Precise and Titratable Regulation of Gene Editing Activity of a Small Cas9 Nuclease by an FDA-approved Small Molecule Drug Using cytoDRiVE® Technology

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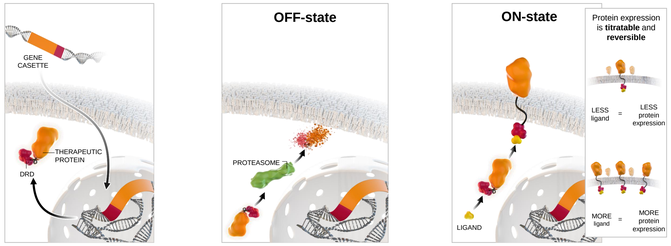
Abstract 531

Abstract

Gene editing has the potential to revolutionize the treatment of many genetic diseases and several *in vivo* gene editing therapies have recently entered the clinic. To increase their safety and efficacy, precise spatial and temporal on-target editing with minimal off-target editing is highly desirable and will require tight intrinsic or extrinsic control of the gene editor. To this end, we have developed small drug-responsive domains (DRDs) as part of our cytoDRiVE® platform that can be fused to Cas9 nuclease. This enables precise control of its expression and activity by exposure to a clinically approved small molecule activating drug. The small size of these DRDs requires minimal vector space and will allow delivery of DRD-Cas9 and guide RNA in a single AAV vector. We show that the DRD-Cas9 fusions have extremely low editing activity in the absence of drug which increases to near constitutive levels of activity upon treatment with activating drug for a panel of guide RNA targets. In addition, we show that fusion of DRDs to Cas9 does not affect target specificity or repair patterns of the nuclease. This level of control demonstrated *in vitro* by our cytoDRiVE® platform supports potential future clinical utility in cell and gene therapies, where tight regulation of DRD-Cas9 would give the physician control of Cas9 activity in the patient. Pausing or stopping administration of the activating drug once edits have been made could limit off target editing activity and reduce the risk of genotoxicity.

cytoDRiVE® technology



Engineer gene cassette for therapeutic protein with DRD tag; package & insert into cell

Cell produces fusion protein with DRD tag that is rapidly degraded by proteasome (basal function is OFF)

Addition of FDA-approved small molecule ligand stabilizes DRD; protein is expressed and accumulates to functional levels in a **titratable** & **reversible** manner

Applications for cytoDRiVE® technology

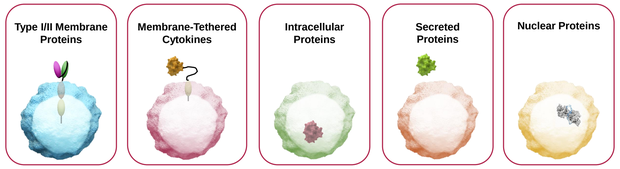


Figure 1. Overview of regulation of therapeutic proteins with cytoDRiVE® technology. Drug responsive domain (DRD) fused to protein of interest (POI) confers titratable and reversible control of the fusion protein using FDA approved drugs. When the DRD-POI fusion protein expressing cells are treated with the activating drug (ON-state), the fusion protein is expressed and POI can function, but in the absence of the drug (OFF-state), the fusion protein is degraded. DRDs have been shown to regulate many different types of therapeutic proteins and have the potential to be applied to multiple therapeutic areas.

Novel FKBP13 DRDs identified by pooled screen

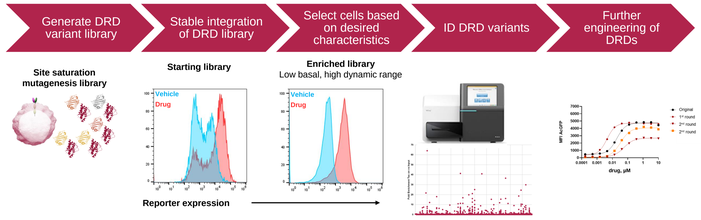
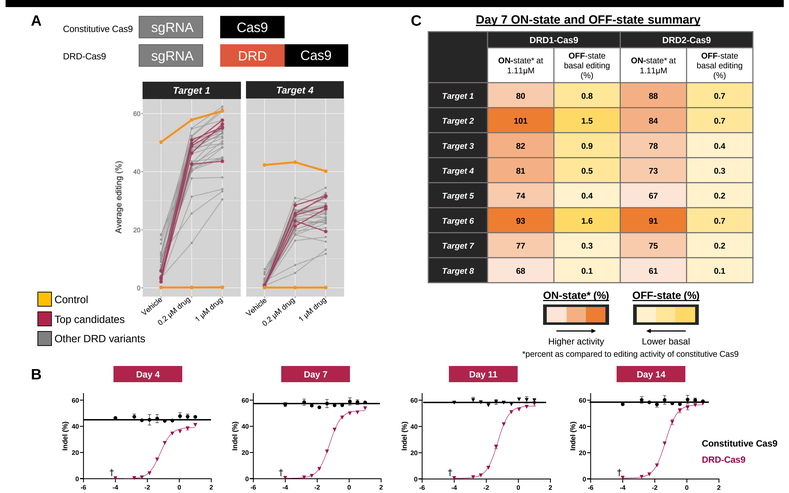


Figure 2. Novel small FKBP13 derived DRDs were identified using saturation mutagenesis library pooled screen. Saturation mutagenesis library of FKBP13, a FK506 binding protein, DRD variants fused to GFP fluorescent reporter was screened in cell line pool by iterative sorting process to select for drug dependent regulation of GFP expression. The hits in the final library were sequenced by NGS to identify enriched DRD variants. The identified DRD variants were engineered further to improve the basal expression and dynamic range.

DRDs tightly regulate Cas9 on-target editing activity



	DRD1-Cas9		DRD2-Cas9	
	ON-state* at 1.11µM	OFF-state basal editing (%)	ON-state* at 1.11µM	OFF-state basal editing (%)
Target 1	80	0.8	88	0.7
Target 2	101	1.5	84	0.7
Target 3	82	0.9	78	0.4
Target 4	81	0.5	73	0.3
Target 5	74	0.4	67	0.2
Target 6	93	1.6	91	0.7
Target 7	77	0.3	75	0.2
Target 8	68	0.1	61	0.1

Figure 3. Novel FKBP13 DRDs regulate Cas9 nuclease activity in dose-dependent manner and DRD-Cas9 maintained low basal editing over 14 days. (A) Novel DRD candidates fused to Cas9 were introduced into HEK293T cells along with sgRNA by lentivirus transduction. Comparing NGS data from vehicle and drug treatment samples for target editing, drug dependent activity of Cas9 was confirmed and top DRDs were identified to be characterized further. (B) Top three DRD candidate-Cas9 fusion proteins were transduced into HEK293T cells along with sgRNA for 8 targets, dosed with titrating dose of the activating drug continuously, samples were collected over 14 days, and editing efficiency was measured by NGS. Example data of one target with one DRD is shown here. †: vehicle treatment sample (concentration of 0.0001 used to allow display on log scale.) (C) Summary table of ON-state editing as compared to constitutive Cas9 and OFF-state editing from dose response data of 1.11uM treatment group at day 7 time point with top 2 DRD-Cas9 candidates and 8 different targets. High level of editing with drug treatment at ON-state and low level at basal OFF-state without drug was observed. Target 1 values for DRD2-Cas9 correspond to data shown in Fig 3B (Day 7).

OFF-state DRDs lower Cas9 off-target editing activity

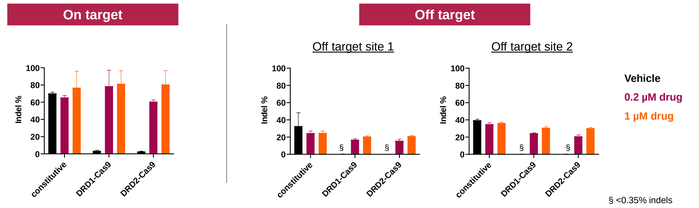


Figure 4. DRD regulated Cas9 results in minimal off-target editing in the OFF-state and similar off-target editing as compared to constitutive Cas9 in the ON-state. Cells expressing constitutive Cas9 or DRD-Cas9 along with a suboptimal VEGF sgRNA known to have high off-target editing were treated with vehicle, low dose drug, or high dose drug for 7 days continuously. Samples were collected and NGS assay was completed to measure the editing efficiency at the on-target site and at previously identified two off-target sites (site 1 and 2) for the gRNA. Vehicle treated DRD-Cas9 samples show below 0.35% editing as measured by indels.

DRDs regulate Cas9 activity by controlling protein expression

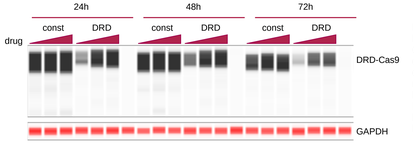


Figure 5. DRD-Cas9 nuclease protein expression is induced within 24 hours of drug treatment. Cells expressing DRD-Cas9 or constitutive Cas9 along with sgRNA were treated with vehicle, low dose, or high dose drug. Samples were collected 24, 48, and 72 hours post treatment and cell lysates were used for ProteinSimple Jess capillary Western blot to measure DRD-Cas9 and GAPDH (housekeeping) protein expression level. This is an example data from a panel of triplicate data with two DRDs. This data along with data showing lack of regulation at the mRNA level (data not shown) suggests that the regulation occurs at the protein level as expected for DRD-POI regulation.

Conclusions

- We have identified **novel small FKBP13 derived Drug Responsive Domains (DRDs)** regulated by an **FDA approved activating drug** using a high throughput screen method
- DRD-Cas9 maintained **low OFF-state activity** for 14 days in the absence of activating drug and achieved **high level of ON-state activity** with drug treatment
- DRD-Cas9 in the OFF-state results in **reduced off-target activity** and in ON-state results in similar off-target activity as compared to constitutive Cas9
- Treatment with activating drug resulted in **increase in DRD-Cas9 protein expression within 24 hours of treatment** and **decrease in protein expression without further treatment** to demonstrate successful drug dependent expression of DRD-Cas9 protein which results in tight control of Cas9 editing activity