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CRISPR/saCas9 and CRISPR/spCas9 systems for combinatorial genetic screens (CRISPR-KO, CRISPRa, CRISPRi)

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Abstract

This study explores the utilization of orthogonal CRISPR-based gene editing/modulation systems for combinatorial genetic screens using CRISPR knockout (CRISPR-KO), CRISPR activation (CRISPRa), and CRISPR interference (CRISPRi) functionalities. S. aureus (sa) Cas9 is an alternative nuclease to S. pyogenes (sp) Cas9 in scenarios where the latter cannot be used, or when multiple independent CRISPR systems need to be simultaneously expressed in the same cell. In this study we set out to explore the feasibility of utilizing different combinations of saCas9 and spCas9 CRISPR systems to achieve the simultaneous inactivation (via CRISPR-KO or CRISPRi) and transactivation (via CRISPRa) of different target genes in the same host cell. For this purpose, a complete set of tools for CRISPR/saCas9 gene editing and gene modulation was developed, compatible with CRISPR/spCas9 coexpression. Specifically, we developed and validated optimized saCas9 and sg(sa)RNA lentiviral and AAV vectors, dual expression (sp)/(sa)sgRNA lentiviral library vectors, as well as (sa)CRISPR-KO, (sa)CRISPRi, (sa) CRISPRa fluorescence-based activity kits for the functional validation of saCas9 expressing cell lines. Results demonstrating the feasibility of the orthogonal screens will be presented, with different combinations of CRISPR-KO, CRISPRa, and CRISPRi systems in multiple cell lines.

Introduction

- Combinatorial genetic screening using a pooled CRISPR-Cas9 library is a powerful approach to identify the genes for drug susceptibility and resistance, tumorigenesis, metastasis, cell differentiation and other biological responses.
- However, to uncover synthetic response, gene redundancies, and more complex gene network interactions, more sophisticated screening approaches that perturb more than one gene target at a time are needed.
- To increase the efficiency of combinatorial screening, we employ orthogonal Cas9 enzymes from S. aureus and S. pyogenes.

Method

Measuring saCas9 Activity in Cells Engineered for CRISPR Knockout

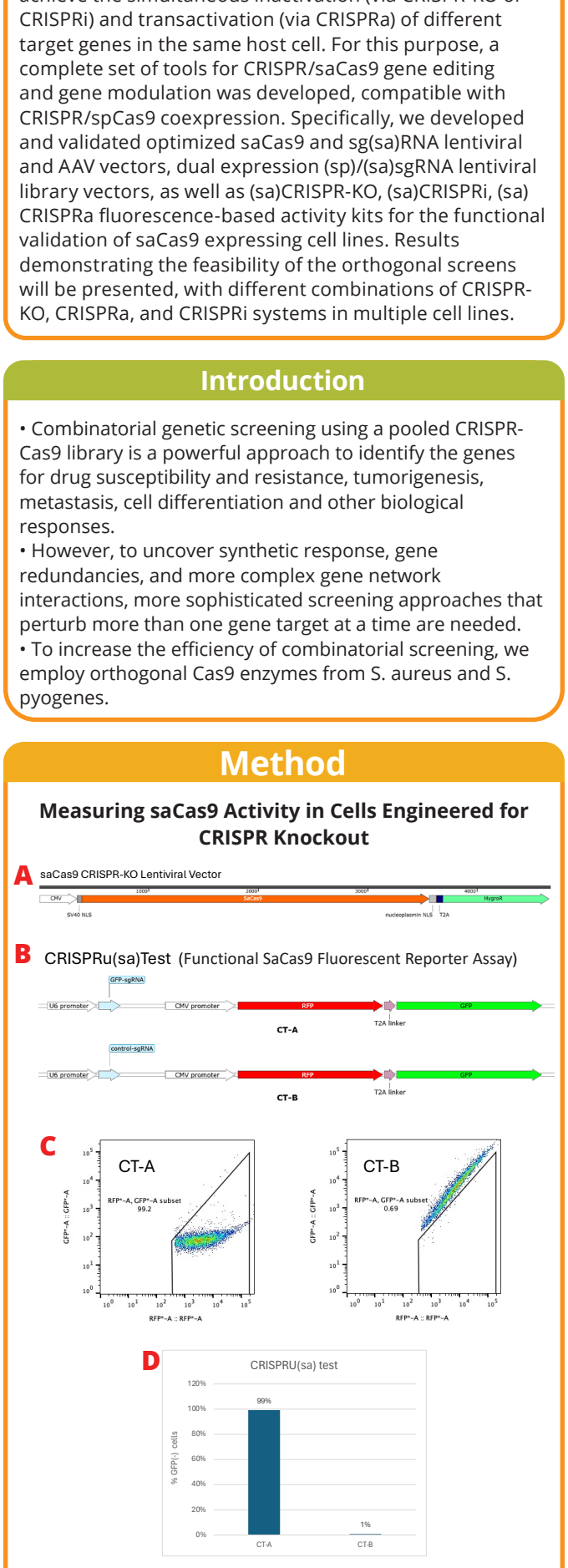

Fig 1: We have developed saCas9 expressing lentivectors, and an assay to measure the CRISPR-KO activity of saCas9 expressing cells **(A).** The assay makes use of two different reporter constructs that express two different (sa)sgRNAs, as well as a polycistronic mRNA for the co-expression of the GFP and RFP markers. One construct expresses a functional sgRNA designed to knock out GFP (GFP-sgRNA)—the other construct, which is used as a control, expresses a non-functional sgRNA (control-sgRNA) **(B).**

Two samples of Cas9-expressing HCT116 cells were each transduced with one of the two reporter constructs. 6 days post transduction, cells were analyzed by flow cytometry. Cells transduced with the functional GFP-sgRNA construct lost GFP fluorescence compared to RFP fluorescence, whereas cells transduced with the control-sgRNA construct did not **(C).** GFP fluorescence was not detected in >98% of the HCT116-(sa)Cas9-Hygro cells transduced with the active GFP-sgRNA construct (CT-A), compared to only 1% GFP+ cell loss in the control-sgRNA cells (CT-B) **(D).**

Method (cont.)

CRISPRi(sa) Assay with (sa)dCas9

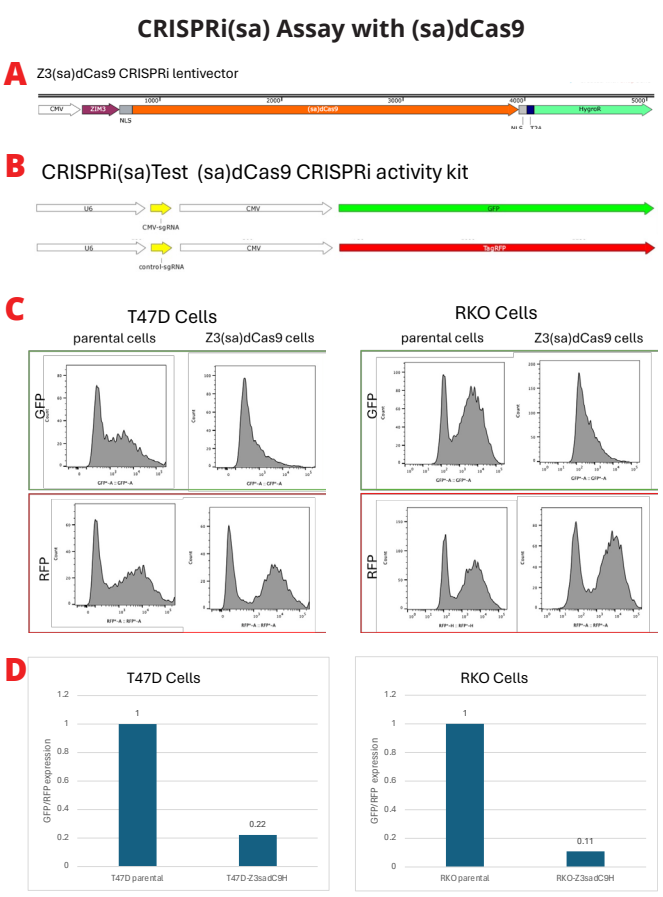

Fig 2: We developed (sa)dCas9-KRAB(ZIM3) expressing lentivectors, and an assay to measure the CRISPRi activity of (sa)dCas9-Repressor expressing cells. The Repressor variant we used was the Zim3-KRAB domain (Alerasool et al, Nat Methods. 2020) fused to the N-Term of the catalytically inactive (sa)dCas9 [Z3(sa)dCas9] **(A).** Similar to the (sa)Cas9 CRISPR-KO assay, the CRISPRi (sa)dCas9-Repressor reporter assay uses two reporter constructs. However, in this case, each construct only has one fluorescent marker **(B).** The reporter construct with GFP expresses sgRNA that targets the CMV promoter which drives expression of the GFP gene. The reporter with RFP expresses a non-targeting sgRNA.

We assayed two cell lines— human T-47D epithelial line and RKO colon cancer line. Each of the two reporter constructs were transduced into each of the two parental cell lines and the derivative populations engineered to express the Z3(sa) dCas9 CRISPRi protein. Within days, the RFP-normalized GFP expression was greatly reduced in the Z3(sa)dCas9 expressing cells vs. the parental lines **(C).** In 4 days, there was 78% knockdown of GFP in the Z3(sa)dCas9 expressing T-47D cells and 89% knockdown of GFP in the Z3(sa)dCas9 expressing RKO cells **(D).**

Assay for S. pyogenes dCas9-VPH CRISPRa Activity in T47D-CRISPRa/CRISPRi cells

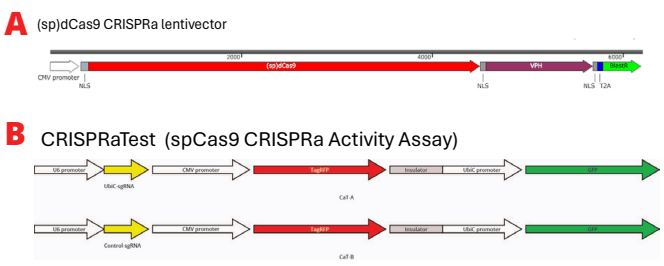

Fig 3: For the purpose of sCRISPRa/CRISPRa orthogonal screens, we also needed to assess the activity of the S. pyogenes (sp) dCas9 CRISPRa activator **(A).** We used our previously developed CRISPRaTest Assay. There are two reporter constructs in this assay—RFP and GFP **(B).** Both fluorescent markers are present on each reporter construct. The GFP gene under control of the UbiC promoter is susceptible to activation by the dCas9-VPH activator complex on the reporter with the UbiC-sgRNA.

With T-47D cells stably expressing (sp)dCas9-VPH-Blast (and Z3(sa)dCas9) 8-fold activation of the GFP fluorescence was seen at 3 days **(C).**

Method (cont.)

Parallel Assay (sp)dCas9-VPH Activation and Z3(sa)dCas9 Repression

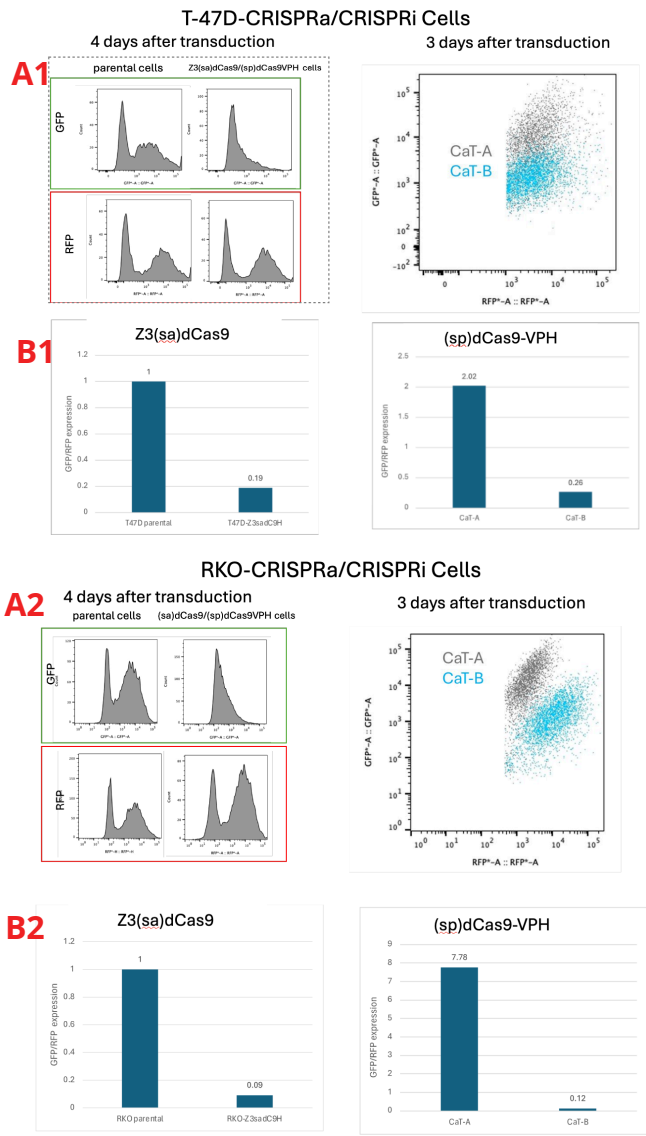

Fig 4: The saCRISPRi and spCRISPRa orthogonal screen requires both CRISPR systems to function in the screened cells. Here we show the flow data of both the Z3(sa)dCas9 inhibition and (sp)dCas9-VPH activation assays in the same cells **(A1, B1).**

Within a few days, a knockdown level of 81% and an activation level of 7.7-fold was observed for the T-47D **(A2)** cells and a knockdown level of 91% and an activation level of 64-fold was observed for the RKO cells **(B2).**

Dual sgRNA Vectors for Complementary Screens

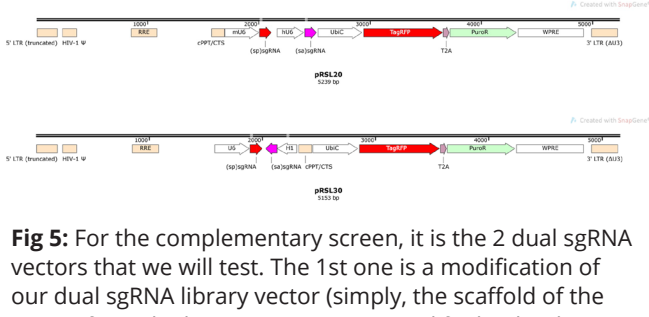

Fig 5: For the complementary screen, it is the 2 dual sgRNA vectors that we will test. The 1st one is a modification of our dual sgRNA library vector (simply, the scaffold of the sgRNA from the hU6 promoter was modified to be the saCas9 sgRNA scaffold). The 2nd one is convergent U6 and H1 promoters, as shown in a published orthogonal screen paper: Orthologous CRISPR-Cas9 enzymes for Combinatorial Genetic Screens - PMC5800952 (nih.gov)

Discussion

- Zim3-(sa)dCas9 works well for CRISPRi.

- Both CRISPRi and CRISPRa function well in single cell line. It is possible to use saCas9 and spCas9 CRISPR systems to achieve the simultaneous inactivation (via CRISPRi) and transactivation (via CRISPRa) in the same host cell.