Directed evolution of Cas9 to reduce identified off-target cleavage

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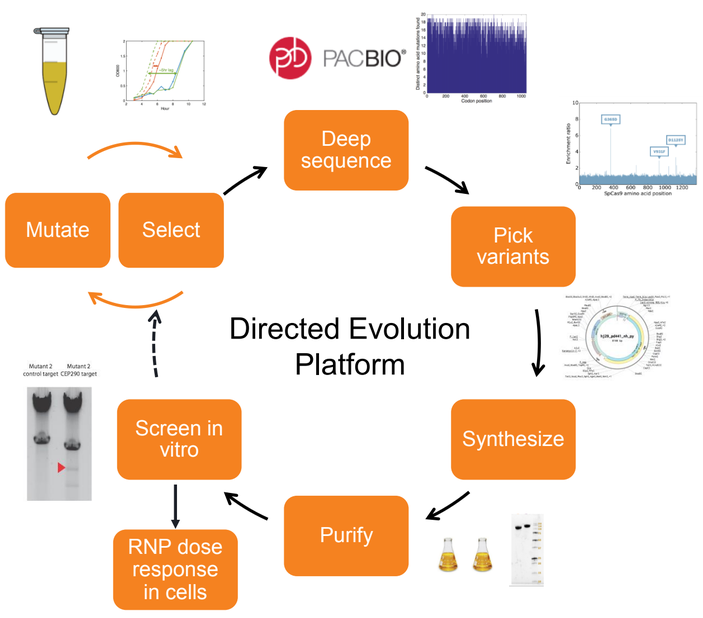
Abstract

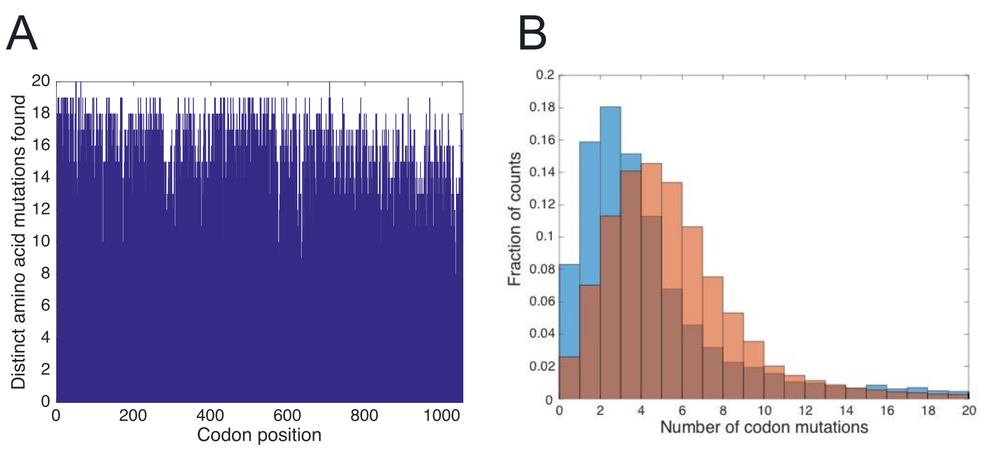
CRISPR/Cas9 has wide reaching scientific and therapeutic applications, but off-target cleavage can potentially represent a safety risk in therapeutic use. Several cas9 variants (Hi-Fi Cas9, e-Cas9) have been engineered to generally increase specificity; however, off-targets may still remain, especially when limited in guide choice. Further these variants are known to have reduced efficacy at some on target sites. In these cases it may be more beneficial to selectively mitigate specific off-targets while maintaining on-target activity. We identified three potential off-targets via GUIDE-Seq when treating T-cells with a given cas9-guide complex. In order to quickly engineer new nuclease variants with decreased cleavage of these off-targets, we developed a directed evolution platform to positively select for DNA cleavage in bacteria at on-targets and negatively select against undesired target cleavage. Our system allows rapid mutation of all amino acids throughout the protein, expanding potential diversity of functional mutants. These mutant libraries are challenged with phage containing target sites in a competitive pool. Using this method, we develop *S. pyogenes* cas9 variants which have maintained on-target cleavage efficiency but have reduced cutting at off-target loci, providing methods to potentially rescue promiscuous guides for therapeutic use.

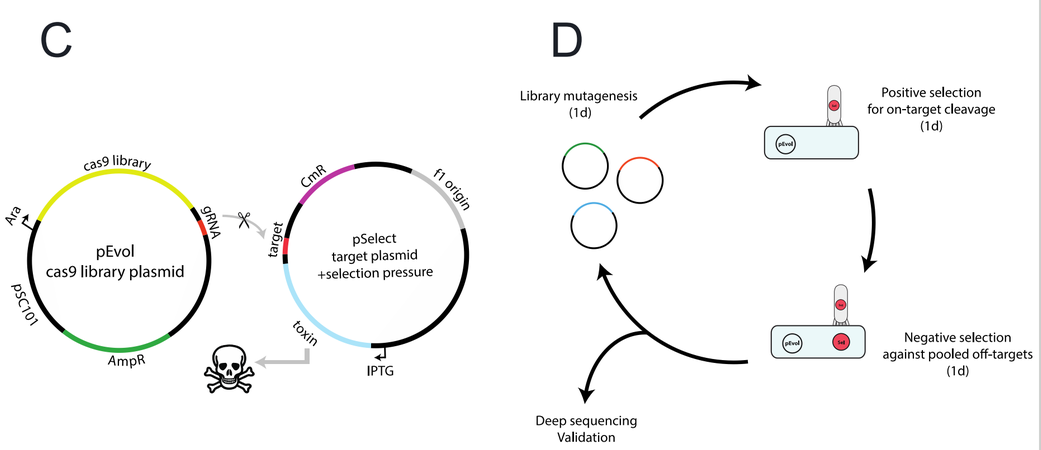
Directed Evolution Overview



Our directed evolution platform begins with generating SpCas9 libraries and subjecting them to several rounds of selection. After deep sequencing of selected libraries, we synthesize and purify mutants of interest for further downstream characterization.

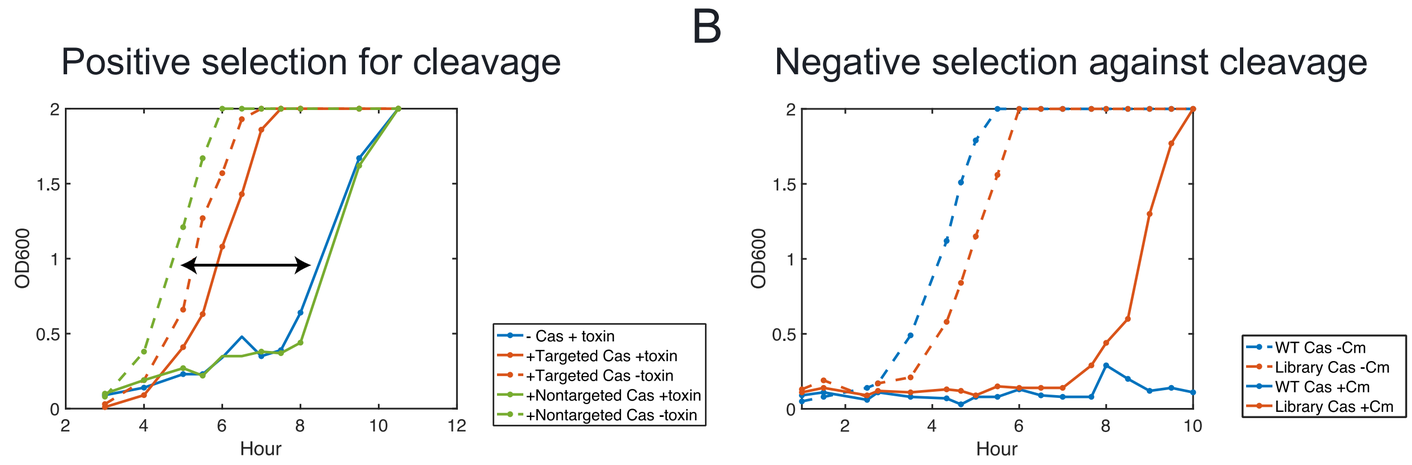
SMART library generation comprehensively mutates every amino acid





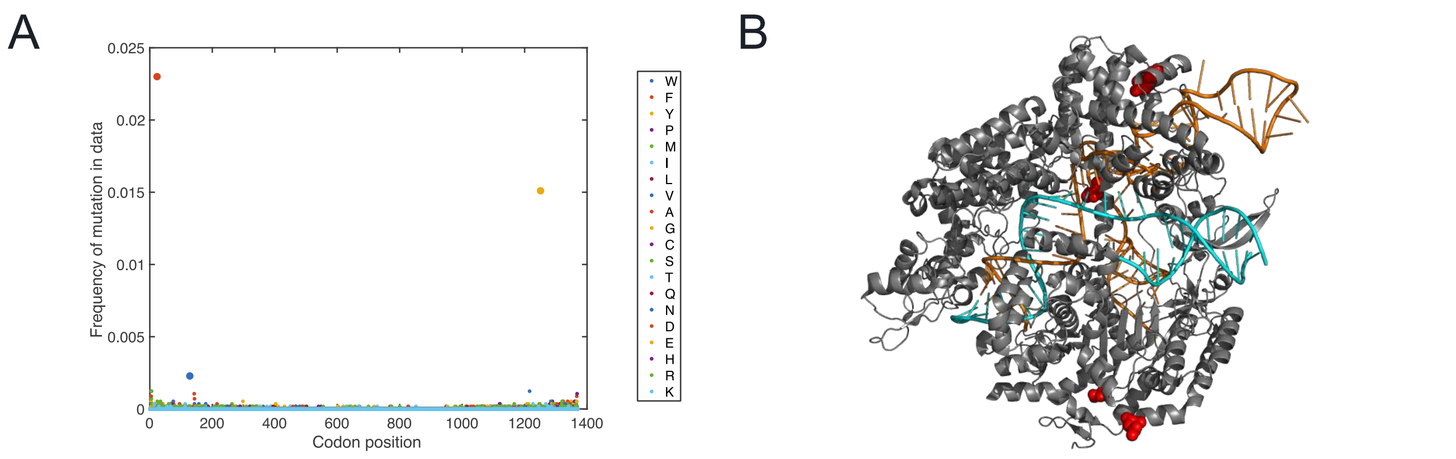
Mutant SpCas9 libraries were engineered using scanning mutagenesis at random targets (SMART). The process begins with a modified PCR step, in which forward oligos, containing codon mismatches, introduce degeneracy at every amino acid position across the protein (**A**). Undesired reaction products were removed from the library with a cocktail of nucleases. The number of mutations can be adjusted depending on reaction conditions (**B**). Libraries were then transformed into *E. coli* and challenged with phage for three rounds of both positive and negative selection (**C, D**).

Selection against off-target cleavage is effective in liquid culture



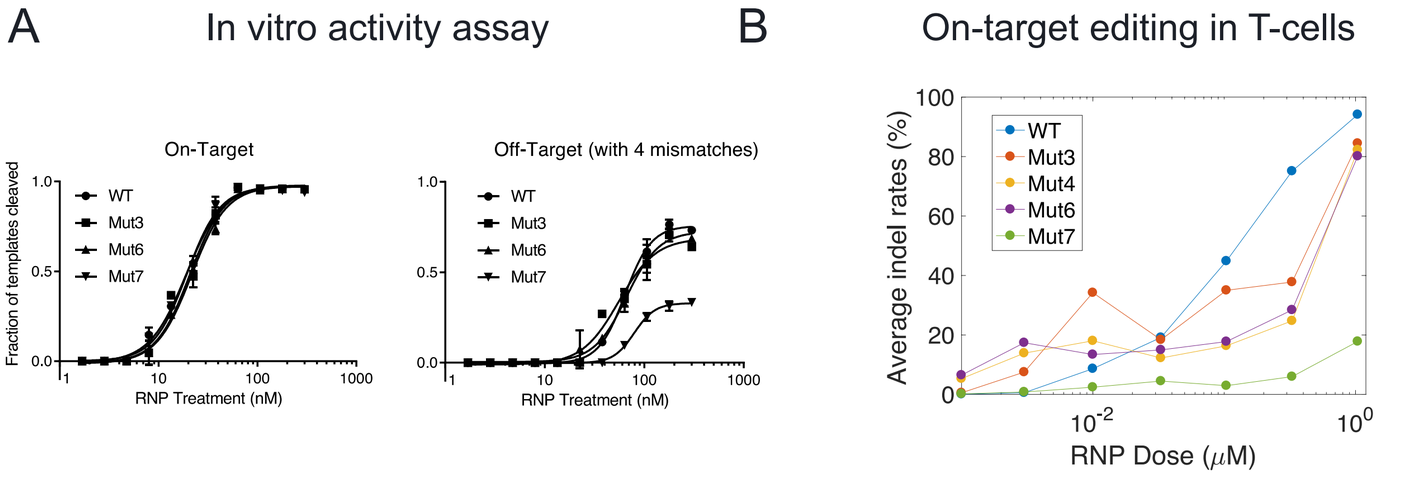
We challenge SpCas9 library plasmids to cleave phage DNA containing a given target. Positive or negative selection can be induced depending on desired activity of cleavage of each target. Growth curves were measured under varying conditions to assess the selection stringency of our system. The toxin greatly reduces the growth rate of cells in liquid media, as indicated by the solid green and blue lines (**A**). Expression of Cas and successful Cas9 cleavage of the target rescues growth and provides a competitive advantage, as shown by the black arrow. Phage can also be used for negative selection by selecting for cells retaining chloramphenicol resistance (**B**) Because our platform allows for competitive selection in liquid culture, we can challenge variants against several selective pressures in a single round. Each round also functions as a true selection rather than a screen for a single cleavage event, as seen in other platforms.

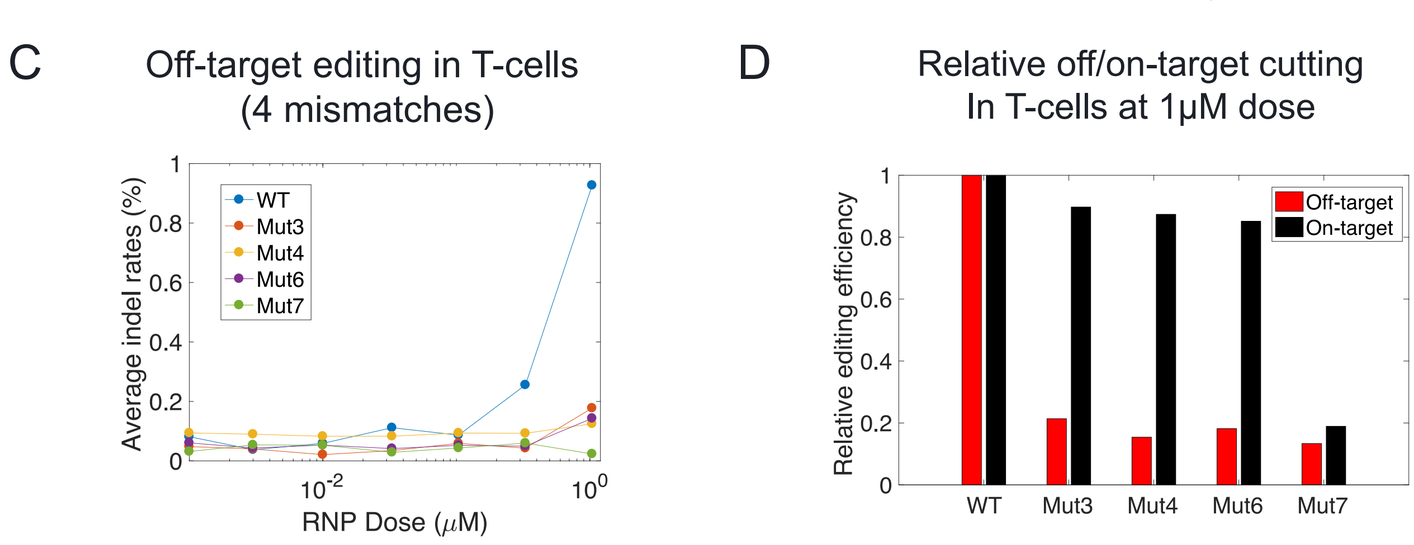
Deep sequencing identifies mutants of interest



After three rounds of directed evolution, libraries were subjected to PacBio next-generation sequencing. Several residues were enriched throughout the protein (as shown in red spheres). Combinations of these mutations were synthesized and tested *in vitro*.

Mutants have reduced off-target editing in T-cells while maintaining similar on-target activity





We synthesized seven mutant proteins to test in vitro (**A**) and in T-cells (**B-D**). Cells were treated with each variant in a dose-response followed by amplification at on- and off-targets and next-generation sequencing to determine indel rates at each locus. All synthesized variants showed decreased relative off-target editing at an off-target site relative to the wildtype enzyme (WT), while many variants maintained comparable levels of editing efficiency at the on-target site. In vitro specific activity indicates that genomic context is important for relative function of these nucleases pointing to the merits of a cellular selection for positive and negative selection for on and off-target editing.