Life Sciences

# Engineered High-Fidelity Cas13 Variants with Minimal Collateral RNA Targeting

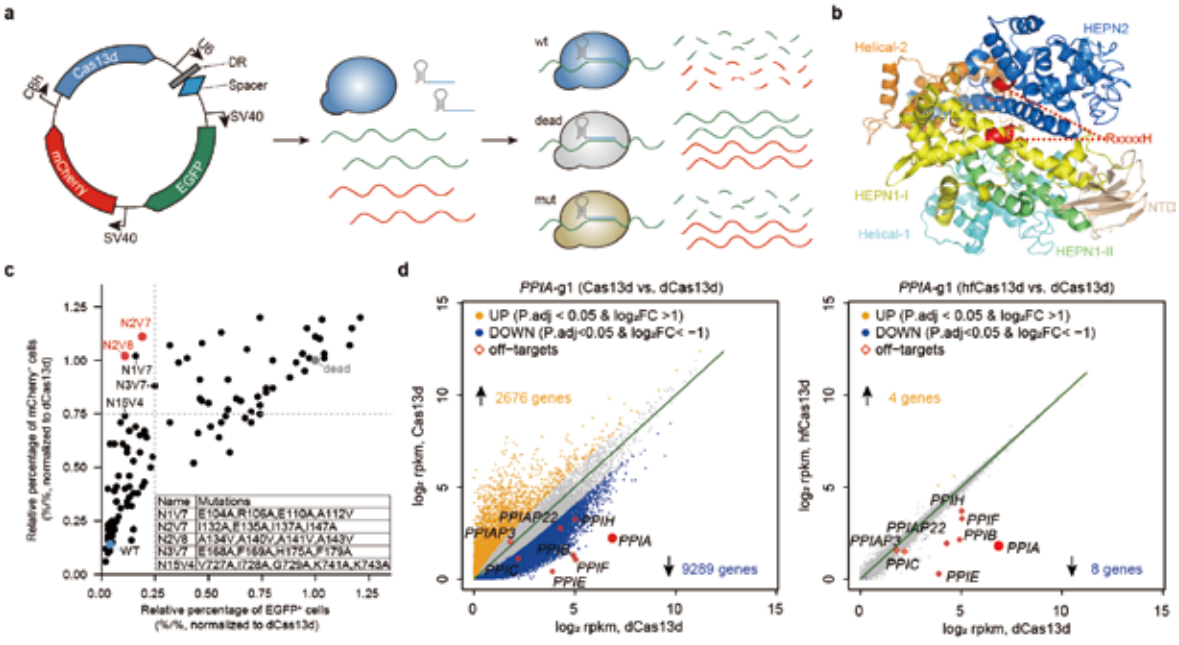
Using CRISPR-Cas13 for targeted RNA degradation is hindered by the collateral damages induced by Cas13. CAS scientists screened hundreds of engineered Cas13 variants and obtained several hits with markedly reduced collateral activity and efficient on-target activity, suitable for in vivo applications.

On August 12, 2022, a *Nature Biotechnology* study revealed the development of high-fidelity Cas13 (hfCas13) variants with markedly reduced collateral effects by mutagenesis. It demonstrated the feasibility of high-fidelity Cas13 (hfCas13) for efficient on-target RNA degradation with almost no collateral damage in mammalian cells and animals.

This work was performed by researchers in Dr. YANG Hui's Lab at the Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology of the Chinese Academy of Sciences, State Key Laboratory of Neuroscience, and the R&D team from HuiGene Therapeutics Co., Ltd.

The CRISPR-Cas13 system is a highly efficient tool for programmable RNA targeting and has been harnessed for nucleic acid detection and RNA manipulations in various cell types and organisms. However, concerns have been raised over the off-targeting effects of Cas13.

Previous structural studies have implicated that upon binding of Cas13 to target RNA, the two HEPN nuclease domains could form a catalytic site on the protein surface, providing the potential for promiscuous




Design, screening, and characterization of engineered high-fidelity Cas13 variants. (Image by YANG Hui's team)

cleavage of bystander RNAs apart from the on-target cleavage of target RNAs.

Due to this so-called collateral effect, Cas13 could degrade both target and non-target RNAs at random, thus making it challenging to design experiments and interpret results when using Cas13. Therefore, attempts to diminish or eliminate promiscuous RNA degradation via engineering approaches are required for basic research and future in vivo applications of the Cas13 system.

The researchers designed a dual-fluorescent reporter (EGFP & mCherry) plasmid system to detect the collateral effects of Cas13 in mammalian cells. In this system, loss of mCherry fluorescent cells is interpreted as successful on-target editing; loss of EGFP fluorescent cells is considered to represent off-target cleavage.

Results showed that either Cas13a or Cas13d could induce significant collateral effects. Then the researchers sought to engineer Cas13d (RfxCas13d or CasRx, Cas13d from Ruminococcus flavefaciens XPD3002) via mutagenesis and screen for variants with minimal collateral effects based on a new well-designed dual-fluorescent reporter system comprised of a single plasmid containing EGFP, mCherry, and EGFP-targeting gRNA, together with each Cas13 variant.

YANG Hui's team designed and generated a mutagenesis library of Cas13d variants and individually transfected them into HEK293 cells. By analyzing reporter fluorescence using flow cytometry, five variants (N1V7, N2V7, N2V8, N3V7, and N15V4) exhibited a relatively low percentage of $EGFP^+$ cells but a high percentage of $mCherry^+$ cells, indicating high on-target activity with reduced collateral activity.

The variant N2V8, high-fidelity Cas13d (hfCas13d) for its highest specificity for RNA degradation, was used for further characterization, including transcriptome-wide off-target analysis using RNA sequencing. The hfCas13d showed a marked reduction in the number of off-target genes targeting several endogenous transcripts, such as PPIA. Moreover, hfCas13 variants showed no detectable collateral damage in cell lines, transgenic animals, and somatic cells, supporting their *in vivo* applications.

Importantly, YANG Hui's team identified the CRISPR-Cas13X system in 2021, the smallest (only 775 amino acids) RNA editing tool. In this new study, the researchers further engineered Cas13X and obtained the hfCas13X variant exhibiting high specificity of on-target RNA degradation but minimal collateral effects. hfCas13X will show great application potential in gene therapy based on RNA editing.

The researchers caution that hfCas13d could still induce mild collateral cleavage activity under conditions whereby hfCas13d or targeted transcripts are expressed at very high concentrations. However, the researchers stated that they could bypass this side effect in most applications or achieve cleavage free of collateral effects via gRNA selection.

"Current versions of the CRISPR-Cas13 system have deficiencies for *in vivo* applications. Here, the authors have performed a comprehensive mutagenesis screen to obtain high-fidelity Cas13 variants that have minimal collateral cleavage activity, making them more suitable for future therapeutic applications," commented the Editorial Team, *Nature Biotechnology*.

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**References**

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