Using TcBuster™ (TcB-M™) transposase for highly efficient and robust delivery of multicistronic therapeutic cargo in immune cells for both RUO and clinical applications

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Introduction

Rapid development of genome engineering tools has driven several immune and stem cell therapies in clinical trials with the goal of generating autologous and allogeneic therapeutics. Many of these therapies use viral vectors for the delivery of therapeutic cargo. However, viral mediated therapies carry the risk of immunogenicity, cargo size limitations, integration site risk, manufacturing delays, and are highly cost prohibitive. While there are two known non-viral transposase-based systems, piggyBac® and Sleeping Beauty™, both are exclusively licensed for cell therapies and are not available for commercial use. TcBuster-M™ (TcB-M™) is a commercially available non-viral transposase-based editing platform that overcomes current viral limitations. TcBuster is found in the red flour beetle and is a member of the hAT family of transposases. Using directed evolution, we engineered a hyperactive mutant (TcB-M) that has improved transposition rates using less mRNA transposase and Nanoplasmid™ DNA transposon. Divergent from the engineering efforts used to build hyperactive enzymes of piggyBac and Sleeping Beauty, we used a novel high-throughput screening platform in mammalian cells. This allowed us to screen a mutant library of >3 million variants, which is much larger than those used to build PiggyBac or Sleeping Beauty. This led to the construction of the most efficient transposase system for engineering primary immune cells. TcB-M allows for rapid cell manufacturing with limited cell manufacturing cost. Current TcB-M timeline from vector map to GMP transposon is ~6-8 months. Since TcB-M is less constrained by cargo size, we can design large multicistronic transposons for robust delivery of multiple proteins in various cell types, including primary T- and NK- cells, mesenchymal stem cells, and induced pluripotent stem cells (iPSCs). Additionally, TcB-M can be easily combined with endonucleases, such as CRISPR reagents, to generate combinatorial knock-out/overexpression edited cell products. The improved TcB-M has resulted in cargo integration rates greater than 60% in primary T-cells and peripheral blood derived NK cells, without sacrificing cell growth or clonal dominance concerns. Finally, we have conducted direct comparisons against lentiviral, piggyBac, and Sleeping Beauty engineered CAR-Ts, demonstrating TcB-M engineered CAR-Ts with equal to higher integration percentage. TcB-M also has a safer integration profile, as it is more randomly integrated into the genome without preference for active sites when compared to lentivirus. Overall, TcB-M is a widely available, proven, non-viral gene editing technology that can deliver large or difficult therapeutic cargos in a variety of cell types. TcB-M reduces many of the viral mediated editing hurdles, allowing faster generation of crucial therapeutics to market.

Mechanism of TcBuster

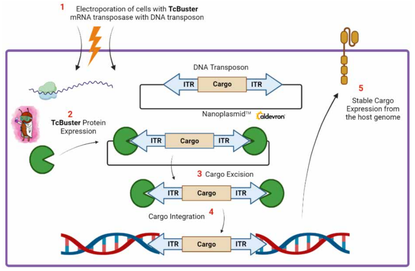


Figure 1. Overview of TcBuster transposition mechanism. TcBuster (TcB-M) is a transposon system similar to piggyBac and Sleeping Beauty. It is a non-viral method to generate stable expression cell lines and can efficiently edit both transformed and primary mammalian cell lines. **(1)** TcBuster mRNA is introduced into the cells via electroporation. **(2)** TcBuster mRNA translates into TcBuster transposase. **(3)** TcBuster excises cargo from Aldeveron's' Nanoplasmid™. **(4)** Cargo is integrated into host cell genome. **(5)** Cargo is stably expressed as protein in cells of interest. Figure made using BioRender. Publication under review.

Developing TcBuster

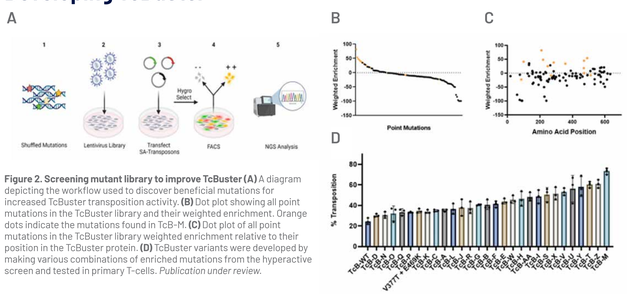


Figure 2. Screening mutant library to improve TcBuster (A) A diagram depicting the workflow used to discover beneficial mutations for increased TcBuster transposition activity. **(B)** Dot plot showing all point mutations in the TcBuster library and their weighted enrichment. Orange dots indicate the mutations found in TcB-M. **(C)** Dot plot of all point mutations in the TcBuster library weighted enrichment relative to their position in the TcBuster protein. **(D)** TcBuster variants were developed by making various combinations of enriched mutations from the hyperactive screen and tested in primary T-cells. *Publication under review.*

TcBuster successfully transposes T cells, NK cells, and iPSCs with single and multicistronic therapeutic cargos

TcB-M for CAR-T Therapies

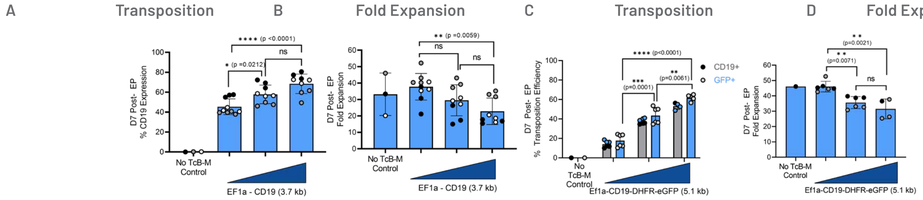


Figure 3. TcB-M efficiently transposes single and multicistronic cargos of varying sizes into T cells CD4+CD8+ cells were activated for two days and electroporated using the Lonza 4D-Nucleofector™: Sizes of both transposons are reported as size integrated into cell genome. **(A)** Graphical representation of transposition efficiency D7 post-electroporation by flow analysis using a titration of EF1a-CD19 in T cells. **(B)** Graphical representation of T cell fold expansion D7 post-electroporation with EF1a-CD19. **(C)** Graphical representation of transposition efficiency D7 post-electroporation by flow analysis using a titration of EF1a-CD19-DHFR-eGFP in T cells. **(D)** Graphical representation of T cell fold expansion D7 post-electroporation with EF1a-CD19-DHFR-eGFP. Experiments were conducted a minimum of twice with n=3 technical replicates in at least 2 biological donors. Figures A, B, D were analyzed using an Ordinary one-way ANOVA, Figure C was analyzed using a 2way ANOVA with Tukey's multiple comparisons test.

TcB-M for CAR-NK Therapies

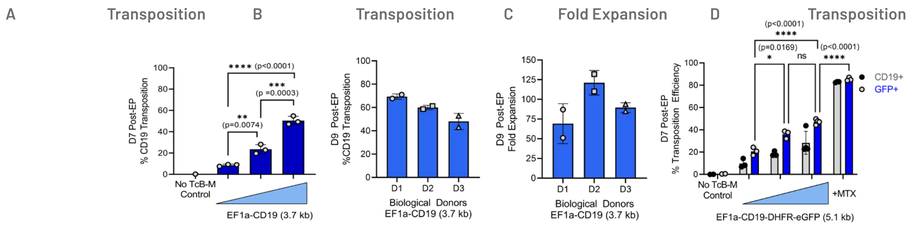
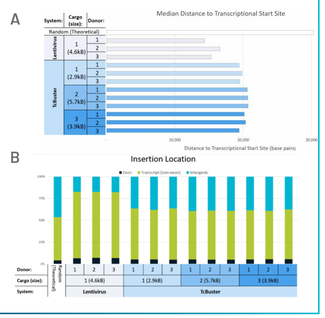


Figure 4. TcB-M efficiently transposes single and multicistronic cargos of varying sizes into NK cells CD3-CD56+ Peripheral Blood (PB)- NK cells were activated using an irradiated K562 feeder line for 4 days and electroporated using the Lonza 4D-Nucleofector™: Sizes of both transposons are reported as size integrated into cell genome. **(A)** Graphical representation of transposition efficiency D7 post-electroporation by flow analysis using a titration of EF1a-CD19 in PB-NK cells. **(B)** Graphical representation of transposition efficiency D9 post-electroporation with EF1a-CD19 in 3 biological donors. **(C)** Graphical representation of fold expansion D9 post-electroporation in 3 biological donors. **(D)** Graphical representation of transposition efficiency D7 post-electroporation by flow analysis using a titration of EF1a-CD19-DHFR-eGFP. Last column took highest concentration of transposon and selected for transposed cells using Methotrexate (MTX). Figures A was analyzed using an Ordinary one-way ANOVA, Figure D was analyzed using a 2way ANOVA with Tukey's multiple comparisons test.

Site Integration Analysis between TcB-M and Lentiviral Transduction

Figure 5. TcB-M has a better safety profile than viral transduction methods. CD4+ CD8+ T cells were either transposed with TcB-M on the MaxCyte® electroporation system or transduced using Lentivirus and compared in 3 different donors using 3 different transposon cargo sizes. **(A)** Graphical representation of the Distance to Transcription Start Site. **(B)** Graphical representation of the insertion location of each transposon when using either TcB-M or lentiviral transduction.



TcB-M for iPSC Gene Replacement Therapies

C

Name	Integration Sites	Exon	Transcript (non-exon)	Intergenic	Median Distance to Transcriptional Start Site (bp)
Random (Theoretical)		4%	48%	47%	31,503
TcB-M Population	2,953	8%	46%	46%	22,567

Clone	Insertion Location	Closest Gene Name	ACCESSION	Category	Copy #
Clone 1	Chr3:152,316,395	muscleblind like splicing regulator 1 (MBNL1)	NM_207297.1	In transcript	3
	Chr11:96,593,393	78.6 kb from long intergenic non-protein coding RNA 2737	NR_135097.1	Intergenic	
	Chr19:30,402,450	zinc finger protein 536 (ZNF536)	NM_001352290.1	In transcript	
Clone 2	chr1:93,309,627	3.2 kb from CCDC18 antisense RNA 1 (CCDC18-AS1), long non-coding RNA	NR_034089.1	Intergenic	5
	chr1:93,472,868	within PREDICTED: formin binding protein 1 like (FNBP1L)	XM_017001533.1	In transcript	
	chr5:78,493,863	within PREDICTED: LHFPL tetraspan subfamily member 2	XR_948226.2	In transcript	
	chr20:12,697,031	23.8 kb from PREDICTED: uncharacterized LOC105372532	XR_937268.2	Intergenic	
	chrX:90,946,113	366.1 kb from PREDICTED: uncharacterized LOC107985700	XR_001759913.1	Intergenic	
Clone 3	chr4:171,668,557	20.8 kb from PREDICTED: uncharacterized LOC107598203	XR_001741464.1	Intergenic	2
	chr10:72,757,325	within PREDICTED: mitochondrial calcium uniporter (MCU)	XM_017019882.2	In transcript	

Figure 6. Site- integration analysis of TcB-M modified iPSCs. Human iPSCs were electroporated with TcB-M mRNA on Lonza's 4D-Nucleofector™ along with a transposon containing a client's therapeutic cargo for autologous therapy **(A)** Distance to Transcription Start Site. **(B)** Insertion Location. **(C)** Copy number analysis and site integration analysis of the transposed cell populations and 3 representative clone.

TcB-M comparison study against competitor transposases

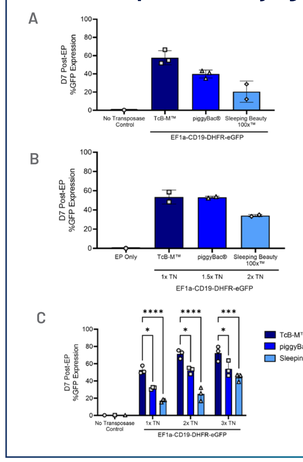
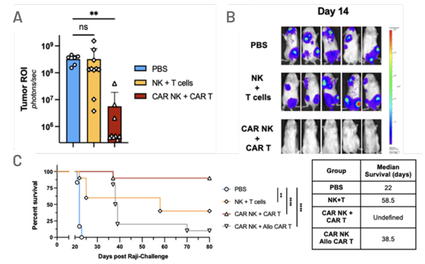


Figure 7. TcB-M outperforms in head-to-head comparison of piggyBac® and Sleeping Beauty 100x™ CD4+CD8+ T cells were electroporated using the Lonza 4D-Nucleofector™ with the EF1a-CD19-DHFR-eGFP transposon (TN) **(A)** Graphical representation of transposition efficiency on D7 post- electroporation using the same concentration of transposon. **(B)** Graphical representation of transposition efficiency on D7 post-electroporation with 1x, 1.5x, or 2x concentration of transposon **(C)** Graphical representation of transposition efficiency using 1x, 2x, or 3x concentration of transposon. Figure C was analyzed using a 2way ANOVA with Tukey's multiple comparison test. Study was conducted using a minimum of 3 different T cell donors. **TcB-M requires less transposon and gentler EP programs to achieve higher transposition compared to competitors.**

TcB-M engineered CAR-T and CAR-NK cell combination therapy effectively control tumor burden in mouse model of human Burkitt's lymphoma

Figure 8. Combination of suboptimal doses of autologous CAR-T and CAR-NK therapy results in control of Raji tumors and significantly improves survival in mice. (A) Graphical comparison of tumor luminance (ROI) across treatment groups. **(B)** IVIS imaging comparison of luminance (ROI) across treatment groups. **(C)** Kaplan-Meier survival of mice bearing Raji tumors following treatment with engineered combination therapy or non-CAR matched donor cells. *Publication under review.*



Group	Median Survival (days)
PBS	22
NK+T	58.5
CAR NK + CAR T	Undefined
CAR NK Allo CAR T	38.5

Conclusions

- TcB-M is a hyperactive engineered transposase for stable integration of genetic cargo into a variety of transformed cell lines and primary immune cells.
- TcB-M displays high transposition efficiencies for both single and multicistronic cargo in primary human T cells, NK cells, and iPSCs.
- TcB-M outperforms in terms of transposition efficiency compared to other transposase competitors.
- TcB-M is a robust, cheaper, faster, and safer non-viral alternative to engineering CAR-NK, CAR-T cells and CAR-iPSCs.
- TcB-M engineered T and PB-NK cells were successfully used in vivo to control Burkitt's lymphoma tumor burden.
- TcB-M can be utilized for other therapy applications such as antibody production bioprocessing.

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