Directed evolution and engineering of CBE-T: next-generation cytosine base editors utilizing TadA

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Genome engineering: CRISPR frontiers
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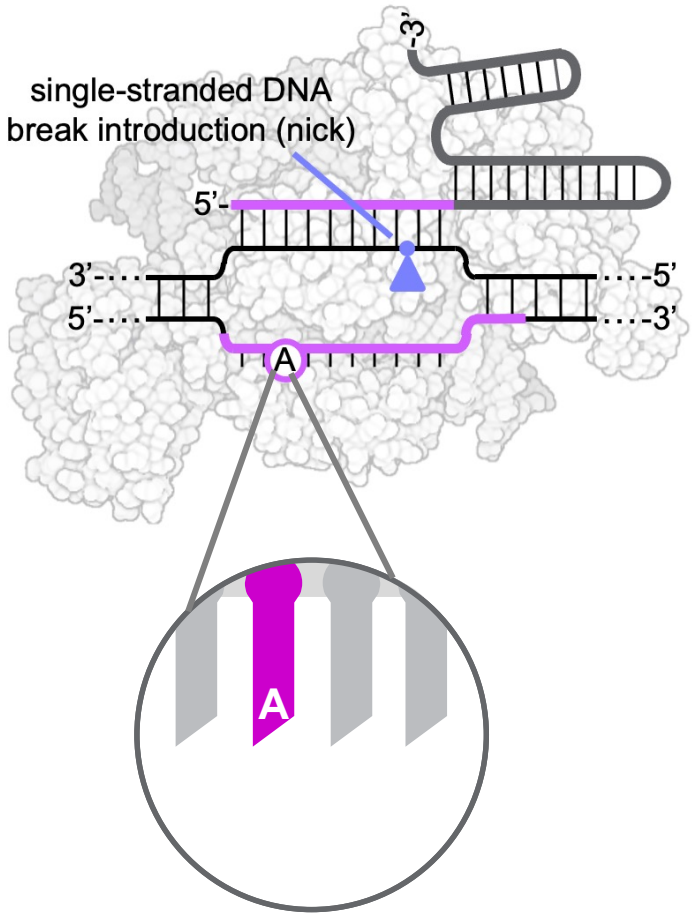
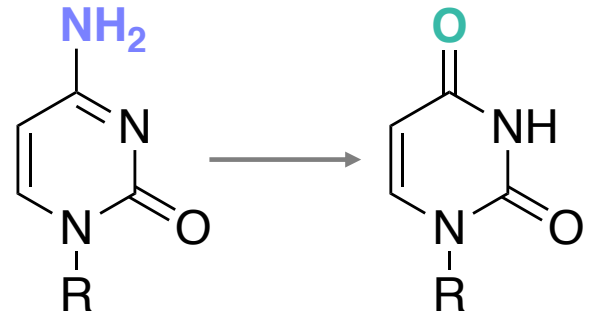
Base editors chemically modify target bases, permanently and predictably

Base editor binds the target DNA and exposes a narrow editing window



Deaminase chemically modifies target base, permanently and predictably

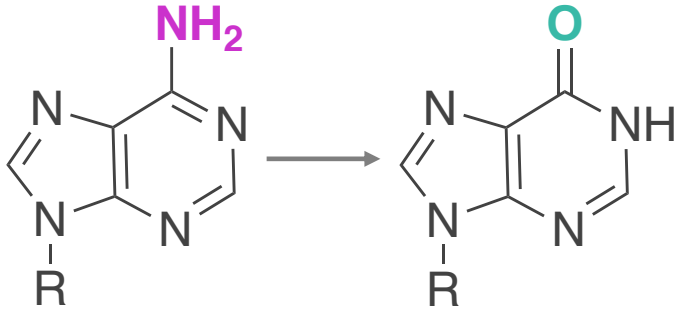
C-to-T base editor ("CBE")



cytosine uracil

Komor, A. C. et. al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420-424 (2016)

A-to-G base editor ("ABE")



adenosine inosine

Gaudelli, N. M. *et al.* Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature* **551**, 464-471 (2017)

Base editors are used for a variety of gene editing applications

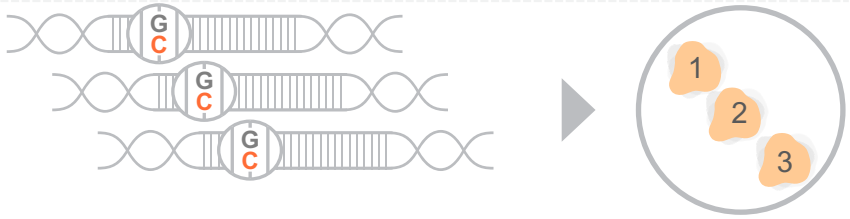
Gene Correction — Directly repair point mutations to restore gene function

Gene Modification — Insert protective clinical variants to prevent or modify risk of disease

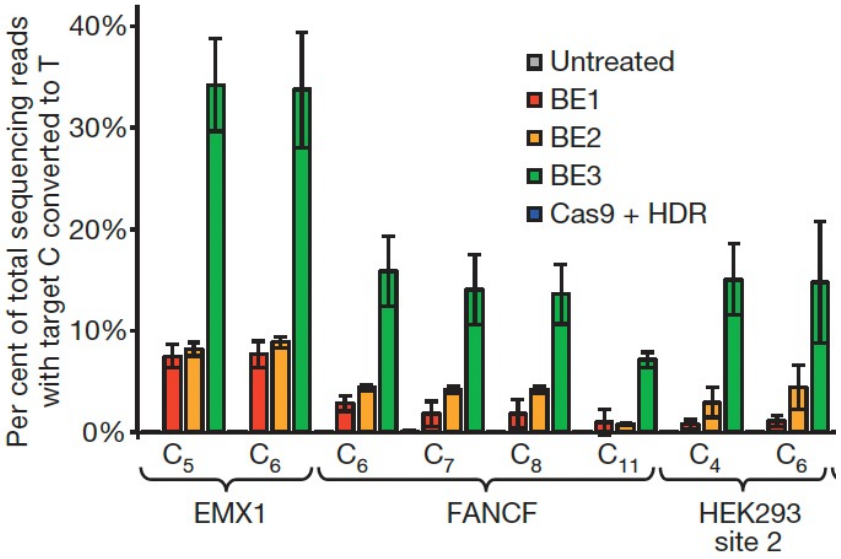
Gene Activation — Edit regulatory elements to reactivate gene expression

Gene Silencing — Edit stop codons or splice sites to silence expression

Multiplex Editing — Editing multiple sites simultaneously, with no detectable translocations



Base editors have been engineered in a variety of ways over the past five years



Komor, Liu *et al. Nature* **533**, 420 (May 19, 2016)

5 years

BEAM-101 IND cleared by FDA for evaluation as a potential treatment for sickle cell disease

Beam Tx (Nov. 8, 2021)

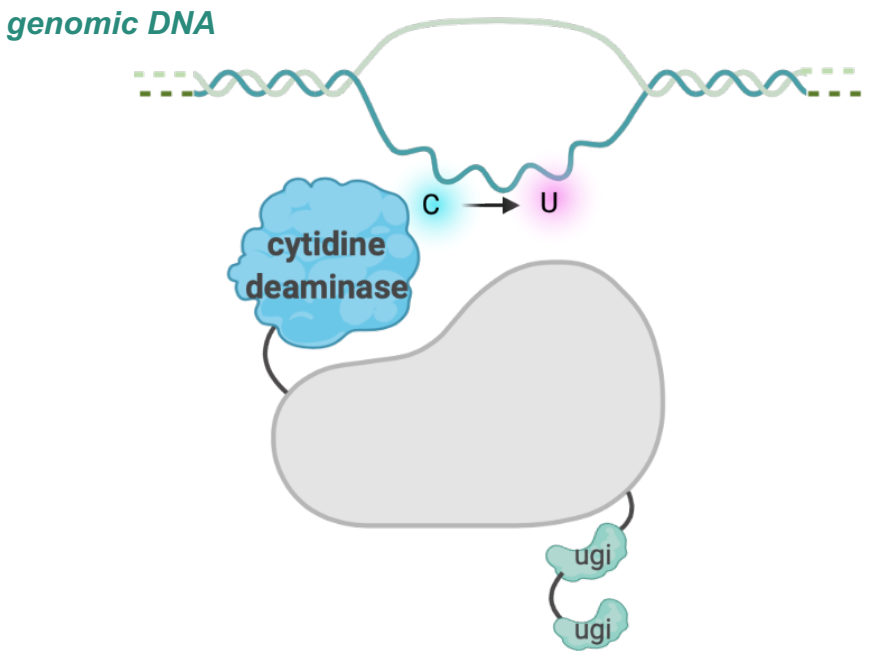
Key base editing milestones

- Development of ABE
  - directed evolution of deaminase
- PAM-variant base editors
- Directed evolution of Cas9 to create non-NGG PAM variants for BEs
- Codon, NLS, and linker optimization
- Circular permutants and inlaid base editors
- Evaluation of DNA off-targets
- Evaluation of RNA off-targets
- Bystander editing minimization
- Guide RNA engineering
- *Ex vivo* and *in vivo* BE delivery
- Engineering BEs with minimized off-target activity
- Ex vivo base editing of HSC, hepatocytes, and T cells
- Cryo-EM structure of ABE
- *In vivo* mouse base editing
- *In vivo* non-human primate editing
- X-ray structure of TadA variants
- Creation of CBEs utilizing TadA as a deaminase for C to U

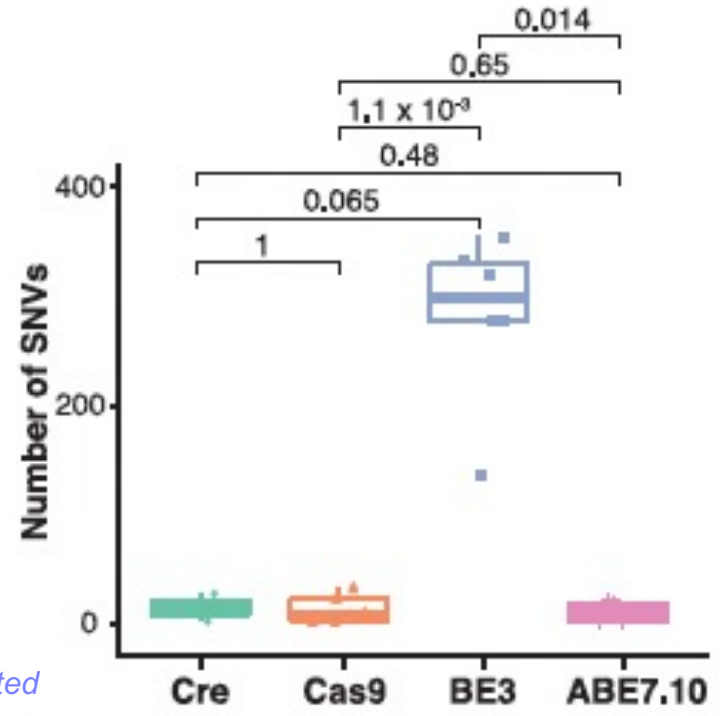
CBE-Ts

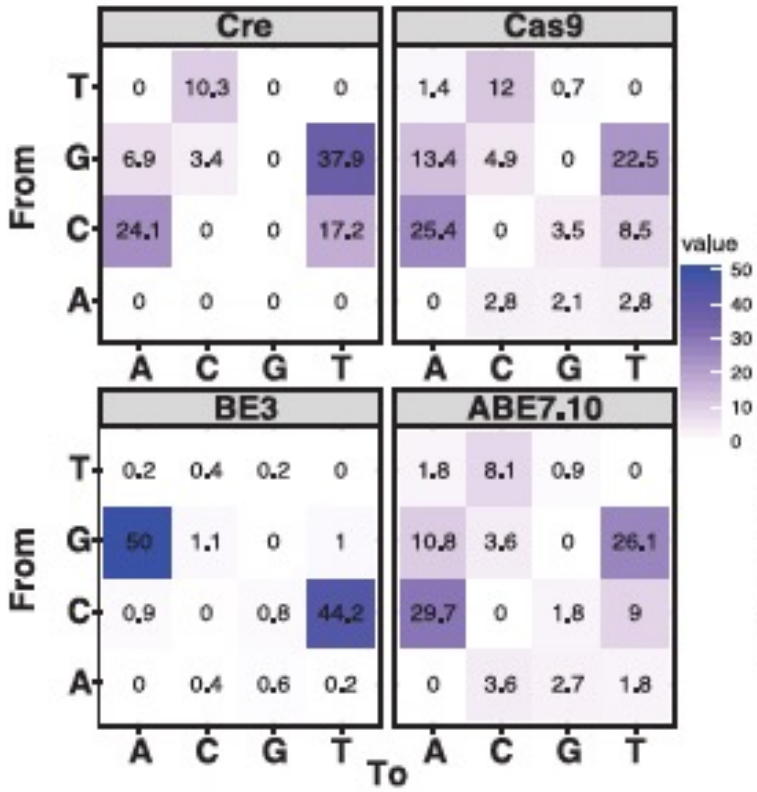
For a review of literature, see:
Anzalone, A.V., Koblan, L.W. & Liu, D.R. Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime editors. *Nat Biotechnol* **38,** 824–844 (2020).

rAPOBEC CBEs are potent C•G to T•A editors and may cause guide-independent off-targets



genomic DNA is vulnerable to CBE deamination when ssDNA is presented





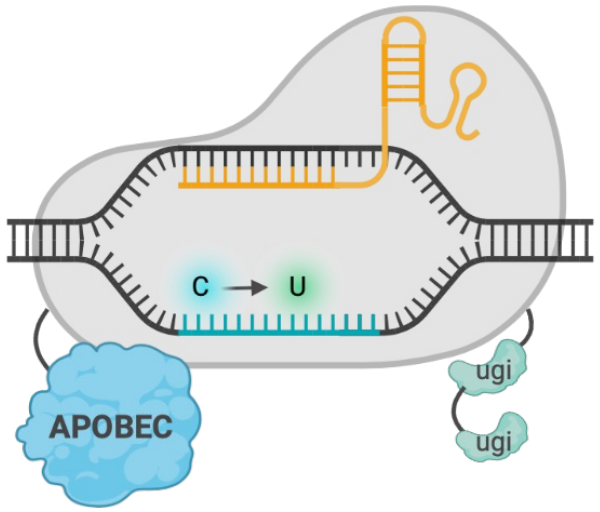
figures adapted from Zuo et al.

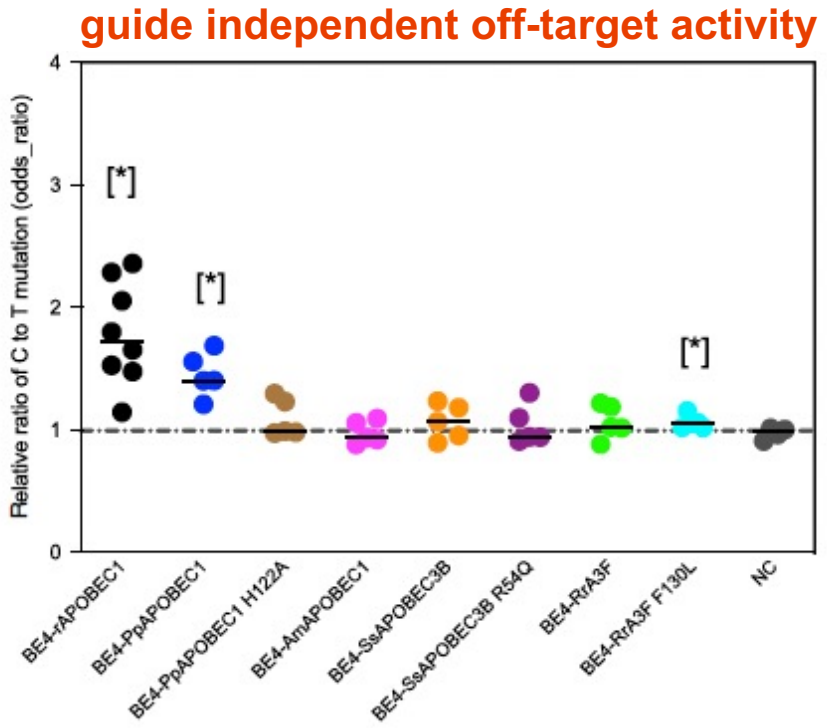
CBEs have been documented to yield guide-independent off-targets

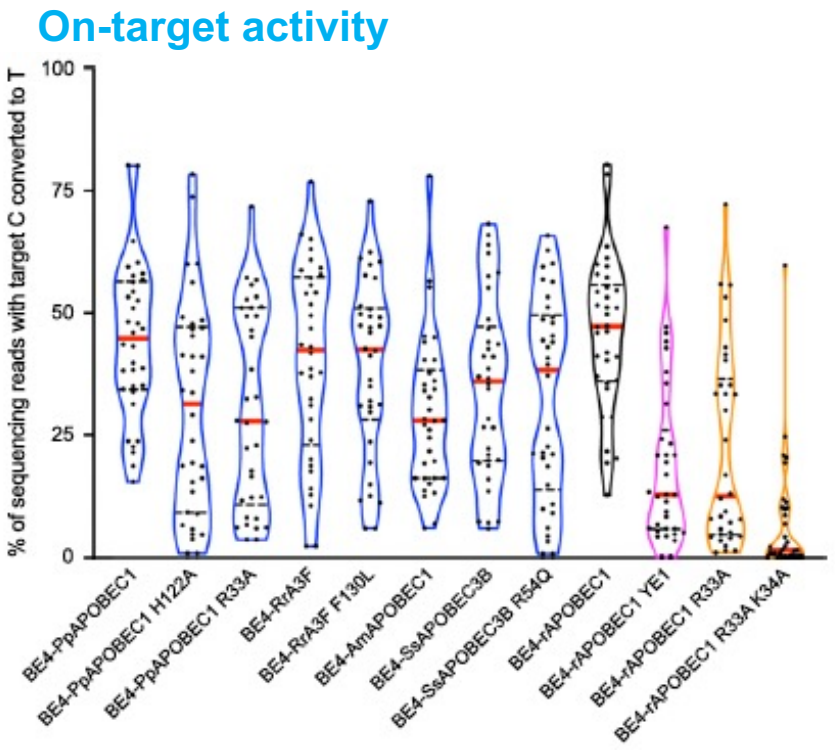
- Reported to have 5x10-8 to 5x10-7 random genome-wide mutations per bp
- APOBECs have intrinsic affinity for ssDNA

Ref: Zuo, E. et al. Cytosine base editor generates substantial off-target single nucleotide variants in mouse embryos. *Science* **364**, 289-292 (2019).
Jin, S. et al. Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. *Science* **364**, 292-295 (2019).
McGrath, E., Shin, H., Zhang, L. *et al.* Targeting specificity of APOBEC-based cytosine base editor in human iPSCs determined by whole genome sequencing. *Nat Commun* **10,** 5353 (2019).

Next-generation CBEs, with mitigated guide-independent OTs have been identified







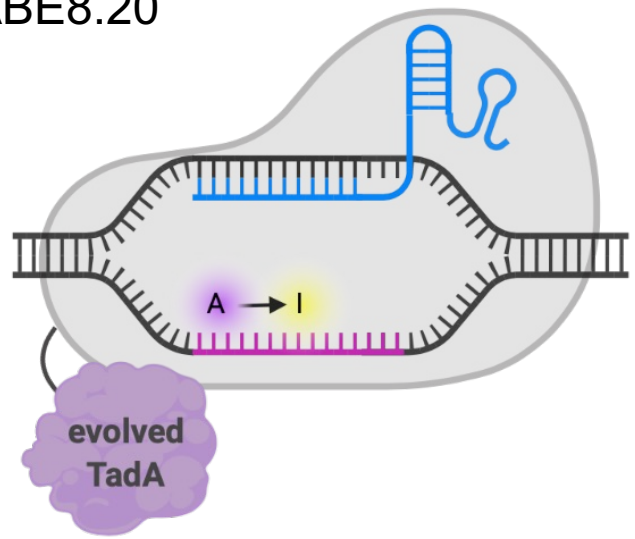
Next generation CBEs to date:

- Use APOBECs
  - Variants of rAPOBEC1
  - Naturally occurring homolog
- Second generation CBEs do achieve lower OT outcomes relative to BE4
- Can have variability in on target editing relative to BE4
  - Weakened enzyme due to mutagenesis
  - Retained sequence specificity

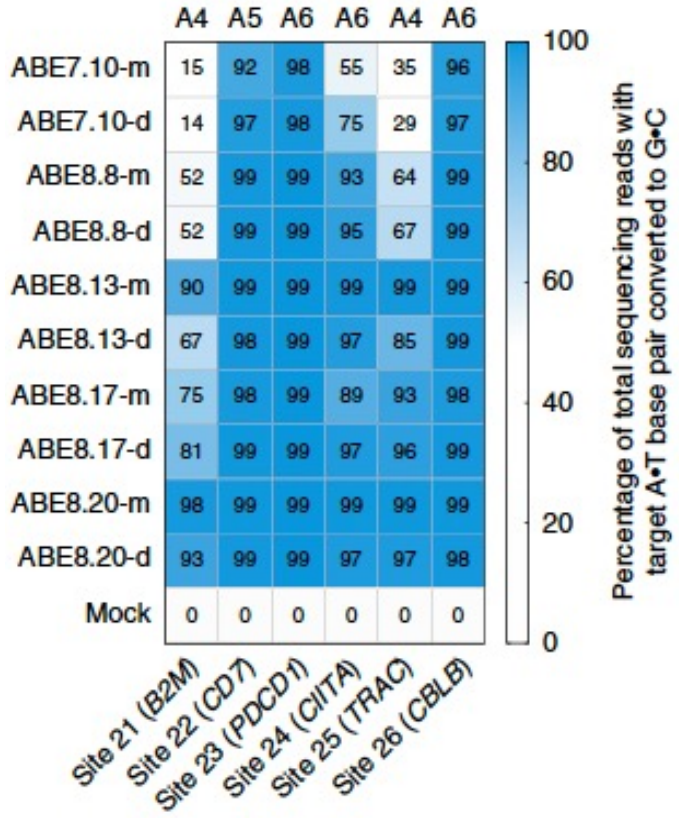
Yu, Y., Leete, T.C., Born, D.A. *et al. Nat Commun* **11,** 2052 (2020).
Doman, J.L., Raguram, A., Newby, G.A. *et al. Nat Biotechnol* **38,** 620–628 (2020).
Zuo, E., Sun, Y., Yuan, T. *et al. Nat Methods* **17,** 600–604 (2020).

ABEs have high on-target activity with low off-targets and have not led to substantially elevated guide-independent mutation rates across the genome

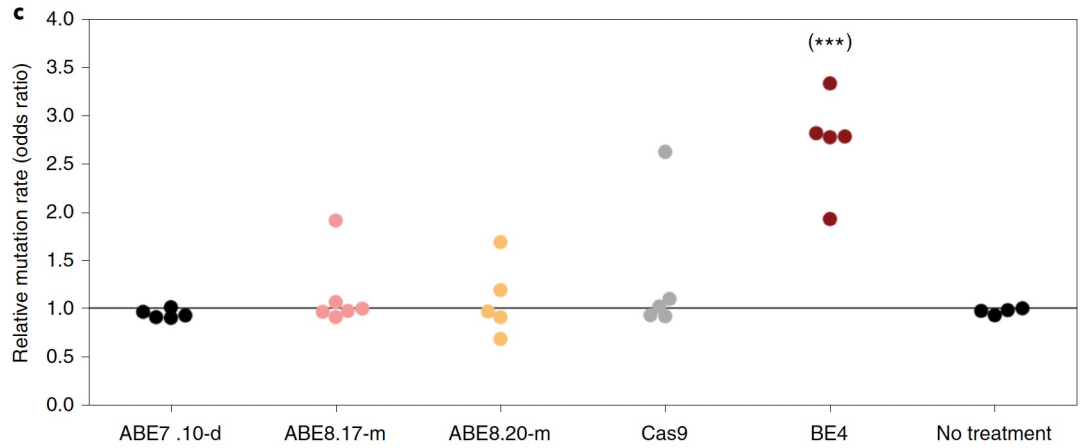
ABE8.20



On-target activity of ABEs



Off-target activity of ABE compared to BE4 (guide independent)



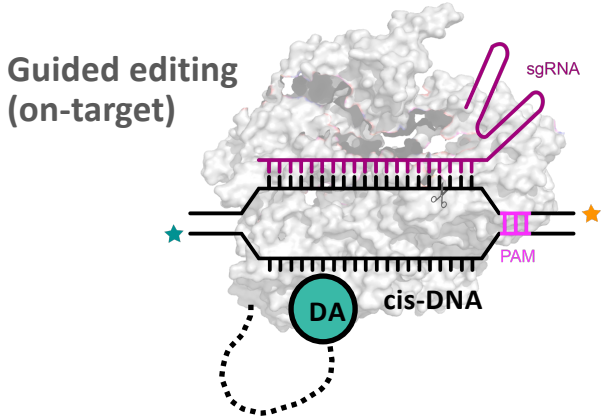
Whole genome sequencing (WGS) reveal cells treated with BE4 have ~3-fold increase in guide-independent off-targets relative to untreated controls

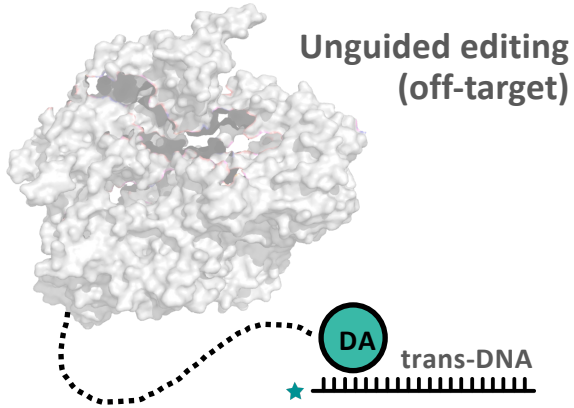
ABE properties to date:

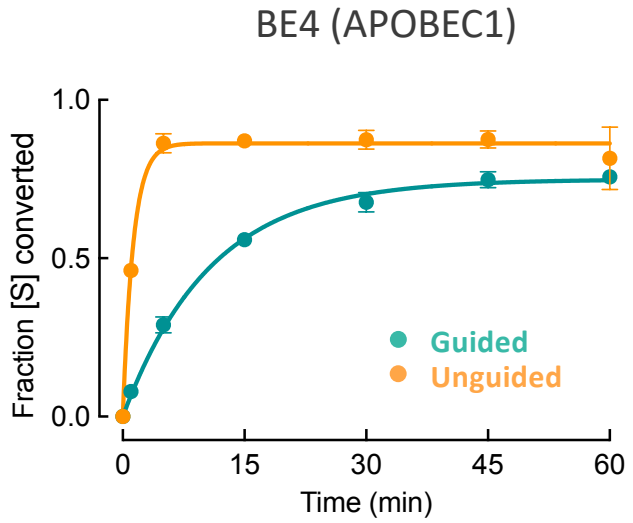
- Use laboratory-evolved TadA
  - Deaminate A to I (not C to U)
- Achieve high on-target base editing outcomes in primary cells
- Beam's ABE8 led to no observable enrichment in guide RNA independent off targets genome-wide
- Editing window ~4-8 (NGG PAM = 21-23)
  - Narrower window relative to CBEs

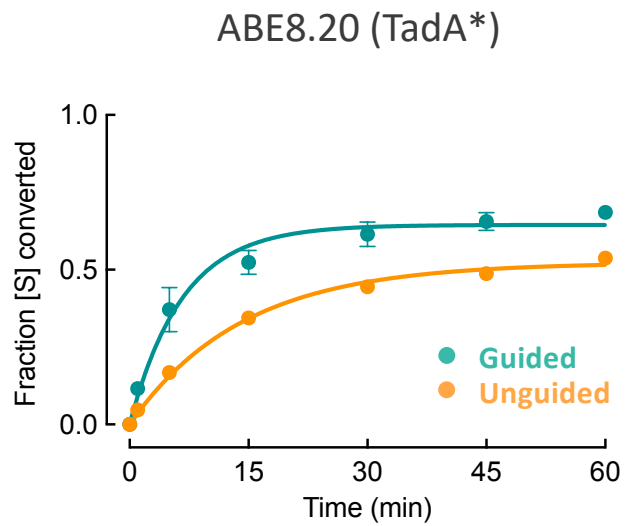
Gaudelli, N.M.. *et al.*. *Nat Biotechnol* **38,** 892–900 (2020)

BE4 led to higher rates of unguided deamination on ssDNA relative to ABE *in vitro*









BE4	K_{app} (mean ± SD, n = 3)
On-target	0.092 ± 0.007 min^{-1}
Off-target	0.78 ± 0.02 min^{-1}

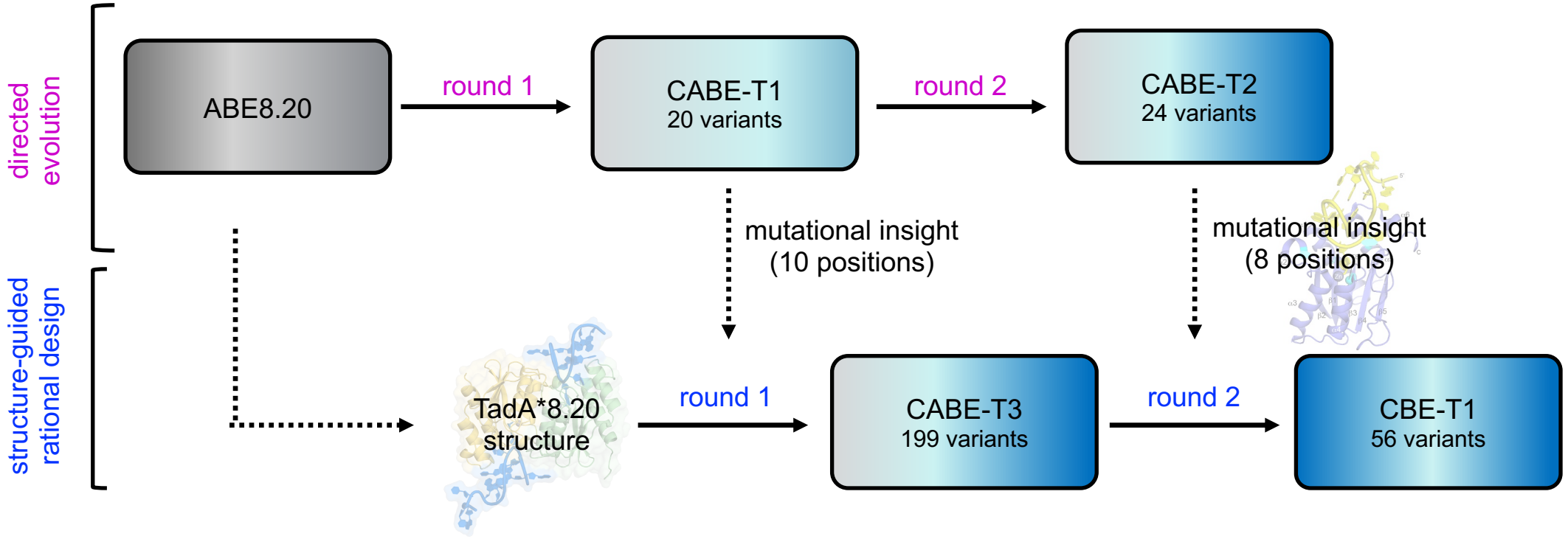
ABE8.20	K_{app} (mean ± SD, n = 3)
On-target	0.17 ± 0.06 min^{-1}
Off-target	0.071 ± 0.005 min^{-1}

APOBEC-derived BE4 caused >10-fold higher rate of unguided deamination compared to ABE8.20 *in vitro*

Lam, D.K., Feliciano, P. R. et. al Gaudelli*, manuscript under peer-review

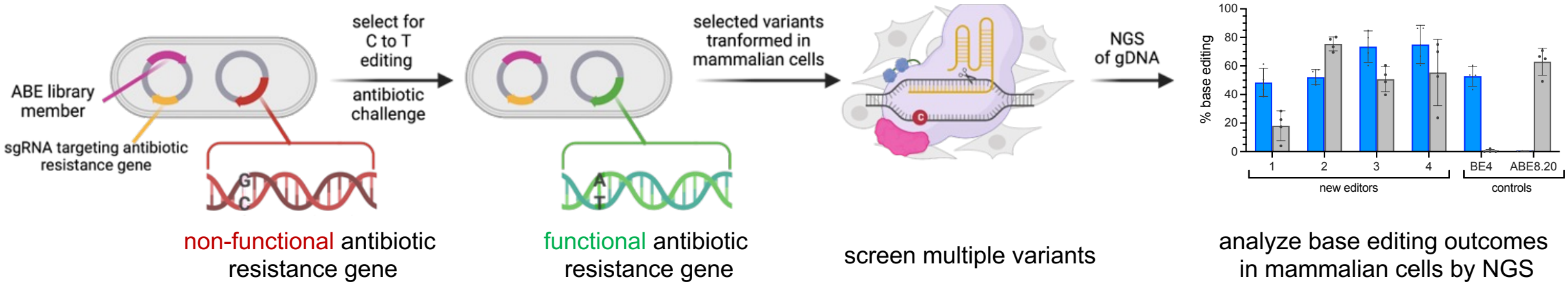
Overview of route to CBE-Ts, starting from ABE

A series of directed evolution and structure-guided combinatorial screens on TadA yielded dual editing (A-to-G & C-to-T) BEs (CABEs) and TadA-derived CBEs (CBE-T1)



Lam, D.K.; Feliciano, P.; … Gaudelli, N.M. (2022) *manuscript under peer review*

Directed evolution of ABE towards C to T activity



non-functional antibiotic resistance gene

functional antibiotic resistance gene

screen multiple variants

analyze base editing outcomes in mammalian cells by NGS

Workflow:

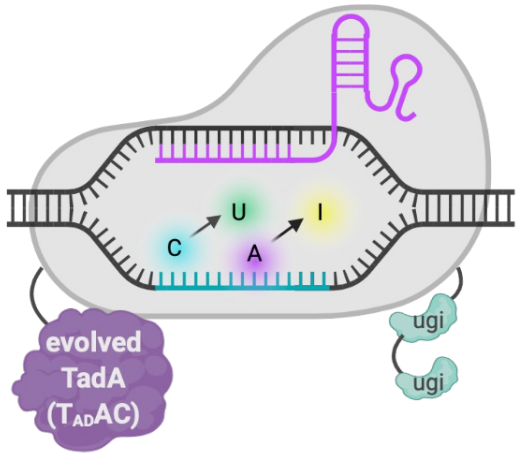
1. Created a library of ABE8.20 (2-3 mutations in TadA per library member).

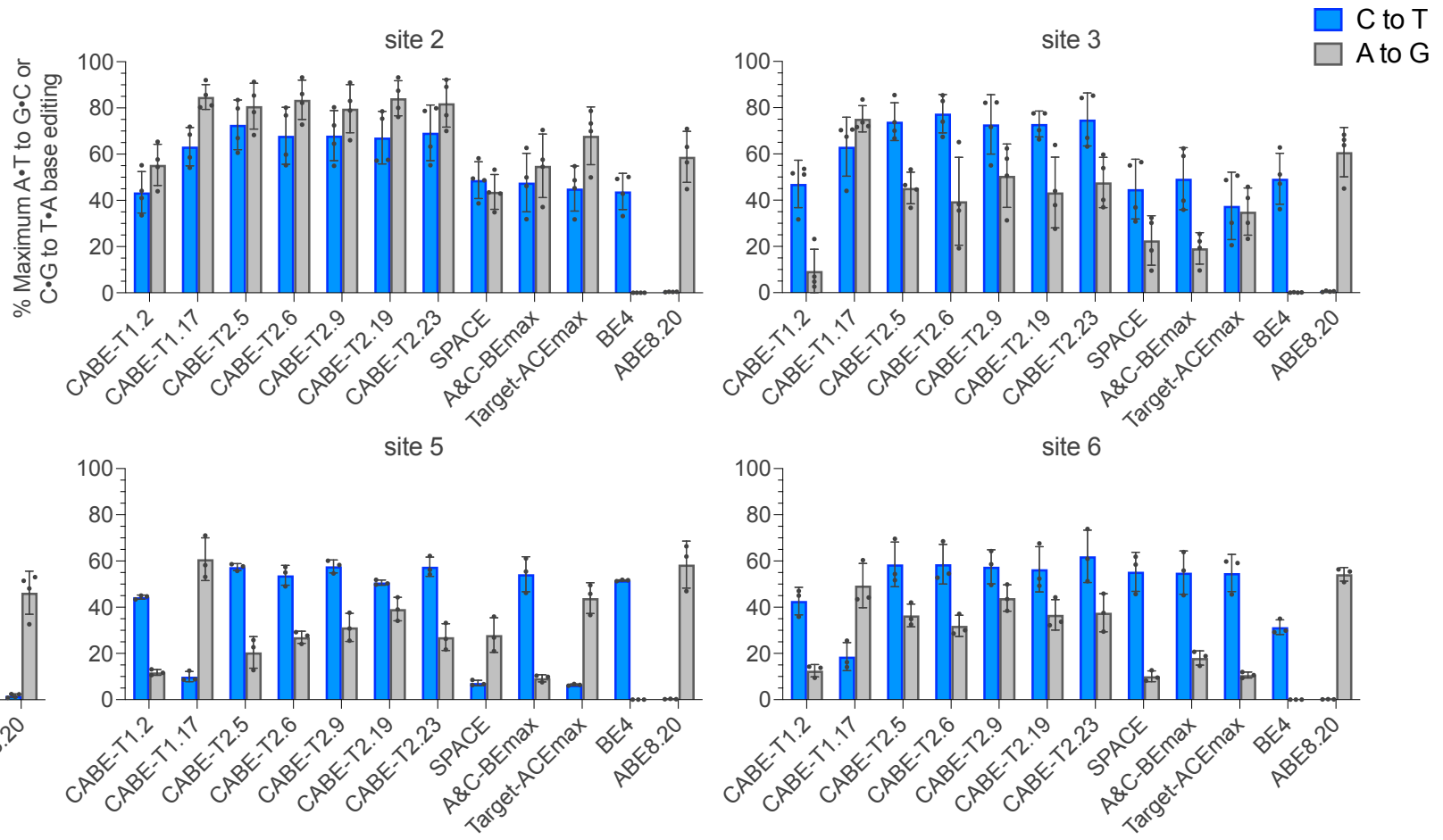
2. Challenged the ABE8.20 library by requiring C to T mutation for survival in a critical antibiotic resistance gene

3. Base editing efficiency on gDNA in mammalian cell evaluated by NGS and top hits are brought forward to the next round of engineering or characterization

Lam, D.K.; Feliciano, P.; … Gaudelli, N.M. (2022) *manuscript under peer review*

CABE-Ts developed from directed evolution of ABE8.20 achieved >50% C-to-T editing efficiency across 22 genomic sites and retained varying levels of A-to-G editing

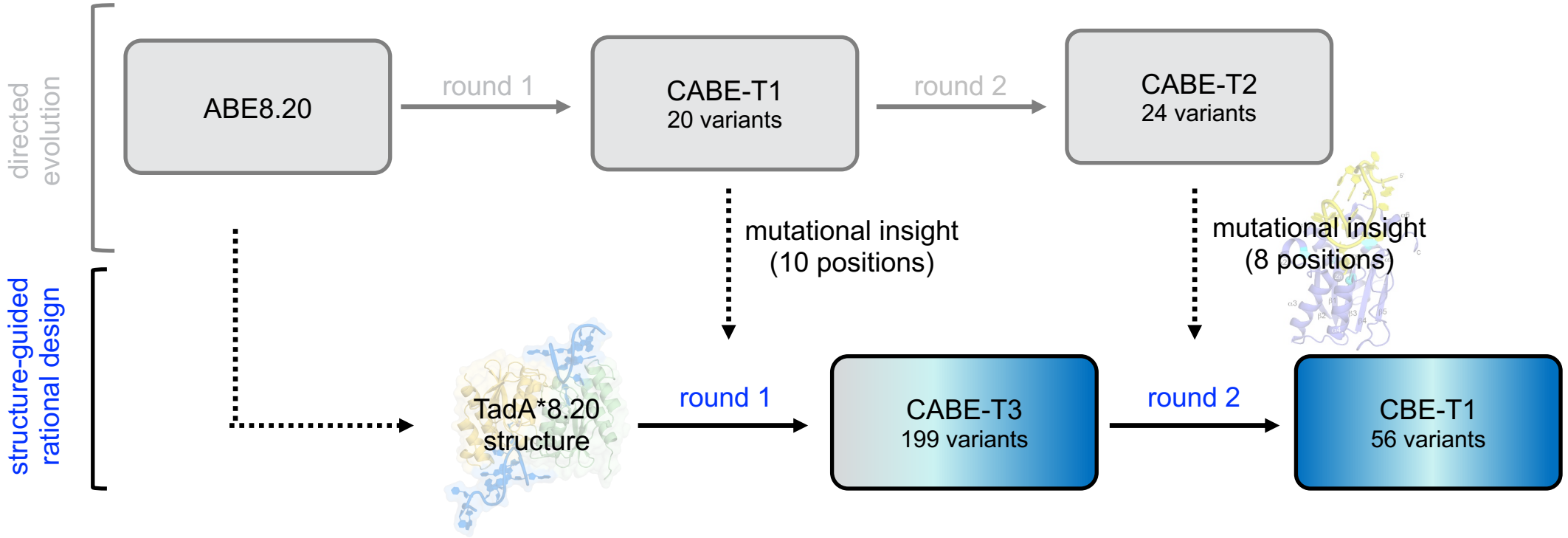




BE4: Komor, A. C., … Liu, D.R. *Sci Adv* **3**, eaao4774, (2017).
A&C-BEmax: Zhang, X. …Li, D. *Nat Biotechnol* **38**, 856-860, (2020).
SPACE: Grunewald, J. … Joung, J.K. *Nat Biotechnol* **38**, 856-860, (2020).
ABE8.20: Gaudelli, N. M. et al. *Nat Biotechnol* **38**, 892-900, (2020).

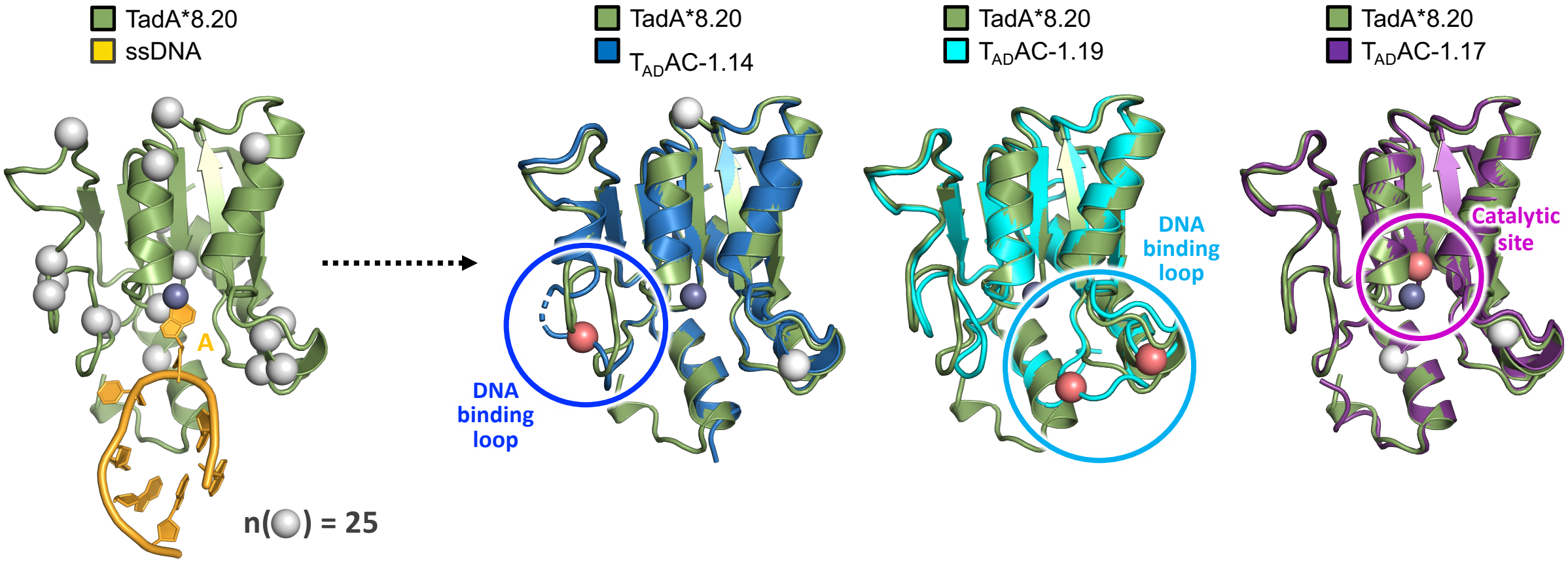
Lam, D.K.; Feliciano, P.; … Gaudelli, N.M. (2022) *manuscript under peer review*

X-ray structural insights from TadA8.20 and T_{AD}AC and directed evolution enabled access to CBE-T



Lam, D.K., Feliciano, P. R. et. al Gaudelli, manuscript under peer-review

Three areas of TadA were identified to be critical towards C-to-U deamination



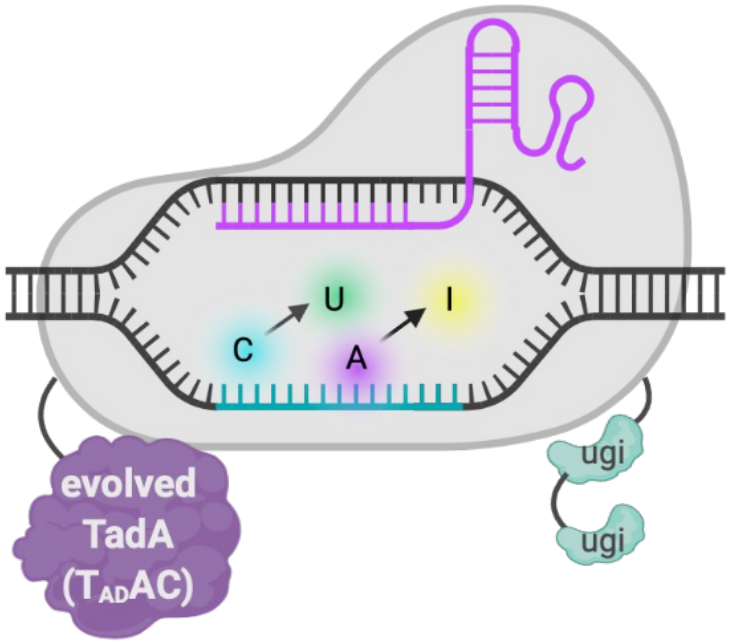
Structural characterization of TadAs in CABE-T1 variants identified mutations potentially critical for rendering C-to-U deamination in DNA binding loops and active site

Structural and mutagenic insights, combined with gene editing outcome of C-to-T conversion, enabled us to make additional mutations in TadA to create CBE-Ts

Lam, D.K., Feliciano, P. R. et. al Gaudelli, manuscript under peer-review

Directed evolution and structure-guided mutagenesis enabled the creation of CBE-Ts

CABE-T

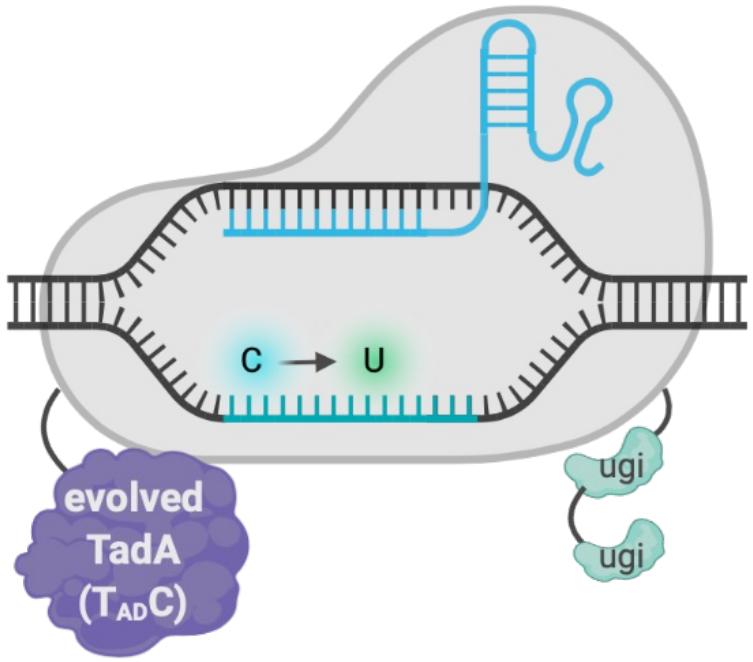


T_{AD}AC: TadA acting on DNA adenines and cytosines

1. x-ray crystallographic structure determination of TadA variants from evolution

2. structure-guided mutagenesis

CBE-T

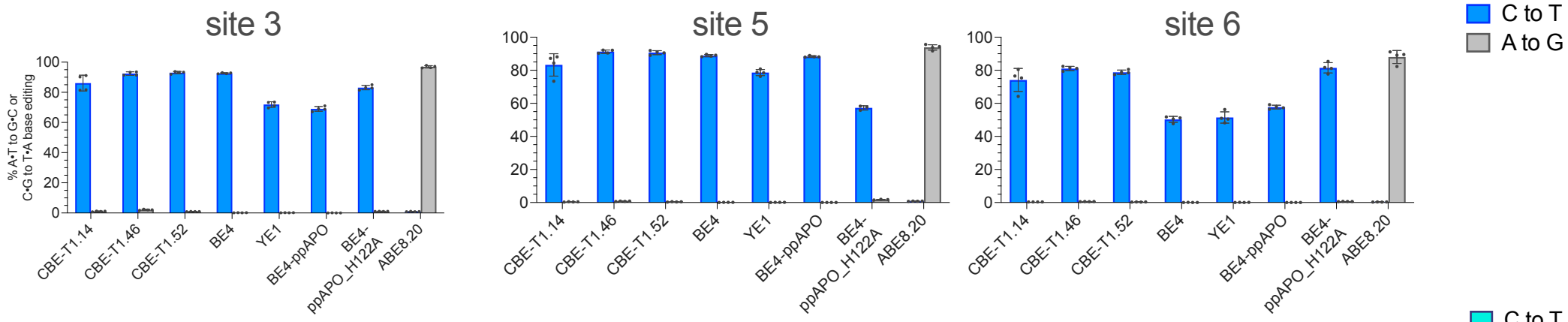


T_{AD}C: TadA acting on DNA cytosines

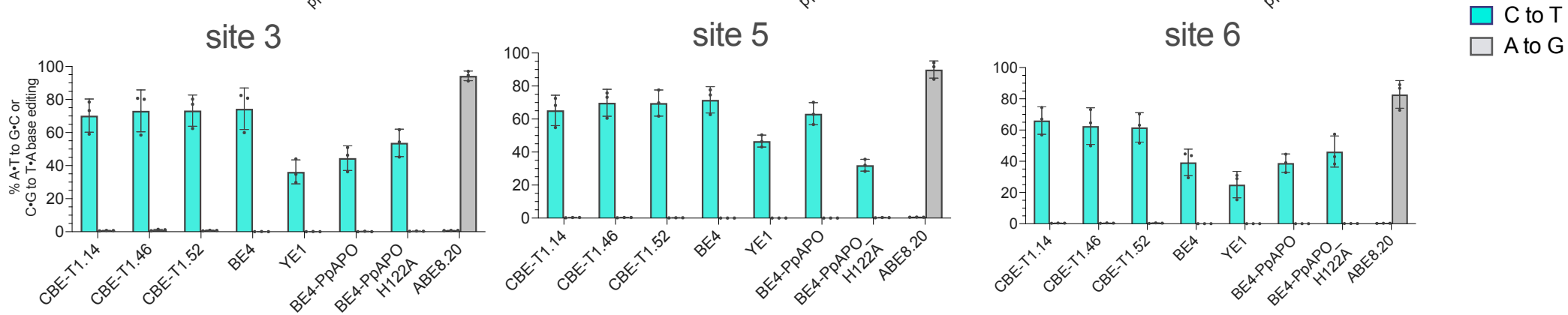
CBE-Ts conduct C-to-T base editing *without* the use of APOBEC, CDA, or AID

Highly-efficient cytosine base editors accessed via engineered TadAs ($T_{AD}C$) optimized for C-to-U deamination

mRNA delivery
+ synthetic gRNA
saturating dose



mRNA delivery
+ synthetic gRNA
sub-saturating dose

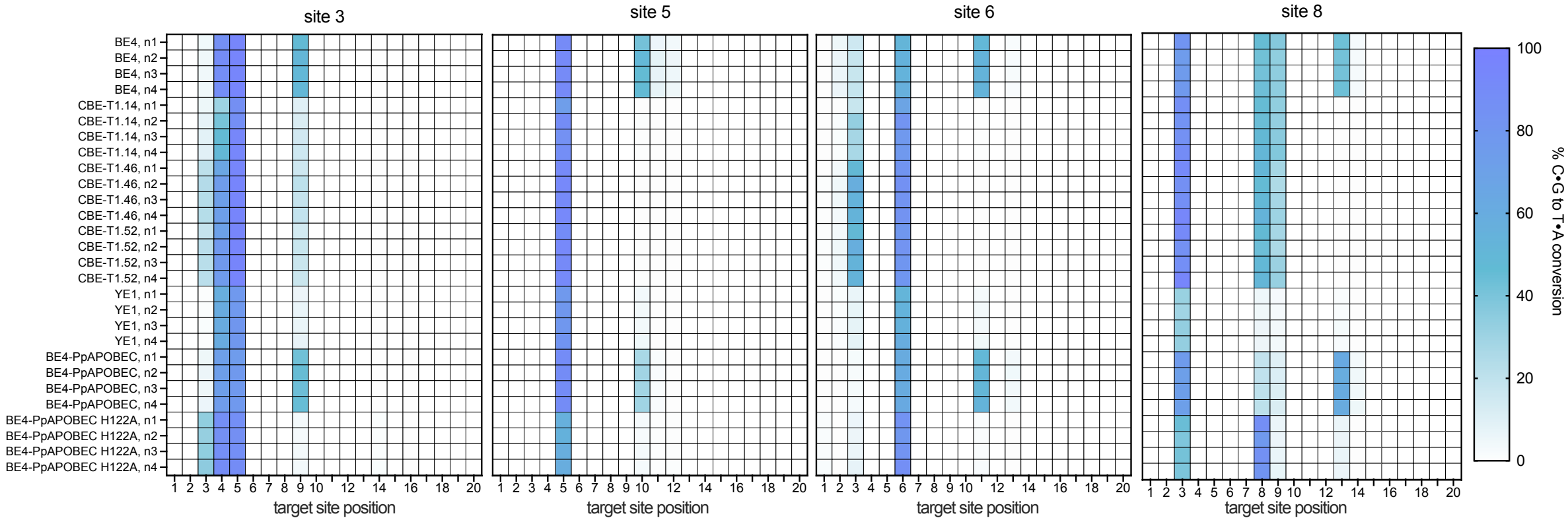


- CBE-Ts conducted C-to-T on target editing with no significant difference to BE4 in C-to-T editing outcomes (p=0.30, two-tailed Wilcoxon–Mann–Whitney U test) *without* the use of APOBEC enzyme
- Across all sites tested, we observed an average 262-fold increase in C-to-T editing relative to ABE8.20
- CBE-Ts enabled higher maximum C-to-T editing than CBEs with mitigated guide- independent outcomes such as YE1 and BE4-PpAPO(H122A).

BE4: Komor, A. C., … Liu, D.R. *Sci Adv* **3**, eaao4774, (2017).
YE1: Doman, J. L., …Liu, D. R. *Nat Biotechnol* **38**, 620-628, (2020)
BE4-PpAPOBEC: Yu, Y. …Gaudelli, N.M. *Nat Commun* **11**, 2052, (2020)

Lam, D.K.; Feliciano, P.; … Gaudelli, N.M. (2022) *manuscript under peer review*

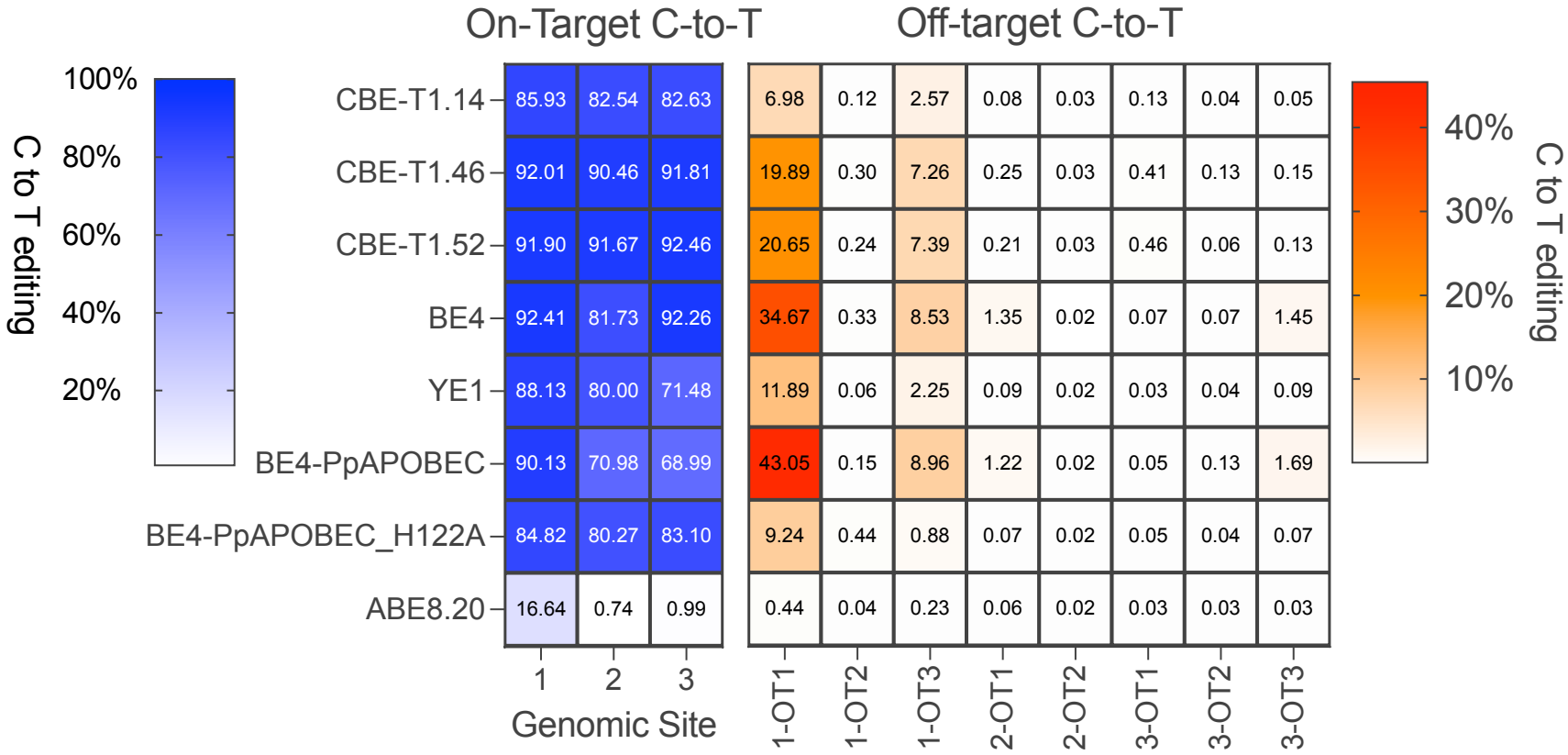
CBE-Ts and CABE-Ts performed C-to-T editing at similar levels to BE4 and had a more precise activity window



CBE-T installed C-to-T base editing within a narrower editing window relative to APOBEC-based CBEs, which may reduce bystander edits

Lam, D.K., Feliciano, P. R. et. al Gaudelli*, manuscript in peer-review

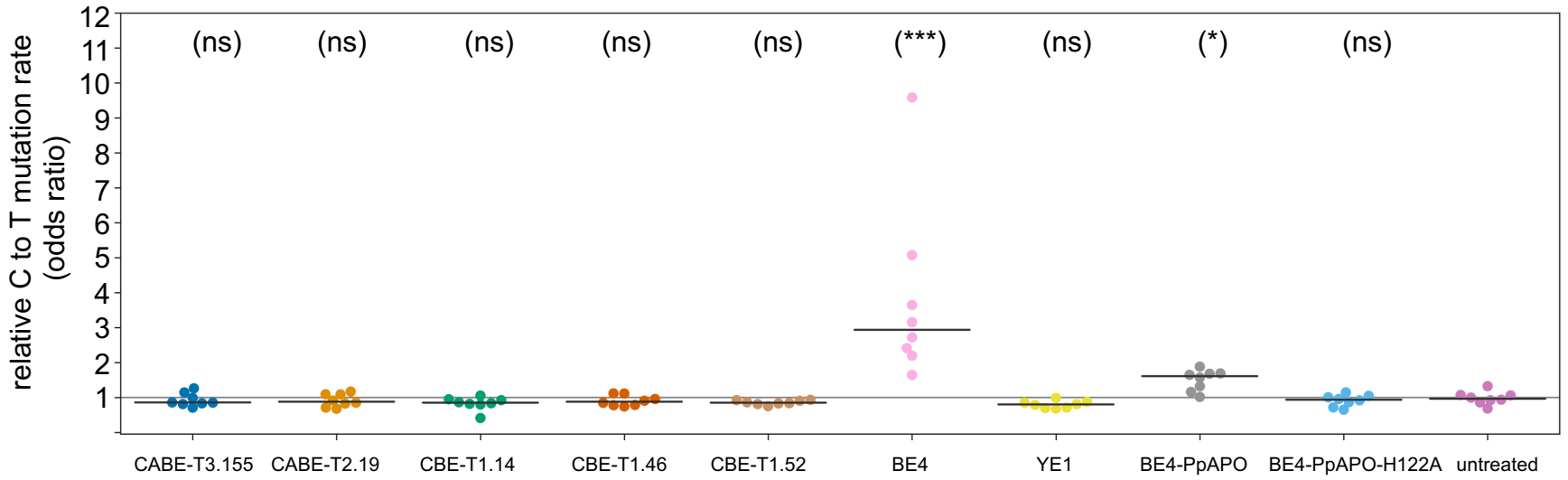
CBE-Ts yielded lower and fewer guide-dependent OT vs. BE4

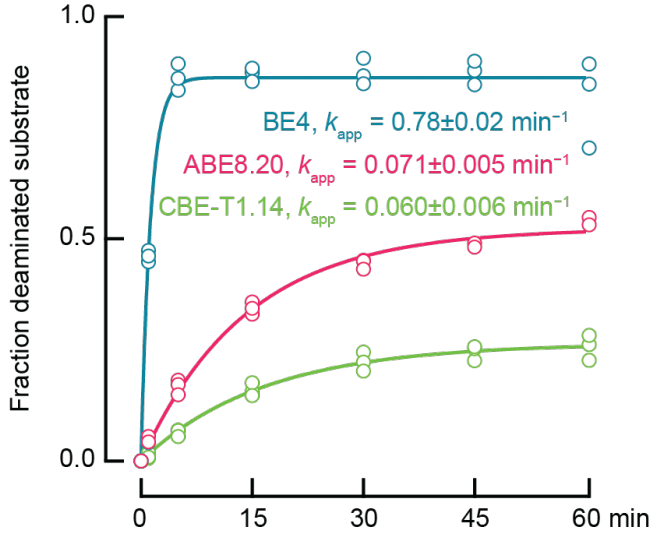


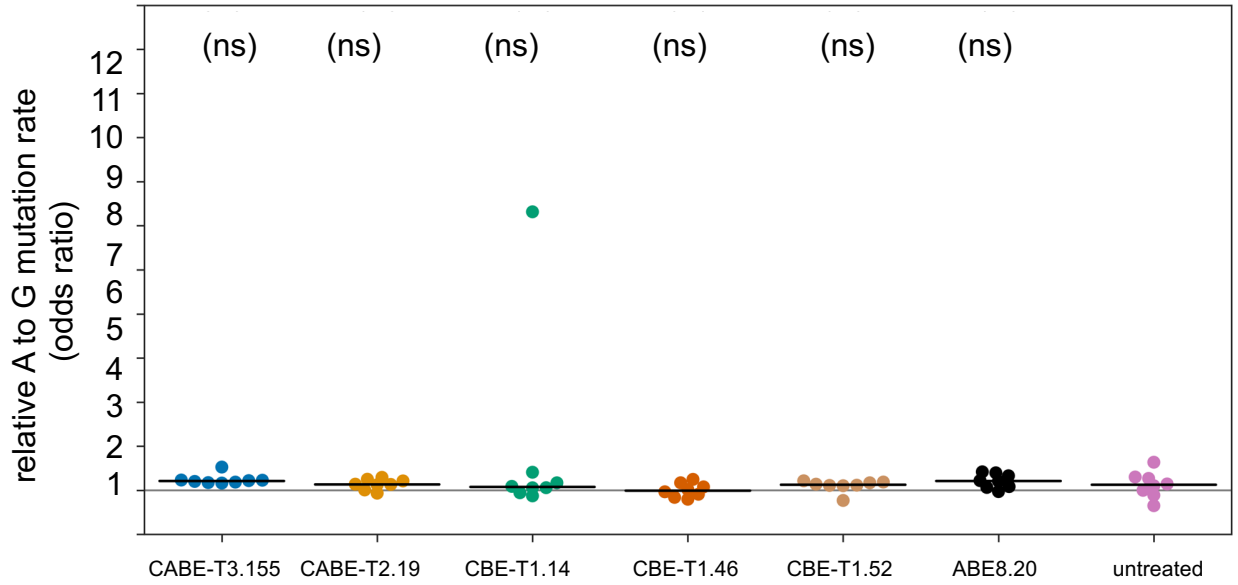
- Compared to BE4, CBE-Ts had a >3-fold decrease in gRNA-dependent C-to-T off-target editing across sites tested.
- Cells treated with CBE-Ts had gRNA-dependent off-target editing outcomes comparable to YE1 and BE4-PpAPO(H122A), previously developed next-generation CBEs with mitigated off-target outcomes

Lam, D.K., Feliciano, P. R. et. al Gaudelli*, manuscript in peer-review

CBE-T and CABE-T caused no significant elevation in genome-wide guide RNA-independent C-to-T or A-to-G mutation rate





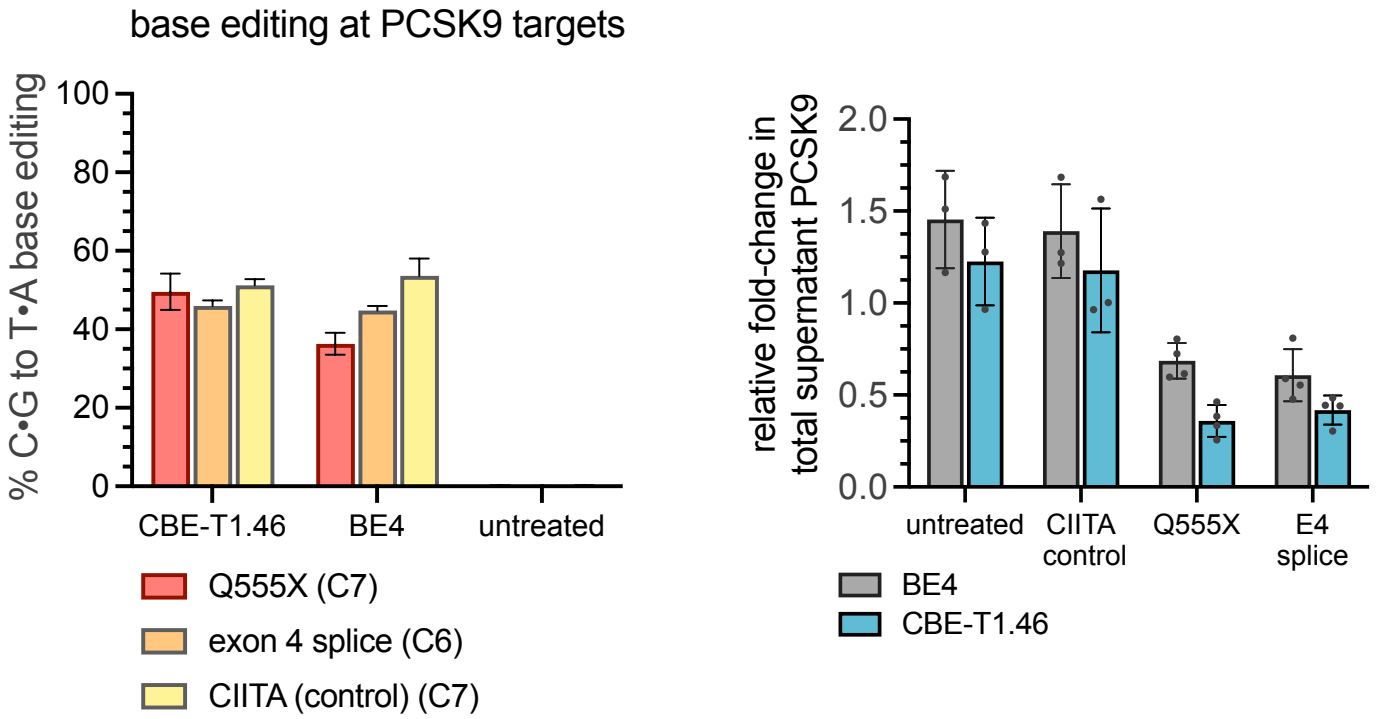


- CABE-T and CBE-T caused no significant elevation in genome-wide C to T SNVs relative to untreated samples (all not significant (ns) p values are > 0.05; one-sided Mann-Whitney U test)
- CABE-Ts and CBE-Ts did not cause significant elevation in genomic A to G SNVs relative to untreated controls (all not significant (ns) p values are > 0.05; one-sided Mann-Whitney U test).

ABE and CBE-T OT *kapp* of deamination on ssDNA were comparable and were ~10x lower than that of BE4

Lam, D.K., Feliciano, P. R. et. al Gaudelli*, manuscript in peer-review

CBE-Ts enabled high C-to-T editing in primary human hepatocytes at PCSK9 targets

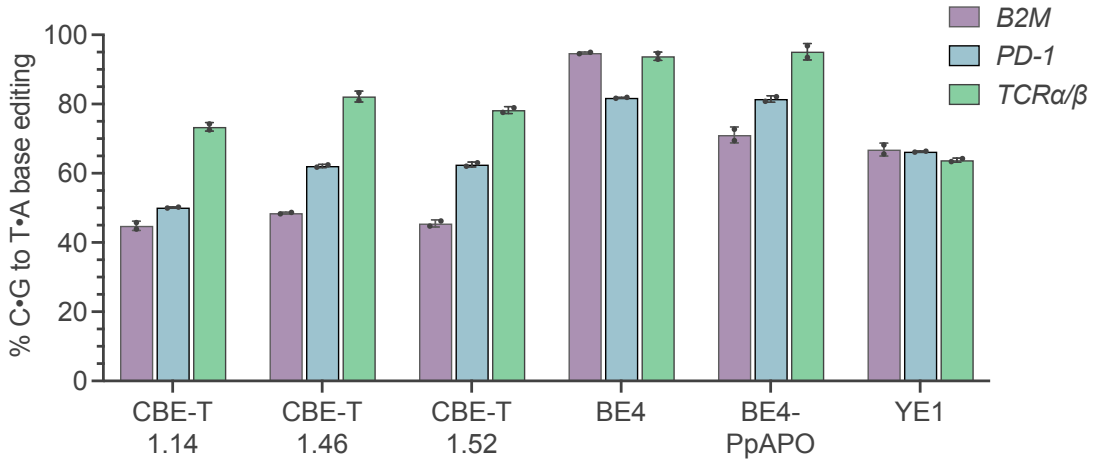


- mRNA transfections of CBE-Ts and BE4 with corresponding synthetic gRNA were performed in primary human hepatocytes (PHH) at different two genomic loci predicted to impact PCSK9 expression
- CBE-Ts performed C-to-T base editing in PHH at levels comparable to BE4 or better. C-to-T editing at Q555X and exon 4 splice site in PCSK9 corresponded to reduction in PCSK9 in supernatant

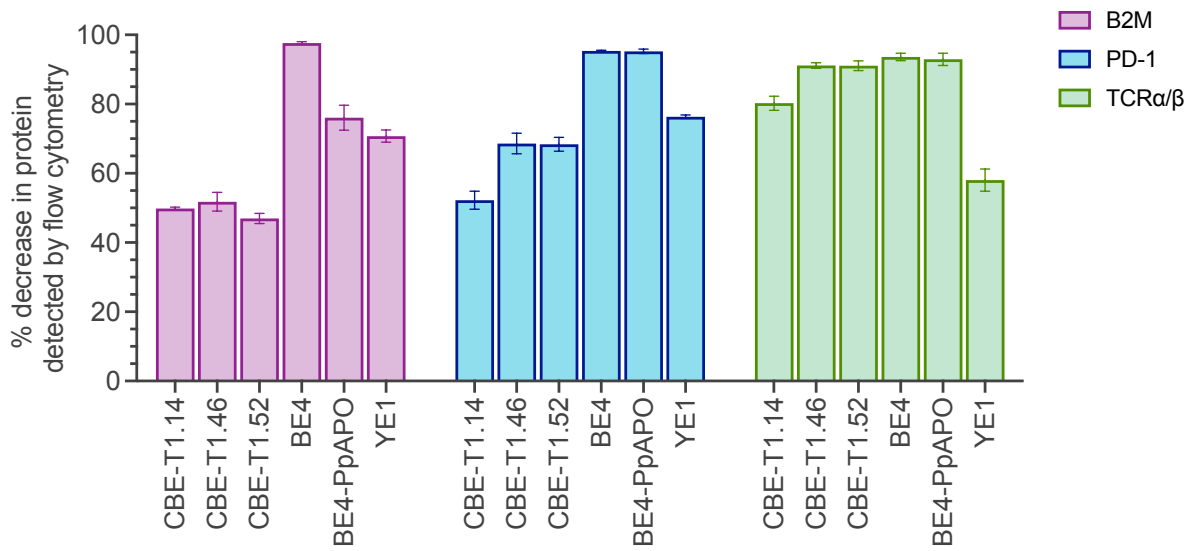
Lam, D.K., Feliciano, P. R. et. al Gaudelli*, manuscript under peer-review

CBE-Ts enabled high C-to-T editing in primary T-cells

multi-plex editing (3 sgRNAs)



protein loss resultant from multi-plex editing



- mRNA encoding CBE were transfected into T-cells with 3 synthetic gRNAs targeting loci of interest for allogenic T cell engineering (e.g. B2M, PD-1, TCR).
- CBE-Ts can disrupt protein expression by targeting splice site donors and through the creation of stop codons.
- CBE-Ts were able to conduct multiplex base editing and C-to-T base edits caused corresponding protein loss.

Lam, D.K., Feliciano, P. R. et. al Gaudelli*, manuscript under peer-review

Summary

- Directed evolution coupled with structure-guided design enabled the development of two new classes of base editors: CABE-Ts and CBE-Ts.

- CABE-Ts conducted C-to-T and A-to-G editing (typically greater than 40% editing for both C-to-T and A-to-G editing) with the use of a single TadA variant per construct capable of cytosine and adenine deamination

- CBE-Ts enabled highly efficient C-to-T editing (typically >80% editing at genomic sites tested), with the use of a TadA variant optimized for cytosine deamination. On target cytosine base editing outcomes were comparable or better than APOBEC-based CBEs (e.g. BE4, YE1).

- CBE-Ts enabled highly efficient C-to-T editing with mitigated off-target outcomes relative to BE4
    - mRNA + sgRNA transfection of CBE-Ts led to highly efficient base editing, comparable or better than C-to-T editing achieved with BE4, with low to no observed A-to-G editing
    - Cells treated with CBE-Ts and CABE-Ts had no elevation in genome-wide mutations relative to untreated cells in WGS experiments.
    - CBE-Ts base edit within a narrower editing window relative to BE4. This resulted in fewer bystander edits and fewer guide-dependent off-targets relative to APOBEC-based CBEs

- CBE-Ts are effective in primary human hepatocytes and can be used for therapeutically relevant targets such as PCSK9. We showed CBE-Ts can introduce stop codons and disrupt splice sites in PCSK9, resulting in loss of protein in serum.

- CBE-Ts were effective tools for multi-plex cytosine base editing in primary T cells at targets relevant for the generation of allogeneic Car-Ts (e.g. B2M, PD-1, TRAC). Disruption of our T-cell targets led to substantial protein knock-out.

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