P0506

Comprehensive Analysis of TALEB Off-target Editing

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Abstract

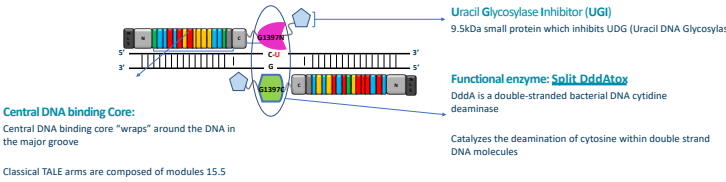
C-to-T TALE base editors (TALEB), composed of TALE DNA-binding domains, split-DddA deaminase halves, and an uracil glycosylase inhibitor (UGI), represent a promising new platform for precision genome editing in cell therapy. These editors induce cytidine deamination in double-stranded DNA, generating uracil intermediates that drive targeted base conversion.

We present a comprehensive evaluation of TALEB off-target activity in the nuclear genome, combining in silico predictions with empirical assays. Our strategy includes: (1) orthogonal off-target mapping using TALEN® as a proxy for TALE binding specificity, (2) genome-wide detection of uracil intermediates via pulldown-based enrichment, and (3) analysis of off-targets overlapping with CTCF transcription factor binding sites.

Our multi-layered approach reveals that off-target editing is influenced by both the design of the base editor and the cellular context. Notably, we find no evidence of bias toward CTCF flanked sequences in TALEB off-target activity. These findings underscore the importance of integrated methodologies for assessing genome-wide specificity and support the continued development of TALEB for therapeutic applications.

#1 Background

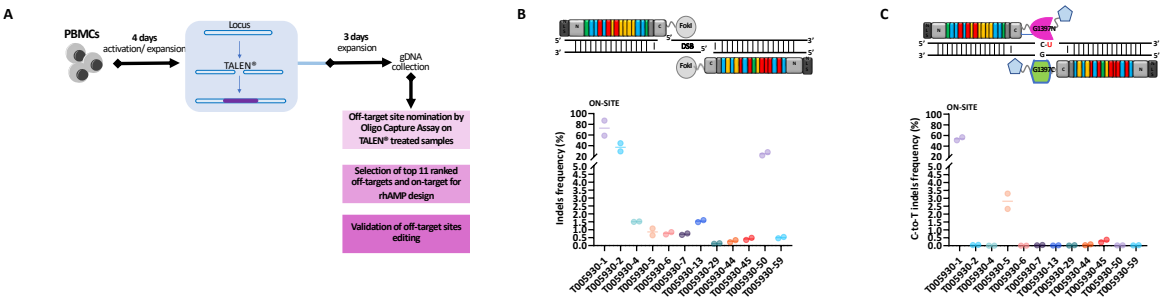
Base editing is an emerging genome engineering approach that enables the precise conversion of specific DNA bases without introducing double-strand breaks. This technology facilitates targeted point mutations—most commonly C>T or A>G transitions—through the deamination of cytosine or adenine, followed by cellular DNA repair mechanisms. A notable implementation involves split-DddA deaminase halves fused to uracil glycosylase inhibitor (UGI), directed to specific genomic loci by TALE DNA-binding domains composed of repeat variable diresidues (RVDs). Due to the irreversible nature of DNA modifications, off-target activity remains a critical concern, particularly in therapeutic contexts. To address this, high-fidelity base editors have been developed, with specificity typically assessed via deep sequencing of computationally predicted off-target sites or by evaluating deamination activity on known off-target sequences. Comprehensive off-target profiling remains a key requirement for the safe and effective clinical application of base editing technologies.



#2 Assessing specificity via orthogonal proxy strategy relying on the use of TALEN®

A. Scheme of the strategy to evaluate the contribution of non-specific TALE binding to TALEB off-target editing. Both a TALEN® and a TALEB were synthetized to target the same region on CD52 locus, ensuring identical RVD composition. Potential off-sites of the TALEN® were identified via an oligo capture assay. Multiplexed amplicon PCR, was performed on the top 11 sites off-sites following Bioinfo ranking plus the on-site, accordingly to base editing criteria. **B.** Next-generation sequencing (NGS) analysis of TALEN®-treated samples shows indels frequencies on the on-site and several off-target sites (T005930-2= 37%± 10.7; T005930-4= 1.5± 0.08; T005930-5= 0.9 ± 0.3; T005930-6= 0.8 ± 0.1; T005930-7=0.7 ±0.1; T005930-13= 1.5 ±0.1; T005930-29= 0.1 ±0; T005930-44=0.3 ±0.1; T005930-45= 0.4 ±0.1; T005930-50= 24.8 ± 4.4; T005930-59= 0.5 ±0.1; each color represent on-target and off-targets). **C.** Next-generation sequencing (NGS) analysis of TALEB-treated samples shows editing at the on-site and only at one site, T005930-5 met these criteria, with a C-to-T editing frequency of 2.8% ± 0.48.

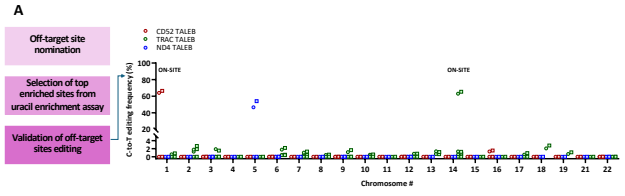
N=2 independent T-cells donors; 4 technical replicates.



#3 Genome-wide unbiased detection of TALEB off-targeting is not biased toward CTCF sites

To broaden our assessment, we implemented a genome-wide, unbiased enrichment of the uracil—transient intermediate generated by TALEB through cytosine deamination events in live cells. To ensure the capture of all the possible off-targets events and so maximize the sensitivity of the strategy, we evaluated the editing kinetics across the three TALEB constructs (two targeting the nuclear loci CD52 and TRAC and one targeting the mitochondrial ND4 gene) and determined the best time point for cell collection as 17h post transfection. Genomic DNA was purified from T cells transfected with TALEB and processed for uracil enrichment and sequencing library preparation, which revealed 19 possible off-targets sites for CD52 TALEB, 148 sites for TRAC TALEB and 59 for ND4 TALEB. Hybrid capture assay probes were designed against all of the sites per each editor. **A.** NGS results obtained by high-throughput hybrid capture enrichment method validated 4 off-targets for CD52 TALEB, 26 off-targets for TRAC TALEB and 1 detected off-target for ND4 TALEB. None of the validated off-targets are flanked by CTCF sites.

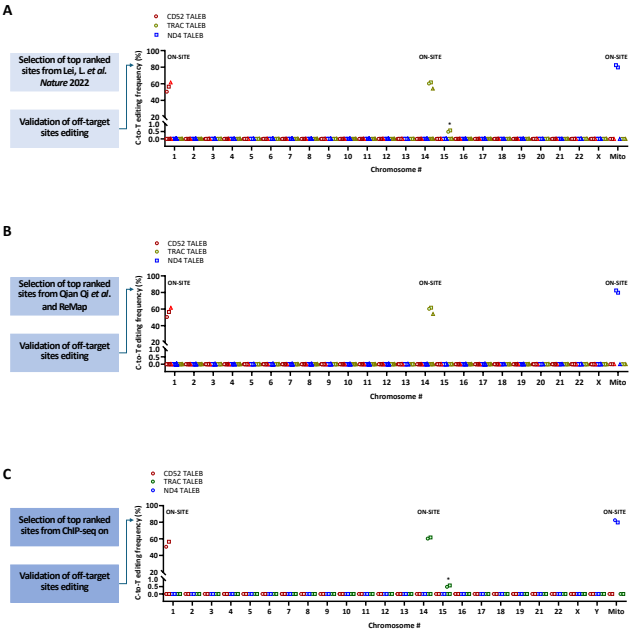
N=2 independent T-cells donors; 5 technical replicates.



#4 Minimal to no detectable editing at potential CTCF TALE-independent off-sites

NGS validation following hybrid capture assay of potential off-targets sites obtained from: **A.** 248 independent TALE binding off-targets sites previously reported in HEK293T transfected with plasmid for mitochondrial base editors. These sites are reported to be universally induced and frequently co-localize with CCCTC-binding factor (CTCF) (Lei, Zhixin et al. "Mitochondrial base editor induces substantial nuclear off-target mutations." *Nature* 2022)*. **B.** 104 T-cell-specific CTCF binding sites compiled from genome-wide occupancy maps in activated T cells, using data from Qian Qi et al. (Qi, Qian et al. "Dynamic CTCF binding directly mediates interactions among cis-regulatory elements essential for hematopoiesis." *Blood* 2021) and the ReMap database. **C.** 229 CTCF binding events in T cells obtained by chromatin immunoprecipitation followed by sequencing (ChIP-seq) before electroporation and at 20 and 40 hours post-electroporation*.

For all panels: N=2-3 independent T-cells donors; 5 technical replicates. *=under further analysis.



#6 Conclusions

We determined that:

- while TALE DNA-binding domains can tolerate mismatches and potentially bind to multiple genomic loci, TALEB-mediated cytosine deamination is highly specific, with minimal off-target editing observed under therapeutically relevant conditions
- TALEB exhibit high specificity in primary T cells, with minimal off-target editing almost exclusively at TALE dependent sites
- transient expression of TALEB via mRNA likely limits its window of activity, reducing the risk of non-specific editing

Together these data reinforce the potential of TALEB as a precise and safe genome editing platform for therapeutic applications, particularly in contexts where off-target effects must be rigorously minimized.