nature portfolio

Peer Review File

Optimization of IS621 recombinase/bridge RNA-directed recombination for precise insertion of large DNA fragments in human cells

Corresponding Author: Professor Jianghuai Liu

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)
Summary

This manuscript describes the rational engineering of the IS621 recombinase system and its cognate bridge RNA (bRNA) to enable site-specific insertion of large DNA fragments in human cells. Through systematic mutagenesis and optimization, the authors develop enIS621-tebRNA, demonstrating reasonable insertion efficiencies across multiple cell types and successfully inserting therapeutically relevant sequences (CD19-CAR and Factor IX). I am especially impressed with how the authors substantially improved the performance of a relatively low-efficiency ortholog, and tested multiple cargo sizes, cell lines, and therapeutic applications. Overall, the engineering strategy is sound and straightforward. While the work represents a valuable contribution to the field, I have concerns that need to be addressed before publication.

Major Comments

- The section “Genome-wide off-target activity assessment” is quite vague overall and should be made much more quantitative. My main concern is with the GUIDE-seq off-target analysis.
- The authors write: “Interestingly, the detected off-target sites exhibited a relatively high number of mismatches to the bRNA target-binding sequence, suggesting that these sites are not bona fide RNA-dependent off-targets (Fig. 3e, f).” The authors do not clearly compare these putative off-targets to the control. Are there more of these off-targets in the treated condition than in the control condition? Is it possible these are all just noise?
- The authors write: “The results revealed that enIS621 exhibits extremely low off-target activity at these sites.” What is meant by extremely low here? Please be quantitative.
- In Fig. 3j, the different cells have numbers in each of them. What do these numbers mean? The legend is unclear.
- The modified GUIDE-seq assay presented may not be appropriate or effective for detecting off-target insertions, and it has not been properly benchmarked in this context. Other assays and computational pipelines have been developed to accurately measure off-target insertions. When applied to related bridge recombinases, numerous off-targets are detected, with human genome-wide on-target specificity ranging from quite low (<10%) to relatively high (~80%). Specificity should be reported in terms % of detected insertions that are on-target. Please see the following publications for examples:
- Systematic discovery of recombinases for efficient integration of large DNA sequences into the human genome (https://www.nature.com/articles/s41587-022-01494-w)
- Megabase-scale human genome rearrangement with programmable bridge recombinases (https://www.science.org/doi/10.1126/science.adz0276)
- The authors should discuss how the exchanged bRNA strategy influences off-targets.
- If you suspect that this engineered system is dramatically more specific than existing tools such as the engineered ISCro4, then this needs to be demonstrated with a direct comparison.
- Overall, the specificity analysis must be significantly improved before publication so that readers have an accurate understanding of the limitations of this system for human genome editing.

Minor Comments

- Fig. 1c indicates that WT reaches ~2% GFP+ cells, but it is much closer to 0% in 1d and 1e. Why is this? Was a proper negative control used?
- Line 159: Fig. 1h should probably be changed to Fig. 1i
- Line 205: “Supplementary Fig. 3a-b” should say “Supplementary Fig. 4a-b”
- Paragraph beginning on line 212: What exactly is meant by “82 bp fragment?” Is this a linear dsDNA? The use of linear donors may increase the risk of DSBs at the insertion site because the product will have a DSB after insertion at the ends of the linear fragment. Indels should be lower when using circular donors.
- The term ISCro4 should be used in place of IS622
- Supplementary Figure 4: It is not clear what the different colors and shades mean.
- I would work on adding more detail to the legends and methods to improve clarity.

Grammar/Optional corrections

- In the abstract, I would change “prokaryotic systems” to “prokaryotes” or “prokaryotic genomes” or “prokaryotic organisms.”
- Lines 62-63: “Alternative transposon-based systems, such as Sleeping Beauty and piggyBac, enable large DNA integration but lack precise control over insertion sites and copy number.”
- I would also mention that these systems are not scarless, and they often insert large unwanted terminal sequences that are required for the transposase to recognize the donor sequence.
- Line 68-69: “This guide-acting RNA is termed as bridge RNA (bRNA)...”
I would change this to “is termed the bridge RNA”
- Line 68,76: “transposes” should be “transposase”
- Line 85: I would rephrase “which presented substantial activities in human cells.”
- Line 135: Would change “Given IS621” to “Given that IS621”

Reviewer #2

(Remarks to the Author)
The authors present a comprehensive study describing the engineering and optimization of the IS621 recombinase and its associated bridge RNA (bRNA) system. Through systematic mutagenesis and screening, they identify an engineered IS621 variant (enIS621) carrying A119R, H138R, and V293Q mutations that markedly enhance recombination activity. In parallel, the authors optimize the bRNA by truncating its stem regions to generate tbRNA, and further exchange the target-binding loop (TBL) and donor-binding loop (DBL) to produce ebRNA. The combination of enIS621 with the truncated and exchanged bRNA (tebRNA) establishes a robust platform enabling efficient and precise site-specific insertion of large DNA fragments in human cells. Off-target effects are systematically assessed using GUIDE-seq, confirming the high specificity of the IS621–bRNA complex. The system’s performance is validated at endogenous loci by inserting CD19-CAR and F9 transgenes into Jurkat and Huh-7 cells, resulting in accurate genomic integration and transgene expression. Overall, by rationally engineering both the IS621 recombinase and its bridge RNA, the authors have developed a versatile enIS621–tebRNA platform that enables efficient and precise insertion of large DNA fragments in human cells. I like to to see this study been published after suitable revision.

Major:
1. In the off-target analysis section, the authors only report data for the enIS621–bRNA/tbRNA systems, whereas the high-activity enIS621–ebRNA/tebRNA platforms are not examined. These should be examined by genome wide analysis.

2. Although the IS621 recombinase system has been previously reported, including a schematic illustration showing how the IS621–bRNA system mediates insertion would greatly improve clarity and accessibility for readers. Further, a set of mechanism related experiments should be performed to distinguish current study, as a similar study has been published.

3. More sites of insertion should be examined to indicate the usages of evolved systems.

Minor:
The description in line 205 appears to incorrectly refer to Supplementary Figure 3a–b, while the relevant data seem to correspond to Supplementary Figure 4a–b. Within the figures (figures 1-4), the subpanels (e.g., a–h) are not arranged in a logical or sequential order consistent with their description in the main text. In the figures (figure 2i-k and supplementary figure 4a-g, 5a-c), multiple colors are used within one group, but their corresponding meanings are not specified. The data mentioned in lines 158–159 and 169, as well as in Figure 1h, are not clearly presented in the corresponding figures.

Reviewer #3

(Remarks to the Author)
This manuscript by Guo et al. reports the rational engineering of the IS621 protein and its cognate bridge RNA (bRNA) to achieve precise and efficient large DNA fragment insertions in human cells. Although this work builds upon the recent studies by Durrant et al. (Nature, 2024) and Hiraizumi et al. (Nature, 2024), the authors demonstrate a substantial improvement in insertion efficiency through rational engineering, making the system considerably more practical and valuable to the genome-editing community. Overall, the study represents a clear advancement beyond prior work, though several aspects would benefit from clarification or additional experimentation to further strengthen the manuscript.

Major:

1. Applicability to non-dividing cells
It is important to evaluate whether the engineered IS621 recombinase and bRNA can function efficiently in non-dividing cells, given their potential therapeutic relevance. The current study examines only cancer cell lines and other immortalized cell types, which are not representative of clinically relevant, post-mitotic cells.

2. Benchmarking against other large-insertion platforms
The manuscript focuses on the improved efficiency of the mutant IS621 recombinase but does not benchmark its performance against other state-of-the-art systems for large-fragment integration, such as TwinPE–Bxb1 recombinase, evoCAST, or CRISPR-associated transposases. Without such comparisons, the presented data are insufficient to establish whether the engineered IS621 provides a distinct advantage over existing technologies.

3. Insertion integrity assessment using long-read sequencing
In addition to ddPCR and deep sequencing, the authors should perform nanopore long-read sequencing to evaluate the integrity and accuracy of large (kilobase-scale) insertions. The current approaches in the study cannot fully resolve insertion structure or detect rearrangements within these long fragments.

4. Off-target analysis
The authors analyzed potential off-target sites identified by GUIDE-seq using deep sequencing and detected low-frequency indels, which they attribute to electroporation-induced genomic instability rather than true off-target events. This explanation is not entirely convincing. The authors should reanalyze the deep-sequencing data to determine whether donor sequences were inserted at these sites. Furthermore, junction PCR (using one primer in the genome and one in the donor) should be performed to test for integration events at the putative off-target loci, and if positive, off-target frequencies should be quantified using ddPCR.

Minor:

1. Reporter assay controls
For the fluorescence reporter assays, please include a donor-only control (e.g., GFP donor only) to demonstrate that observed fluorescence signals depend on IS621 activity.

2. Citations and context
The authors should carefully review and update citations throughout the manuscript. Several key references are missing. For example, when stating “Recent studies have shown that IS621, a member of the IS110 family, encodes a 326-amino-acid recombinase…”, the appropriate references should be cited.

3. Title specificity
The title should be made more specific to reflect that this study focuses on the engineering and optimization of an existing technology rather than the discovery of a completely new system.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Overall I am pleased with this revision. The modified UDITAS assay is the correct approach, although it would have been better if they had proper UMIs. I don't think the results here were too overstated, although I think it should emphasize that clearly more specific systems are still needed. I continue to be very impressed with their therapeutic examples.

The comparisons with ISCro4 and evoCAST are good, although the error bars are quite wide. I am impressed and I want to thank the authors for their hard work here.

Reviewer #2

(Remarks to the Author)
No more comments

Reviewer #3

(Remarks to the Author)

The authors have addressed all of my previous concerns, and the manuscript is now much improved.

I only have one minor comment regarding data presentation. Several datasets are based on n = 2. It is recommended to avoid displaying error bars for such small sample sizes(n≤2). In addition, please clearly indicate the exact sample size (n) in

the figure legends.

Overall, I support publication after these minor revisions.

Point-by-point responses

We sincerely thank the reviewers for their careful evaluation of our manuscript, originally titled "Harnessing bridge RNA-guided recombination for precise insertion of large DNA fragment in human cells." The reviewers' comments were highly insightful and constructive, and they have substantially helped us to improve both the scientific rigor and the overall logical clarity of the manuscript. Below, we provide a point-by-point response to each comment raised by the reviewers. The reviewers' original comments are shown in black, and our responses are provided in blue. Some references are cited when necessary. In addition, the revisions are marked by blue color in the accompanying Main Manuscript and Supplementary Information.

Response to Reviewer #1

Reviewer #1 (Remarks to the Author):

Summary

This manuscript describes the rational engineering of the IS621 recombinase system and its cognate bridge RNA (bRNA) to enable site-specific insertion of large DNA fragments in human cells. Through systematic mutagenesis and optimization, the authors develop enIS621-tebRNA, demonstrating reasonable insertion efficiencies across multiple cell types and successfully inserting therapeutically relevant sequences (CD19-CAR and Factor IX). I am especially impressed with how the authors substantially improved the performance of a relatively low-efficiency ortholog, and tested multiple cargo sizes, cell lines, and therapeutic applications. Overall, the engineering strategy is sound and straightforward. While the work represents a valuable contribution to the field, I have concerns that need to be addressed before publication.

Response: We sincerely thank the reviewer for the overall positive assessment of our study. We also appreciate the favorable comments on our approaches leading to advances of the bridge RNA technology, as well as on the scope of the work. At the same time, we fully acknowledge and carefully consider the additional concerns raised by the reviewer. We address each point in detail below and, where appropriate, provide new experimental data and analyses to further strengthen the scientific rigor of the manuscript and the robustness of our conclusions.

Major Comments

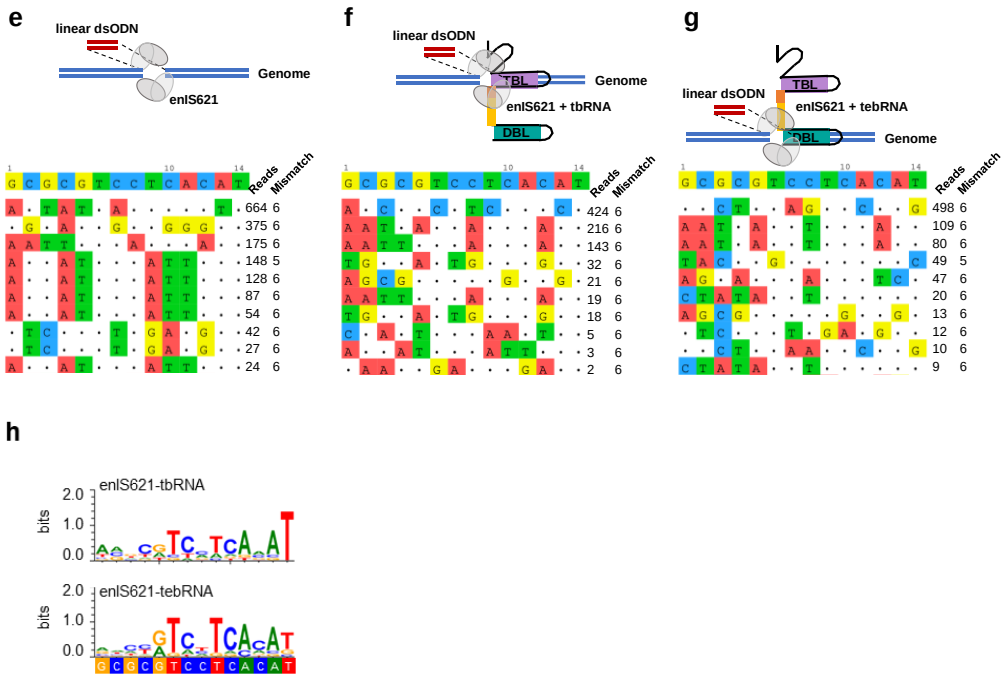
- The section "Genome-wide off-target activity assessment" is quite vague overall and

should be made much more quantitative. My main concern is with the GUIDE-seq off-target analysis.
- The authors write: “Interestingly, the detected off-target sites exhibited a relatively high number of mismatches to the bRNA target-binding sequence, suggesting that these sites are not bona fide RNA-dependent off-targets (Fig. 3e, f).” The authors do not clearly compare these putative off-targets to the control. Are there more of these off-targets in the treated condition than in the control condition? Is it possible these are all just noise?
- The authors write: “The results revealed that enIS621 exhibits extremely low off-target activity at these sites.” What is meant by extremely low here? Please be quantitative.

Response: We thank the reviewer for this series of comments, and have made efforts to deliver clear-cut analyses in the off-target section. Firstly, we resorted to another more established methodology[1] to detect potential off-target integration events (current Fig. 3a-d, discussed in the one after the next response). This should help to address a major concern from multiple reviewers. Furthermore, we have now focused on applying GUIDE-seq (with only the linear dsODN tag) to capture potential cleavage (DSB) events (current Fig. 3e-h). The descriptions to these experiments can be found in the text (Line 325-396). As suggested by the reviewer, the GUIDE-seq experiment was reperformed with the inclusion of untreated control, which would correspond to the baseline signals.

Cells were electroporated with enIS621 expression plasmids and either of the two higher-activity bRNA configurations (± tbRNA or tebRNA), together with a double-stranded oligodeoxynucleotide tag (dsODN). High-throughput sequencing was then used to identify the sites of dsODN incorporation (Fig. 3e-g). GUIDE-seq detected DSB signals at only a small number of genomic loci in tbRNA and tebRNA groups (Fig. 3f, g). In addition, the general abundance of such enIS621-associated GUIDE-seq reads was in a similar scale as those from the control group (no bRNA, Fig. 3e), indicating the rarity of true cleavage events. Interestingly, motif analysis of the top-ten sites from the bRNA groups (but not those from the control group) showed some overall similarities to the target DNA sequence, yet without obvious selection of the CT core motif (Fig. 3h). The GUIDE-seq-nominated sites would be subsequently tested by targeted sequencing (see our next response).

Current Fig. 3e-h



e-g: Top GUIDE-seq sites from control, tbRNA and tebRNA groups, and their sequence alignments. **h:** The top-ten GUIDE-seq-nominated sites from the tbRNA and tebRNA groups are subjected to motif analyses.

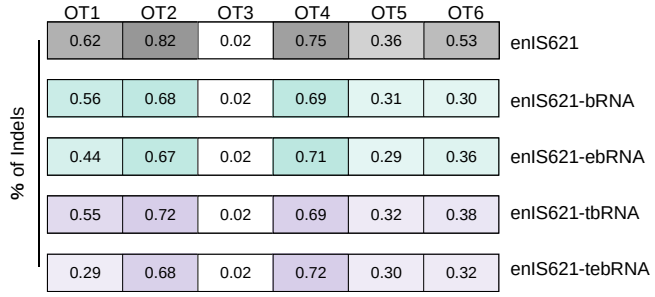
- In Fig. 3j, the different cells have numbers in each of them. What do these numbers mean? The legend is unclear.

Response: We thank the reviewer for this comment, and apologize for having not provided sufficient information in the legends. The targeted sequencing experiments have been carried out again together with the control sample (current Fig. 3i), based on the new GUIDE-seq analyses (collecting the top three hits in tbRNA and tebRNA groups). For each potential cleavage site, the data are presented as % of indels in total reads. The numbers are now clearly defined in the figure legend. The text has been updated (Line 386-396).

Only background levels of indels could be observed at these sites in samples from all enIS621-bRNA groups (bRNA, ebRNA, tbRNA and tebRNA) (Fig. 3i). Together, although GUIDE-seq captured some weak signal of bRNA-associated off-target cleavage, our deep sequencing results at the candidate sites indicated that the extent of DSB risk by enIS621-bRNA was minimal. This proposition is consistent with the reported DSB-independent mechanism of IS621 even at the target site, where the cleavage of the second target strand only occurs upon completion of the first strand exchange[2].

Current Fig. 3i:

i



i: The levels of indels at six GUIDE-seq-nominated sites in control (no bRNA) and different enIS621-bRNA variants-transfected cells.

- The modified GUIDE-seq assay presented may not be appropriate or effective for detecting off-target insertions, and it has not been properly benchmarked in this context. Other assays and computational pipelines have been developed to accurately measure off-target insertions. When applied to related bridge recombinases, numerous off-targets are detected, with human genome-wide on-target specificity ranging from quite low (<10%) to relatively high (~80%). Specificity should be reported in terms % of detected insertions that are on-target. Please see the following publications for examples:
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- Megabase-scale human genome rearrangement with programmable bridge recombinases (https://www.science.org/doi/10.1126/science.adz0276)
- The authors should discuss how the exchanged bRNA strategy influences off-targets.

Response: We thank the reviewer for these important comments. Based on the recommendation, we resorted to this more established method[1, 3] (involving tagmentation and unidirectional sequencing) to detect off-target integration events (current Fig. 3a-d). The original GUIDE-seq assay for off-target integration (which may not be an appropriate assay) were removed, and replaced with new results from detection of cleavage events (see two responses above).

Following the reviewer's suggestion, we adapted the strategy used in *"Systematic discovery of recombinases for efficient integration of large DNA sequences into the human genome"* paper[1] to reassess genome-wide off-target integration by enIS621. A modified UDiTaS approach was applied to samples from HEK293T cells transfected with various bRNA formats. Such a method allows unbiased detection of donor integration sites via tagmentation, target-enrichment, followed by high-throughput sequencing analysis (Fig. 3a)[1]. We opted to focus on transiently transfected cells (instead of following weeks of selection), reasoning that it would lead to detection of

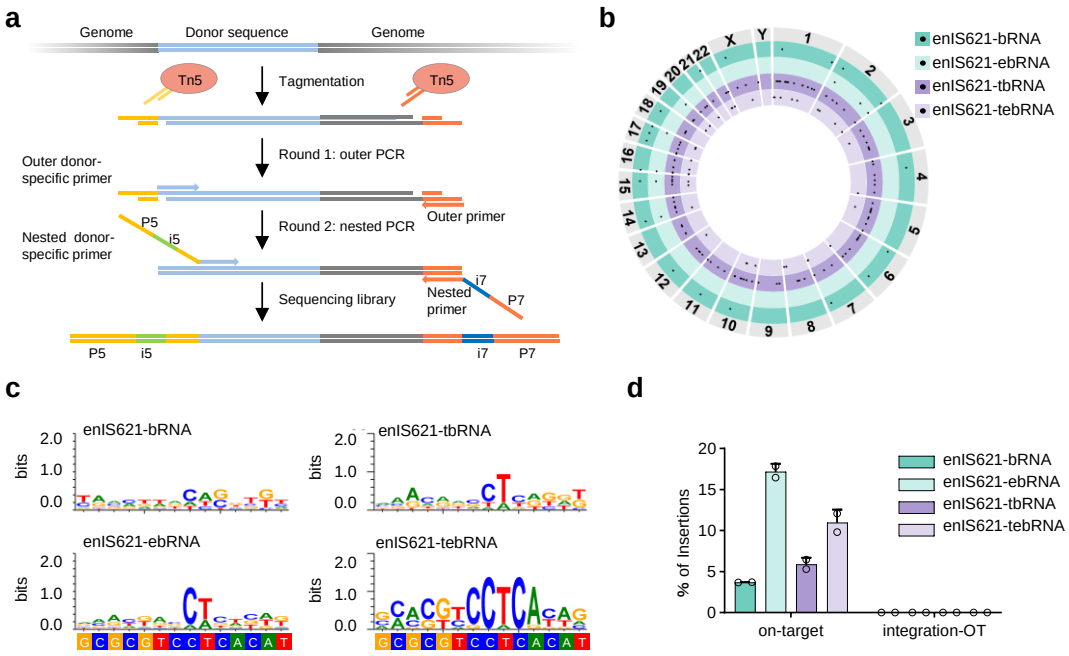
off-target events in cell populations directly under the influences of the delivered recombinases. The contaminating reads corresponding to transiently transfected donor plasmid are filtered by removing reads containing a distal plasmid sequence.

The remaining reads in different bRNA groups could be mapped to the on-target locations, as well as to dozens to >100 other locations (current Fig. 3b and Supplementary Data 1). Specifically, 42, 23, 139, and 48 off-target integration sites were detected for bRNA, ebRNA, tbRNA, and tebRNA groups, respectively (Fig. 3b). Motif analysis of the top ten sites ranked by read abundance highlights a frequent CT dinucleotide core, supporting that the captured integration sites contain *bona fide* off-targets by the recombinase (Fig. 3c). Notably, a clear similarity of tebRNA motif to the desirable target site can also be recognized, providing further confidence to this analysis pipeline. The increased numbers of off-target integration sites by the two-part bRNA variants (tbRNA and tebRNA) compared with the single-unit bRNA configurations (bRNA and ebRNA) point to the decreased targeting specificity upon splitting the TBL and DBL, as suggested by a very recent study on another active IS110 family member, ISCro4[3]. This may represent a potential applicational tradeoff for the more active tbRNA and tebRNA. The relevant descriptions can be found in the main text (Line 325-355).

Despite the detection of off-target integration by all bRNA formats, as we did not include UMI in the donor plasmid, our modified UDiTaS protocol might be influenced by PCR biases and therefore might not be quantitative. For quantitation of off-target activities, we performed ddPCR analyses. We chose the on-target site, as well as a high-rank UDiTaS-nominated off-target site across all experimental groups. The ddPCR results validated on-target integration in all bRNA groups. In contrast, ddPCR analyses at the UDiTaS-nominated OT site did not reveal detectable integration (Fig. 3d). Although the high sensitivity and comprehensiveness of the UDiTaS method is clearly instrumental to probe the risk of off-target integration, our results above suggest that some of the sites might be false-positives or arising from rare events, especially in transiently transfected cell populations. However, we maintain the importance of active investigations into the potential off-targeting aspects. The relevant descriptions can be found in the main text (Line 361-373).

Effects by exchanging the binding specificity of DBL and TBL: We thank the reviewer for this important question. From our results, the visibly reduced numbers of off-target integration sites for ebRNA and tebRNA (23 and 48, *vs* the TBL-target binding configuration in bRNA and tbRNA: 42 and 139, respectively) suggest that DBL-dependent selection of target sites improved integration specificity. Such an observation is consistent with those from the very recent study on ISCro4[3] (Line 356-360).

Current Fig. 3a-d



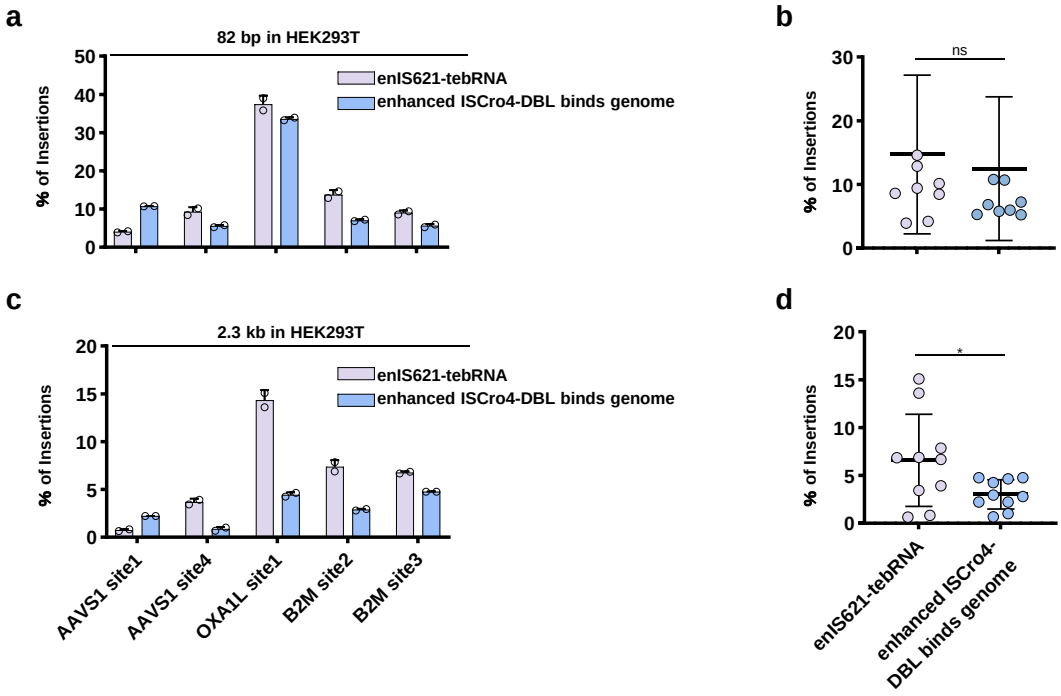
a: Schematic illustration for the modified UDiTaS strategy. **b:** The genomic distribution of integration sites in each condition is presented in Circos plots. **c:** Motif analysis of the top ten sites ranked by read abundance. **d:** The on-target site, as well as a high-rank UDiTaS-nominated off-target site were subjected to ddPCR analyses.

- If you suspect that this engineered system is dramatically more specific than existing tools such as the engineered ISCro4, then this needs to be demonstrated with a direct comparison.

Response: Given the recency of the reported ISCro4 application in human cells[3, 4], we considered parallel analyses of enIS621 and ISCro4's activity profiles a priority (Supplementary Fig. 9, Line 285-301). Insertion efficiencies were systematically evaluated at five endogenous genomic loci. For the 82 bp donor sequence, enIS621-tebRNA mediated an insertion efficiency of 14.71% ± 12.44% (average ± SD). In parallel, the ISCro4 system was tested using the configuration reported to yield the highest activity in the literature, namely the engineered enISCro4 recombinase combined with a split bRNA in which the donor-binding loop targets the genomic sites. Under these conditions, ISCro4 achieved an insertion efficiency of 12.49% ± 11.29% (Supplementary Fig. 9a, b). For a 2.3 kb donor sequence, enIS621-tebRNA achieved an insertion efficiency of 6.57% ± 4.81%, whereas the ISCro4 system yielded an efficiency of 3.03% ± 1.53% (Supplementary Fig. 9c, d). Therefore, enIS621 appears to perform at efficiencies equivalent or sometimes higher than ISCro4. Notably, enIS621 and ISCro4 showed some correlations in activity patterns across different sites, consistent with their similarity in protein sequences as well as in editing mechanisms. Future comparisons of specificities between IS621 and ISCro4 systems are highly warranted, especially given the anticipated future activity upgrades in either system. Such a point

has been added to the discussion (Line 488-489).

Current Supplementary Figure 9



a-d, ddPCR analysis of insertion efficiency for an 82 bp donor or 2.3 kb donor at five endogenous loci in HEK293T cells mediated by enIS621 with tebRNA or the active ISCro4 format. Insertion efficiency comparisons across five endogenous loci (**a, c**). Summary dot plots are presented (**b, d**).

- Overall, the specificity analysis must be significantly improved before publication so that readers have an accurate understanding of the limitations of this system for human genome editing.

Response: We thank the reviewer for stressing this important point. We agree and have further strengthened the specificity and quantitative analyses in the revised manuscript so as to provide readers with a more accurate and objective understanding of the performance of this system in human genome editing (current Fig. 3).

Minor Comments

- Fig. 1c indicates that WT reaches ~2% GFP+ cells, but it is much closer to 0% in 1d and 1e. Why is this? Was a proper negative control used?

Response: We thank the reviewer for carefully going over the details of our work. Fig.

1c shows the actual percentage of GFP-positive cells in IS621-transfected cells (mCherry), in which the WT group reaches approximately 2%. Fig. 1d and 1e are from an independent experiment. While Fig. 1d shows the normalized activities (WT level set to 1), Fig. 1e presents the % of GFP-positivity in transfected cells. In Fig. 1e, the WT group value was measured to be ~ 0.6%. The low activity of WT IS621 contributed such "drifts" of baselines in independent experiments. The differences in the scale of Y axis in Fig. 1c and 1e also amplified the visual differences between 2% and 0.6%. To clearly present these low values, we adjusted Fig. 1c and 1e to the same Y scale, and denoted the WT values in the figures (current Fig. 1c and 1e). The figure legends are revised to provide more details.

- Line 159: Fig. 1h should probably be changed to Fig. 1i

Response: We apologize for this error. The label has been corrected in the revised manuscript.

- Line 205: "Supplementary Fig. 3a-b" should say "Supplementary Fig. 4a-b"

Response: We apologize for this error. The label has been corrected in the revised manuscript.

- Paragraph beginning on line 212: What exactly is meant by "82 bp fragment?" Is this a linear dsDNA? The use of linear donors may increase the risk of DSBs at the insertion site because the product will have a DSB after insertion at the ends of the linear fragment. Indels should be lower when using circular donors.

Response: We apologize for causing confusion. We would like to clarify that all experiments assessing insertion efficiency in this study were performed using circular double-stranded DNA donors, including the 82 bp fragment mentioned here. Indeed, our original wording could be misleading. The "82 bp fragment" has been changed into "82-bp circular DNA donor".

- The term ISCro4 should be used in place of IS622

Response: We thank the reviewer for pointing out this nomenclature issue. The corrections are made throughout the text.

- Supplementary Figure 4: It is not clear what the different colors and shades mean.

Response: We apologize for having not provided sufficient annotations in these figures.

The color schemes in the original Supplementary Fig. 4 correlated with those in Fig. 2. We now provide the color schemes for all panels (current Supplementary Fig. 6).

- I would work on adding more detail to the legends and methods to improve clarity.

Response: We thank the reviewer for this valuable suggestion. We have carefully reviewed and updated the Figure legends and Methods section. In the revised manuscript, the figure legends now include additional experimental details to help readers better understand the conditions and measurements shown, and the Methods section has been supplemented with clarifying information to better guide the readers.

Grammar/Optional corrections

- In the abstract, I would change "prokaryotic systems" to "prokaryotes" or "prokaryotic genomes" or "prokaryotic organisms."

Response: We appreciate the suggestion. The correction is made.

- Lines 62-63: "Alternative transposon-based systems, such as Sleeping Beauty and piggyBac, enable large DNA integration but lack precise control over insertion sites and copy number."
- I would also mention that these systems are not scarless, and they often insert large unwanted terminal sequences that are required for the transposase to recognize the donor sequence.

Response: We thank the reviewer for suggesting the mention of other limitations with the traditional transposon-based vectors. Such information is now added.

- Line 68-69: "This guide-acting RNA is termed as bridge RNA (bRNA)..."
I would change this to "is termed the bridge RNA"

Response: We appreciate the correction. The sentence has been updated in the text.

- Line 68,76: "transposes" should be "transposase"

Response: We apologize for the mistake. The correction has been made.

- Line 85: I would rephrase "which presented substantial activities in human cells."

Response: We thank the reviewer for this suggestion. To avoid overstating the activity of enIS621 in human cells, we have changed “substantial” to “evident”.

- Line 135: Would change “Given IS621” to “Given that IS621”

Response: We apologize for the mistake. The correction has been made.

Response to Reviewer #2

Reviewer #2 (Remarks to the Author):

The authors present a comprehensive study describing the engineering and optimization of the IS621 recombinase and its associated bridge RNA (bRNA) system. Through systematic mutagenesis and screening, they identify an engineered IS621 variant (enIS621) carrying A119R, H138R, and V293Q mutations that markedly enhance recombination activity. In parallel, the authors optimize the bRNA by truncating its stem regions to generate tbRNA, and further exchange the target-binding loop (TBL) and donor-binding loop (DBL) to produce ebRNA. The combination of enIS621 with the truncated and exchanged bRNA (tebRNA) establishes a robust platform enabling efficient and precise site-specific insertion of large DNA fragments in human cells. Off-target effects are systematically assessed using GUIDE-seq, confirming the high specificity of the IS621-bRNA complex. The system’s performance is validated at endogenous loci by inserting CD19-CAR and F9 transgenes into Jurkat and Huh-7 cells, resulting in accurate genomic integration and transgene expression. Overall, by rationally engineering both the IS621 recombinase and its bridge RNA, the authors have developed a versatile enIS621-tebRNA platform that enables efficient and precise insertion of large DNA fragments in human cells. I like to to see this study been published after suitable revision.

Response: We thank the reviewer very much for the comprehensive summary of our work and for the positive overall assessment. In response to other helpful comments and suggestions below, we have made according revisions to the manuscript.

Major:
1. In the off-target analysis section, the authors only report data for the enIS621-bRNA/tbRNA systems, whereas the high-activity enIS621-ebRNA/tebRNA platforms are not examined. These should be examined by genome wide analysis.

Response: We thank the reviewer for this important suggestion on the inclusion of the high-activity enIS621-tebRNA group in off-targeting assessment.

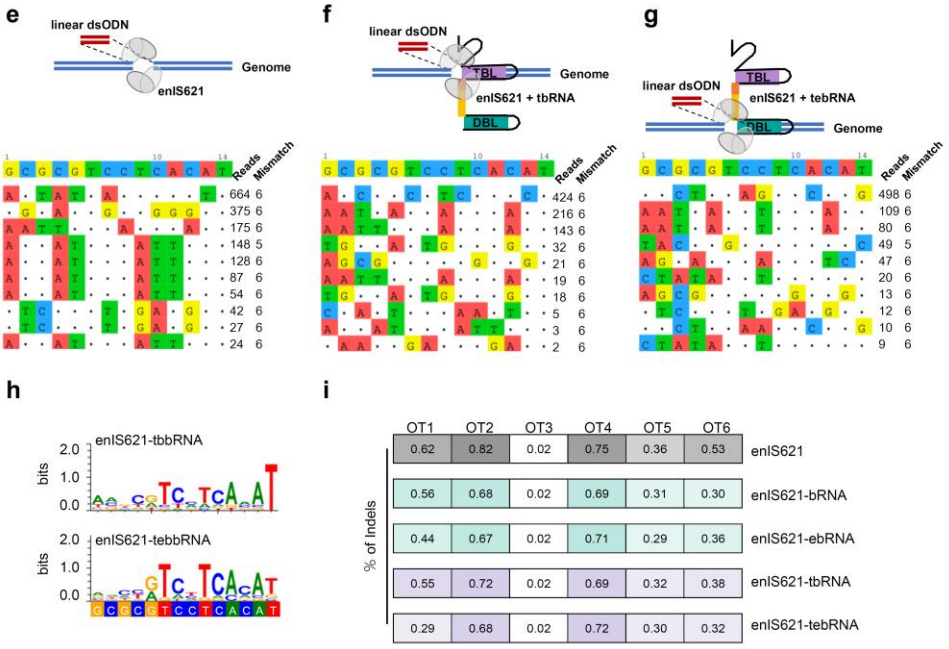
As pointed out by Reviewer #1, our original GUIDE-seq-dependent workflow for might not be suitable for genome-wide detection of off-target integration events. Therefore, we resorted to another more established methodology[1] to detect potential off-target integration events (current Fig. 3a-d). Second, we have now focused on applying GUIDE-seq (with only the linear dsODN tag) to capture potential cleavage (DSB) events (current Fig. 3e-h). In both analyses, we included the high-activity tbRNA and tebRNA groups. Here we will briefly describe our results. Largely similar contents may be found in our responses to other reviewers.

To track the events of donor DNA integration in the genome, we applied a modified UDiTaS strategy[1] (Fig. 3a). Such a method allows unbiased detection of donor integration sites via tagmentation, target-enrichment, followed by high-throughput sequencing analysis. Cells were co-transfected with enIS621-bRNA, enIS621-ebRNA, enIS621-tbRNA, or enIS621-tebRNA targeting OXA1L site1 together with a 2.3 kb circular donor DNA, and the samples were subsequently analyses via the modified UDiTaS work flow. The results revealed that different bRNA configurations influenced off-target integration profiles. A total of 42, 23, 139, and 48 off-target integration sites were detected for bRNA, ebRNA, tbRNA, and tebRNA groups, respectively. The genomic distribution of integration sites in each condition is presented in Circos plots (Fig. 3b). Motif analysis of the top ten sites ranked by read abundance highlights a frequent CT dinucleotide core, supporting that the captured integration sites contain *bona fide* off-targets by the recombinase (Fig. 3c). A clear similarity of tebRNA motif to the desirable target site can also be recognized, providing further confidence to this analysis pipeline. Together, the different numbers of UDiTaS-nominations for different bRNA format groups suggest that while the two-part bRNA format reduce targeting specificity, the DBL-dependent selection of target sites improved integration specificity. These observations are consistent with those from the very recent study on ISCro4[3]. The descriptions of the experiments in the current Fig. 3a-c have been added to the text (Line 325-360). The relevant figure panels may be found in our responses to Reviewer #1 and #3.

The GUIDE-seq analysis now serve to detect potential off-target cleavage (DSB-forming) events. This series of experiments included control (no bRNA), tbRNA and tebRNA groups (dsODN co-transfected). The results in the original manuscript have been replaced (Fig. 3e-h). Only a small number of genomic loci were captured by GUIDE-seq in tbRNA and tebRNA groups. In addition, the general abundance of such enIS621-associated GUIDE-seq reads was in a similar scale as those from the control group (no bRNA) (Fig. 3e-g). Interestingly, motif analysis of the top-ten sites from the bRNA groups (but not those from the control group) showed some overall similarities to the target DNA sequence, yet without obvious selection of the CT core motif (Fig. 3h). Subsequently, the top three GUIDE-seq-nominated loci from the tbRNA and

tebRNA groups were collected (OT1 to OT6) for further testing. Only background levels of indels could be observed at these sites in samples from all enIS621-bRNA groups (bRNA, ebRNA, tbRNA and tebRNA) (Fig. 3i). Together, although GUIDE-seq captured some weak signal of bRNA-associated off-target cleavage, our deep sequencing results at the candidate sites indicated that the extent of DSB risk by enIS621-bRNA was minimal. The descriptions of the experiments in the current Fig. 3e-i are updated into the text (Line 374-396).

Current Fig. 3e-i

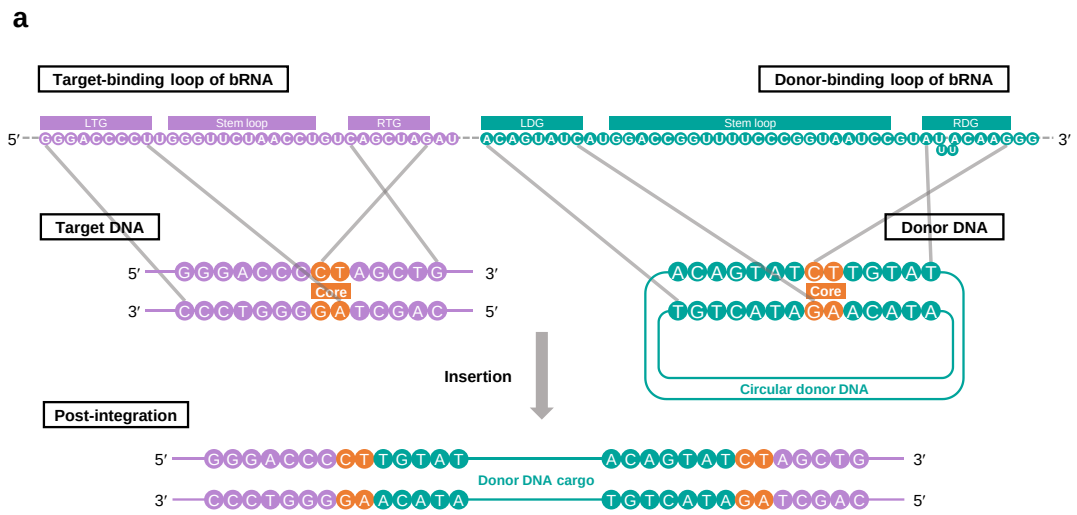


e-g: Top GUIDE-seq sites from control, tbRNA and tebRNA groups, and their sequence alignments. **h:** The top-ten GUIDE-seq-nominated sites from the tbRNA and tebRNA groups are subjected to motif analyses. **i:** The levels of indels at six GUIDE-seq-nominated sites in control (no bRNA) and different enIS621-bRNA variants-transfected cells.

2. Although the IS621 recombinase system has been previously reported, including a schematic illustration showing how the IS621-bRNA system mediates insertion would greatly improve clarity and accessibility for readers. Further, a set of mechanism related experiments should be performed to distinguish current study, as a similar study has been published.

Response: We thank the reviewer for the constructive suggestions. First, to aid readers' comprehension of IS621-bRNA-dependent insertion, we have added a schematic illustration in current Supplementary Fig. 1a, which provides a more intuitive overview on the bifunctional interactions of bRNA with the circular donor and with the target DNA, leading to donor insertion at the target site.

Revised Supplementary Fig. 1a

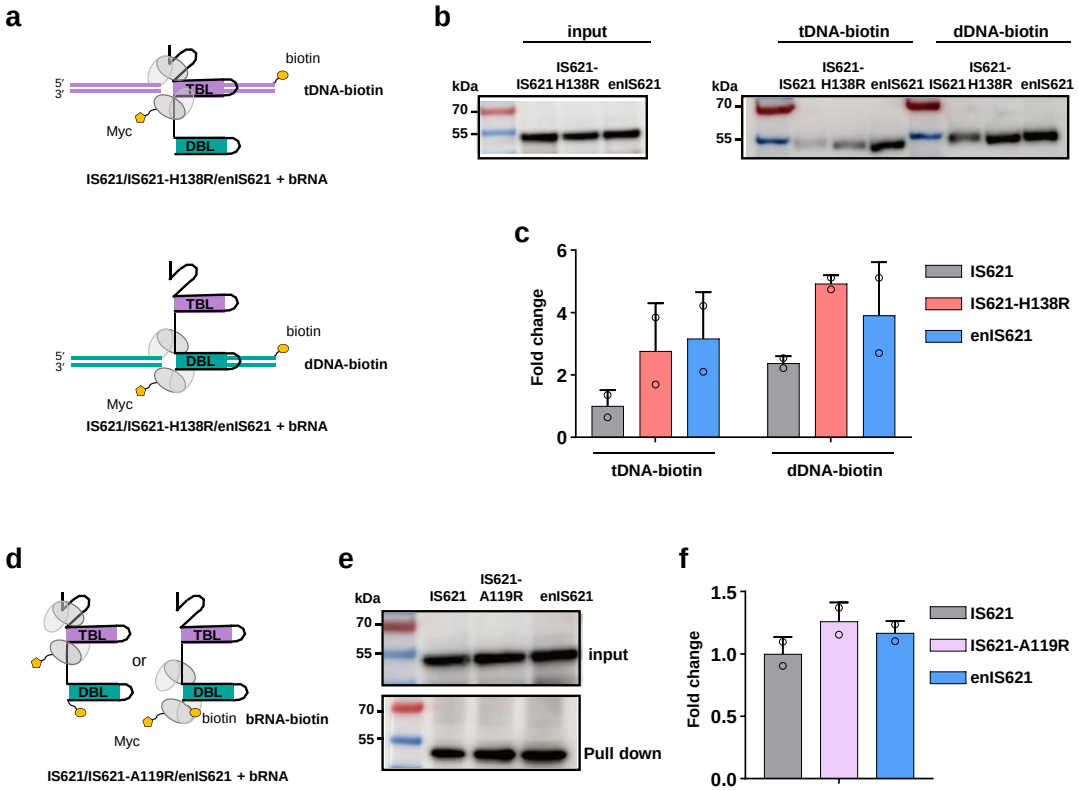


a: Schematic illustration of base-pairing interactions between the bRNA and tDNA or dDNA, and the resulting DNA insertion mediated by the IS621 recombinase.

Second, in response to the reviewer's suggestion to include mechanism-related experiments that further distinguish this study from previous work, we sought to probe the basis for the enhanced activities by enIS621. To this end, we investigated the characteristics of the selected IS621 variants to complex with the nucleic acid components. Given the proximity of H/R138 to the substrate DNAs (see Fig. 1g), we tested the interaction of WT-, H138R- and enIS621-bRNA with DNA. Target DNA (tDNA) and donor DNA (dDNA) were biotinylated and separately immobilized onto streptavidin-magnetic beads, which were subsequently used to pull down IS621 variants in recombinase/bRNA-expressing cell lysates (Supplementary Fig. 5a). Western blot analyses of the captured fraction revealed enhanced binding of IS621-H138R and enIS621 to both target DNA and donor DNA (Supplementary Fig. 5b, c).

Furthermore, based on the proximity of A/R119 to bRNA (see Fig. 1g), we performed a similar experiment on bRNA interaction with WT, A119R and enIS621. The biotinylated bRNA was used to capture IS621 variants from recombinase-expressing cell lysates (Supplementary Fig. 5d). The results showed that IS621-A119R and enIS621 displayed increased binding to bRNA, compared with wild-type IS621 (Supplementary Fig. 5e, f). These results suggest that improved binding of enIS621 to substrate DNA and to the bRNA in the recombination synaptic complex contributes its enhanced activities. The descriptions of the experiments are added to the text (Line 156-168).

Current Supplementary Fig. 5

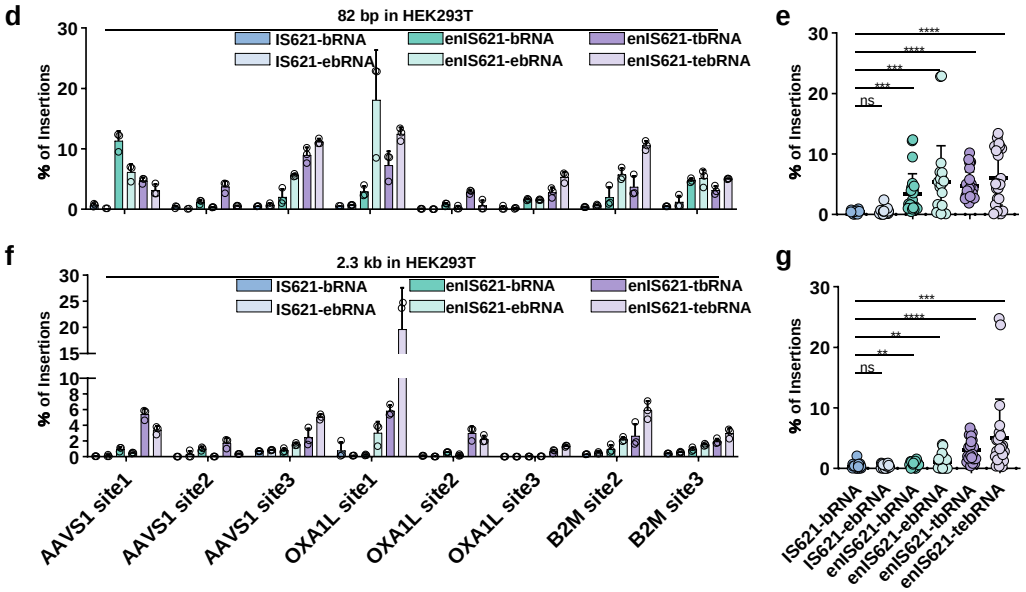


a, Schematic representation of tDNA- or dDNA-based pull-down assays. **b,** Western blot analysis of IS621, IS621-H138R, and enIS621 proteins from substrate DNA pull-down assays. **c,** Quantification of fold changes (over WT levels) in IS621 variants bound to tDNA or dDNA. **d,** Schematic representation of bRNA-based pull-down assays. **e,** Western blot analysis of IS621, IS621-A119R, and enIS621 proteins from bRNA pull down assays. **f,** Quantification of fold changes (over WT levels) of IS621 variants bound to bRNA.

3. More sites of insertion should be examined to indicate the usages of evolved systems.

Response: We thank the reviewer for this important suggestion. To further demonstrate the applicability of the IS621-bRNA system across different genomic contexts, we expanded our analysis to include additional endogenous insertion sites in the revised manuscript, with the corresponding results presented in current Fig. 2d-g. Specifically, we extended the analysis to eight distinct endogenous genomic loci and systematically compared the performance of WT-IS621 and enIS621 in combination with different bRNA formats (bRNA, ebRNA, tbRNA, and tebRNA) for both short (82 bp, Fig. 2d, e) and large (2.3 kb, Fig. 2f, g) DNA payloads. The overall patterns for the current Fig. 2d-g are highly consistent with those in the original version with fewer representations. The text has been updated (Line 213-233).

Current Fig. 2d-g



d-g: Deep sequencing analysis of insertion efficiency for an 82 bp donor or 2.3 kb donor at eight endogenous loci mediated by IS621 or enIS621 recombinases with various bRNA formats. The data for insertion efficiency comparisons across eight endogenous loci are shown in (**d, f**). The dot plot summarizing results from all tested sites are shown in (**e, g**).

Minor:
The description in line 205 appears to incorrectly refer to Supplementary Figure 3a-b, while the relevant data seem to correspond to Supplementary Figure 4a-b. Within the figures (figures 1-4), the subpanels (e.g., a-h) are not arranged in a logical or sequential order consistent with their description in the main text. In the figures (figure 2i-k and supplementary figure 4a-g, 5a-c), multiple colors are used within one group, but their corresponding meanings are not specified. The data mentioned in lines 158-159 and 169, as well as in Figure 1h, are not clearly presented in the corresponding figures.

Response: We thank the reviewer for pointing out these issues, and apologize for causing confusions. The mistake in the labeling of original Supplementary Fig.4 is now corrected (Supplementary Fig. 6). The panels in all figures have now been rearranged logically and sequentially. The column annotations issues in original Fig. 2, Supplementary Figure 4, 5 are now resolved. The mislabeling of Fig. 1i (by Fig. 1h) in the original text is corrected. Fig. 1i and its legend have also been improved for clarity.

Response to Reviewer #3

Reviewer #3 (Remarks to the Author):

This manuscript by Guo et al. reports the rational engineering of the IS621 protein and its cognate bridge RNA (bRNA) to achieve precise and efficient large DNA fragment insertions in human cells. Although this work builds upon the recent studies by Durrant et al. (Nature, 2024) and Hiraizumi et al. (Nature, 2024), the authors demonstrate a substantial improvement in insertion efficiency through rational engineering, making the system considerably more practical and valuable to the genome-editing community. Overall, the study represents a clear advancement beyond prior work, though several aspects would benefit from clarification or additional experimentation to further strengthen the manuscript.

Response: We sincerely thank the reviewer for the overall positive appraisal on our work. We also greatly appreciate that the reviewer has commended the major advances in our work. In the meantime, we are thankful to the suggestions for further clarifications and analyses.

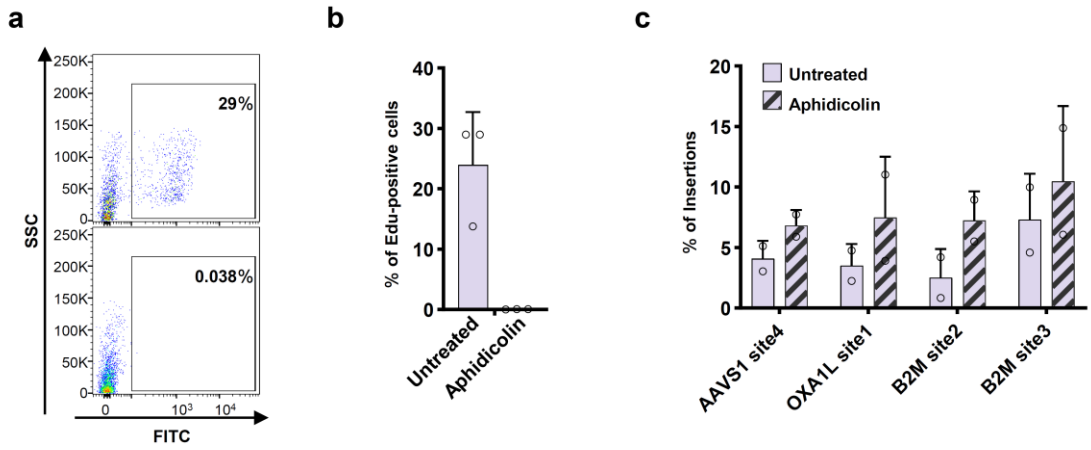
Major:

1. Applicability to non-dividing cells

It is important to evaluate whether the engineered IS621 recombinase and bRNA can function efficiently in non-dividing cells, given their potential therapeutic relevance. The current study examines only cancer cell lines and other immortalized cell types, which are not representative of clinically relevant, post-mitotic cells.

Response: We thank the reviewer for raising this important point regarding the applicability of the engineered IS621 recombinase system in non-dividing cells. To this end, we employed RPE1 cells, a diploid immortalized cell line commonly used for investigation of cell cycle regulation and for studies of genome editing[5, 6]. RPE1 cells were arrested in S-phase by aphidicolin treatment, which was confirmed by inhibition of EdU incorporation (Supplementary Fig. 8a, b). Following ±aphidicolin pre-treatment, cells were transfected with enIS621-tebRNA and the donor. The aphidicolin was removed 3 days following transfection, to avoid extensive cellular toxicity. One day later, the cells were harvested for analyses of donor insertion. Across four endogenous loci, the insertion efficiency (average ± SD) was 4.34% ± 2.84% in untreated cells, and 8.00% ± 3.55% in aphidicolin-treated cells (Supplementary Fig. 8c). These results indicate that enIS621-mediated donor insertion does not require active cell cycle progression. The description has been added to the main text (Line 273-284).

Current Supplementary Figure. 8



a-b: Flow cytometry analysis of EdU incorporation in RPE-1 cells treated for 6 h with aphidicolin. The quantitation is provided in (b). **c:** Four days after transfection, ddPCR analysis was performed to determine the insertion efficiency (of a 2.3 kb donor) in control or Aphidicolin-treated cells.

2. Benchmarking against other large-insertion platforms

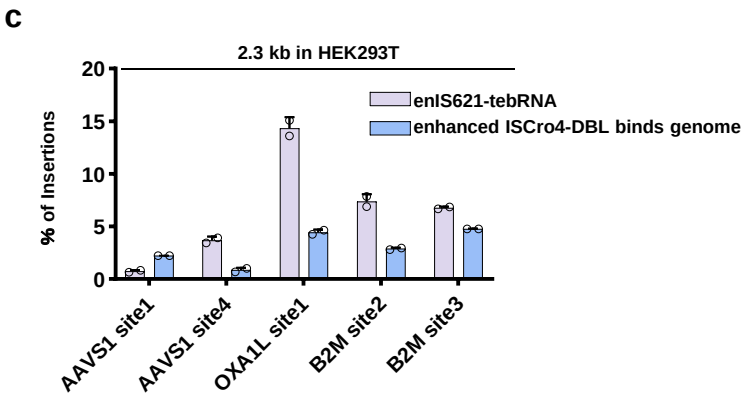
The manuscript focuses on the improved efficiency of the mutant IS621 recombinase but does not benchmark its performance against other state-of-the-art systems for large-fragment integration, such as TwinPE-Bxb1 recombinase, evoCAST, or CRISPR-associated transposases. Without such comparisons, the presented data are insufficient to establish whether the engineered IS621 provides a distinct advantage over existing technologies.

Response: We thank the reviewer for raising this important point regarding benchmarking against other large-fragment integration platforms. We considered to compare the performances of enIS621-tebRNA to another very recent CRISPR-associated transposase tool, the evoCAST system[7], under largely identical experimental settings (Supplementary Fig. 10a).

Specifically, we employed evoCAST to insert a 2.3 kb exogenous DNA fragment at four endogenous genomic loci also under test with enIS621 and ISCro4 (see Supplementary Fig. 9c). The stoichiometry of all evoCAST plasmids was used according to the optimized format[7], as the total amount per transfection was set up to contain a similar input of donor DNA plasmid (600 ng) as in the enIS621 experiment (500 ng). We found that across all four loci, the insertion efficiencies achieved by evoCAST were below 1% (Supplementary Fig. 10b), which is substantially lower than the efficiencies observed with enIS621-tebRNA (see Supplementary Fig. 9c). Furthermore, the cross-site pattern of evoCAST activities did not correlate with those of enIS621, most likely determined by the distinct editing mechanisms for IS110 recombinases and CRISPR-associated transposases. From the comparison, we also discuss the minimal scar formation and compositional simplicity as important positive attributes of enIS621-tebRNA. The description and discussion are added to the text

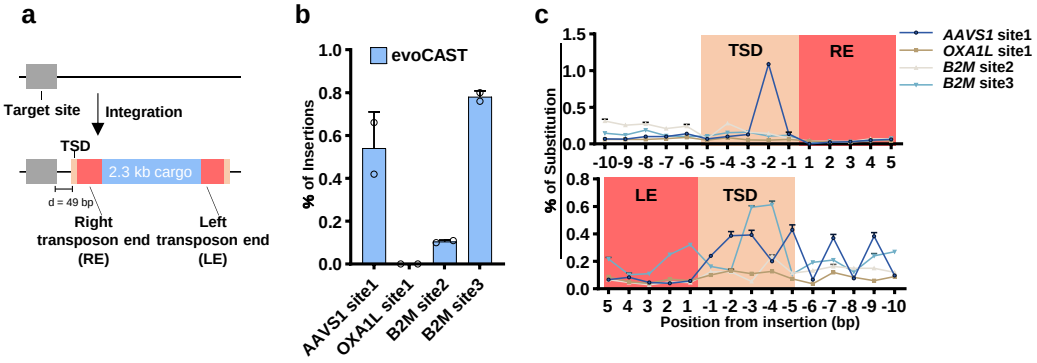
(Line 302-323).

Current Supplementary Fig. 9



c, ddPCR analysis of enIS621- and ISCro4-dependent insertion of a 2.3 kb donor at various endogenous loci in HEK293T cells.

Current Supplementary Fig. 10



a, Schematic illustration of evoCAST-mediated insertion editing. **b,** ddPCR analysis of evoCAST-dependent insertion of a 2.3 kb donor at endogenous loci in HEK293T cells. **c,** Quantification of substitution mutations associated with evoCAST insertions across four genomic target sites within a 15 bp window at the genome-transposon right-end and left-end junction (deep sequencing).

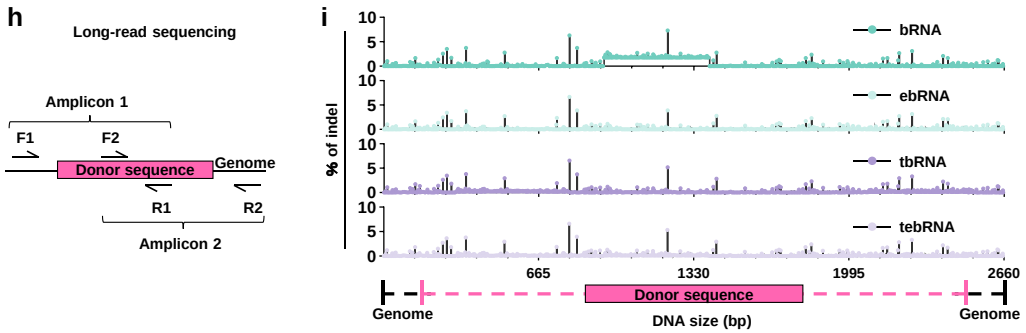
3. Insertion integrity assessment using long-read sequencing

In addition to ddPCR and deep sequencing, the authors should perform nanopore long-read sequencing to evaluate the integrity and accuracy of large (kilobase-scale) insertions. The current approaches in the study cannot fully resolve insertion structure or detect rearrangements within these long fragments.

Response: We thank the reviewer for this important suggestion. We agree that long-read sequencing is suitable to probe the potential, integration-associated structural

abnormalities. Subsequently, we assessed the structural integrity of the integration products by long-read sequencing. To this end, two partially overlapping cross-junction amplicons (Amplicon 1 and Amplicon 2) were prepared to span the integrated payload and the flanking genomic sequences (Supplementary Fig. 6h). The amplified products were subjected to PacBio long-read sequencing. Analysis of the resulting reads from all bRNA groups revealed no detectable structural abnormalities or high-frequency mutations within the donor sequence or at the donor-genome junctions (Supplementary Fig. 6i). The distribution of indels across the integration region was comparable across groups corresponding to different bRNA formats. These results indicate that enIS621-mediated recombination proceeds with high precision and preserves the structural integrity of the target site in mammalian cells. The descriptions have been added in the main text (Line 246-262).

Revised Supplementary Figure. 6h, i



h, Schematic of long-read sequencing strategy used to evaluate the integrity of integration products. Two PCR amplicons spanning the donor sequence and flanking genomic regions were generated using primer pairs F1/R1 and F2/R2 to ensure full coverage of the integration locus. **i**, Per-base indel frequency across the integration region determined from long-read sequencing data. Each dot represents the percentage of reads containing an insertion or deletion at the corresponding position.

4. Off-target analysis

The authors analyzed potential off-target sites identified by GUIDE-seq using deep sequencing and detected low-frequency indels, which they attribute to electroporation-induced genomic instability rather than true off-target events. This explanation is not entirely convincing. The authors should reanalyze the deep-sequencing data to determine whether donor sequences were inserted at these sites. Furthermore, junction PCR (using one primer in the genome and one in the donor) should be performed to test for integration events at the putative off-target loci, and if positive, off-target frequencies should be quantified using ddPCR.

Response: We sincerely thank the reviewer for these important comments. We agree that the off-target integration section needed to be strengthened. As similar comments

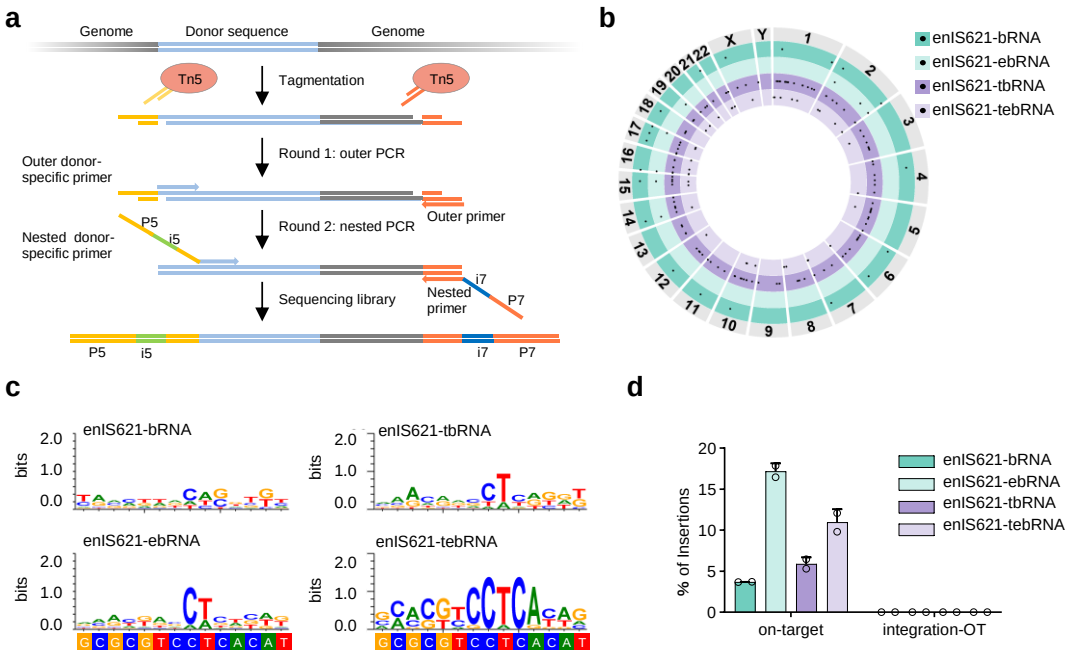
had been raised by multiple reviewers, we had summarized the key points from all reviewers and accordingly designed further analyses.

With the input from Reviewer #1, we noted that the off-targeting events by recombinases are often measured by a tagmentation-based approach (commonly referred to as UDiTaS)[1, 3]. Therefore, we have decided to adapt this methodology to detect potential off-target integration events (current Fig. 3a-c). We also take the suggestion from you to apply ddPCR for quantitation of potential off-target integration (current Fig. 3h). On the other hand, we have now focused on applying GUIDE-seq (with only the linear dsODN tag) to capture potential cleavage (DSB) events (current Fig. 3e-h). Here we will briefly describe our results. Largely similar contents may be found in our responses to other reviewers.

To track the events of donor DNA integration in the genome, we applied a modified UDiTaS strategy[3] (Fig. 3a). Such a method allows unbiased detection of donor integration sites via tagmentation, target-enrichment, followed by high-throughput sequencing analysis. Cells were co-transfected with enIS621-bRNA, enIS621-ebRNA, enIS621-tbRNA, or enIS621-tebRNA targeting OXA1L site1 together with a 2.3 kb circular donor DNA. Our new analysis of genome-wide integration events revealed that different bRNA configurations influenced off-target integration profiles. A total of 42, 23, 139, and 48 off-target integration sites were detected for bRNA, ebRNA, tbRNA, and tebRNA groups, respectively. The genomic distribution of integration sites in each condition is presented in Circos plots (Fig. 3b). Motif analysis of the top ten sites ranked by read abundance highlights a frequent CT dinucleotide core, supporting that the captured integration sites contain *bona fide* off-targets by the recombinase (Fig. 3c). A clear similarity of tebRNA motif to the desirable target site can also be recognized, providing further confidence to this analysis pipeline. Together, the different numbers of UDiTaS-nominations for different bRNA format groups suggest that while the two-part bRNA format reduce targeting specificity, the DBL-dependent selection of target sites improved integration specificity. These observations are consistent with those from the very recent study on ISCro4[3].

For quantitation of off-target activities, we performed ddPCR analyses. We chose the on-target site, as well as a high-rank UDiTaS-nominated off-target site across all experimental groups. The ddPCR results validated on-target integration in all bRNA groups. In contrast, ddPCR analyses at the nominated OT site did not reveal detectable integrations (Fig. 3d). Although the high sensitivity and comprehensiveness of the UDiTaS method is clearly instrumental to probe the risk of off-target integration, our results above suggest that some of the sites might be false-positives or arising from rare events, especially in transiently transfected cell populations. However, we maintain the importance of active investigations into the potential off-targeting aspects. The descriptions of the experiments in the current Fig. 3a-d have been added to the text (Line 325-373).

Current Fig. 3a-d



a: Schematic illustration for the modified UDiTaS strategy. **b:** The genomic distribution of integration sites in each condition is presented in Circos plots. **c:** Motif analysis of the top ten sites ranked by read abundance. **d:** The on-target site, as well as a high-rank UDiTaS-nominated off-target site were subjected to ddPCR analyses.

The GUIDE-seq analysis now serve to detect potential off-target cleavage (DSB-forming) events. This series of experiments included control (no bRNA), tbRNA and tebRNA groups. The results in the original manuscript have been replaced (Fig. 3e-h). The relevant figure panels may be found in our responses to Reviewer #1 and #3. Here, the cells were electroporated with enIS621-bRNAs, together with a double-stranded oligodeoxynucleotide tag (dsODN). The GUIDE-seq workflow was then used to detect DSB signals. Together, although GUIDE-seq captured some weak signal of bRNA-associated off-target cleavage, our deep sequencing results at the candidate sites indicated that the extent of DSB risk by enIS621-bRNA was minimal. The descriptions of the experiments in the current Fig. 3e-i are updated into the main text (Line 374-396).

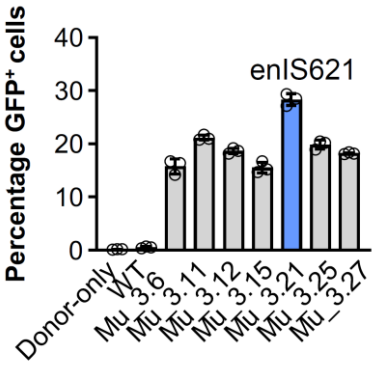
Minor:

1. Reporter assay controls

For the fluorescence reporter assays, please include a donor-only control (e.g., GFP donor only) to demonstrate that observed fluorescence signals depend on IS621 activity.

Response: We thank the reviewer for pointing out this important control, and apologize for having not included this data in our original figure. The updated Fig. 1e now contain this control and the figure legend is updated accordingly.

Current Fig. 1e



2. Citations and context

The authors should carefully review and update citations throughout the manuscript. Several key references are missing. For example, when stating "Recent studies have shown that IS621, a member of the IS110 family, encodes a 326-amino-acid recombinase…", the appropriate references should be cited.

Response: We thank the reviewer for pointing out this issue, and apologize for our negligence in this aspect. We have checked and placed proper citations throughout the text.

3. Title specificity

The title should be made more specific to reflect that this study focuses on the engineering and optimization of an existing technology rather than the discovery of a completely new system.

Response: We thank the reviewer for this helpful suggestion. We have revised the title to: "Optimization of IS621 recombinase/bridge RNA-directed recombination for precise insertion of large DNA fragment in human cells."

1. Durrant MG, *et al.* Systematic discovery of recombinases for efficient integration of large DNA sequences into the human genome. *Nat Biotechnol* **41**, 488-499 (2023).

2. Hiraizumi M, *et al.* Structural mechanism of bridge RNA-guided recombination. *Nature* **630**, 994-1002 (2024).

3. Perry NT, *et al.* Megabase-scale human genome rearrangement with programmable bridge

recombinases. *Science* **391**, eadz0276 (2026).

4. Pelea O*, et al.* Programmable genome editing in human cells using RNA-guided bridge recombinases. *Science* **391**, eadz1884 (2026).

5. Zhang R*, et al.* Amplification editing enables efficient and precise duplication of DNA from short sequence to megabase and chromosomal scale. *Cell* **187**, 3936-3952 e3919 (2024).

6. Rega C*, et al.* High resolution profiling of cell cycle-dependent protein and phosphorylation abundance changes in non-transformed cells. *Nat Commun* **16**, 2579 (2025).

7. Witte IP*, et al.* Programmable gene insertion in human cells with a laboratory-evolved CRISPR-associated transposase. *Science* **388**, eadt5199 (2025).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Overall I am pleased with this revision. The modified UDITAS assay is the correct approach, although it would have been better if they had proper UMIs. I don't think the results here were too overstated, although I think it should emphasize that clearly more specific systems are still needed. I continue to be very impressed with their therapeutic examples.

The comparisons with ISCro4 and evoCAST are good, although the error bars are quite wide. I am impressed and I want to thank the authors for their hard work here.

[Authors Response]
We sincerely thank the reviewer for the positive evaluation and constructive comments. Indeed, we had clarified the limitation of the modified UDiTaS assay without implementation of UMIs. We now also emphasize that future works to explore and develop systems with more stringent integration specificities are warranted (at the end of the paragraph related to the UDiTaS analysis).

Reviewer #2 (Remarks to the Author):

No more comments

[Authors Response]
We thank the reviewer for his/her approval of our revision.

Reviewer #3 (Remarks to the Author):

The authors have addressed all of my previous concerns, and the manuscript is now much improved.

I only have one minor comment regarding data presentation. Several datasets are based on n = 2. It is recommended to avoid displaying error bars for such small sample sizes($n \leq 2$). In addition, please clearly indicate the exact sample size (n) in the figure legends.

Overall, I support publication after these minor revisions.

[Authors Response]
We sincerely thank the reviewer for the positive evaluation and support for publication. Following the reviewer's suggestion, we have removed error bars from datasets with n = 2 and have clearly indicated the exact sample size for each dataset in the corresponding figure legends.