs and that could ultimately trigger undesired effects. We have discussed this point raised by the reviewer in lines 587-589. However, although comparable to the collateral activity, we can not unequivocally conclude that the toxic effect from IVTcd gRNAs is indeed collateral activity. The collateral activity is first triggered by CRISPR-RfxCas13d (and CRISPR-DjCas13d but with a less severe effects) by an on-target depletion of extremely abundant mRNAs in zebrafish as we described and characterized in Figure 5 and Extended Data Figures 7 and 8. Notably, IVTcd gRNAs we have showed in the manuscript that induced toxic effects are targeting mRNAs that due to their low or medium-low levels of expression (*si:dkey-93m18.4* and *brd4* mRNA: 7.69 and 67.14 average TPM between 0-6 hpf, Medina-Muñoz SG et al., 2021) are not triggers of collateral activity. Furthermore, the equivalent chemically synthesized gRNAs of these IVTcd gRNAs from Figure 2 do not generate toxicity and therefore demonstrates that this toxicity is specifically associated to the IVT reaction, and it is not related to with the targeted sequence. Indeed, to avoid potential confounding variables, all gRNA used in Figure 5 to characterize the collateral activity were chemically synthesized. Interestingly, it has been demonstrated that IVTcd and not chemically synthesized

gRNAs from CRISPR-SpCas9 or CRISPR-Cas12a can trigger immune/interferon responses in mammalian cells (Kim S et al., 2018; Wienert B et al., 2018). The activator of this response is the 5'-triphosphate from IVT that is absent in chemically synthesized gRNAs (5'OH). Notably, these two reports showed that using IVT gRNAs treated with calf intestinal alkaline phosphatase (CIP) reduced or abolished the observed immune response. However, we have not detected cytotoxicity in zebrafish embryos using thousands of IVT gRNAs from CRISPR-SpCas9 (or its variants SpG/SpRY) or CRISPR-Cas12a systems (Moreno-Mateos MA et al., 2015 Nature Methods; Moreno-Mateos MA et al., 2017 Nature Communications; Vicencio J & Sanchez-Bolaños C et al., Nature communications 2022) and many papers from other laboratories have never reported a similar toxic effect.

Nevertheless, to address the reviewer concern, we have performed a set of experiments to test whether this specific toxic effect from IVT gRNAs could be due to the 5'-triphosphate or to the IVT reaction specifically mediated by T7 RNA polymerase. First, we treated toxic IVT gRNAs with CIP, but this did not ameliorate the cytotoxicity during zebrafish embryogenesis. Second, to test whether this was a specific effect from T7 RNA polymerase we generated new IVT gRNAs using T3 RNA polymerase that maintained the level of depletion triggered by those gRNAs *in vitro* transcribed by T7 RNA polymerase, but they did not reduce the toxicity. Third, we performed a transcriptomic analysis in those embryos injected with IVT (T7) gRNAs or their chemically synthesized counterparts and showed that there was not a significant interferon response triggered by IVT gRNAs supporting the hypothesis that the toxicity mediated by some IVT gRNAs for RfxCas13d is not due to the 5' triphosphate. All these results are now included in **New Figure 2 and Extended Data Fig. 3** and mentioned and discussed in lines 223-235, 238-240 and 575-594.

Finally, we hypothesized that this particular IVT gRNA toxicity related to CRISPR-RfxCas13d could be associated to other CRISPR-Cas RNA-targeting systems. As reviewer suggested, we used IVT gRNAs from CRISPR-DjCas13d system targeting the same mRNA sites previously analyzed when employing IVT gRNAs for RfxCas13d approach in Fig. 2 and Extended Data Fig. 3. Then, we compared IVT gRNAs for DjCas13d with their equivalents for RfxCas13d and also with their chemically synthesized versions from both systems (CRISPR-RfxCas13d and CRISPR-DjCas13d). All tested IVT gRNAs from CRISPR-DjCas13d system showed a significant level of mRNA depletion comparable to their chemically synthesized equivalents and to their CRISPR-RfxCas13d gRNAs counterparts and, more importantly, they induced similar developmental phenotypes, without toxic effects. The results from these new experiments have been included in Extended Data 12 (lines 475-487) and discussed in lines 596-603. Although these results suggest that this effect could be specifically associated to CRISPR-RfxCas13d gRNAs, we cannot exclude the possibility that with the continued use of CRISPR-DjCas13d technology, gRNAs used with this system could induce toxicity.

Nevertheless, our aim was to i) report to the scientific community that some IVT gRNAs of the CRISPR-RfxCas13d system can trigger toxicity in zebrafish embryos ii) provide a guidance to test this IVT gRNA toxicity in advance and iii) a protocol to pre-select high quality IVT gRNAs. In addition, instead if a particular region within a mRNA and no other needs to be targeted using IVT gRNAs for RfxCas13d that cause toxicity, another potential alternative is to use CRISPR-DjCas13d since we have

demonstrated that IVTed gRNAs for this system can safely replace those toxic IVTed gRNAs for RfxCas13d.

4. In Fig. 1E and Extended Data Fig. 1B, whether gRX3 and gRX3-1 are the same gRNA? If it's the same, the combination of RfxCas13d mRNA and cm-gRNA is more efficient than RNP form in *rx3*, *albino* and *tyr*. Why did this happen?

We thank the reviewer and apologize for the lack of clarity that could be confusing. gRX3 is a combination of three gRNA targeting *rx3* mRNA while gRX3-1 is one of these gRNAs that has been used alone on that experiment (as well as gRX3-2 and gRX3-3, Extended Data Fig. 1C). Although we have used individual gRNAs to target *rx3* mRNA, we realized that we have missed one condition to be compared to RNP: mRNA-gRX3 and mRNA-cm-gRX3 that we have now included it for further clarity in Extended Data Fig. 1C. We have also included RNP with gGOLDEN that it was not shown (Extended Data Fig. 1B). Altogether, and as detailed in reviewer's question 2, our results demonstrate that the combination of RfxCas13d mRNA-gRNA and specially RfxCas13d mRNA and cm-gRNA is more efficient than RNP when targeting *rx3*, *tbx6*, *golden*, *albino* and *tyr* all genes whose highest expression occurs after 7-8 hpf (see Figure 1 and Extended Data Fig. 1B-C). We believe that this may be due to the lower stability of Cas13d protein vs mRNA and mention it in the Discussion, lines 505-507. We have reviewed the text to ensure that this message is clearer adding more details related to these results (lines 152-167).

5. Except for *nanog*, *no-tail*, *rx3* phenotypes, the authors should give more specific phenotype from other knock-down genes, Including evaluating other unknown genes by this system. Moreover, for the purpose of RNA-targeting, compared to morpholino, the advantages of RfxCas13d should be explored.

We appreciate reviewer comment, but we respectfully believe that the analysis of the efficacy of CRISPR-RfxCas13d system is out of the scope of this manuscript since it has been already well-demonstrated in Kushawah et al and other reports. Indeed, in Kushawah et al, we targeted *tbxta*, *nanog*, *dnd1*, *smad5*, *alk8*, *oep*, *smad2*, *tyr* and *prp1* recapitulating their loss-of-function phenotypes. Further, we targeted other mRNAs from endogenous or reporter genes such as *szrd1*, *vcla*, *nano-luciferase*, *rfp* and importantly *brd3a*, *brd3b* and *brd4*, revealing, for first time, that that Brd3a was specifically required during early development and demonstrating that the technology can be used to uncover novel molecular functions. Using qPCR and/or RNA-seq we measured CRISPR-RfxCas13d efficiency for 14 out of 16 mRNA targeted in that report with a depletion average of 76%. As mentioned, the competence of this technology has been further consolidated by other reports not only in zebrafish but in other vertebrate embryos (References 10-21) including our data targeting *rx3* mRNA in medaka or *rfp*, *ubtf* and *emg1* mRNAs in mouse embryos (Fig. 5 Kushawah et al). Moreover, we have recently showed that CRISPR-RfxCas13d can be used for maternal mRNA screenings, and we have targeted 49 mRNAs coding kinases and phosphatases uncovering the crucial role for one of these post-translational regulators, Bckdk, during MZT (Hernandez-Huertas L. et al., 2024 Biorxiv, PMID: 38826327 and under second revision in Nature Struct. Mol. Biol., Editors: Dimitrios Typas and Carolina Perdigoto). Furthermore, in this preprint we have also shown that with a set of gRNA

optimizations described by Wessels HH. et al., 2020 and implemented in our previous work (Hernandez-Huertas L et al., 2022 Star Protocols: a 1 nt stem loop disruption of the gRNA and/or spacers of 23 nt long instead of 22 nt), the targeting efficiency could be further increased up to a median of 92% depletion (although with a variable activity between gRNAs or set of gRNAs as observed here as well). Furthermore, Dr. Bazzini lab, a collaborator in this study, has recently showed the depletion of more maternal RNAs. Three of these KD showed early developmental defects that were additionally rescued (Kushawah G et al. 2024, BioRxiv, PMID: 39574587).

In sum, over the last four years we have demonstrated the KD of more than 75 RNAs which known and novel phenotypes (Kushawah G. et al., 2020 Dev. Cell; Hernandez-Huertas L. et al., 2022 Star Protocols; da Silva Pescador G et al., 2024 Cell Reports; Hernandez-Huertas L. et al., 2024 BioRxiv; Kushawah G. et al., 2024 BioRxiv). Some of these KD were rescued and, by qPCR or RNA-seq, we additionally showed the high efficacy of CRISPR-RfxCas13d targeting those mRNAs.

But more importantly, here in this manuscript, we have targeted and measured the phenotype of more genes besides *rx3*, *no-tail (tbxta)* and *nanog*. Using our diverse set of CRISPR-RfxCas13d optimizations, we have targeted and recapitulated (with different level of penetrance) the loss-of-function phenotype of *smad5*, *noto*, *tbx6*, *tyr*, *golden*, *albino*, *brd4* and *miR-430* loss-of-function and for five of them (*tbx6*, *noto*, *tyr*, *brd4* and *miR-430*) we have measured the mRNA levels upon the KD. Additionally, we have also depleted *si:dkey-93m18.4*, *U4atac snRNA*, *hnrnpa0l*, *hmga1a*, *hspa8* and *gfp* reporter (10 pg injected per embryo) mRNAs. The targeting of these mRNAs showed a mild or absent loss-of-function phenotype during the first 6-7 hpf but we quantified their depletion levels and observed a high targeting efficiency by qPCR for all of them. Therefore, the competence of CRISPR-RfxCas13d to knockdown mRNAs and recapitulate known (or reveal new) developmental phenotypes has been extensively shown not only in this work but in other reports by our and other laboratories (References 10-21).

Besides, a comparison with morpholinos as knockdown system was already performed in Kushawah et al 2020 (Fig. 3F and Fig. S3F,G,I,K and M) where we showed that we were able to recapitulate known developmental and molecular phenotypes derived from morpholinos-based knockdown (for example *nanog*) but with a technology that is more flexible, cost-effective and easier to validate than morpholinos. These and other aspects comparing morpholinos and CRISPR-RfxCas13d system were discussed in Kushawah et al.

For all these reasons, we respectfully think that it is not part of this work to re-evaluate these particular points that the reviewer raised. Importantly, and as explained in detailed above, the specific aim of this manuscript was to further enhance CRISPR-Cas RNA-targeting technology in zebrafish solving a set of issues detected in our previous reports or along our continued work with this approach and that they needed to be addressed to expand the *in vivo* applications of this system.

6. How to choose the target sequence?

We thank the reviewer for this question that could not be clear enough. RNA accessibility *in silico* was initially used to choose gRNAs (Abudayyeh et al., 2017 Nature; Konermann et al., 2018 Cell) and we chose the targets using RNAfold algorithm

selecting regions where the probability of intramolecular interactions was low to ultimately facilitate the accessibility of RfxCas13d-gRNA complex (Kushawah G et al., 2020, Hernandez-Huertas L et al., 2022). This was explained in Methods section, but we have further clarified it (Guide RNA design section). However, this approach has been later showed not to be the most accurate to select efficient gRNAs and we have now established that computational prediction models such as RNAtargeting (Wei J et al., 2023 Cell Systems) developed from mammalian cell culture experiments could help to choose active gRNAs *in vivo* using transient approaches such as RNP yet with a modest accuracy. Nevertheless, we believe that computational models based on data from transient approaches *in vivo* will help to increase the accuracy when designing highly efficient CRISPR-RfxCas13d gRNAs as we discussed in the manuscript (lines 547-553).

If targeting the same gene on different domains, the phenotype is the same or not?

CRISPR-RfxCas13d RNA-targeting activity can be influenced by distinct RNA molecular features and several laboratories have integrated this information from data in mammalian cell cultures to generate different computational models to ultimately predict gRNA activity. We have analyzed the accuracy of these computational models in zebrafish embryos in our manuscript as it is detailed in Fig. 4 and Extended Data Fig. 6. Nevertheless, it has been demonstrated (Kushawah G et al., 2020, Wessels HH et al 2020) that the targeting within the UTRs is less efficient than within the ORF. Therefore, all gRNAs we have used in this study were designed within the ORF (with the exception on non-coding RNAs such as miR-430 and u4atac snRNA). We have now described this in Methods (Guide RNA design section).

Beyond, once an mRNA is cleavage in two or more molecules from the activity of a CRISPR-Cas RNA targeting system, the endogenous RNA degradation machinery will quickly eliminate this cut RNA and in the unlikely case that this RNA degradation was slow, no translation from this mRNA will occur because the lack of 3' polyA and/or 5' CAP in those molecules. Therefore, different targeted sites could influence the RNA depletion efficiency due to the reasons described earlier but independently on what is encoded in those mRNA regions.

7. On line 264, is there significant difference between 10 or 25 gRNAs as a set to detect highly efficient gRNAs? Why choose 25 gRNAs as a set to evaluate the KD efficiency?

We agree with the reviewer that using sets of 10 gRNAs would have been equally useful than injections with 25 gRNAs together to ultimately detect efficient gRNAs. We initially used a pool of 10 gRNAs together in a concentration that guaranteed the detection of a highly efficient gRNAs (Extended Data Fig. 5, fold change > 3.5, q4 and q5: 4 most active gRNAs in the set of 10 gRNAs). Then, we demonstrated that these highly active gRNAs could be also detected as part of the most efficient group (q4 and q5) in a set of 25 gRNAs. Therefore, we decided to carry out the experiment using sets of 25 gRNAs because it notably reduced the cost of transcriptomics and number of injections (from 20 to 8) to analyze the efficacy of our approximately 200 gRNAs. We have included a sentence clarifying this (lines 303-305).

8. Some detailed information should be added to figures. Such as in Fig. 3B, 3D, *mRfxCas13d* or *pRfxCas13d* should be showed as a control.

We thank the reviewer for pointing this out. All controls with different *mRfxCas13d* versions (with or without NLS) alone that can be applied to Fig. 3B and Fig. 3D (now Fig. 3F) or the whole Fig. 3 are detailed in Extended Data Fig. 4. None of these controls showed any significant developmental defect during the first 48 hpf, where miR-430 loss-of-function phenotypes were observed. We decided to have these controls in a separate figure to avoid overcrowded panels. Nevertheless, we have reviewed the whole manuscript and figure panels and added some detailed information that could be missed in the original version.

Reviewer #2 (Remarks to the Author):

The manuscript by Moreno-Sanchez et al. is an excellent example of a comprehensive characterization of a new CRISPR-related tool for research in zebrafish. The authors performed an impressive amount of high-quality work that will hopefully allow the whole zebrafish field to start using this tool.

We thank very much the reviewer positive comments on our work and appreciate their kind words.

Below are some comments to further improve the manuscript.

Major:

- In Summary, please indicate what Cas13d generally does.

Thanks for the suggestion. We have added “RNA-targeting” to the first sentence: CRISPR-Cas13 “RNA-targeting” systems are widely used in basic and applied sciences.

- Line 281: why do you have to classify the whole set? Are there cases when individual guide RNAs are classified well, but the whole set is somehow defective? Can you mention what was wrong with the other 3 sets?

We appreciate reviewer points about our analysis of different computational models’ performance. Our idea was to compare in 8 different experiments how these computational models classified the activity of each set of gRNAs. This allowed us to reveal that RNAtargeting was the most efficient model to predict RNP activity *in vivo*. Nevertheless, we agree with the reviewer that the most important output from this analysis is that those gRNAs that were predicted as high active were, indeed, efficient gRNAs *in vivo*. Notably, when we specifically studied highly or poorly active gRNAs from different sets and examined how these models performed to predict their efficacy, RNAtargeting was also the most accurate differentiating between highly and poorly active with the lowest p-value when Non-parametric Mann-Whitney U statistical tests were applied (Fig. 4C-E). Furthermore, we have now individually examined the top 5 most efficient gRNAs per set to determine whether these gRNAs

were still predicted as highly active in sets where the correlation between activity and score was less precise (set 8, 5 and specially 7, Fig. 4B-E and Extended Data Fig. 6C-F). From this new specific analysis (Extended Data Fig. 6D-F), we have observed that RNAtargeting still detected some gRNAs from these sets as highly efficient. These gRNAs had a score above the first quartile of highly active gRNAs selected by RNAtargeting *in vivo* (Score >0.55 Extended Data Fig. 6D-F). Indeed, we found 1 gRNA from set 8 and set 5 within this group while no one from set 7, with the poorest Pearson correlation coefficient, was detected (Fig. 4B and Extended Data Fig. 6C). Moreover, analyzing gRNAs with the lowest activity we also observed how in set 7, 1 gRNA was predicted as highly efficient by RNAtargeting (Score >0.55 Extended Data Fig. 6D-F), suggesting that the lack of correlation of this set came from the poor prediction accuracy for both high and low active gRNAs. We have added this new analysis in the Results section (lines 326-341).

Nevertheless, our aim was to help to elucidate what computational model, among the algorithms developed in mammalian cell culture, RNAtargeting can be used for *in vivo* CRISPR-RfxCas13d activity prediction when RNP are employed. However, RNAtargeting prediction competence is limited, and we have highlighted it in the manuscript (lines 338-341, 530-534 and 545-546).

We acknowledge that further analysis should be done to better understand the molecular features controlling CRISPR-RfxCas13d activity *in vivo* and through transient approaches. Now that we have optimized an approach to measure the activity of hundreds of gRNAs in a straight-forward and cost-effective manner, a high-throughput experiment could help to develop a specific model for our optimized conditions as we discussed (lines 547-553)

- Lines 291-292: The phrase "to moderately classify CRISPR-RfxCas13d activity delivered *in vivo* as RNP complexes" has a few issues: "moderately" does not have a precise meaning here, the second one is that you are classifying the guide RNAs, not the protein activity.

We appreciate reviewer's correction. We have changed the text as follows (lines 338-341): "Together, our results validate the use of RNAtargeting to classify CRISPR-RfxCas13d system activity delivered *in vivo* as RNP complexes yet with a modest level of accuracy." We have added the term system after CRISPR-RfxCas13d to clarify that it is not the protein activity but the whole system efficiency what we were testing, and we believe that the last part of the sentence clarifies that RNAtargeting can be used but it is not as accurate as in mammalian cell culture systems. We further considered this in the Discussion section.

- Phenotype-based verification of miR430-targeting guideRNAs needs to be significantly substantiated by molecular measures of targeting.

- Figure 3A: It is unclear if your guide RNAs actually targeted inside the miRNA encoding parts of the transcript.

- Figure 3: There should be verification of mature miR430s levels after the knockdown

We agree with the reviewer on these points all about Figure 3. We have modified our scheme in the paper and added more details in Figure Legends and

Methods (Guide RNA design section) to clarify that gRNAs were designed specifically within pri-miR430 without targeting pre-miRNA or mature miR-430.

We have performed an experiment to analyze miR-430 levels upon CRISPR-RfxCas13d KD. Using qPCR we demonstrated that mRfxCas13d-2C-NLS (and not mRfxCas13d-NLS or mRfxCas13d) together with gRNAs targeting pri-miR430, significantly depleted this transcript and consequently the levels of mature miR-430 decreased (Fig. 3D-E). These results are in agreement with all other results from Fig. 3B,C,F and further support the conclusion that mRfxCas13d-2C-NLS allows now the possibility to target nuclear RNAs using transient approaches in zebrafish embryos.

- Line 554: The phrase "together with translational inhibitors" is unclear. How would this be done in practice without killing the fish?

We thank the reviewer for this correction. We meant catalytically dead versions of Cas13 fused to translational inhibitors. We have changed it in the text, lines 635-636: "catalytically dead versions of Cas13 fused to translational inhibitors to silence specific mRNA expression rather than depleting the transcript". This approach would allow translational inhibition of particular mRNAs and not the whole transcriptome as it wrongly stated in the original manuscript.

Minor:

- Line 46: "RNA-targeting" should be 2 words in this case. Otherwise, "RNA-targeting" is appropriate as an adjective.*
- Line 58: Please indicate the origin of RfxCas13d and its history prior to this study, especially its usage in zebrafish.*
- Line 125: The phrase "later during development after 7-8 hours post-fertilization" is somewhat confusing. Please make the timing more precise and the sentence grammatically correct.*
- Line 132: It is unclear what was combined with RfxCas13d. Please revise.*
- Line 224: "it" is not needed.*
- Line 236-238: need to indicate the type of experiment and that 203 is not a sample number.*
- Line 313: The phrase "KD of 20 or more pg of gfp mRNA" is not suitable for a scientific publication. Please describe things more precisely.*
- Line 361: "eliminated" is not the right word, "targeted" may be better.*
- Line 371: "a reduced or lack of" can be shortened to "little or no".*
- Line 445: incorrect usage of "allow", you could write "allow us to ...".*
- Line 468: "that" should be "than". "approaches with a constitutive expression" should be "approaches using constitutive expression".*
- Line 498-499: check the grammatical usage of "despite" and correct the sentence.*
- Line 516: After "chemically synthesized" should be a noun and the phrase itself should be "chemically-synthesized".*
- Line 566: "being" is at the wrong position in the sentence.*
- Line 567: "to a lower degree" is the correct version.*
- Line 568-569: "broadening and enhancing" is the correct usage.*

We really appreciate all text and figure corrections that reviewer pointed out. We have gone through all of them and corrected them or adding more details to the text for clarifying these minor issues.

We answer here some specific points beyond the changes we have made in the text:

- *Line 170-171: The phrase "was lethal with previous developmental defects even by 2 hpf" is unclear. In what sense defects are previous?*

Here, we referred to the fact that this IVTed gRNA triggered an extremely deleterious effect. This toxicity was very evident at 2 hpf with most of the embryos showing an altered development that ultimately translated into a very low survival with severe epiboly defects at 6 hpf. Using other IVTed gRNAs that also triggered toxicity, the toxicity was not clearly observed until later during early development between 4-6 hpf. We have modified the text as follows (lines 181-183): "revealed even earlier developmental defects that were observed by 2 hpf and with most of the embryos dead within the first 6 hpf"

- *Line 302: do you mean that ribosomes are targeted?*

We meant that ribosomal RNA was fragmented. To clarify it, we have removed the term "subunit" from the text (line 352).

- *Line 315-316: Please check is "data not shown" is allowed.*

Initially, we did not add this data because all embryos died between 0-6 hpf. Nevertheless, we agree with the reviewer that providing specific information will be always more convenient and appropriate. We have added now the details within the text as follows (lines 364-366): "100 pg per embryo caused a massive embryo death, n=107 embryos from two independent experiments".

- *Line 416: down-regulation should be indicated by negative log values or small numbers less than 1.*

We have now indicated the mRNA reduction in %, as we did it in other sections of the manuscript, for further clarity and consistency.

- *For all figures, please indicate whether a protein or mRNA RfxCas13d was used such as mRfxCas13d or pRfxCas13d*

We thank the reviewer for this suggestion that will help to better understand the manuscript. We have added this information in all panels where was missing, including Figure 6 where we analyzed other CRISPR-Cas RNA-targeting systems.

Reviewer #3 (Remarks to the Author):

This manuscript by Moreno-Sanchez, Hernandez-Huertas, Nahon-Cano and colleagues benchmarks the performance of various RNA-targeting CRISPR systems in zebrafish. Specifically, they improve the performance of the RfxCas13d system via use of chemically-modified gRNAs and optimized placement of nuclear localization signals. They report interesting discrepancies in silencing efficiency of temporally-restricted transcripts when RfxCas13d and its guide are delivered via RNP or mRNA-gRNA formulations. The thorough comparison of on-target efficiency and collateral activity of RfxCas13d, DjCas13d and Cas7-11 is useful for the CRISPR community.

We appreciate the positive reviewer's comments on our work.

Major point:

In Figure 2, the authors show convincing data that certain IVTted gRNAs induce toxicity in zebrafish embryos, whereas chemically synthesized gRNAs do not. In the Discussion section, the authors state that the difference in toxicity between chemically synthesized and IVTted gRNAs is "remains to be determined".

It is known that T7 in vitro transcription results in the production of RNAs that carry 5'-triphosphate groups. This 5'ppp can trigger a RIG-I-mediated innate immune responses which may lead to cell death, particularly in primary mammalian cells (Wienert et al., 2018, PLOS Biology; Kim et al., 2018, Genome Biology). The extent of the innate immune response is protospacer sequence-specific, which may be why the authors see different toxicities between gRNAs 1-3. Additionally, T7 RNA polymerase is reported to have some RNA-dependent RNA transcription activity, which can lead to the generation of dsRNAs via anti-sense transcription and/or spurious "turn-around" transcription in cis. dsRNA can also activate innate immune responses.

Removal of the 5'ppp via e.g. phosphatase treatment has been shown to prevent such an immune response and thereby rescue loss in cell viability when using IVTted gRNAs. It doesn't appear as though this step is included in the IVT protocol. Did the authors profile the interferon response following administration of the IVTted gRNAs? Does 5'ppp removal rescue this toxic effect, as it does in mammalian cells?

We thank the reviewer for bringing this hypothesis. As reviewer mentioned, it has been demonstrated that IVTted and not chemically synthesized gRNAs from CRISPR-SpCas9 or CRISPR-Cas12a can trigger immune/interferon responses in mammalian cells (Kim S et al., 2018; Wienert B et al., 2018). The activator of this response is the 5'-triphosphate from IVTted that is absent in chemically synthesized gRNAs (5'OH). Notably, these two reports showed that using IVTted gRNAs treated with calf intestinal alkaline phosphatase (CIP) reduced or abolished the observed immune response. However, we have not detected cytotoxicity in zebrafish embryos using hundreds of IVTted gRNAs from CRISPR-SpCas9 (or its variants SpG/SpRY) or CRISPR-Cas12a systems (Moreno-Mateos MA et al., 2015 Nature Methods; Moreno-Mateos MA et al., 2017 Nature Communications; Vicencio J & Sanchez-Bolaños C et al., Nature Communications 2022) and many papers from other laboratories have never reported a similar toxic effect. Nevertheless, we performed a set of experiments to test whether this specific toxic effect from IVTted gRNAs could be due to the 5'-triphosphate (Fig. 2

and Extended Data Fig. 3) or to the IVT reaction specifically mediated by T7 RNA polymerase. First, we treated toxic IVTed gRNAs with CIP, but this did not ameliorate the cytotoxicity during zebrafish embryogenesis. Second, to test whether this was a specific effect from T7 RNA polymerase we generated new IVTed gRNAs using T3 RNA polymerase that maintained the level of depletion triggered by those gRNAs *in vitro* transcribed by T7 RNA polymerase, but they did not reduce the toxicity. Third, we performed a transcriptomic analysis in those embryos injected with IVTed (T7) gRNAs or their chemically synthesized counterparts and showed that there was not a significant interferon response triggered by IVTed gRNAs supporting the hypothesis that the toxicity mediated by some IVTed gRNAs for RfxCas13d is not due to the 5' triphosphate. All these results are now included in **New Fig. 2 and Extended Data Fig. 3** and mentioned and discussed in lines 223-235, 238-240 and 575-594.

Fourth, *in vitro* transcription reactions may generate byproducts that are not the *in silico* designed gRNAs and that ultimately trigger undesired effects. We speculate that a potential explanation could be that RNA byproducts from the IVT reaction (Triana-Alonso FJ et al., 1995 JBC) could trigger this deleterious effect. However, this is something that remains to be determined. We have also discussed this point in lines 587-589.

Finally, we hypothesized that this particular IVTed gRNA toxicity related to CRISPR-RfxCas13d and could be associated to other CRISPR-Cas RNA-targeting systems. We used IVTed gRNAs from CRISPR-DjCas13d system targeting the same mRNA sites previously analyzed when employing IVTed gRNAs for RfxCas13d in Fig. 2 and Extended Data Fig. 3. Then, we compared IVTed gRNAs for DjCas13d with their equivalents for RfxCas13d and also with their chemically synthesized versions from both systems (CRISPR-RfxCas13d and CRISPR-DjCas13d). All tested IVTed gRNAs from CRISPR-DjCas13d system demonstrated significant mRNA depletion levels comparable to their chemically synthesized equivalents and CRISPR-RfxCas13d gRNA counterparts. More importantly, they induced similar developmental phenotypes without exhibiting any toxic effects. The results from these new experiments have been included in **Extended Data Fig. 12** (lines 479-487) and discussed in lines 596-603. Although these results suggest that this effect could be specifically associated to CRISPR-RfxCas13d gRNAs, we cannot rule out the possibility that with the continued use of CRISPR-DjCas13d technology, gRNAs used with this system could induce toxicity.

Minor points:

- Recent reports have directly benchmarked RfxCas13d against PspCas13b in mammalian cells, concluding that PspCas13b exhibits comparable on-target activity but superior specificity due to the absence of noticeable collateral activity. (Hu et al., NSMB, 2024). It is obviously not feasible to test all orthologues in every study, but I am curious as to whether authors have tested PspCas13b in the zebrafish system? It would be interesting to see how this compares with RfxCas13d, and particularly with DjCas13d, in terms of both on-target efficiency and collateral activity.

We agree with the reviewer that this proposed comparison is very reasonable. However, this particular question was addressed in our previous work (Kushawah G et al., 2020 Dev. Cell). We showed that PspCas13b and PguCas13b mRNA injected alone

in zebrafish embryos caused toxicity at concentrations where RfxCas13d did not, even though both Cas13b proteins were less efficiently produced (Fig. 1B and Supp. Fig. 1A,C in Kushawah G et al., 2020 Dev. Cell). Consequently, we did not continue working with these two systems and focused on RfxCas13d.

- *“One-cell stage zebrafish embryos were injected with 300 pg of RfxCas13d mRNA or 3 ng of RfxCas13d protein together with 1 ng of a mix of three gRNAs (~300 pg from each gRNA).” How were the doses of mRNA vs protein selected? Do these doses lead to approximately equal Cas13 expression level? Does delivering a higher or lower dose of Cas13 affect the targeting window (i.e. early- vs -late transcripts)?*

This is a great point, but it was also addressed in our previous work (Kushawah G. et al., 2020 Dev. Cell Fig. 1E Supp. Fig 1D, Fig 4B and Supp. Fig. 4B-D). We tested the maximal amount of mRNA and purified RfxCas13d protein that could be injected in zebrafish embryos without triggering toxicity. For example, 400 or 600 pg of RfxCas13d mRNA per embryo caused developmental defects and using 200 or 300 pg of mRNA we observed an efficient production of the protein *in vivo* (Extended Data Fig. 9D and Supp. Fig 1A in Kushawah et al., 2020 Dev. Cell). We have observed this in other CRISPR-Cas systems such as SpG and SpRY (Vicencio J et al., 2022, Nature Communications) where high concentrations of mRNAs (600 pg per embryo) coding these endonucleases significantly reduced embryo viability. In addition, our optimized concentration of purified RfxCas13d protein triggers a highly efficient maternal and early-zygotically expressed mRNA depletion as shown here, in Kushawah G. et al., 2020 and our recent report deposited in Biorxiv. In that manuscript, we targeted 49 maternal mRNAs coding kinases and phosphatases with a median of mRNA depletion of 92% which ultimately allowed us to uncover the crucial role for one of these post-translational regulators, Bckdk, during MZT (Hernandez-Huertas L. et al., 2024 Biorxiv, PMID: 38826327 and under second revision in Nature Struct. Mol. Biol., Editors: Dimitrios Typas and Carolina Perdigoto). Therefore, we previously optimized the CRISPR-RfxCas13d-targeting (delivered as RNP or mRNA-gRNA) conditions that we used in this manuscript.

- *The authors convincingly show that the RNAtargeting algorithm performs better at predicting gRNAs efficiency vs TIGER or DeepCas13. Why does it perform better for some guide sets (i.e. sets 1-4) but worse for others (e.g. set 5, 7). What happens if you look at individual guide performance rather than pooled guide sets?*

We thank the reviewer for highlighting that our analysis has helped to clarify what computational model developed in mammalian cell culture was the most efficient to predict CRISPR-RfxCas13d activity *in vivo* using transient approaches. Our idea was to compare in 8 different experiments how these computational models classified the activity of each set of gRNAs. This allowed us to reveal that RNAtargeting was the most efficient model to predict RNP activity *in vivo*. We have now individually analyzed the most (top 5) efficient gRNAs per set to test whether some of these were still predicted as highly active in sets where the correlations between activity and score were not highly precise (set 8, 5 and specially 7, Fig. 4B-E and Extended Data Fig. 6C-F). From this new specific analysis (Extended Data Fig. 6D-F), we observed that

RNA targeting still detected gRNAs from these sets as highly efficient with a score above the first quartile of highly active gRNAs selected by this algorithm *in vivo* (Score >0.55 Extended Data Fig. 6D-F). Indeed, we found 1 gRNA from set 8 and set 5 within this group while no one from set 7, with the poorest Pearson correlation coefficient, was detected (Fig. 4B and Extended Data Fig. 6C). Furthermore, analyzing gRNAs with the lowest activity we also observed how in set 7, 1 gRNA was predicted as highly efficient by RNA targeting (Score >0.55 Extended Data Fig. 6D-F), suggesting that the lack of correlation of this set came from the poor prediction accuracy for both high and low active gRNAs. We have added this new analysis in the Results section (lines 326-341).

Nevertheless, our aim was to help to elucidate what computational model, among the algorithms developed in mammalian cell culture, RNA targeting can be used for *in vivo* CRISPR-RfxCas13d activity prediction when RNP are employed. However, RNA targeting prediction competence is limited, and we have highlighted it in the manuscript (lines 338-341, 530-534 and 545-546).

Unfortunately to perform an individual analysis with hundreds of gRNAs *in vivo* is not feasible due to time-consuming and economic cost limitations since it would require assessing individual gRNA activity from independent and single injections per gRNA. That was the reason to optimize an experiment where multiplexing CRISPR-RfxCas13d targeting could detect efficient gRNAs. Thus, we initially used a pool of 10 gRNAs together in a concentration that guaranteed the detection of a highly efficient gRNAs (Extended Data Fig. 5, fold change > 3.5, q4 and q5: 4 most active gRNAs in the set of 10 gRNAs). Then, we demonstrated that these highly active gRNAs could be also detected as part of the most efficient group (q4 and q5) in a set of 25 gRNAs. Therefore, we decided to carry out the experiment using sets of 25 gRNAs because it notably reduced the cost of transcriptomics and number of injections (from 20 to 8) to analyze the efficacy of our approximately 200 gRNAs. We have included a sentence clarifying this (lines 303-305).

We acknowledge that further analysis should be done to better understand the molecular features controlling CRISPR-RfxCas13d activity *in vivo* and through transient approaches. Now, that we have optimized an approach to measure the activity of hundreds of gRNAs in a straight-forward and cost-effective manner, a high-throughput experiment could help to develop a specific model for our optimized conditions as we discussed (lines 547-553)

- In Figure 6B, injection of Cas7-11 alone shows a decently strong epiboly phenotype (in ~30% of embryos), even in the absence of gRNA. This is not the case for the no-tail phenotype in Figure 6C. Why is Cas7-11 alone affecting the epiboly phenotype?

We thank the reviewer to bring this point. We believe that this is essentially related to the variability in the viability and quality of different zebrafish embryos clutches. Indeed, a similar control as this mentioned by the reviewer in Figure 6B is shown in Figure 6D and 6J targeting other 4 different mRNAs and the level of epiboly defects was much lower, below ~20% of affected embryos that is considered relatively normal.

Stylistic points:

- The phenotype severity for noto, rx3 and tyrosinase are ordered as least-to-most sensitive from left-to-right, but nanog is ordered from most-to-least severe.*
- In this reviewer's opinion, it would be beneficial to show all four stages from Figure 1B (maternal, early zygotic, mid-zygotic and late-zygotic) in panels 1D and 1E, for both protein and mRNA. This would make the contrast between delivery modes at each timepoint clearer, and saves the reader flicking between the main and supplementary figures.*
- The data showing collateral targeting of the DsRed reporter in the context of high-abundance eGFP targeting is an important control and a relevant finding. I recommend the authors include this data in the main figures, rather than in the supplementary data.*
- Please also include the colour code legend (from 6A) for the various proteins in Extended Data Fig 9a.*

We appreciate reviewer's suggestions. Indeed, we have done all modifications that further clarify our manuscript. However, when we combined all DsRed data together with GFP panels in the same figure the result was not as clear as we could initially anticipate since those main figures were already too full. Therefore, we kept these figures as originally designed.