Accurate Integration of PiggyBac Transposon into Defined Genome Locus by using Engineered PBase fused to Cas9

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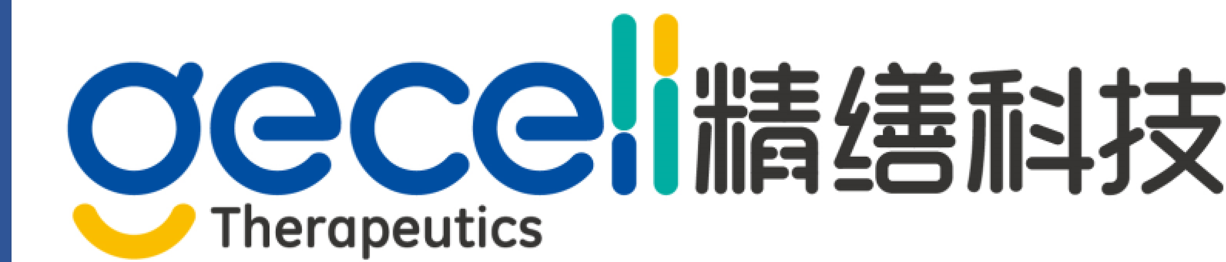


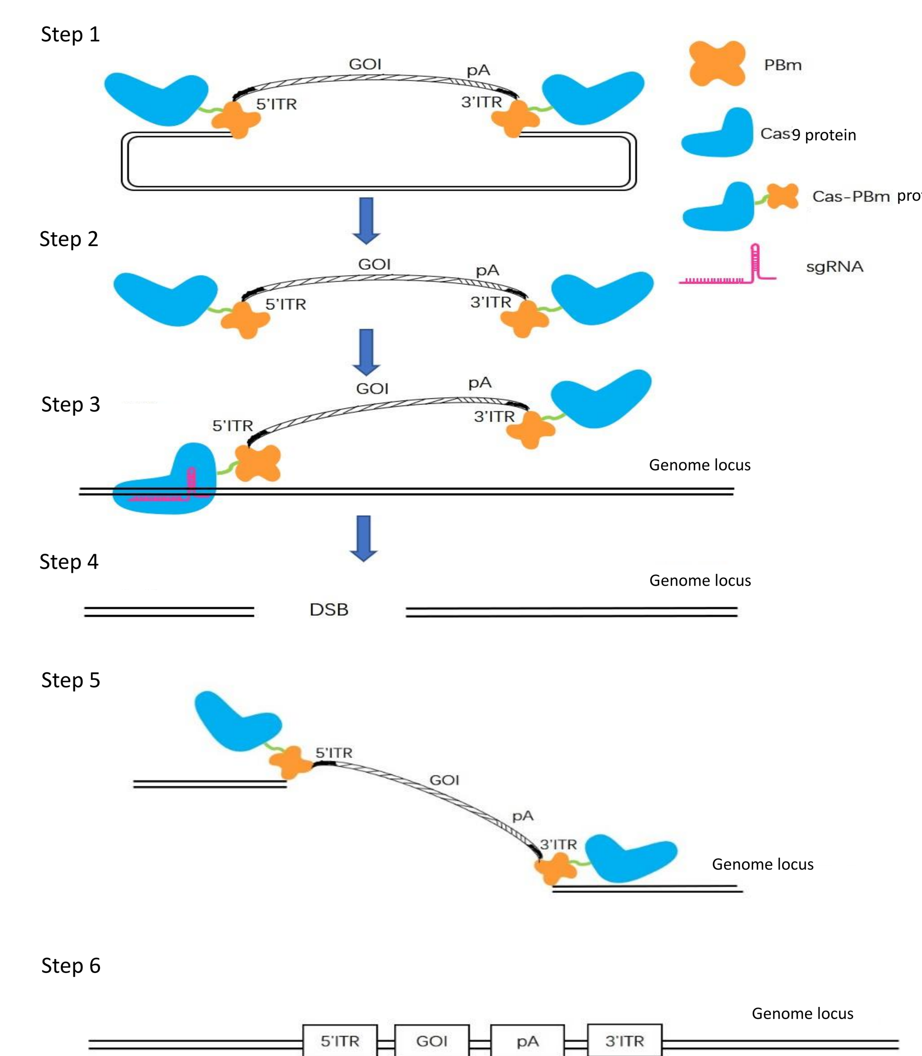
ABSTRACT

Gene editing has achieved much advance in the past decade, especially the development of CRISPR-Cas system and its derived tools such as base editors and prime editors. These game-changing tools allow precise gene editing in genome and transform the cell & gene therapy field. However, precise integration of a large DNA fragment into a specific site of mammalian genome remains challenging.

The piggyBac (PB) transposon is widely used to efficiently integrate large cargo into mammalian genome in the "TTAA" dependent manner. PB is almost randomly integrated as it displays little selectivity for particular genome regions. Excision competent yet integration defective (Exc+Int-) PB mutants have little "TTAA" dependent integration. Fusion of Exc+Int- PB mutants to Cas9 takes the advantages of the precision cleavage of Cas9 and the high integration efficiency of PB.

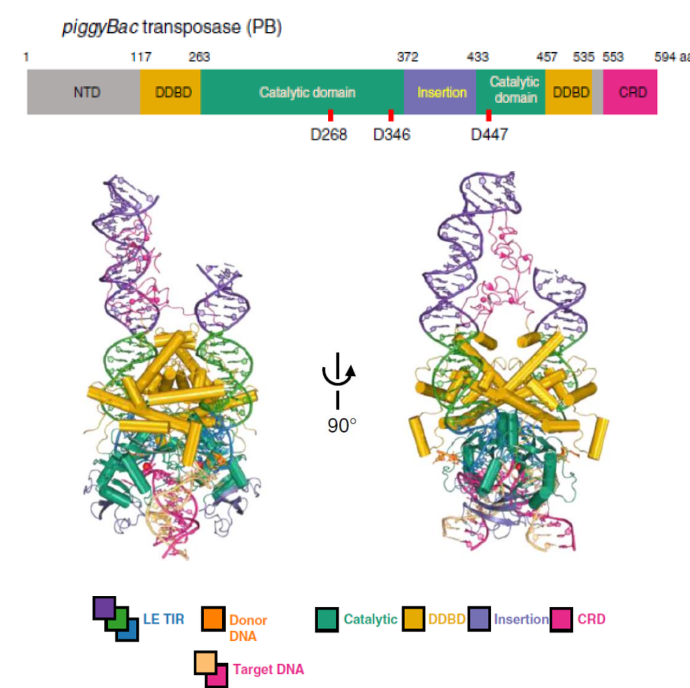
We have developed several Exc+Int- PB mutants with high excision but little integration activity in comparison with the WT PB. Fusion of these mutants to Cas9 results in a brand new tool for ACcurate Transposon into Genome (ACTinG) with double strand breaks generated by Cas9-sgRNA cleavage. This tool can efficiently integrate transposon carrying a large cargo (>10kb) into a precise genome locus. The integration efficiency is about 4-8 folds higher than that of previous reported Cas9-PBase (R372A_K375A_D450N) and HDR methods. The on-target integration efficiency can be even higher as much as 60% - 80% with a modified donor sequence. These results demonstrate that ACTinG is a robust and efficient tool for precise integration of large DNA cargos into mammalian genome. Further characterization and optimization of this gene editing tool will potentially increase its therapeutic value in different areas.

Schematic illustration of ACTinG technology



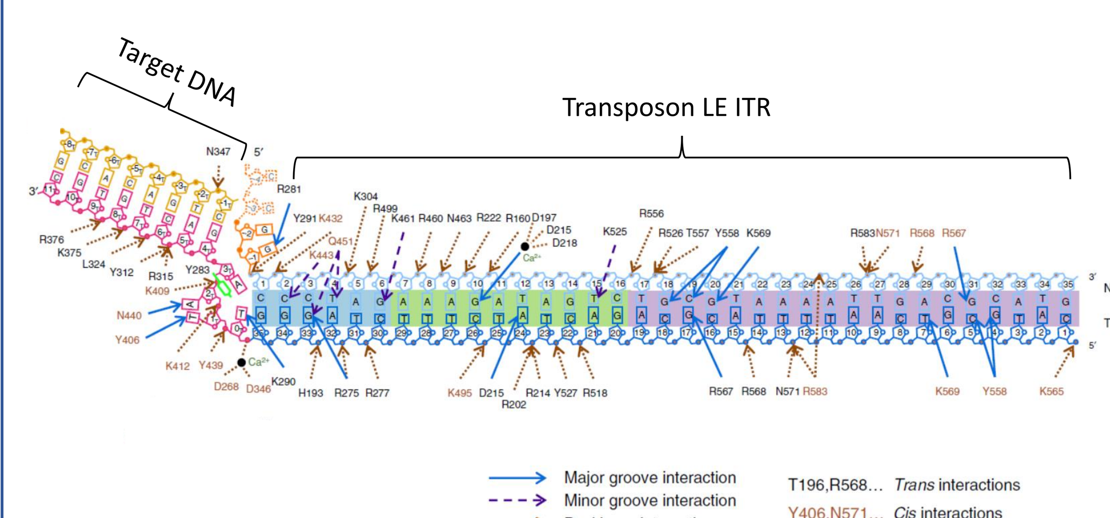
RESULTS

Fig 1. Structure of the strand-transfer complex



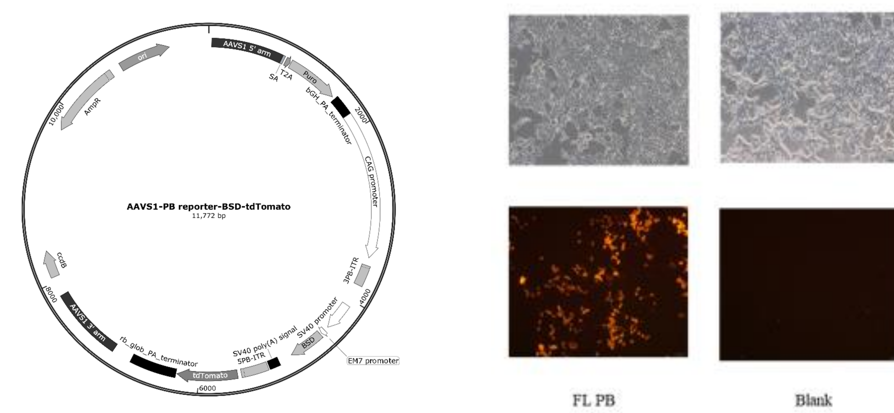
The strand-transfer complex comprises a PBase dimer, which bind to ITRs and the target DNA. (Cited from *Nat Commun*. 2020;11(1):3446.)

Fig 2. PB residues binding to target DNA and LE ITR



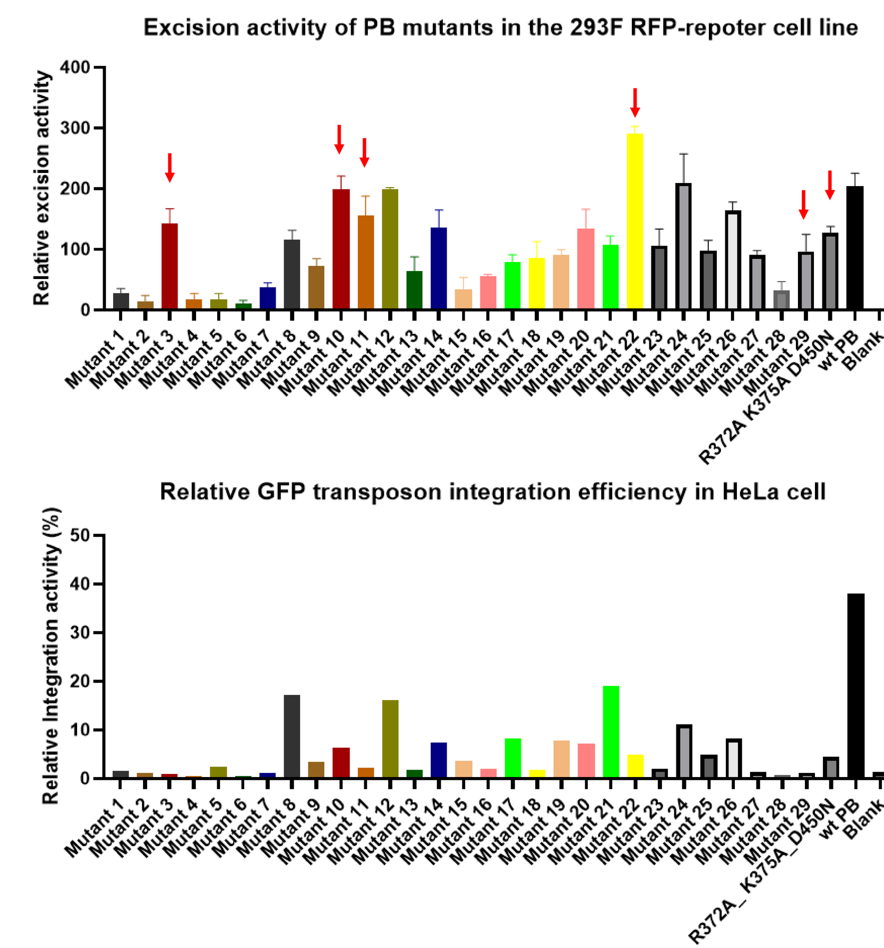
PB residues binding to target DNA (Y312, R315, L324, N347, K375, R376) and transposon ITR were schematically labeled. (Adapted from *Nat Commun*. 2020;11(1):3446.)

Fig 3. RFP reporter system to test excision activity



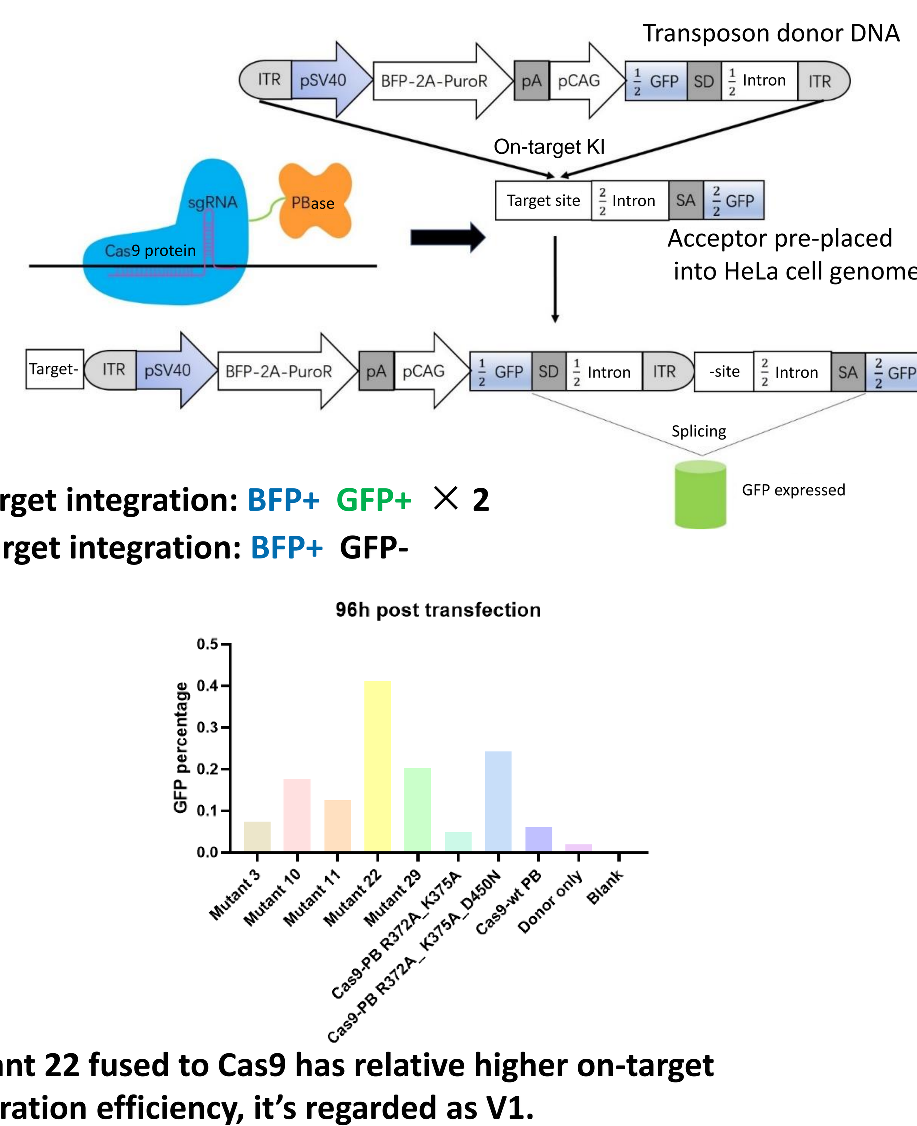
RFP reporter was pre-placed to the AAVS1 site of 293F cells. RFP expression is interrupted by the piggyBac transposon and would be restored when the transposon is excised out. Excision activities of PB mutants are calculated as RFP⁺ cell numbers/Total cell numbers.

Fig 4. Excision and integration activities of PB mutants



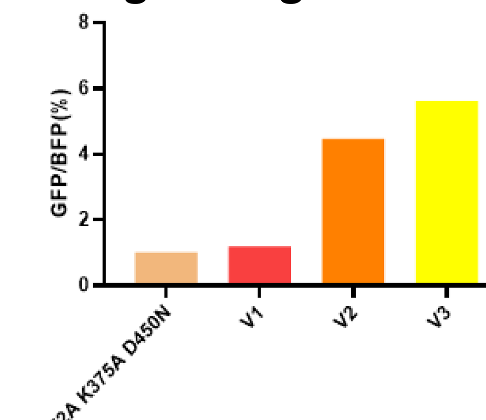
Excision (upper) and integration activities (lower) of respective PB mutants. Excision activity was measured with the RFP reporter system. Integration activity was measured with GFP-expressing transposon in HeLa cells. Integration activity=Day 14 GFP FACS /Day 3 GFP FACS. Exc+Int- mutants are indicated by red arrows.

Fig 5. Cas9-PB mutants show on-target integration activity assayed by the split GFP system



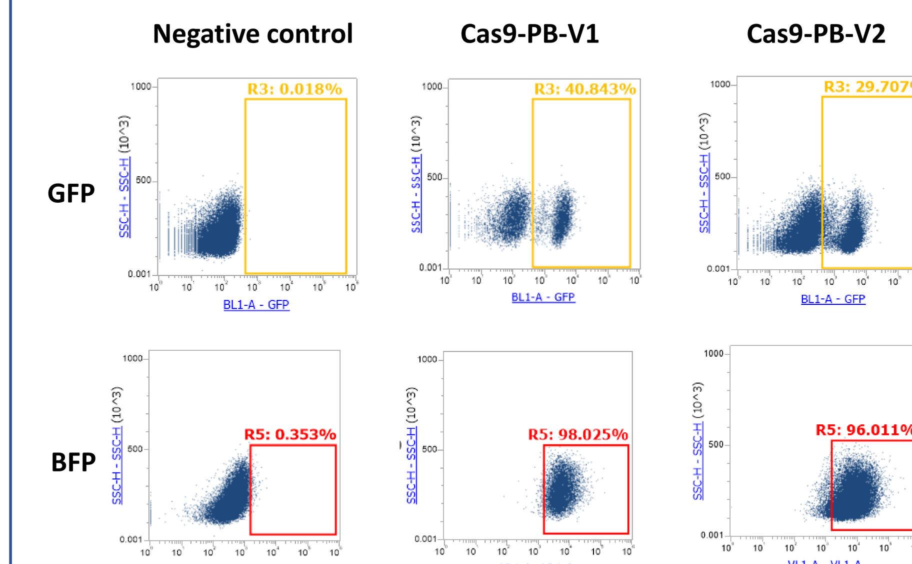
Mutant 22 fused to Cas9 has relative higher on-target integration efficiency, it's regarded as V1.

Fig 6. New Cas9-PB mutant versions with improved on-target integration efficiency



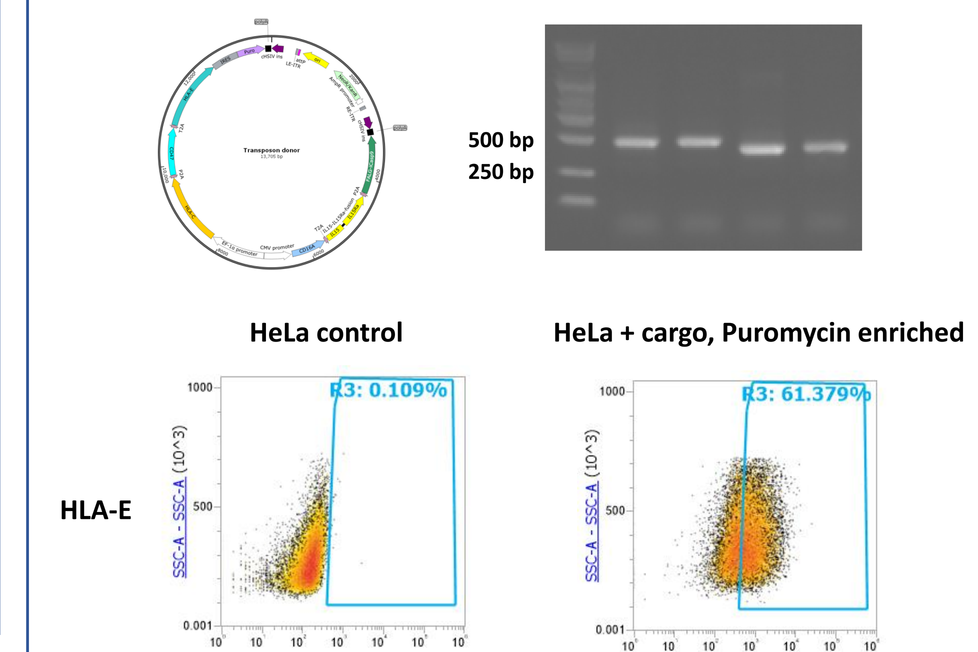
V3 and V2 mutants improved the on-target integration efficiency assayed by the Split-GFP system.

Fig 7. On-target ratio reaches up to 60%-80%



Split-GFP transposon donor (6 kb) and Cas9-PB plasmids were co-transfected into HeLa acceptor cells. Puromycin selection enriched total integrated cells. Half of on-target integrated cells express GFP (only forward integration results in GFP expression).

Fig 8. Integration of a large cargo (11.8kb)



11.8kb transgene cargo was successful integrated to CIITA locus in HeLa cells, which is validated by the junction PCR between transposon ITR and CIITA locus genomic DNA. Transgene expression is confirmed by HLA-E antibody staining.

Fig 9. Random small indels in junction between transposon ITR and genomic DNA

The junction between RE-ITR and genomic DNA is sequenced. Representative reads from different single cell clones are aligned to reference sequence. Random small indels exist in the junction.

CONCLUSIONS

1. Key PB residues binding to target DNA are selected and mutated to disrupt "TTAA" dependent integration.
2. We generated several Exc+Int- PB mutants which reserve high donor excision activity but show little "TTAA" integration activity.
3. ACTinG combines Cas9 and Exc+Int- PB mutants for the purpose of on-target integration into mammalian cell genome .
4. Cas9-PB-V3 shows higher on-target knock-in efficiency into mammalian genome.
5. On-target integration efficiency of ACTinG tech is about 60-80%.
6. ACTinG successfully inserted 11.8kb large cargo into genome as we already tested.
7. Random small indels exist in the junctions between ITR and genomic DNA.