

Supplementary Materials for

**Megabase-scale human genome rearrangement with programmable bridge recombinases**

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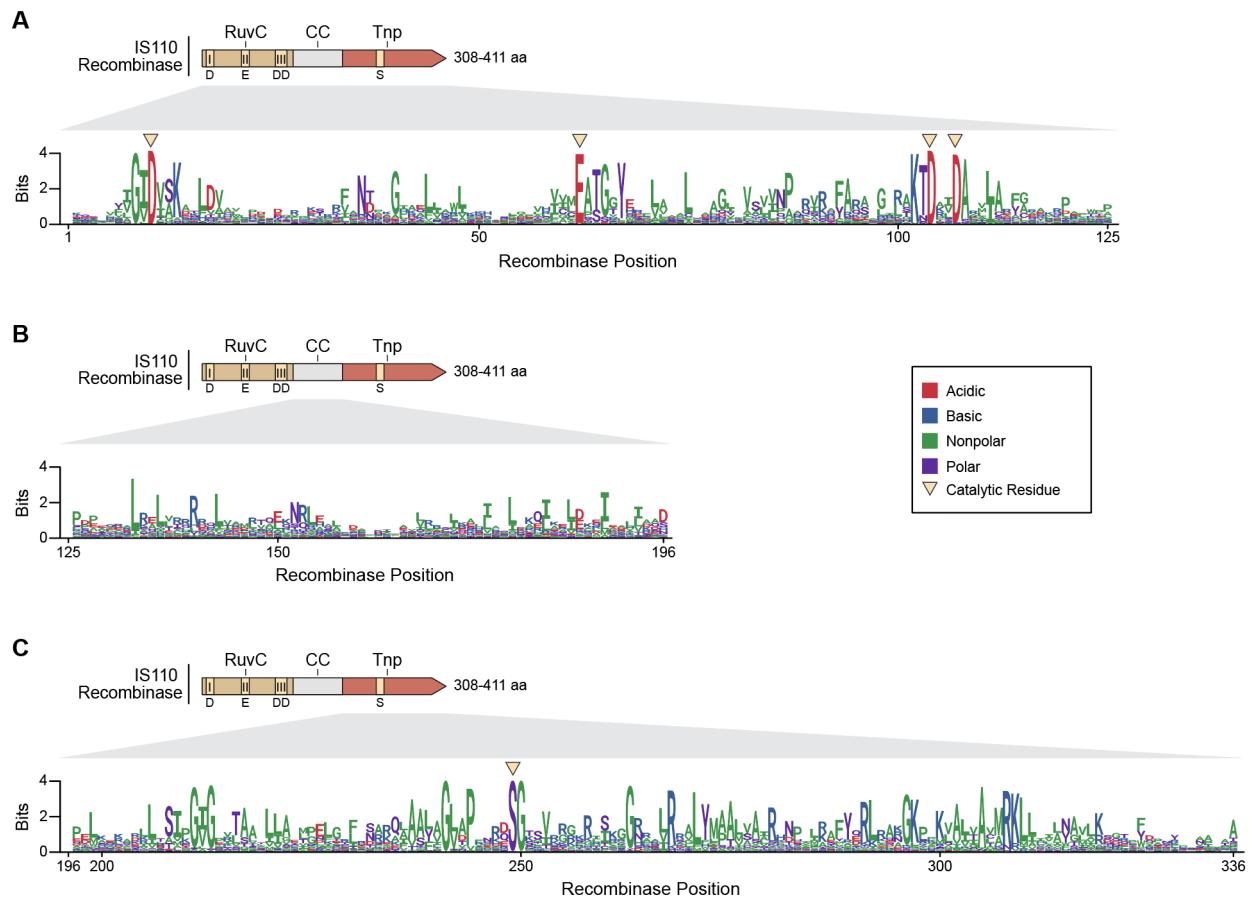
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**The PDF file includes:**

Figs. S1 to S14  
Tables S1 to S4

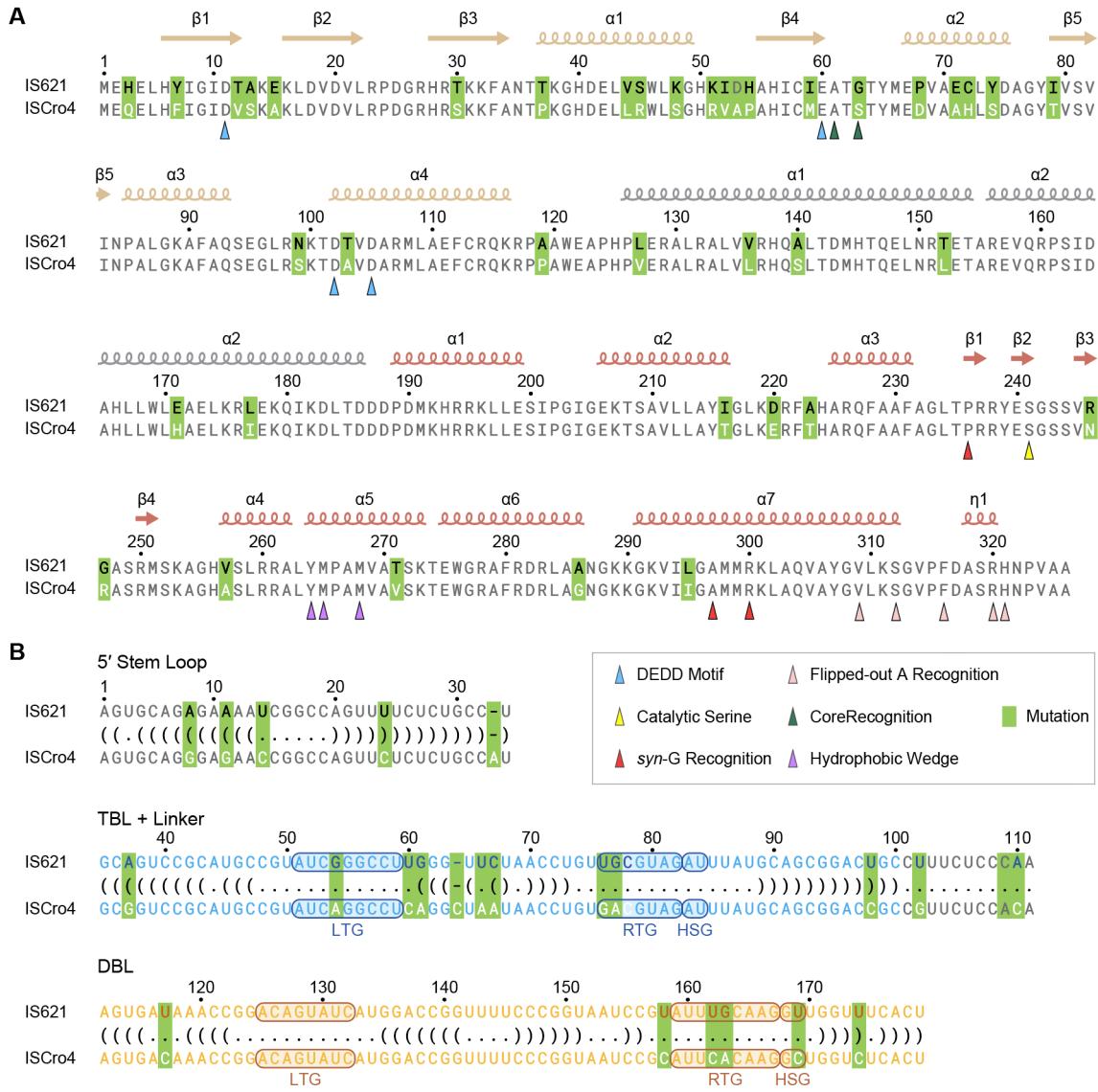
**Other Supplementary Material for this manuscript includes the following:**

MDAR Reproducibility Checklist



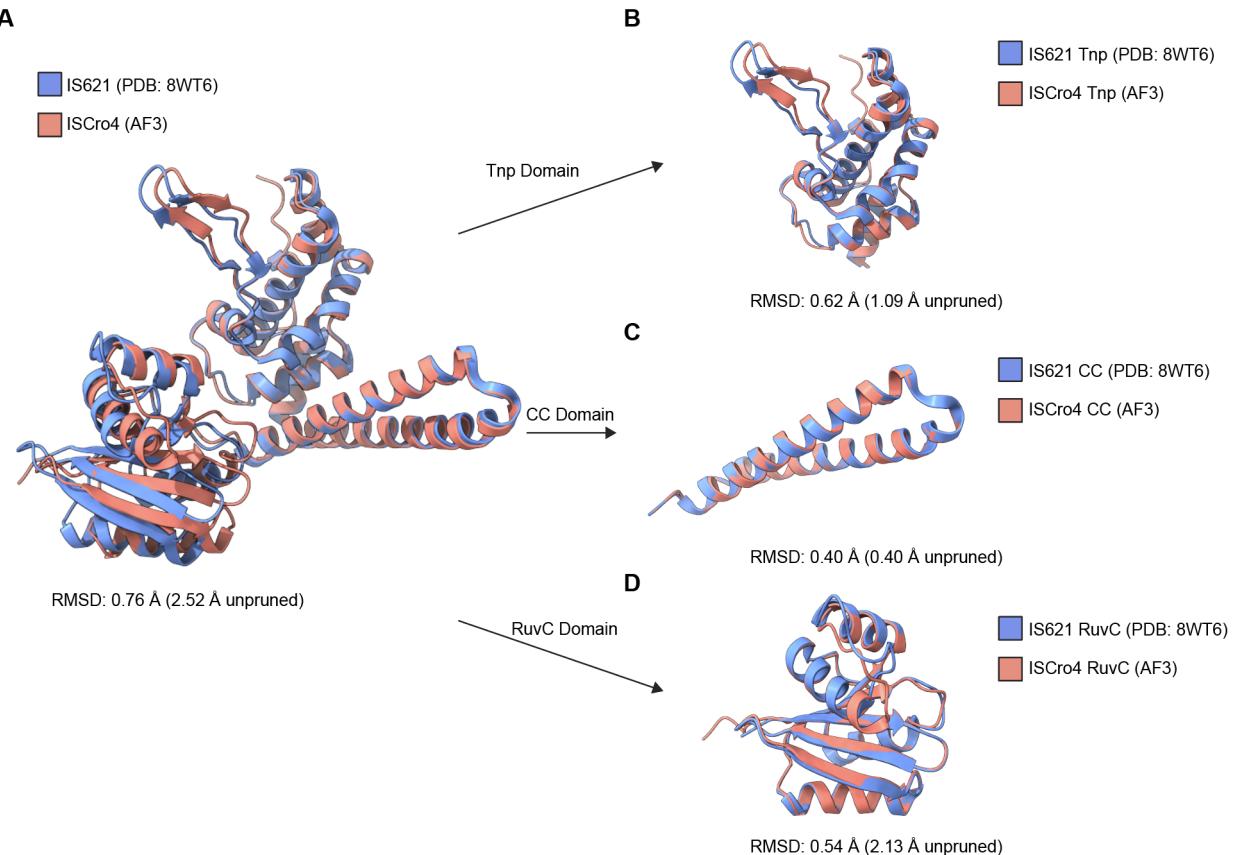
**Figure S1: Diversity of tested bridge recombinases**

**(A-C)** Sequence conservation of 72 bridge recombinases in the RuvC domain (A), CC domain (B), and Tnp domain (C). Alignment columns with more than 75% gaps are filtered out for clarity.



**Figure S2: Sequence comparison between IS621 and IS Cro4**

(A) Alignment of IS621 and IS Cro4 recombinases. Secondary structures are shown above the sequences for IS621 (PDB: 8WT6). Differences are highlighted in green. (B) Alignment of IS621 and IS Cro4 bRNAs. Dot-bracket notation of bRNA structure based on IS621 bRNA structure. Differences highlighted in green and gold.

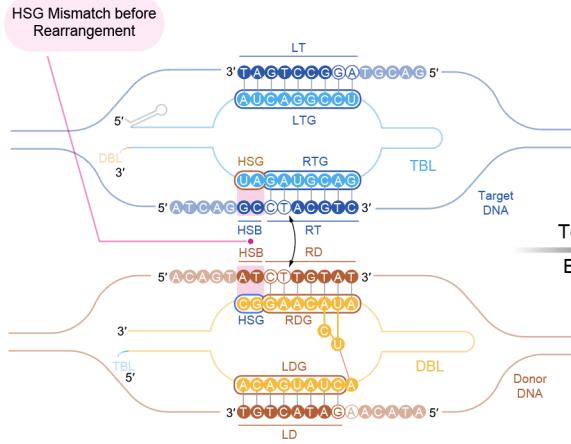


**Figure S3: Comparison of the IS621 structure with the predicted model of ISCro4**

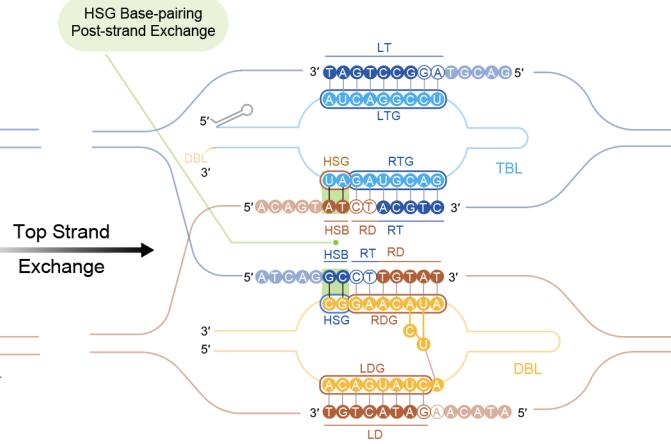
(A) Structural alignment of IS621 recombinase monomer (PDB: 8WT6) with ISCro4 recombinase monomer predicted with AlphaFold 3. (B-D) Structural alignment of individual domains of IS621 and ISCro4 for the Tnp domain (B), CC domain (C), and RuvC domain (D).

**A**

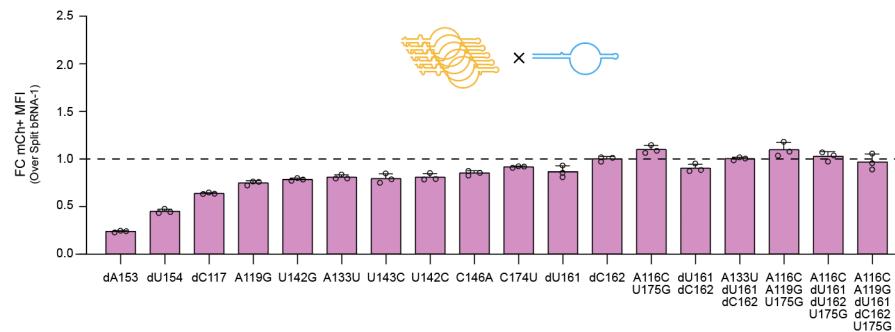
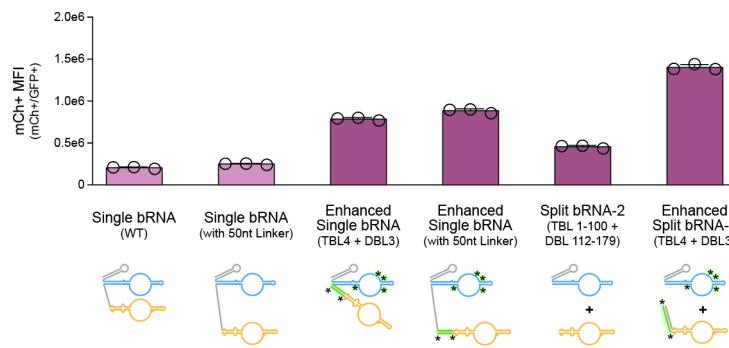
## Pre-exchange bRNA-DNA Complexes

**B**

## Holliday Junction Intermediate

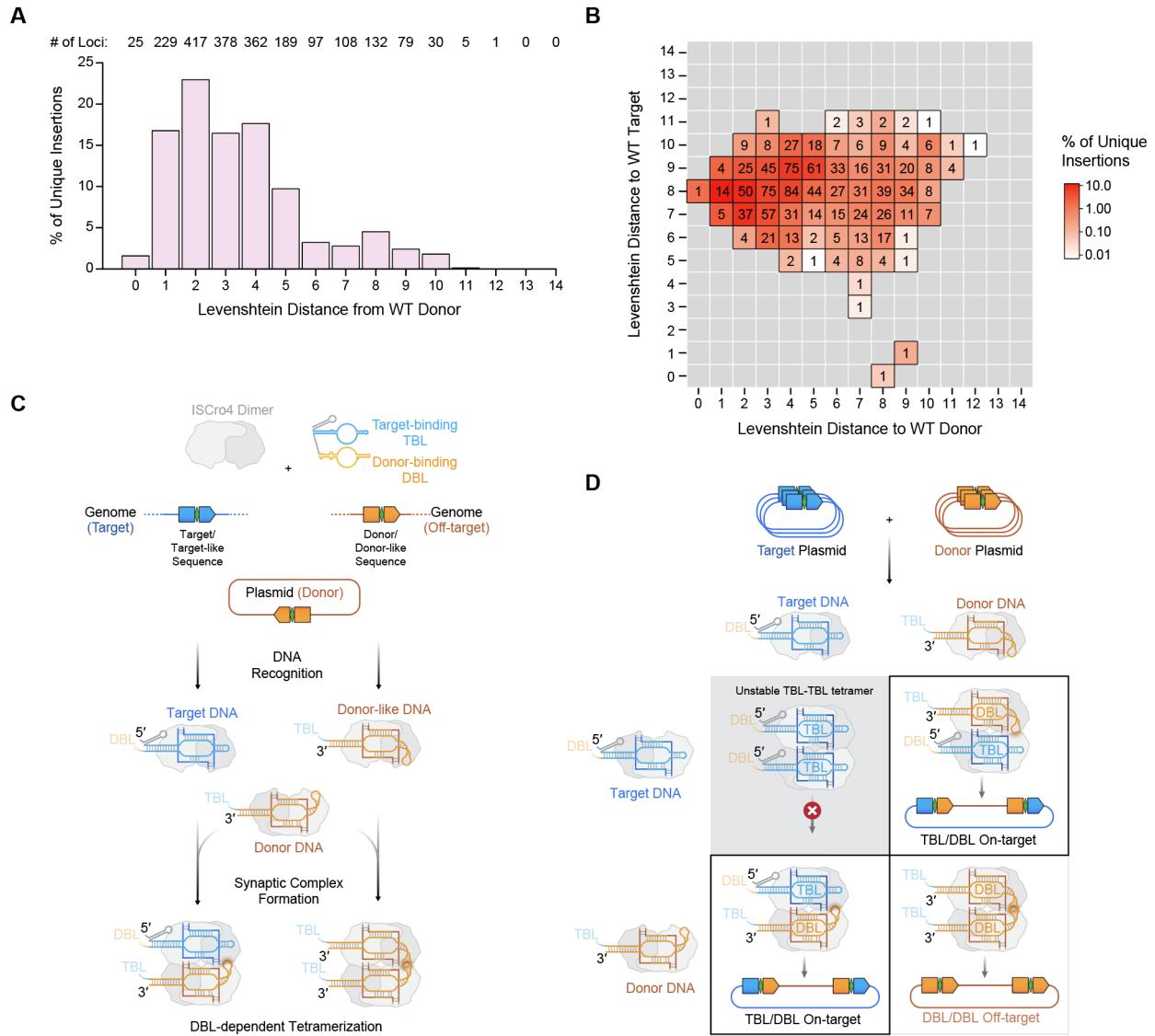
**Figure S4: Handshake guide base-pairing mechanism**

**(A-B)** Model of TBL and DBL base-pairing with target and donor, pre- **(A)** and post- **(B)** strand exchange. Handshake guides mismatch with DNA upstream of core sequences prior to strand exchange and base pair with DNA after strand exchange. LTG, left target guide; RTG, right target guide; LDG, left donor guide; RDG, right donor guide; TBL, target-binding loop; DBL, donor-binding loop; HSG, handshake guide; HSB, handshake bases.

**A****B**

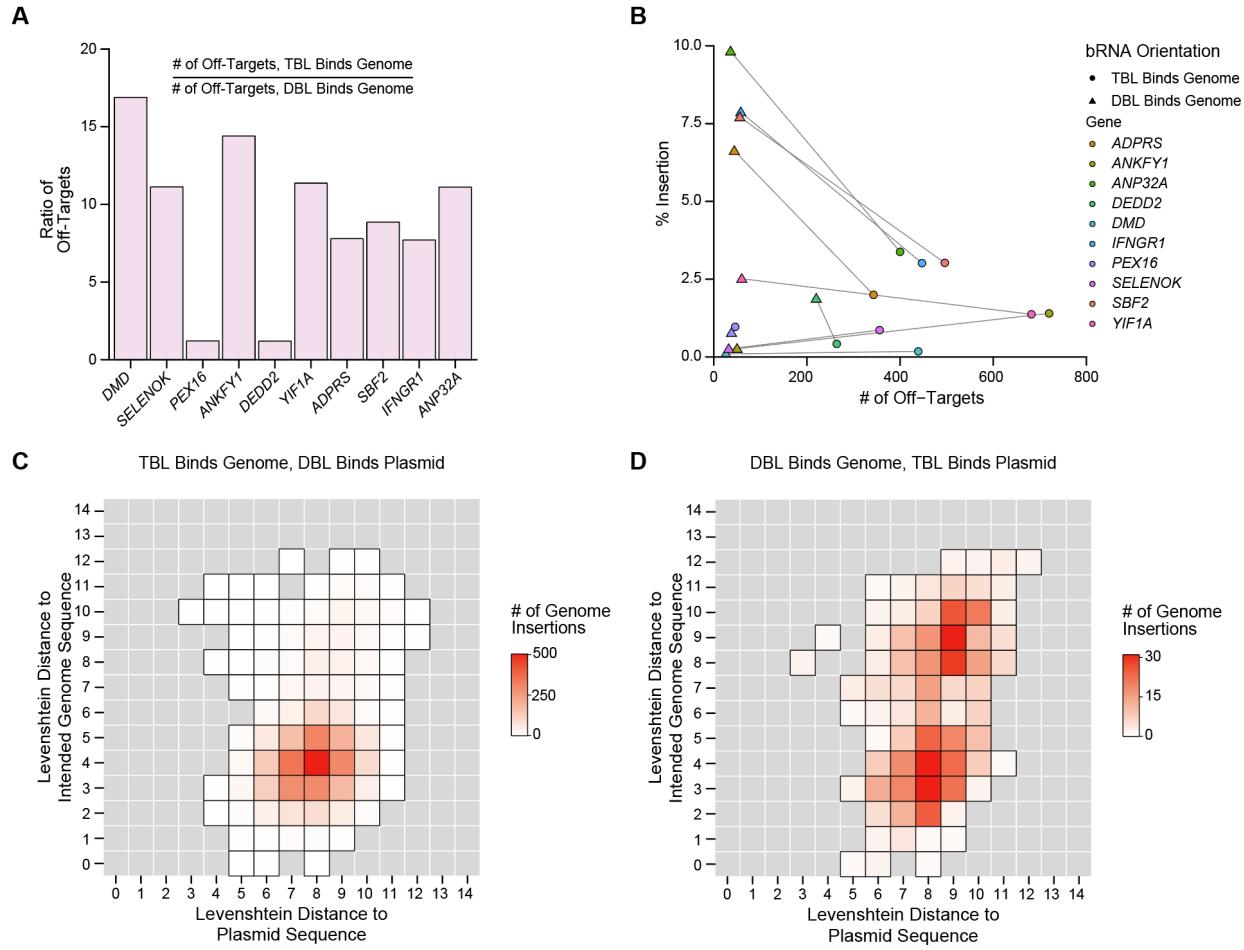
**Figure S5: Donor binding loop rational mutagenesis and bridge RNA linker extension**

(A) Efficiency of recombination of DBL mutants in inversion reporter assay. DBL mutants tested with boundaries of 112-179 and paired with a TBL with boundaries of 35-100. Mean  $\pm$  s.d. of three biological replicates. (B) Efficiency of recombination with bridge RNAs featuring extended linker regions. Mean  $\pm$  s.d. of three biological replicates.



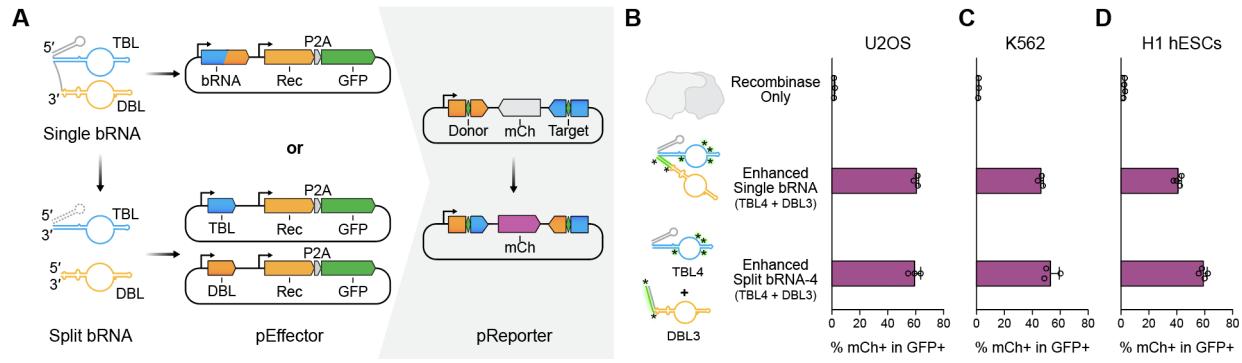
**Figure S6: Assessment and mechanism of DBL-DBL guided off-targets**

**(A)** Levenshtein distance of off-targets from the use of a WT ISCro4 bRNA with a WT donor plasmid. Percentage of unique insertions based on the total number of recovered UMIs across all insertions genome wide. The number of loci represented within each bar is shown at the top. **(B)** Heatmap of Levenshtein distance of off-targets in comparison to WT target and WT donor. The number of loci represented are shown in the center of each cell. **(C)** Mechanism of tetramerization with one TBL and one DBL or between two DBLs in the context of human genome insertion. **(D)** General mechanism of possible dimer-dimer interactions between DNA-bound TBL and DBL dimers. TBL-TBL tetramers are destabilized by the lack of a long hairpin reaching between dimers.



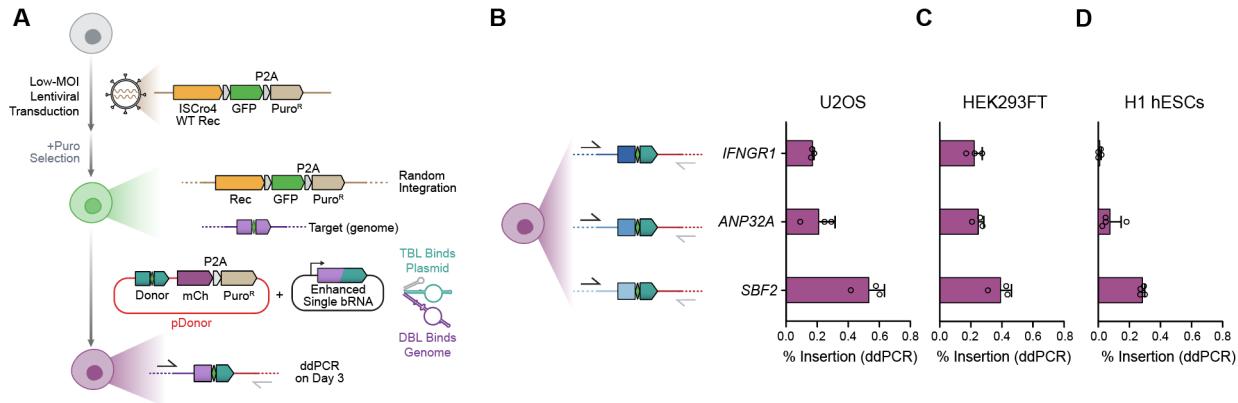
**Figure S7: Specificity and off-target analysis of programmable gene insertion**

(A) Ratio of the number of off-targets when the bRNA is designed for the TBL to bind the genome versus for the DBL to bind the genome. (B) Comparison of efficiency and number of off-targets across all genes between TBL or DBL bound to the genome. (C-D) Heatmap of Levenshtein distance of off-targets in comparison to expected genome site or plasmid encoded sequence when the TBL (C) or DBL (D) is encoded to bind the genome.



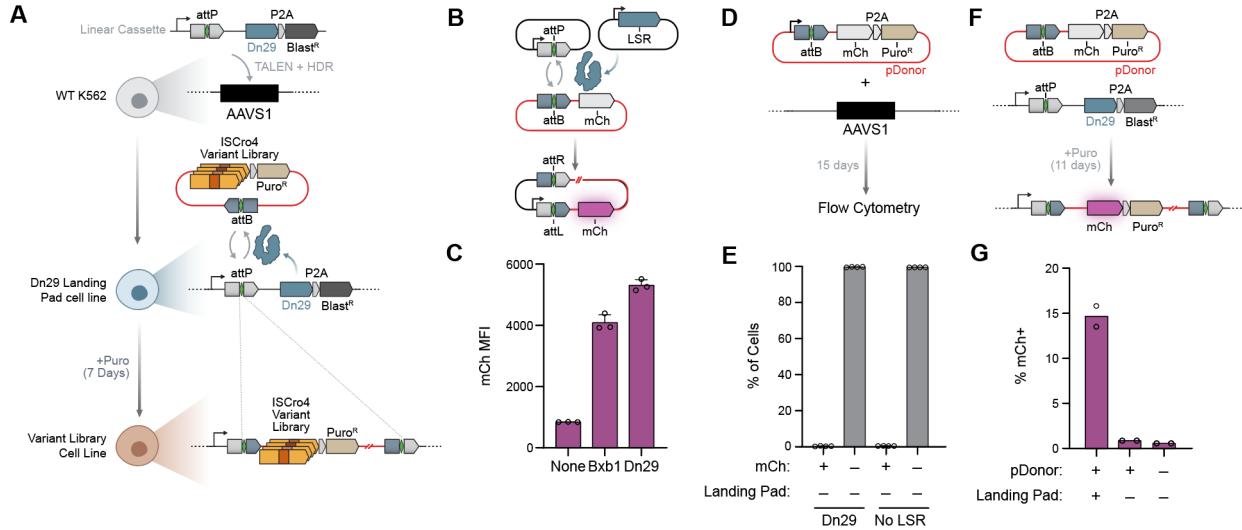
**Figure S8: Activity of engineered bridge RNAs in other cell types**

(A) Overview of recombinase and bRNA expression plasmids with mCherry inversion reporter. (B-D) Inversion efficiency with single and split enhanced bridge RNAs in U2OS (B), K562 (C), and H1 human embryonic stem cells (hESCs) (D). Mean  $\pm$  s.d. of three or four biological replicates.



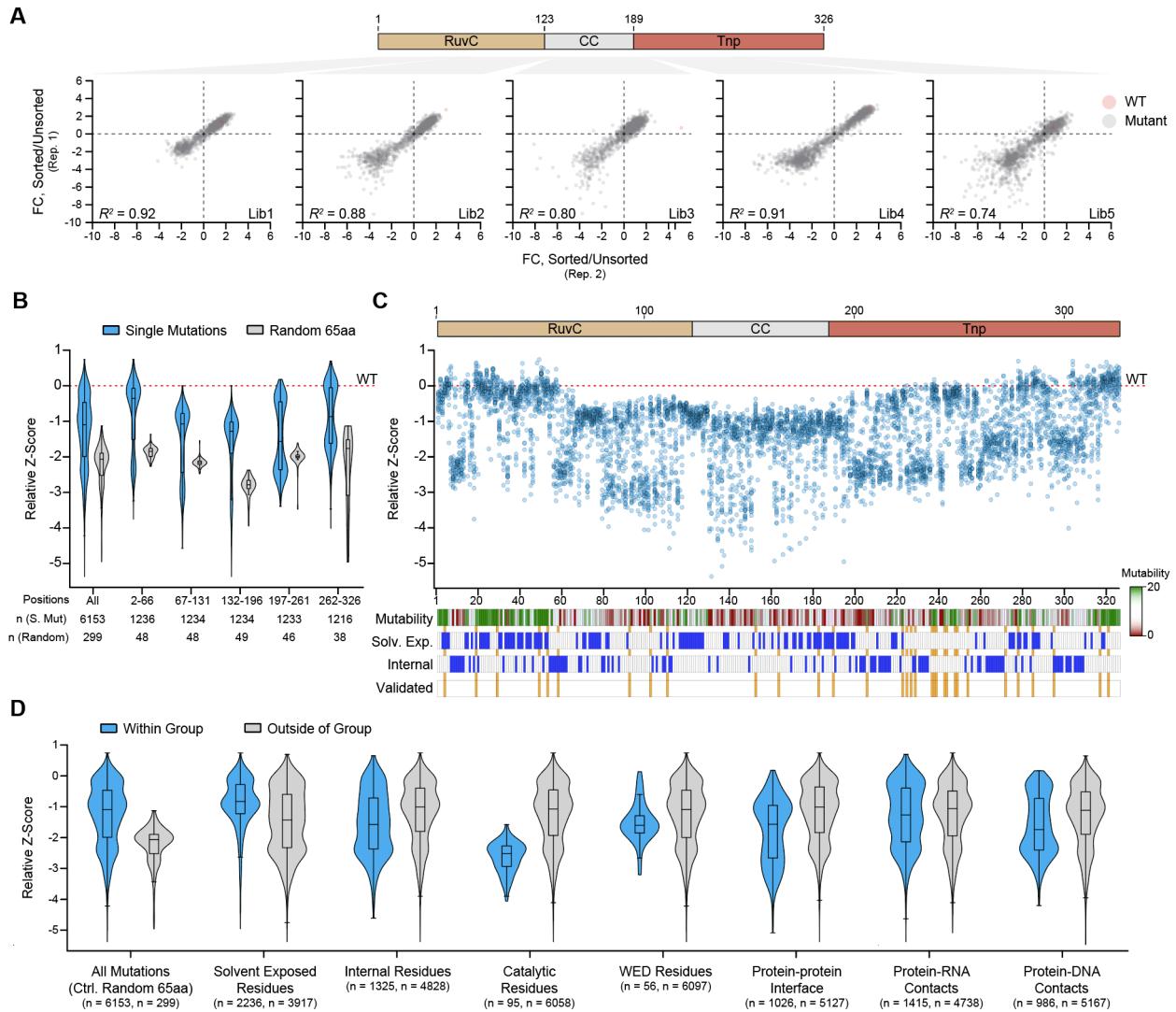
**Figure S9: Genome insertion with lentivirally integrated bridge recombinase**

(A) Schematic of lentiviral production, recombinase cell line generation, and delivery of bRNA and donor to cell lines. (B-D) Genome insertion efficiency into several genes in U2OS (B), K562 (C), and human embryonic stem cells (hESCs) (D). Mean  $\pm$  s.d. of three or four biological replicates.



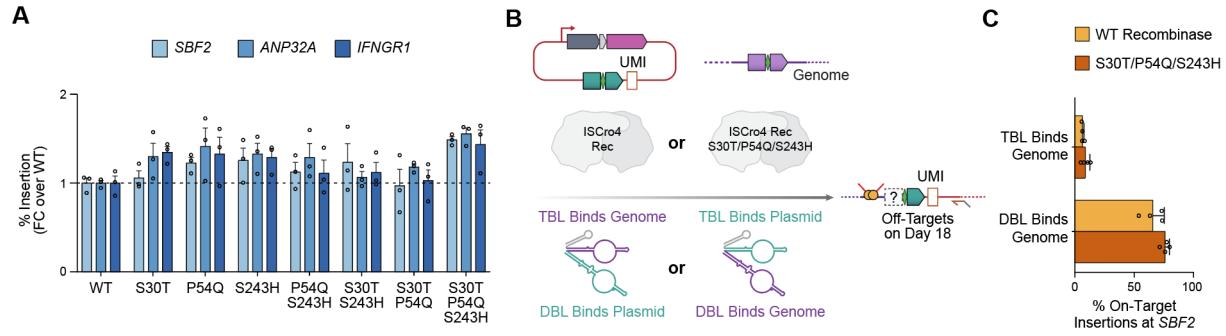
**Figure S10: Generation and validation of Dn29 landing pad cell line**

**(A)** Schematic of cell line creation. attP-Dn29-P2A-Blast<sup>R</sup> was knocked into AAVS1 using TALENs. AttB-containing plasmids are subsequently knocked into the AAVS1 locus via Dn29 mediated attP/attB recombination. **(B)** Schematic of plasmid recombination assay for evaluating LSR efficiency. **(C)** Relative recombination efficiency of Bxb1 and Dn29 LSRs. Mean  $\pm$  s.d. of three biological replicates. **(D)** Schematic of off-target insertion assay. AttB-containing plasmids were nucleofected into WT K562 cells, and mCherry expression was measured after 15 days in culture. **(E)** Percentage of WT K562 cells bearing mCherry knock-in after 15 days. Mean of four biological replicates. **(F)** Schematic of landing pad cell line insertion efficiency assay. mCherry-P2A-Puro<sup>R</sup> is knocked into the genome and cultured under puromycin selection for 11 days. **(G)** Percentage of Dn29 landing pad cells with mCherry knock-in after 15 days.



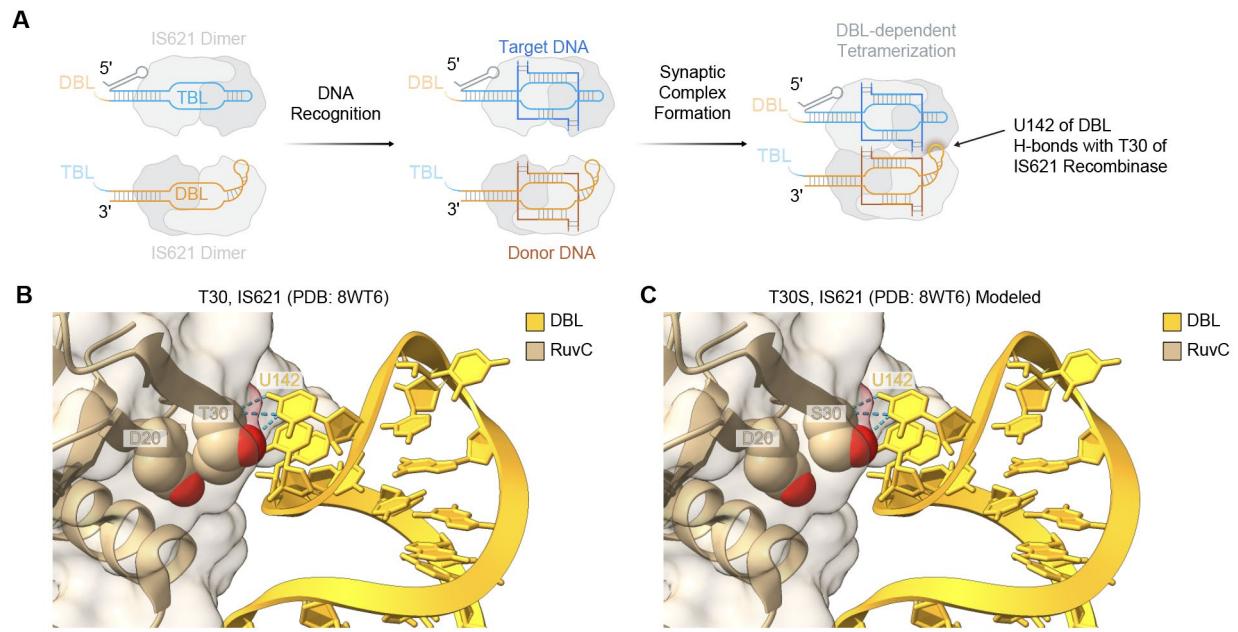
**Figure S11: Deep mutational scan distributions and mutant validations**

**(A)** Replicate correlation for each 65 aa segment of deep mutational scan. WT recombinase highlighted in red. FC, fold-change. **(B)** Distribution of all mutations compared to distributions of random 65 aa negative controls. **(C)** Distribution of all mutations per position. Mutability score, solvent exposure, internal, and validated residues are highlighted. **(D)** Distribution of mutations within and outside of various residue groups. Groups determined based on IS621 transpososome structure (PDB: 8WT6).



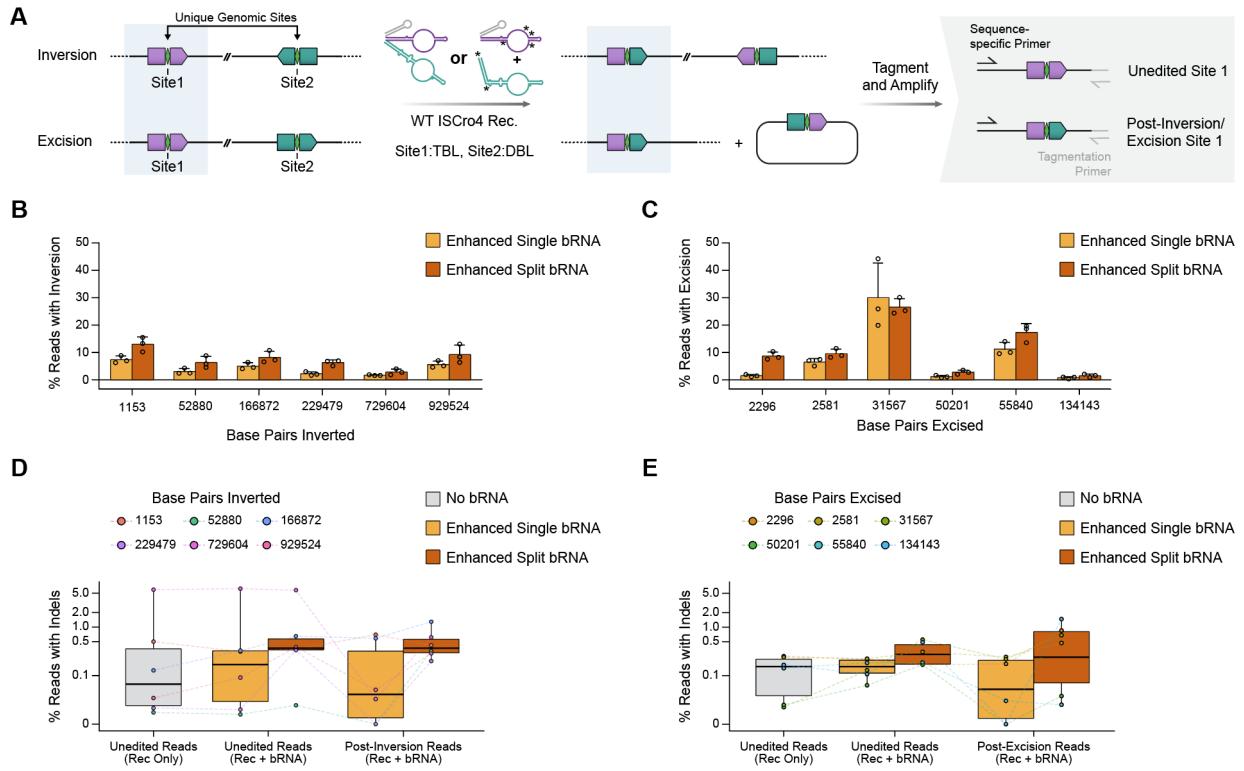
**Figure S12: Efficiency and specificity of ISCr04 multi-mutants**

(A) Relative insertion efficiency across genes with double and triple mutants. (B) Schematic showing recombinases and bridge RNAs evaluated for genome insertion specificity. (C) Genome insertion specificity into *SBF2* for combinations of bRNA orientations and recombinase sequences. Mean  $\pm$  s.d. of four biological replicates.



**Figure S13: Modeling of ISCro4 T30S mutation on the IS621 structure**

(A) Overview of bridge recombinase synaptic complex assembly, based on IS621. TBL, target-binding loop; DBL, donor-binding loop. U142 of the DBL contacts S30 of the IS621 recombinase. (B-C) Recognition of the DBL U142 by T30 (B) and T30S (model) (C) in the IS621 structure (PDB: 8WT6). Contacts between D20 and X30 (T30 or S30) are compared.



**Figure S14: Precision of genome rearrangement with enhanced single and split bRNAs**

**(A)** Schematic of performing intra-chromosomal recombination. Enhanced bRNAs in both orientations are used to yield inversion or excision, which is measured by tamentation and next-generation sequencing. **(B-C)** Efficiency of genome inversion **(B)** and excision **(C)** across 14 sequence pairs with either enhanced single or split bRNA. Mean  $\pm$  s.d. of three biological replicates. **(D-E)** Rate of indel formation pre- and post- inversion **(D)** and excision **(E)** with or without bRNAs. Points represent the rate of indel formation across three biological replicates **(Methods)**.

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