Directed evolution platforms and applications for engineering RNA-guided nucleases

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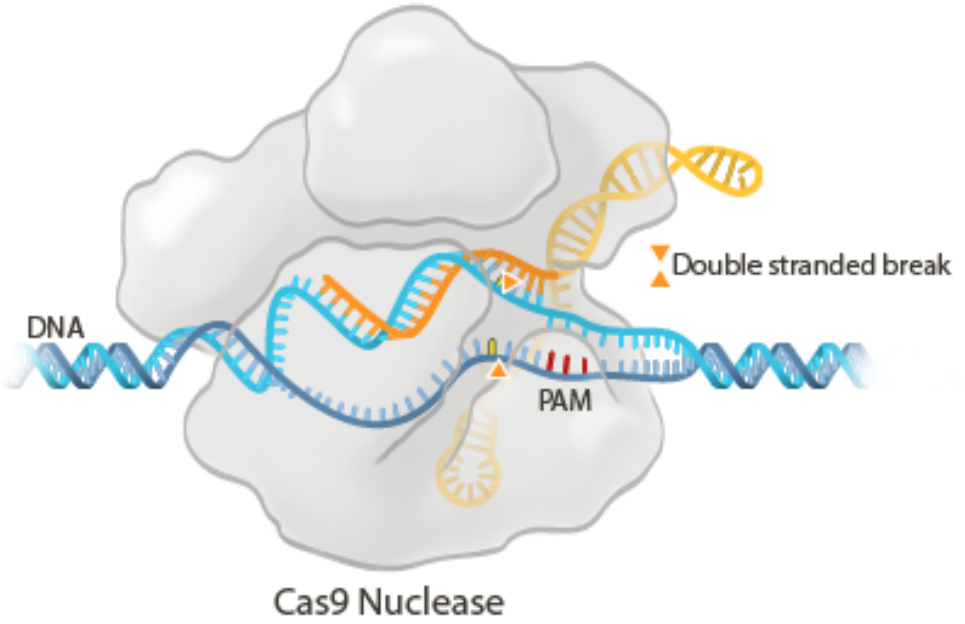
Directed evolution to improve gene editing

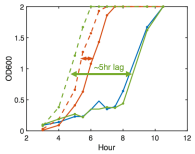
- CRISPR is an amazing tool with great potential in the clinic, but there are improvements to make
- Rational design is often difficult
- Design can be slow as more gene editing tools are discovered
- We are developing a directed evolution platform for quickly and effectively engineering gene editing tools



Building new systems for directed evolution of nucleases

SMART libraries	▪ Improving mutagenesis over whole proteins
Phage-based selection	▪ Competitive and fast evolution in liquid culture
Specificity evolution	▪ Application to designing against identified off-targets
PAM evolution	▪ Application to changing PAM preferences of enzymes

Directed evolution pipeline



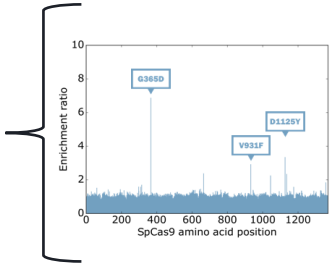
Mutate

Select

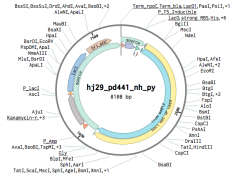
Deep sequence

PACBIO

Pick variants



SMART evolution platform



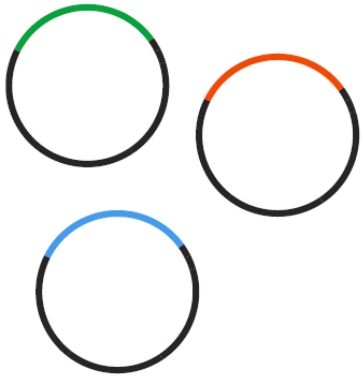
Synthesize

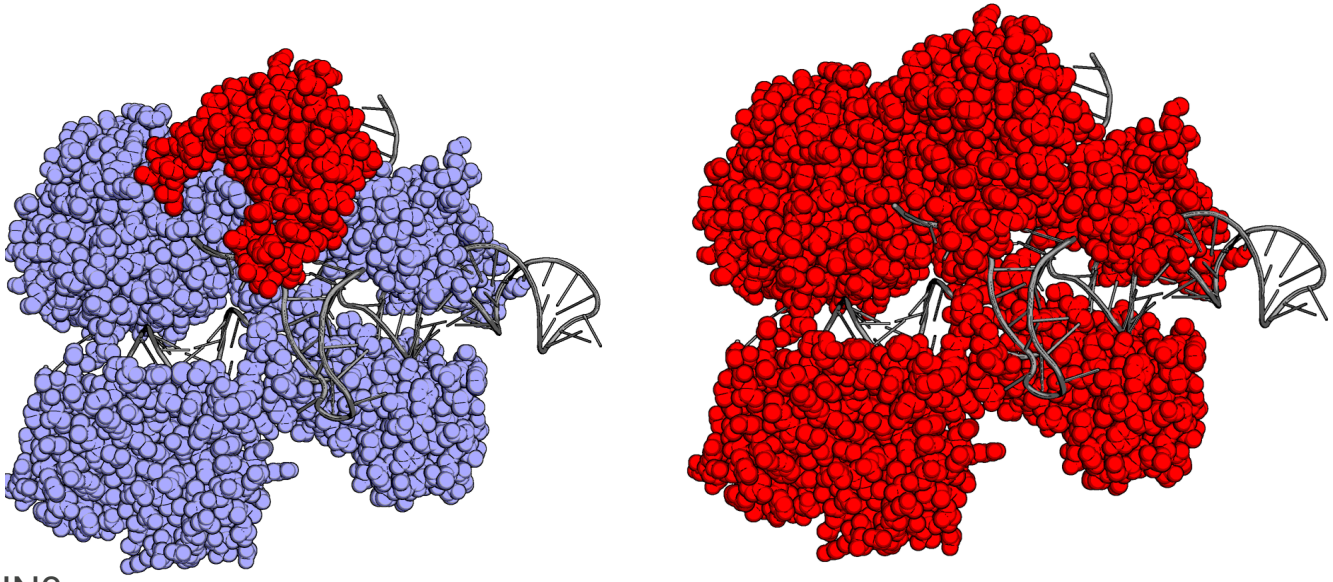
Purify

Screen in vitro

Mutagenesis: SMART library creation

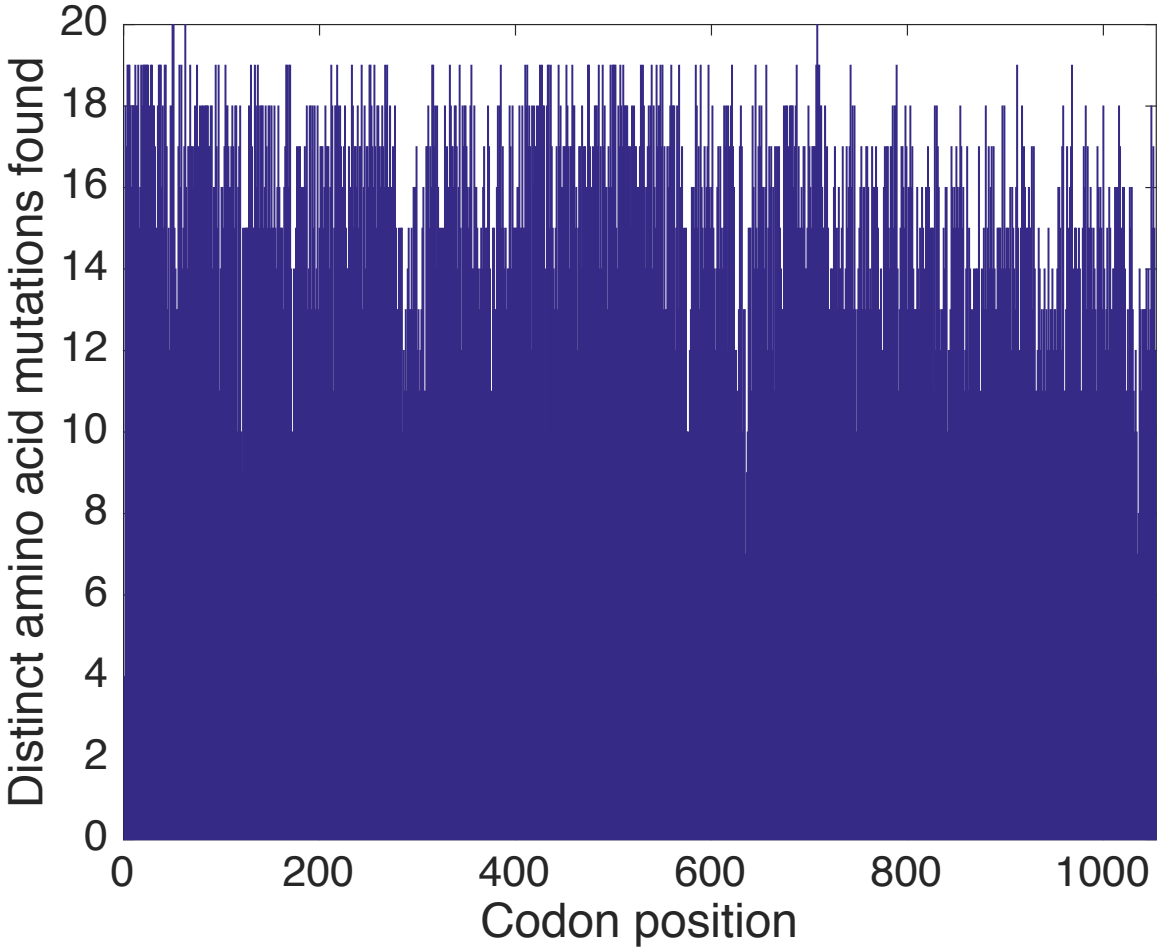
- **S**canning **m**utagenesis **a**t **r**andom **t**argets
- Codon mutations along entire *cas9* gene
- Can be targeted to specific regions
- One-pot ~6hr protocol on dsDNA template
- High yield





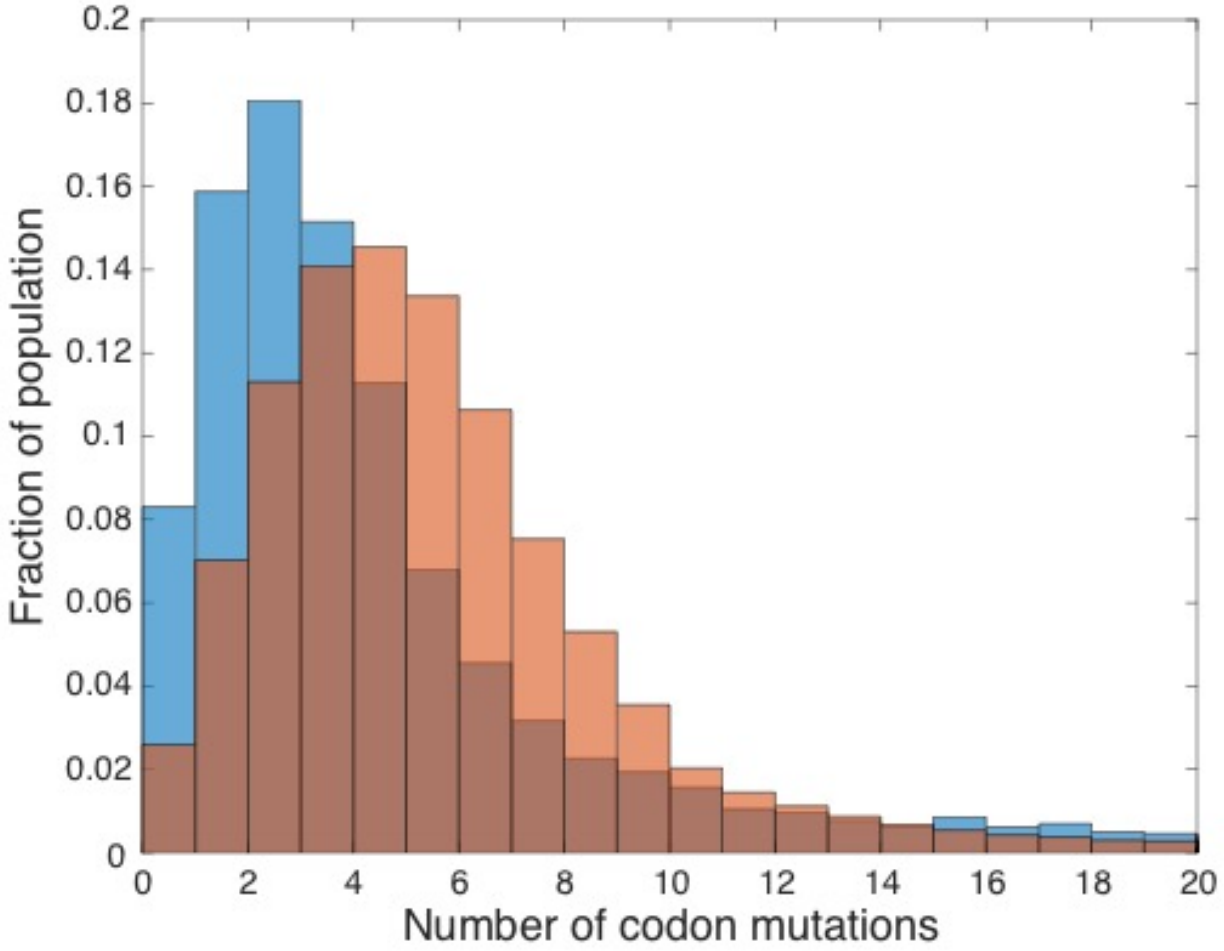
pdb:4UN3

SMART accesses all potential amino acid mutations throughout the protein



We can infer we are making every possible mutation, but is diversity of libraries overwhelming?

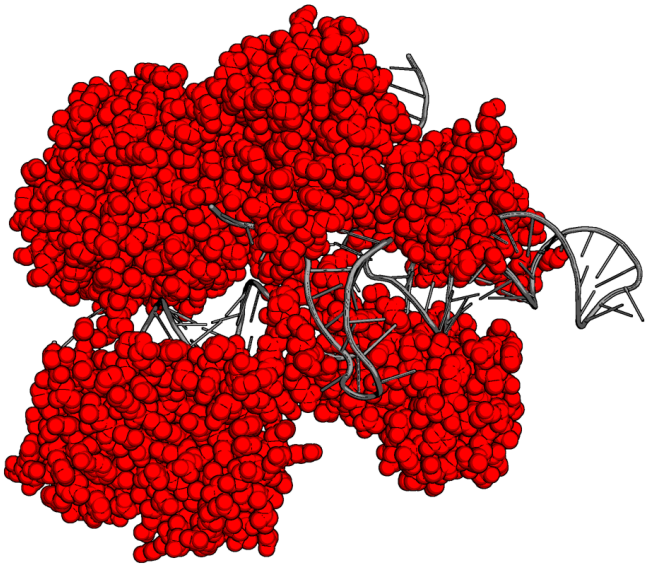
SMART mutation rate is tunable



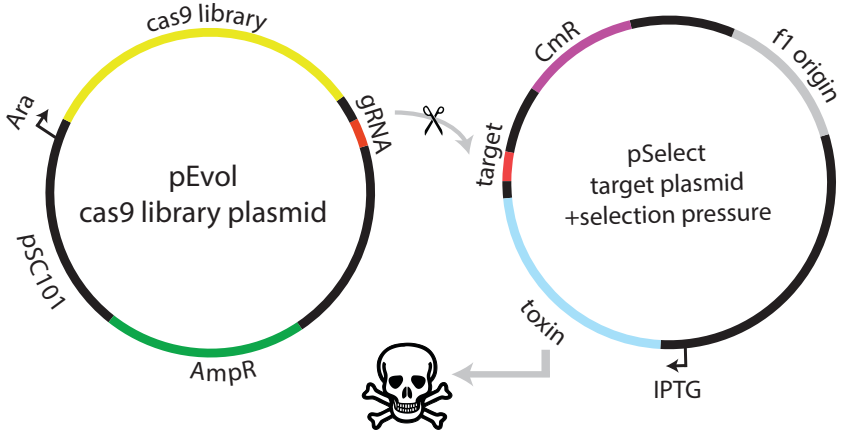
With a million transformants, we have 10X coverage of all single mutants and sample other mutations broadly

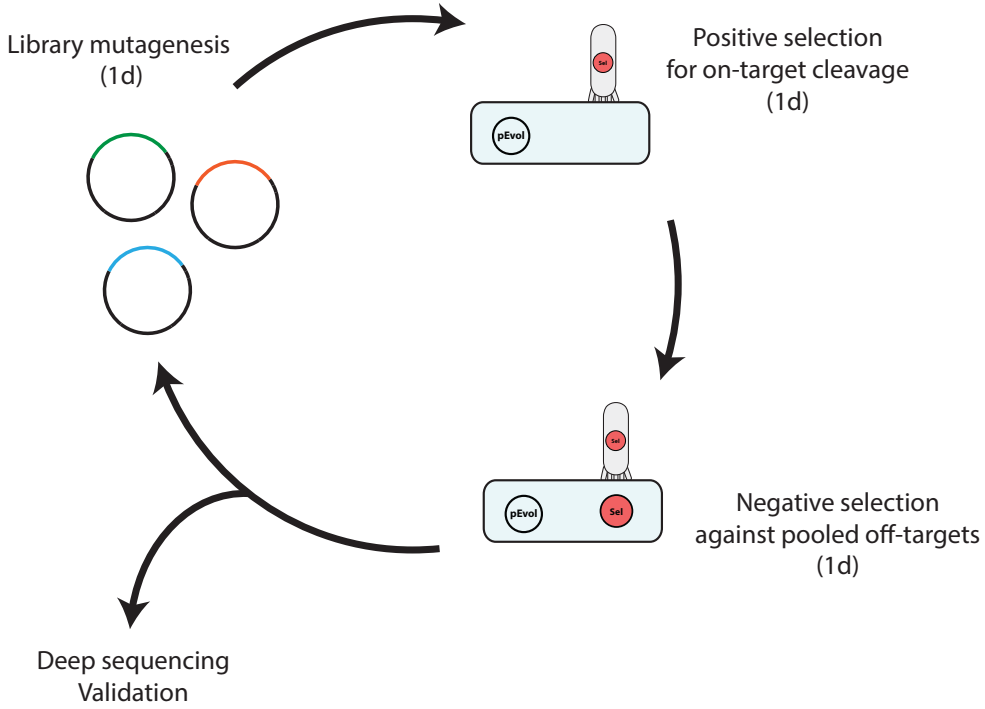
SMART libraries are comprehensive

- Comprehensive libraries containing every single amino acid mutation
- Not limited by region
- Using NNN codon replacement, but can create non-random mutations
- **Better libraries lead to better hits**

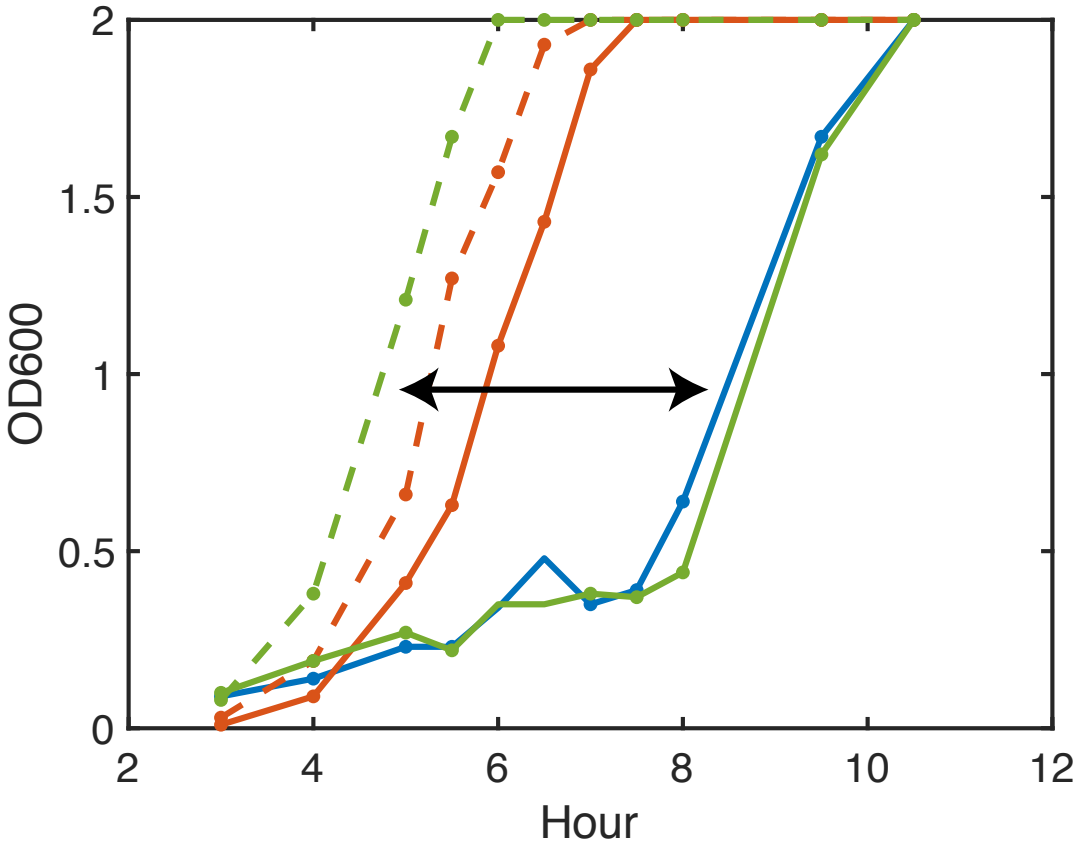


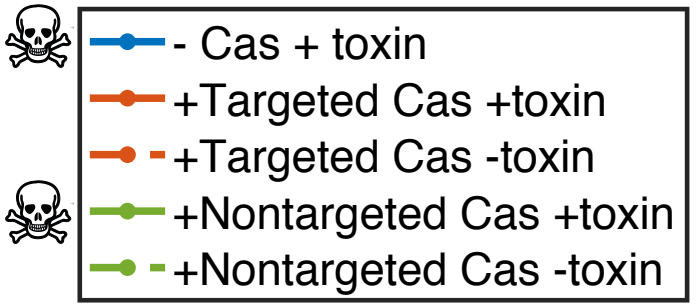
Phage-based selection for or against cleavage



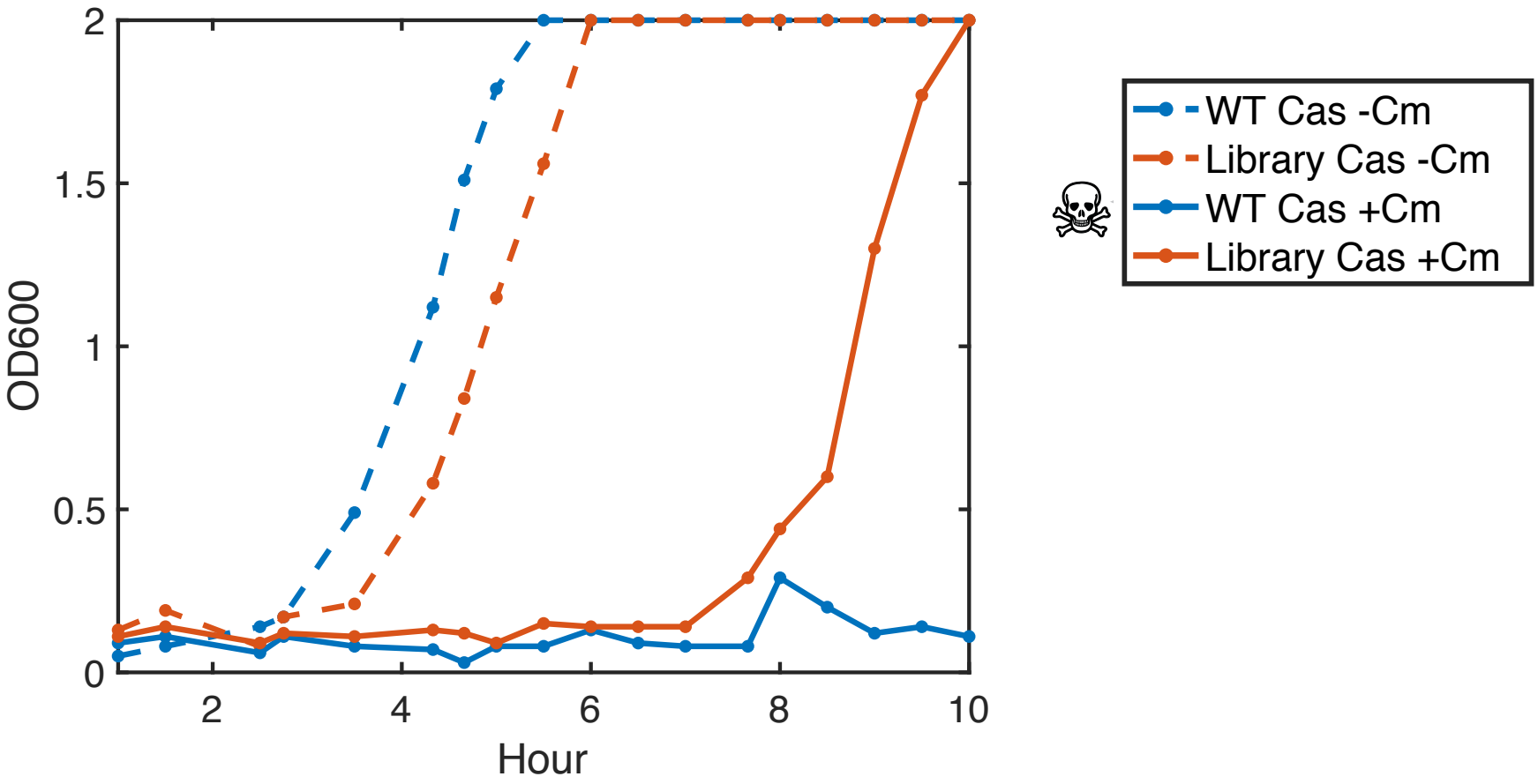


Positive selection is effective in liquid culture



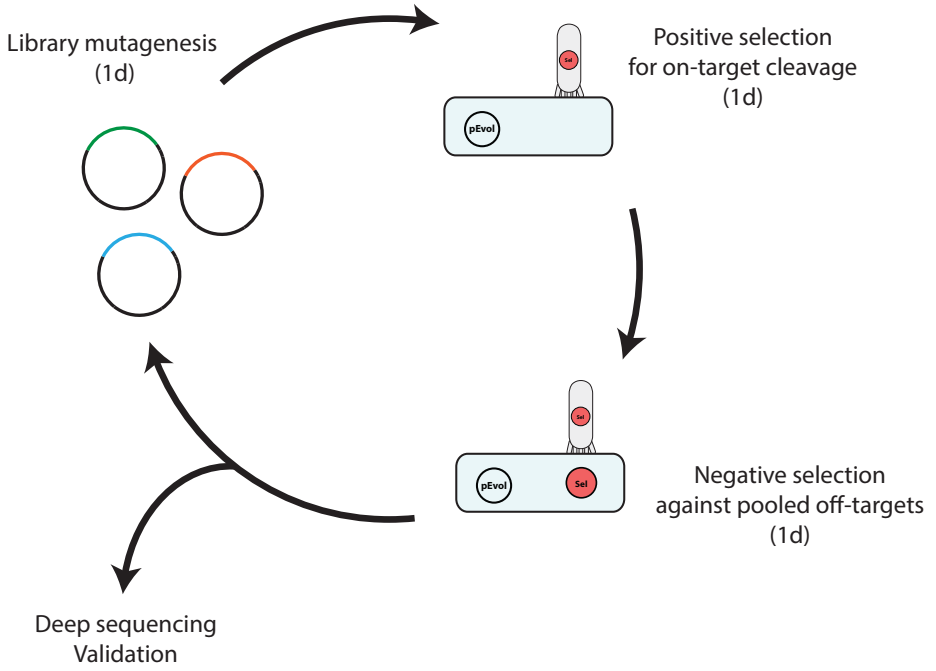


Negative selection is effective in liquid culture



Effective phage-based selection for nuclease activity

- Highly modular and fast
- Can handle large libraries
- Not a screen – competition increases selective events
- Multiple selective conditions can be pooled together



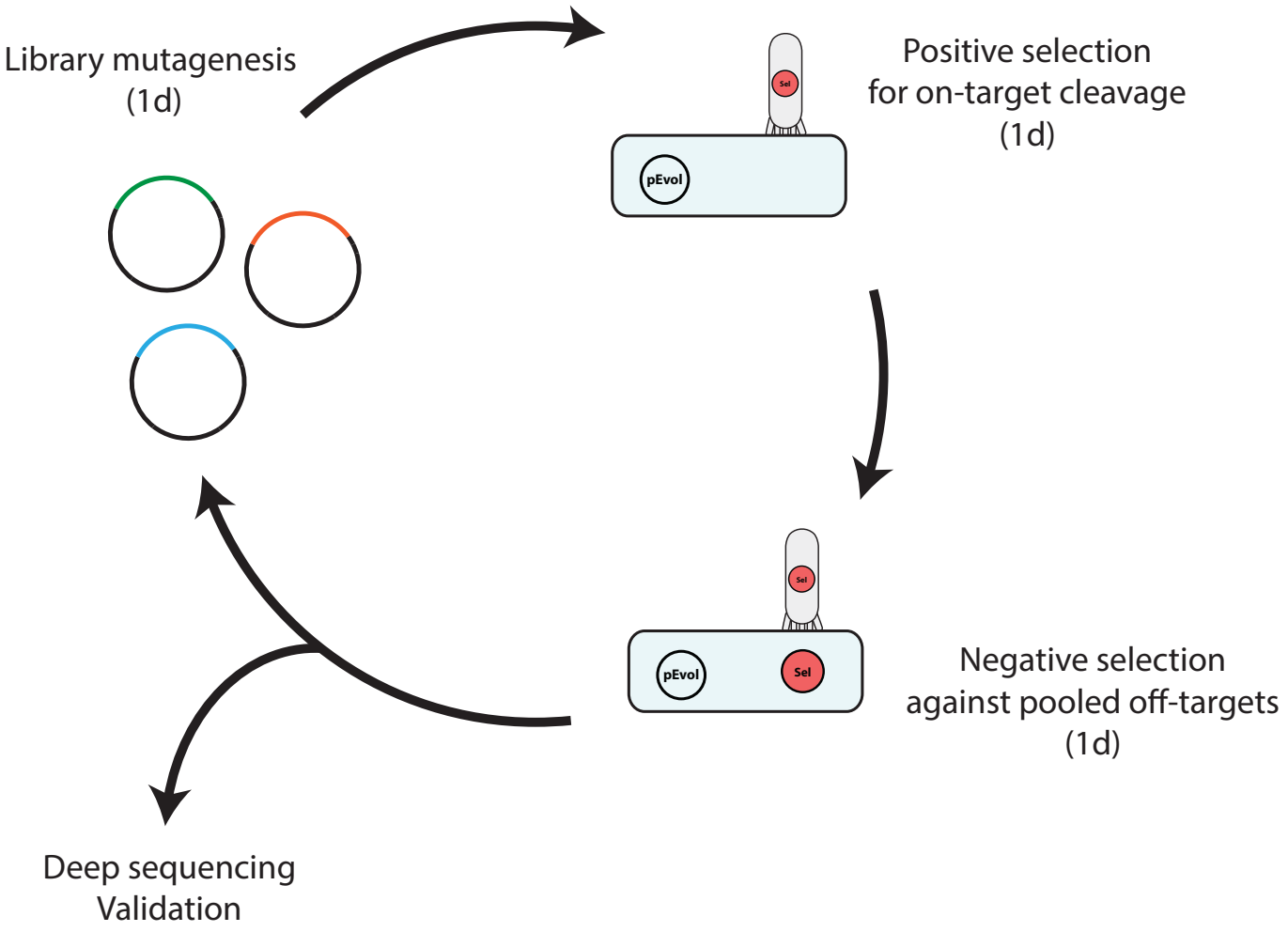
Building new systems for directed evolution of nucleases

SMART libraries	▪ Improving mutagenesis over whole proteins
Phage-based selection	▪ Competitive and fast evolution in liquid culture
Specificity evolution	▪ Application to designing against identified off-targets
PAM evolution	▪ Application to changing PAM preferences of enzymes

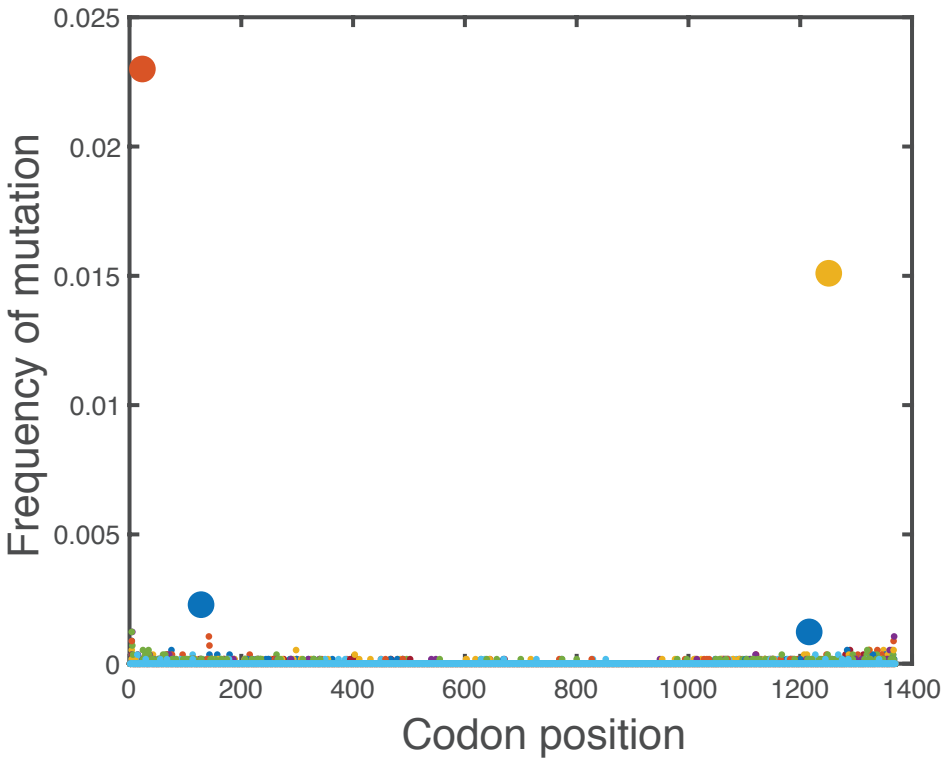
Application:
Selection against identified off-targets in Sp. Cas9

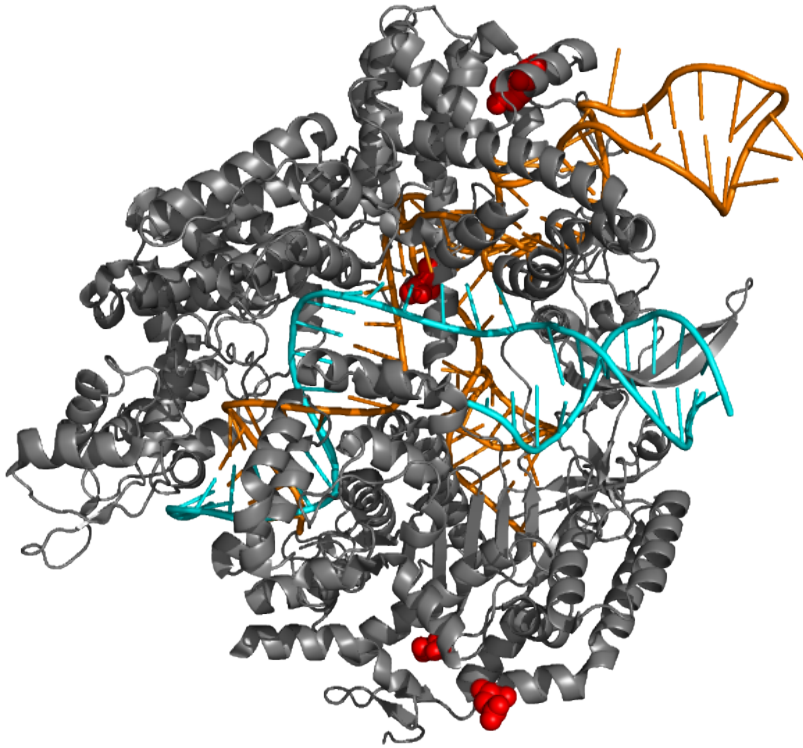
- Four off-targets were identified in edited T-cells
- We wanted to decrease off-target cutting while maintaining on-target efficacy
- Approach should be enzyme- and target-agnostic and tailorable to a specific guide
- Does not require structural information

Selection against identified off-targets with phage



Identified enriched cas9 mutations via deep sequencing



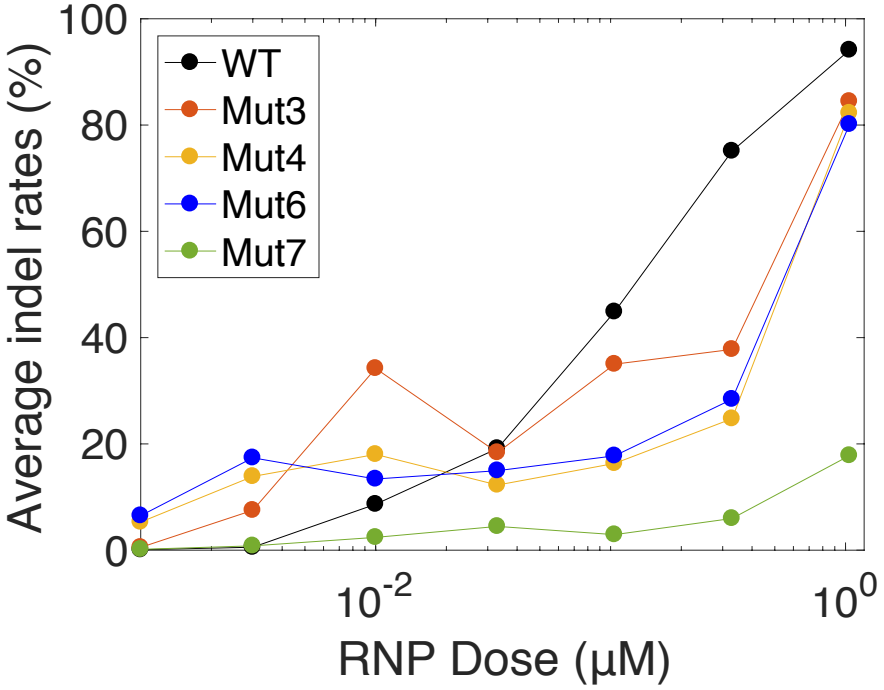


DNA
RNA
Enriched mutations

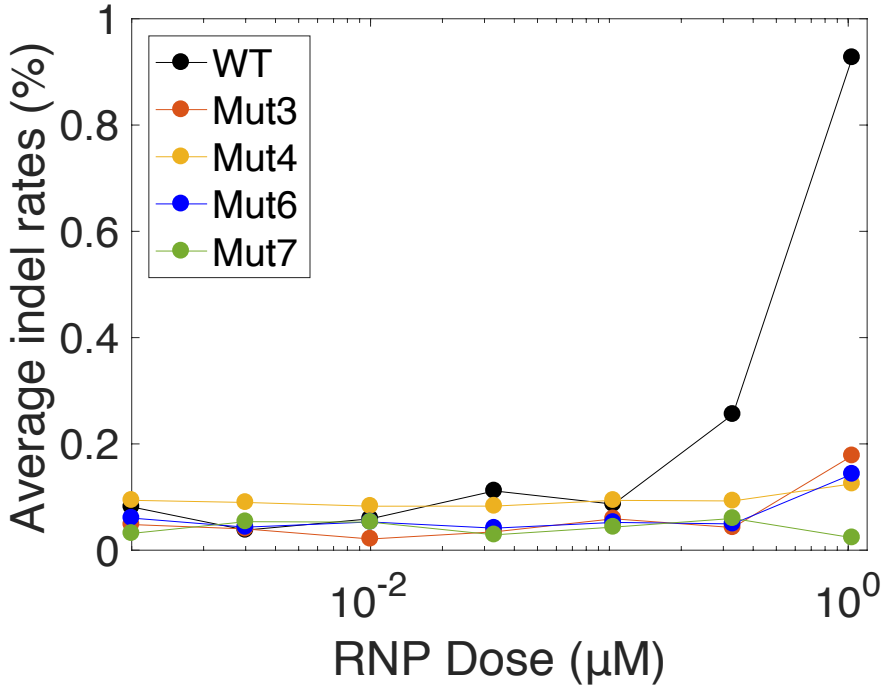
pdb:4UN3

Mutant proteins show decreased off-target editing and maintain on-target efficacy in T-cells

On-target editing



Off-target editing (4 mismatches)



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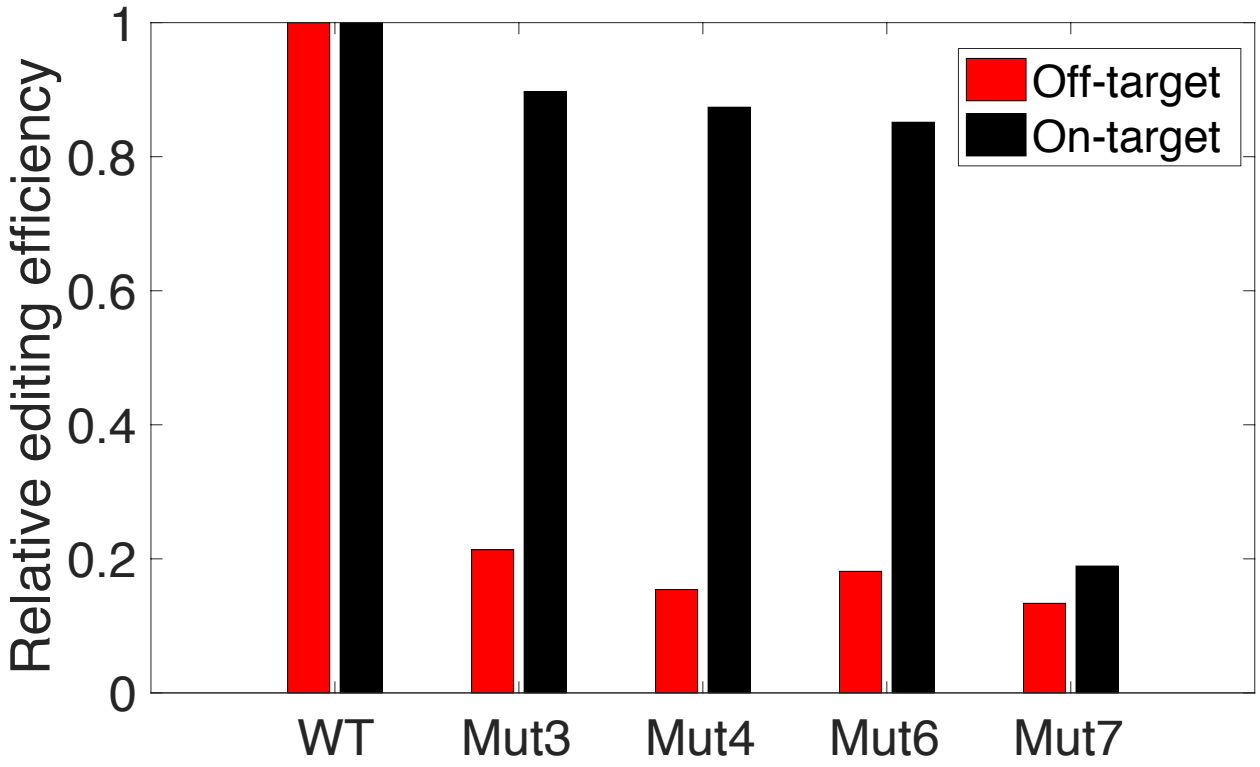
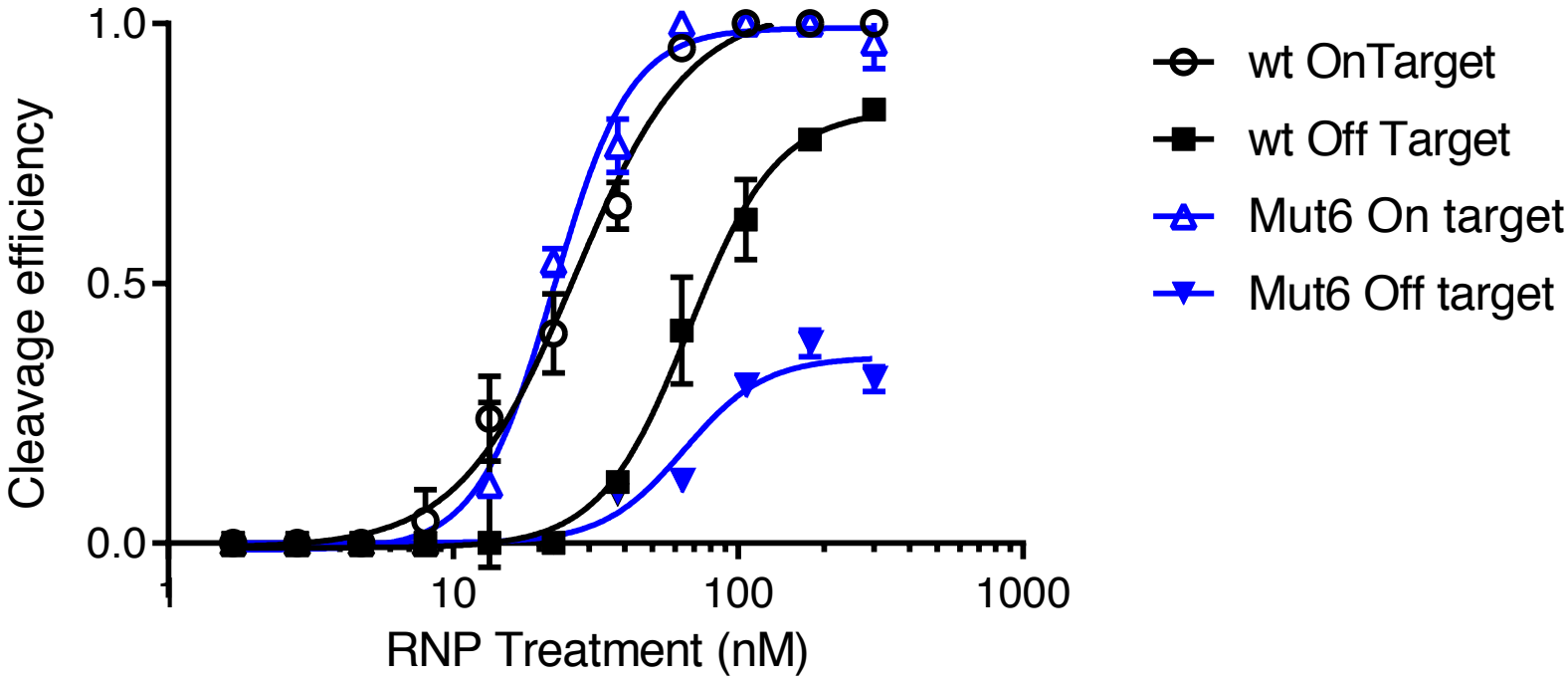
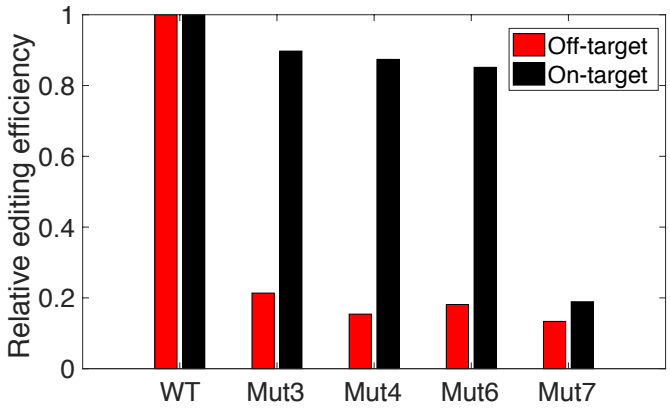


Combinations of enriched mutations reduce off-target cutting in vitro



Evolution against identified off-targets

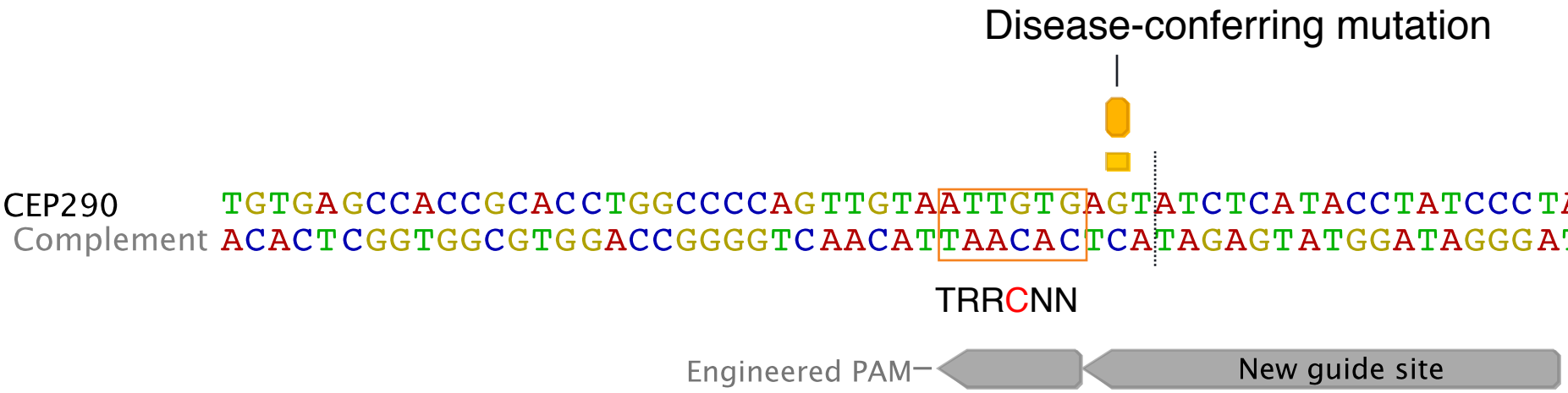
- We can evolve cas9 variants to mitigate identified off-targets
- Multiple off-targets can be selected against in each round
- We maintain on-target cleavage efficiency
- In vitro tests confirm lower max cutting – may indicate competitive inhibition



Application:
Evolution for alternate PAMs in Sa. cas9

Wildtype Sa. Cas9 requires NNGRRT adjacent motif to cleave

We wanted to evolve a change to NNCRRT to target a specific cut



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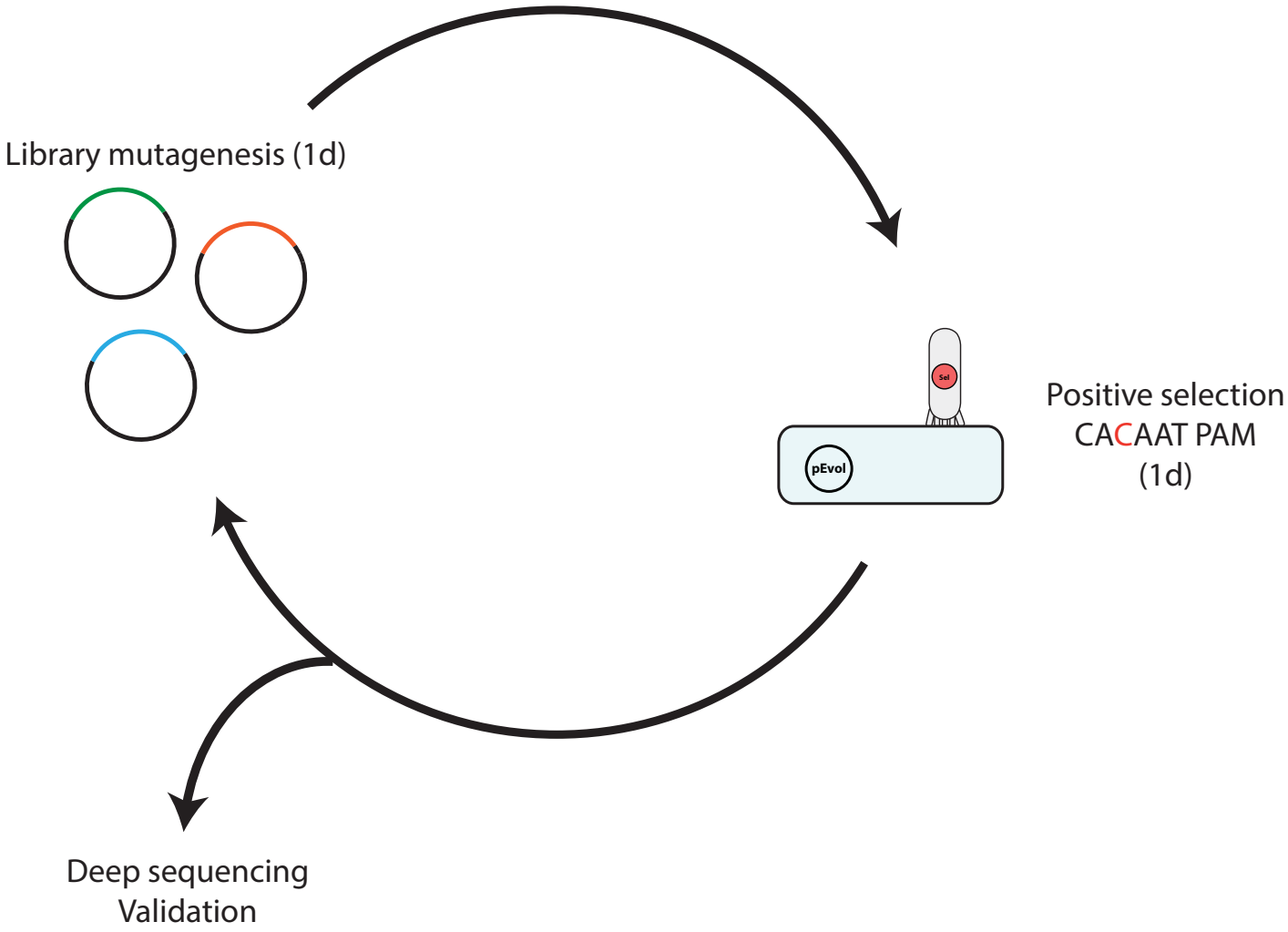
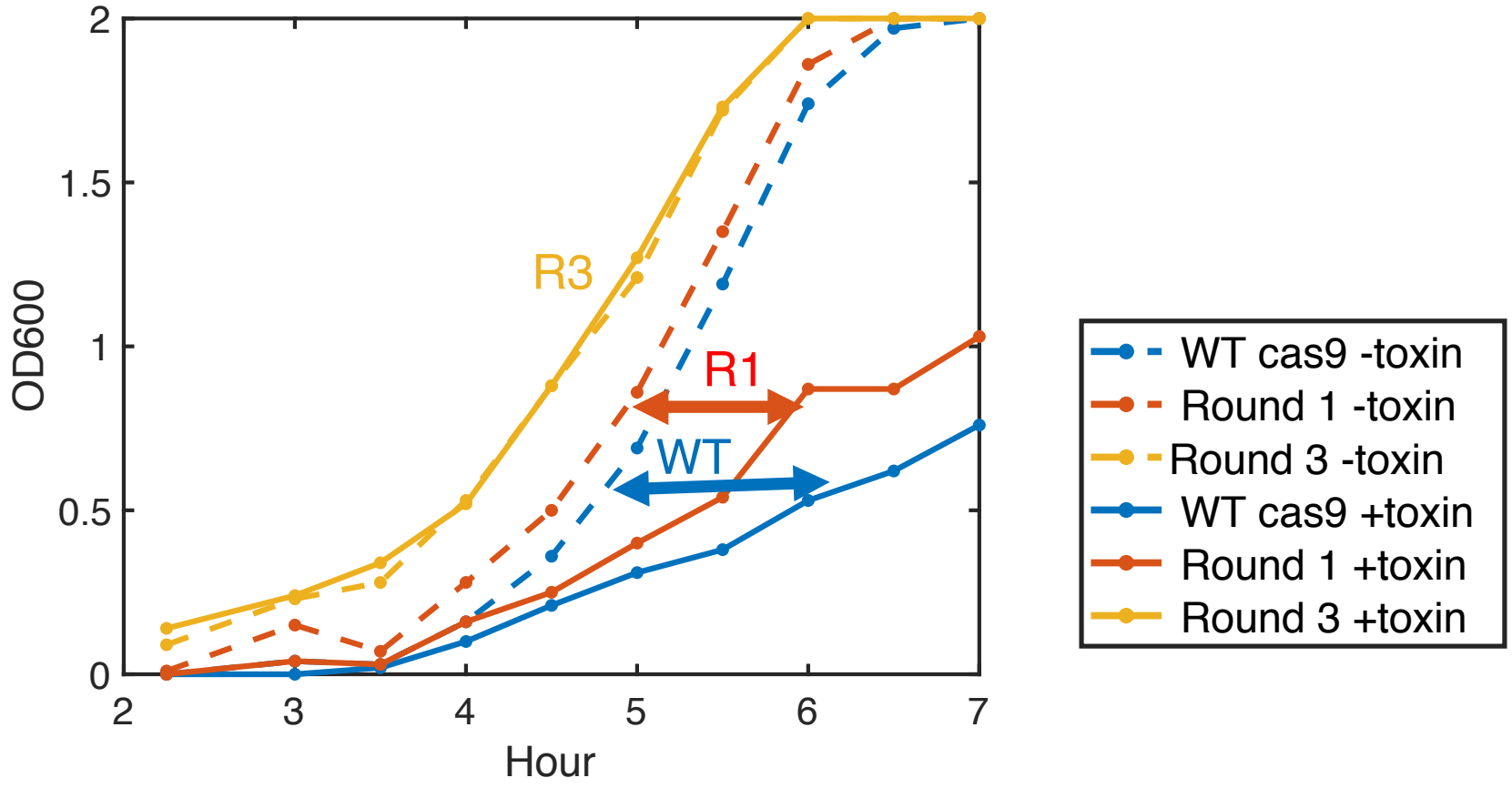
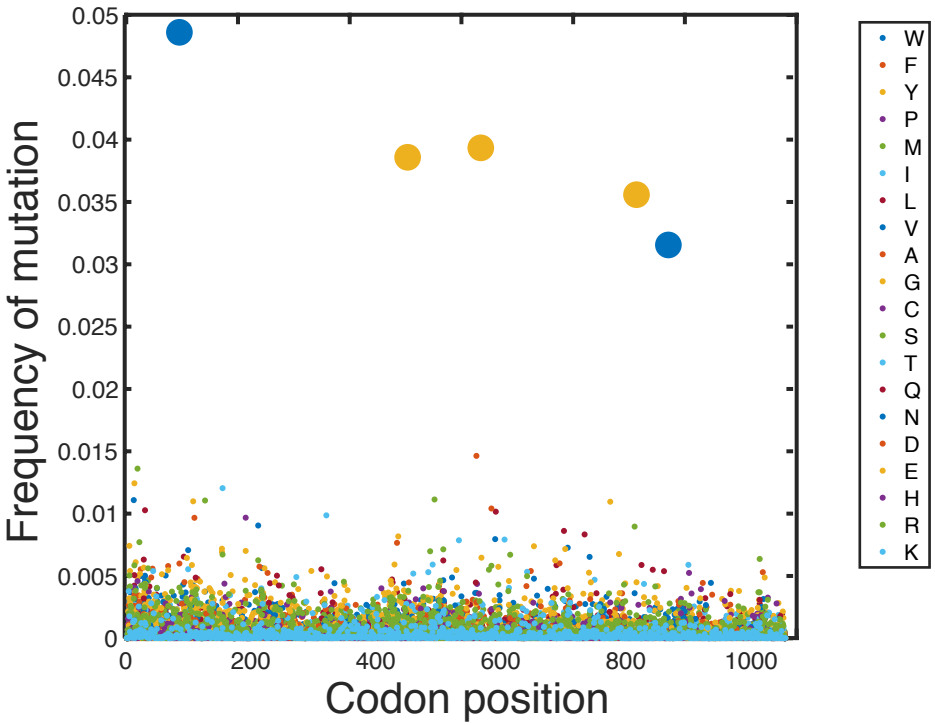
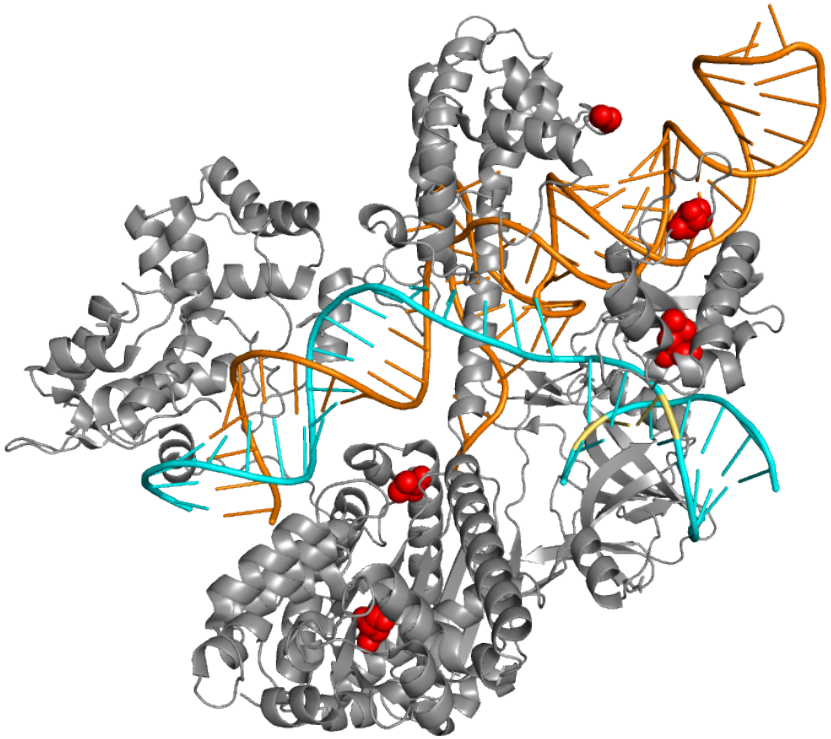


Libraries have increasing fitness during evolution



Identified hits via deep sequencing

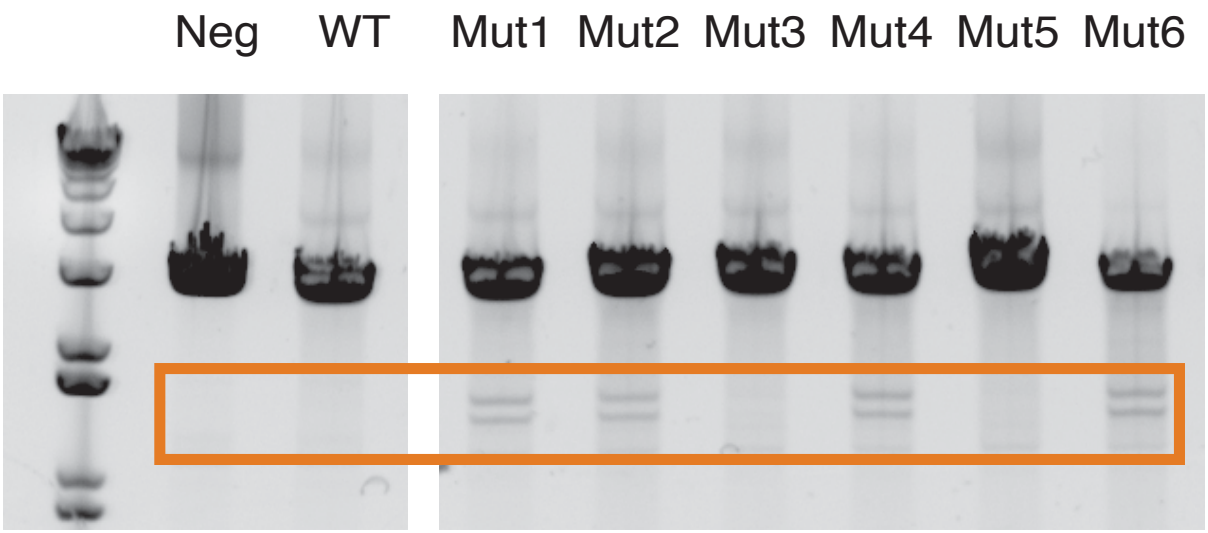




DNA
RNA
Enriched mutations
Altered PAM base

pdb:5AXW

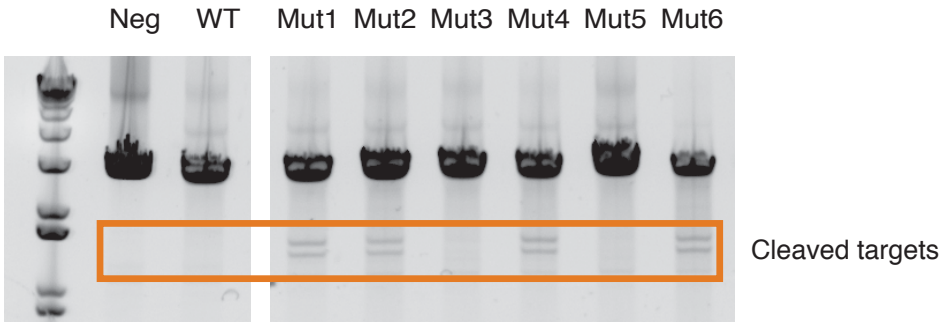
Combinations of enriched mutants are functional for novel PAM cleavage

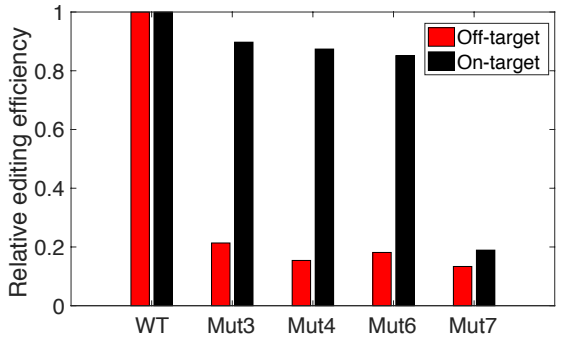


We can evolve towards new sequence dependencies

Conclusions

- SMART mutagenesis creates highly diverse libraries
- Phage can be used in competitive evolution of nuclease activity
- We can selectively dial-down off-target activity while retaining on-target efficacy
- We can engineer new PAM-changing enzymes





Acknowledgements

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SMART libraries have low bias