Metagenomic Discovery of Type VI-D CRISPR Effectors for Transcriptome Engineering

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ABSTRACT

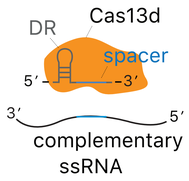
The development of CRISPR-Cas9 into a programmable DNA-editing tool is enabling researchers to control the genetic code at the heart of all life. Recently, our group mined metagenomic data to identify a new subtype of CRISPR effectors that instead cleave RNA, known as Cas13d. Members of this family include CasRx, a highly active ortholog that mediates robust RNA-knockdown with few off-target effects. We also developed assays which show that these members exhibit distinct cytotoxic profiles in human cells. Understanding these behaviors is necessary before Cas13d can be developed into a useful tool. For instance, some variants become highly toxic when paired with a targeting sgRNA; others, like CasRx, appear basally toxic but in a non-targeting-dependent manner. This motivates the search for new orthologs that may express the right properties suitable for downstream application.

Here, I outline a framework for the discovery and characterization of these new effectors, via a metagenomic extension of the Cas13d family. This consists of two parts: a computational phase to algorithmically discover putative orthologs, and an experimental phase to assess their activity and cytotoxicity as RNA-editors. Results from this project will aid in developing Cas13d into a truly versatile transcriptome engineering tool.

INTRODUCTION & BACKGROUND

Microbes get sick too!

- CRISPR is a prokaryotic immune system (Clustered Regularly Interspaced Short Palindromic Repeats)
- Fingerprint-like stretches of invader DNA, called spacers, are integrated into host genome
- Spacers are separated by direct repeats (DRs)
- Upon attack, host ncRNAs recognize foreign nucleic acids and guide endogenous Cas (CRISPR associated) enzymes to destroy them
- Cas13d uniquely targets RNA

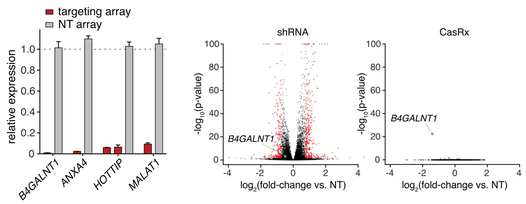


Why transcriptome engineering?

- Impermanent, unlike gene editing with Cas9
- Transcripts are tissue- and cell-dependent; DNA is not
- RNA-knockdown, mRNA splice modulation, base editing

Why Cas13d?

- Robust (1) and highly specific (2) RNA-knockdown



1. Strong knockdown of 4 target transcripts in HEK-293FTs. qPCR relative to GFP.

2. mRNA knockdown specificity using RNAi and CasRx. RNA-seq relative to non-targeting shRNA/guide.

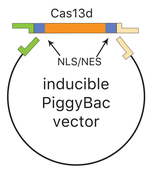
- Compact enough (~930 a.a.) for transduction with A.A.V.
- Simplicity: single-effector (Class II) complex, processes its own array
- Harness cytotoxic profiles for various applications

ACTIVITY

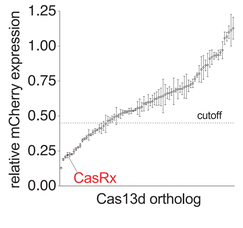
METHODS

Fluorescent reporter knockdown (mCherry) or immunoprecipitation (CD81, CD58)

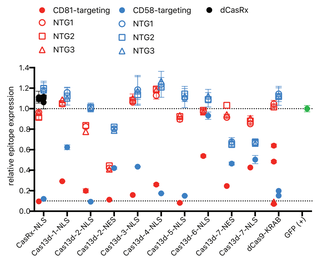
- For each ORF/DR, clone inducible constructs with NLS/NES, guides
- Constructs integrate into K562 cells upon addition of PiggyBac transposon; in 293FTs, can transiently transfect
- Quantify via flow cytometry (FACS)
- Non-targeting controls



RESULTS



1. mCherry-targeting activity panel in 293FTs, relative to NT. CasRx is one of the most active orthologs.



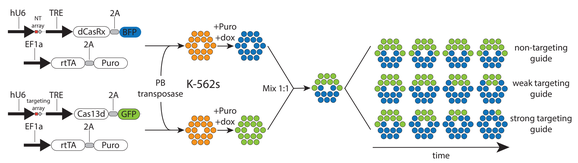
2. CD81 & CD58-targeting activity panel in K562s, relative to GFP. Cas13d-1, -3, and -6 exhibit weak activity.

CYTOTOXICITY

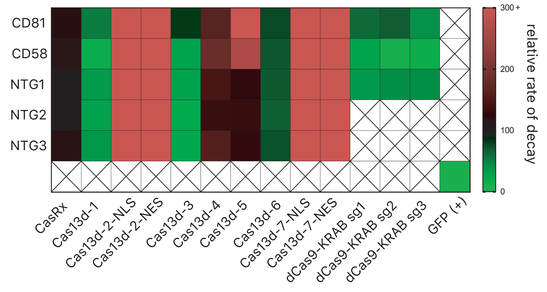
METHODS

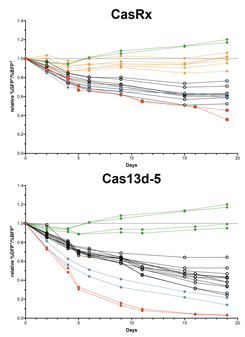
Depletion-in-competition assay over 1 month

- Assess basal toxicity with EF1a-BFP-2A-Puro control



RESULTS





Cytotoxic Profile No. 1
Somewhat basally toxic, but no strong target-dependence

- Suitable for pooled transcriptome screens, some imaging/sensing
- Targeting condition will not confound data

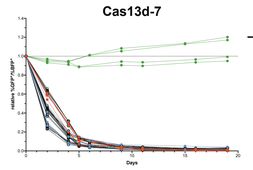
Improve: less basal toxicity

Cytotoxic Profile No. 2
Highly stratified clustering, with clear target-dependence

- Therapeutic that destroys diseased cells expressing an ectopic transcript (e.g. HPV)
- Selection agent for cell culture

Improve: less basal toxicity, stronger clustering

CYTOTOXICITY, cont'd

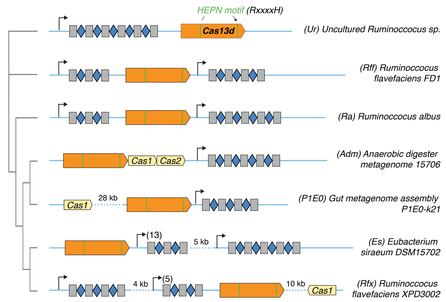


Cytotoxic Profile No. 3
Highly toxic in all cases. Unsuitable for further development

Along with "Profile Zero", the ideal non-toxic ortholog safe for in-vivo work, there are myriad applications for Cas13d

COMPUTATIONAL PIPELINE

We previously identified Cas13d by mining assembled prokaryotic genomes for motifs associated with CRISPR loci.



Now that this family is known, it can be expanded by searching sequence data for structurally similar proteins, using bioinformatics tools:

Download assembled prokaryotic genome + metagenome data from online repositories (e.g. NCBI WGS)

↓ >20 Tb, .fasta format

Create BLAST-compatible databases. Identify putative orthologs using TBLASTN and known effector sequences

↓

Tabulate results; extract contigs containing hits

↓

Search contigs with CRISPRFinder to identify direct repeats (DRs)

↓

Dedupe (vs. previously identified effectors)

↓

Cluster with multiple sequence alignment and build maximum-likelihood phylogenetic tree

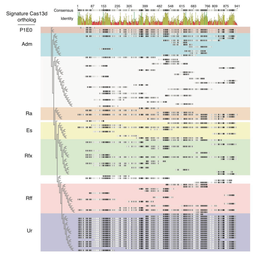
↓ ClustalOmega, PhyML

Select candidate orthologs to synthesize and characterize

Candidates will be chosen by comparison with known behaviors of sequence-similar effectors. Additional criteria will include e.g. size (affects transduction capability)

Preliminary results: when run on a 180 Gb subset (<1% current total), identified 82 DRs

NEXT STEPS



1. Finalize pipeline and synthesize candidate effectors

2. Clone PiggyBac constructs for subsequent experiments

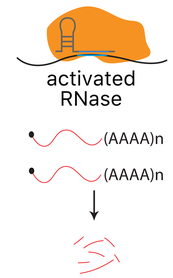
- Gibson/Golden Gate Assembly
- Tet/dox-inducible expression, including GFP
- Flanking NLS, NES motifs
- Puromycin resistance

3. Assess ribonuclease activity

- CD81, CD58, NTG guides
- Integrate into K562s (PB transposase)
- Puro selection to establish cell line
- Induce expression with doxycycline; immunoprecipitate after 72 hours
- Quantify knockdown with FACS

4. Assess cytotoxicity

- Transfer cell line to competitive depletion assay
- Calculate non-linear regression to determine relative rates of decay
- Compare cytotoxic profiles



5. Analyze results; develop orthologs with high activity and desired cytotoxic behavior for RNA-targeting applications

DISCUSSION & FUTURE DIRECTIONS

Cas13d is a promising new tool for the robust modulation of gene expression at the RNA level, including:

- Precise RNA-knockdown
- Transcript imaging and sensing

By harnessing ortholog-specific cytotoxic behavior, we can further develop it for a variety of applications, such as:

- Transcriptome-wide genetic screens
- Targeted cell destruction for therapeutic use
- A selection agent in cell culture
- Ideally, in-vivo work

Ultimately, we hope that by characterizing new orthologs, these possibilities will one day make Cas13d a versatile and powerful addition to the bioengineering toolkit.

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