A Compact Zinc Finger Architecture for Highly Efficient Base Editing in Human Cells

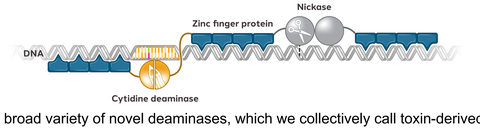
Abstract #560
Board No. TU-65

Friedrich Fauser, PhD, Bhakti N. Kadam, MS, Sebastian Arangundy-Franklin, PhD, Jessica E. Davis, PhD, Nicola J. Schmidt, Vishvesha Vaidya, MS, Colman Ng, Yuanyue Zhou, MS, Nicholas A. Scarlott, MS, Garrett Lew, Jason Eshleman, PhD, Yuri R. Bendaña, MS, David A. Shivak, MS, Andreas Reik, PhD, Patrick Li, PhD, Gregory D. Davis, PhD, Jeffrey C. Miller, PhD

Sangamo Therapeutics, 7000 Marina Blvd, Brisbane, CA

Introduction

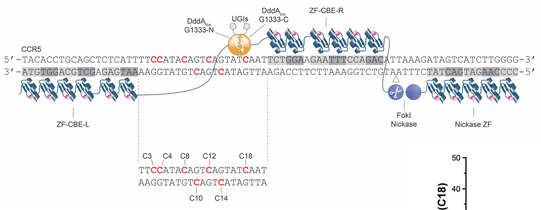
- Nucleobase editors represent an emerging technology that enables precise single-base edits to the genomes of eukaryotic cells. Most conventional cytosine base editors (CBEs) are too large to be packaged into a single adeno-associated virus (AAV) vector. The performance of AAV-compatible CBEs at chromosomal target sites also limits their use for therapeutic applications.
- Most CBEs use deaminase domains that act on single-stranded DNA and require RNA-guided proteins such as Cas9 to unwind the DNA prior to editing[1,2]. However, the most recent class of CBEs uses a deaminase domain derived from an interbacterial toxin called double-stranded DNA cytidine deaminase toxin A ($DddA_{tox}$) that can act on double-stranded DNA and thus allows the use of more compact DNA targeting domains, such as transcription activator-like effectors (TALE)[3] or zinc fingers (ZF)[4], that do not need to unwind DNA prior to editing.
- Here we use $DddA_{tox}$ fragments and FokI DNA cleavage domains fused to arrays of engineered ZFs to create a highly efficient CRISPR-free base editing architecture that can nick the non-deaminated DNA strand and achieves similar editing efficiencies as BE3[1], a CRISPR-nickase-derived CBE.



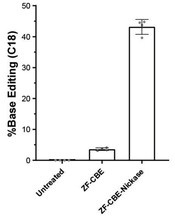
- We also identify a broad variety of novel deaminases, which we collectively call toxin-derived deaminases (TDDs), that allow us to fine-tune properties such as targeting density and specificity. TDD-derived ZF base editors enable up to 69% base editing in T cells with good cell viability.
- This new construct architecture also promises to be compact enough to be packaged in a single AAV vector.

Results

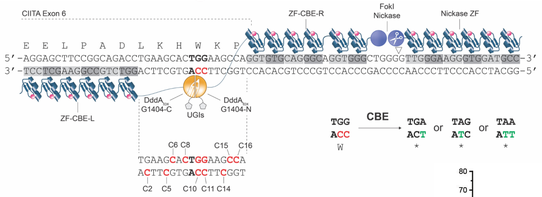
Development of ZF-CBE-nickases



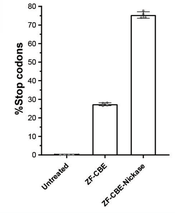
- We developed ZF-CBEs for a site within the human CCR5 gene that was previously targeted with DddA-G1333-derived TALE-CBEs[3].
- To create a ZF-CBE-nickase, we fused a copy of the FokI cleavage domain to the right ZF-CBE arm and fused a second copy of the FokI cleavage domain containing the D450N mutation[5] to a third ZF array.
- Our ZF-CBE-nickase construct architecture substantially increased base editing efficiencies in human K562 cells.



ZF-CBE-nickases can be targeted to the CIITA locus

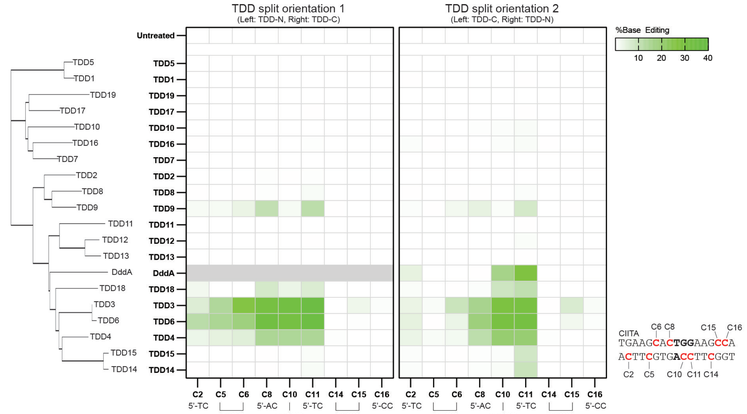


- We used a new $DddA_{tox}$-G1404 split variant to design a ZF-CBE-nickase to knock out expression of the human class II transactivator (CIITA), a step used in the generation of allogeneic chimeric antigen receptor T cells[6].
- DddA's strong 5'-TC sequence-context constrained target site selection.
- We achieved up to ~75% base editing that yielded the desired stop codons in human K562 cells.



Results

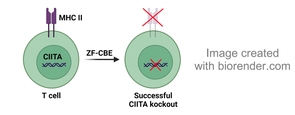
Discovery of new deaminase domains with diverse editing profiles

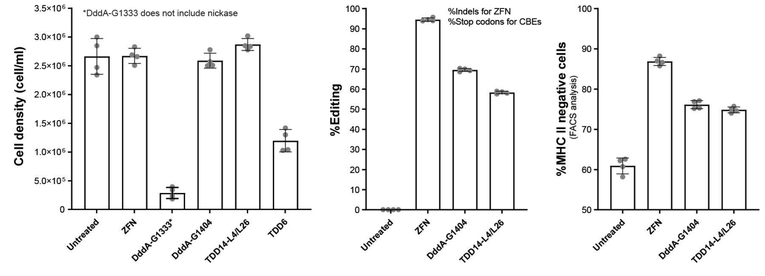


- We identified and explored novel TDDs with the goal of finding $DddA_{tox}$ alternatives with no strict 5'-TC substrate preference and less activity at unintended cytosines near the intended target.
  - TDD6 edits 5'-DC substrates at the tested CIITA site (D=A, G, or T).
  - TDD14 has a very narrow base editing window at the tested CIITA site.
- Additional construct variations, such as different ZF-TDD linkers, support fine-tuning of base editing properties (data not shown; shorter L4 linker used in T cell studies).

CIITA can be successfully knocked out in T cells

- Given the results in an immortalized cell line (human K562 cells), we decided to compare the performance of TDD6 and TDD14 with $DddA_{tox}$ for knocking out the CIITA gene in primary human T cells.



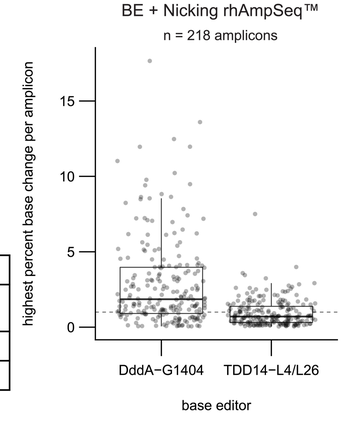


- We noted varying levels of cell viability across the tested deaminase domains where $DddA_{tox}$-G1333 and TDD6 showed the strongest negative impact on growth, while cell growth in T cells transfected with $DddA_{tox}$-G1404 and TDD14 was indistinguishable from that of untreated samples.
- For constructs with good cell viability, we observed editing activity levels similar to that in K562 cells using a PCR-based NGS assay.
- CIITA is a transcriptional coactivator that regulates γ-interferon-activated transcription of major histocompatibility complex (MHC) class I and II genes. Thus, we confirmed that the stop codons introduced into the CIITA transcript diminished the percentage of cells expressing an MHC class II cell surface protein.

Genome-wide specificity profiling

- To assess the specificity of these constructs, we performed a genome-wide unbiased assay to detect ZF-CBE off-target sites outside of a cellular context.
- We then assayed cellular base editing activity in human K562 cells at 218 candidate off-target sites using RNase H-dependent multiplexed PCR[7] and sequencing (rhAMPSeq™).
- TDD14 has a favorable specificity profile.

		Off-target sites	
Base Editor	**On-target editing (CIITA)**	**≥1% editing**	**≥10% editing**
DddA-G1404	69.2%	157	7
TDD14-L4/L26	58.1%	84	0



Conclusions

- We have developed a compact base editor that can be targeted with high precision and specificity using ZFs, is small enough for packaging into relevant viral vectors and achieves high levels of editing that are suitable for therapeutic applications.
- In this study, we first developed a highly efficient base editor architecture using $DddA_{tox}$ fragments and FokI DNA cleavage domains fused to arrays for engineered ZFs.
- Next, we identified and characterized novel cytidine deaminases capable of generating efficient CBEs without the need to unwind dsDNA. This allows these deaminase domains to be fused to any non-RNA guided DNA binding protein such as ZFs or TALEs for efficient editing of eukaryotic genomes.
- TDD14 enabled up to ~58% editing of the CIITA gene in human T cells with low levels of indels, good cell viability, and a favorable genome-wide specificity profile when delivered in conjunction with a ZF nickase.
- We envision that construct architecture improvement strategies that were previously described in the context of ZF nucleases[8,9] will guide future development of therapeutic ZF-CBEs.
- ZF-CBEs are well-suited to knocking out multiple genes at once due to the reduced probability of chromosomal translocation events between simultaneous DNA double-strand breaks.
- Notably, the compact construct architecture of ZF-CBE-nickases can make it possible to package all 3 components in a single AAV vector and thus shows promise for therapeutic application in vivo.
  - For example, the size of the TDD14-derived ZF-CBE-nickases can be reduced to ~3.5 kb.
  - Individual components could be separated by self-cleaving peptides and therefore could be controlled by the same regulatory elements.

References

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Author disclosures

All authors are full-time employees of Sangamo Therapeutics.

Contact: Friedrich Fauser ffauser@sangamo.com

Presented at the ASGCT 25th Annual Meeting, 2022