A protein-guided modular integrase (MINT) platform enables efficient targeted integration of compact therapeutic payloads in primary human T cells

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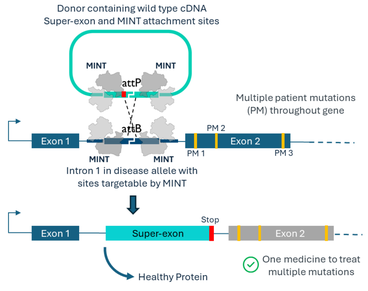
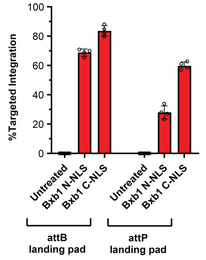
Poster #648

Introduction

- Here we describe the development of a Modular Integrase (MINT) platform, a versatile protein-guided genome editing method that achieves site-directed integration of large synthetic DNA constructs into user-specified chromosomal target sites.
- We envision using the MINT platform as a single step strategy to overcome genetic disorders characterized by an array of mutations in the diseased allele by efficiently integrating a correct coding sequence for the gene into its natural locus upstream of the disease-causing mutations.
- Bxb1 is a Large Serine Integrase (LSI) capable of efficiently and precisely integrating large synthetic DNA constructs into its native DNA recognition site or into copies of this site that have first been pre-installed into the genome as a "landing pad".
- To obviate the need for this prior insertion of the Bxb1 landing pad, we reprogrammed the sequence recognition characteristics of Bxb1 while also including activity-increasing mutations and zinc finger fusions to optimize integration efficiency and specificity.

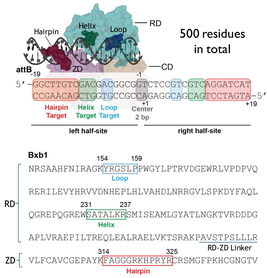


Bxb1 activity in human landing pad cells



Bxb1-DNA interaction mapping

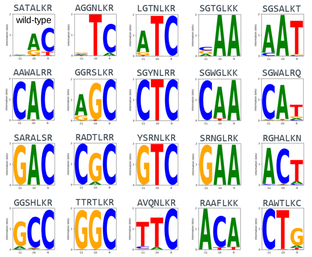
- Use of LSIs as genome editing reagents has been hampered by the difficulty in reprogramming them to recognize new DNA target sequences in therapeutically relevant loci.
- We used a combination of structural modeling and experimental characterization of systematic mutations to both Bxb1 and its DNA target site to map critical Bxb1-DNA interaction domains.
- We hypothesized that if Bxb1 recognized its target sites in a modular fashion then we could reprogram its DNA target site specificity by performing directed evolution on each interaction domain (hairpin, helix, loop) using Bxb1 libraries in which the residues that make sequence-specific DNA contacts are randomized.



Changing the molecular specificity of Bxb1

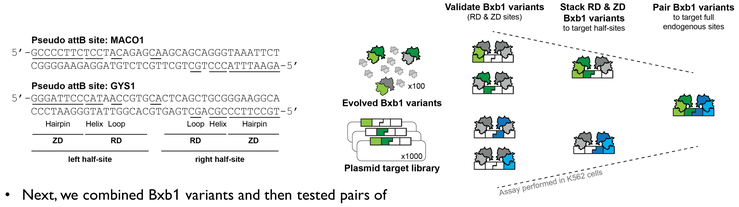
- We developed a directed evolution system in which a recombinase variant present in a library is enriched when it can restore the open reading frame of an antibiotic-resistance gene if it acts on a new target sequence.
- We carried out 64 separate selections using our library of Bxb1 helix variants and each possible DNA triplet sequence.
  SATALKR wild-type Bxb1 residues 231-237
  XXXXLXX helix variant library
- We characterized the target site specificity of the wild-type helix (SATALKR) which yielded a preference for NAC (N = G/A/T/C) DNA binding motifs consistent with natural DNA target sites.
- Many of the selected helices were able to recognize alternative DNA triplets with high molecular specificity and the data provide insights into Bxb1 protein-DNA interaction patterns.

Novel Bxb1 helices and their DNA triplet specificity



Performance of Bxb1 variants at endogenous human sites

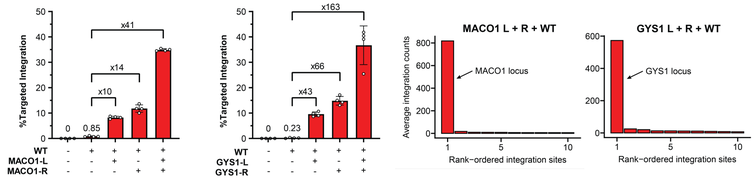
- We identified Bxb1 pseudo-sites in the human genome using both computational and experimental methods.
- We then generated a plasmid target library consisting of the regions of each pseudo-site recognized by the RD or ZD domains of Bxb1 and tested evolved Bxb1 variants against this library of DNA target sites.



- Next, we combined Bxb1 variants and then tested pairs of variants against their chromosomal target sites in K562 cells.
- This resulted in variant pairs that substantially outperformed wild-type Bxb1 at the same pseudo-site.
- A genome-wide integration assay revealed that for two separate target sites in the MACO1 and GYS1 genes respectively, the intended target site was the most preferred integration site in the human genome by 47-fold and 23-fold.

Bxb1 peptide sequence derived from directed evolution

	Loop	Helix	Hairpin
Wild-type Bxb1	YRGSLP	SATALKR	FAGGGRKHPRYR
MACO1-L	YRGSLP	SATALKR	MAGGRRKQALYR
MACO1-R	YRGSLP	RAWSLKR	MAGGRRKGRYR
GYS1-L	YRGSLP	HGWSLKV	LARGSRKLALYR
GYS1-R	YRGSLP	HGCTLKR	NARGNRKRGRYR



Reprogramming Bxb1 to integrate at clinically relevant sites

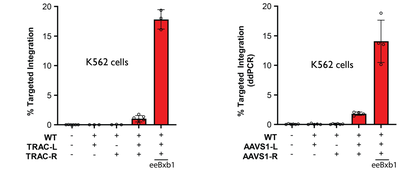
- Encouraged by our success targeting pseudo-sites where wild-type Bxb1 was already active, we proceeded to the more difficult challenge of targeting clinically relevant regions of the human genome such as TRAC and AAVS1.
- We also added activity-increasing mutations[1] from eeBxb1 (V74A, E229K, V375I) to further improve activity.

TRAC: attB site
5′-TGGTGTCCAGGAGCCGAGGTATCGGTCCTGCCAGGGCC
ACCACAGGTCCTCGGCTCCATAGCCAGGACGGTCCCGG-5′

AAVS1: attB site
5′-CTGAGCGCCTCCTCCTGGGCTTGCCAAGGACTCAAACCC
GACTCGCGGAGAGGACCCGAACGGTTCCTGAGTTTGGG-5′
Hairpin Helix Loop Loop Helix Hairpin

Bxb1 peptide sequence from directed evolution			
	Loop	Helix	Hairpin
Wild-type Bxb1	YRGSLP	SATALKR	FAGGGRKHPRYR
TRAC-L*	YRGGLP	YGSALKQ	LARGPRKRAGYK
TRAC-R	YRGGLP	SQWALKC	RARGKRKYAYYQ
AAVS1-L	YRGGLP	YPWSLRR	KARGSRKTRLYR
AAVS1-R	YRGGLP	AGGNLKR	MARGGRKSAIYY

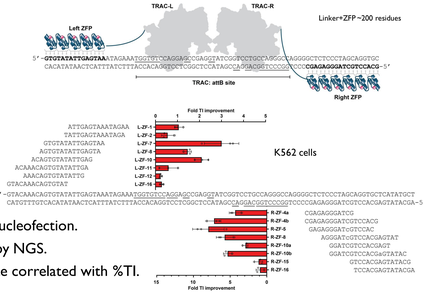
*also includes D257K mutation

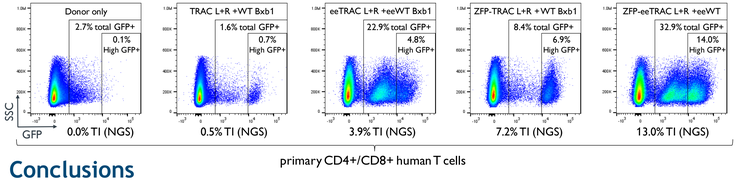


[1]Pandey et al. *Nat. Biomed. Eng.* 2025

Fusions to engineered zinc fingers further improves performance

- We fused a panel of engineered zinc finger arrays to our LSI variants that target TRAC.
- We varied the position of the ZF binding site, the linker length, and the number of ZFs per array and tested in K562 cells.
- The most promising ZF fusions were tested in primary human T cells.
- To mimic therapeutic conditions, we used a nanoplasmid donor that expressed a functional protein (GFP).
- Donor and LSI mRNA delivered by Lonza nucleofection.
- Targeted integration (TI) assayed at Day13 by NGS.
- Flow cytometry at Day14 with high GFP gate correlated with %TI.





Conclusions

- We have demonstrated the direct reprogramming of the LSI Bxb1 and further improved performance via addition of activity-increasing mutations and fusions of zinc finger arrays to the LSI.
- We showed the therapeutic potential of this single-step, non-landing pad approach by stably integrating an expression cassette at the TRAC locus in primary human T cells with up to 13% efficiency.
- In addition to human therapeutics, the MINT platform has a variety of potential applications in biomanufacturing, drug discovery, agricultural biotech, and basic research.

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