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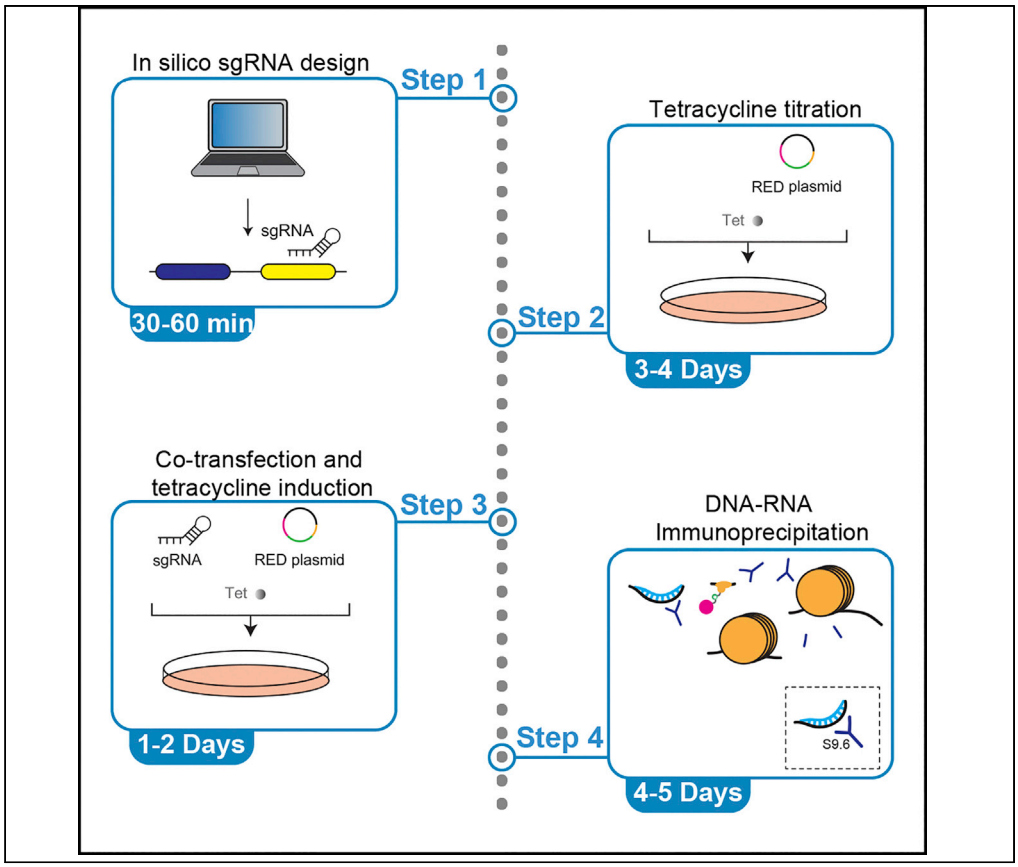
Protocol to use RNaseH1-based CRISPR to modulate locus-associated R-loops



Modulating R-loop triplex nucleic acid structures reveals their roles across the genome. However, common approaches cannot ascribe functions to R-loops in a locus-associated manner. This protocol presents the use of a locus-associated R-loop-modulating system (dubbed LasR), which employs an inducible RNaseH1-EGFP-dCas9 chimaera. We detail the *in silico* design of sgRNAs and their transfection with the chimaera, and outline steps confirming RNaseH1-EGFP-dCas9 expression, localization, locus-targeted association, and R-loop modulation in *cis* or *trans* using immunoblotting, microscopy, and chromatin and DNA-RNA immunoprecipitation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol uses RNaseH1-EGFP-dCas9 in a locus-associated R-loop-modulation system (LasR)

LasR allows for targeted R-loop modulation in cis or trans

Optimize inducible R-loop targeting by altering tetracycline concentration

Confirm R-loop modulation by LasR using DNA-RNA immunoprecipitation

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Protocol to use RNaseH1-based CRISPR to modulate locus-associated R-loops

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SUMMARY

Modulating R-loop triplex nucleic acid structures reveals their roles across the genome. However, common approaches cannot ascribe functions to R-loops in a locus-associated manner. This protocol presents the use of a locus-associated R-loop-modulating system (dubbed LasR), which employs an inducible RNaseH1-EGFP-dCas9 chimaera. We detail the *in silico* design of sgRNAs and their transfection with the chimaera, and outline steps confirming RNaseH1-EGFP-dCas9 expression, localization, locus-targeted association, and R-loop modulation in *cis* or *trans* using immunoblotting, microscopy, and chromatin and DNA-RNA immunoprecipitation.

For complete details on the use and execution of this protocol, please refer to Abraham et al. (2020).

BEFORE YOU BEGIN

The LasR system described here can be used to modulate R-loop levels at a given genetic locus via one of two distinct types of application. First, the chimeric RED or dRED proteins can be targeted to a given locus for the modulation of R-loop levels of another spatially proximal locus (Figure 1) (Abraham et al., 2020). This modulation of R-loop levels in *trans* is facilitated by natural chromosome loops that depend on factors including the CCCTC-binding factor (CTCF) and Cohesin complex (Abraham et al., 2020; Rowley and Corces, 2018). Such loops have been extensively studied using chromosome conformation capture, chromatin immunoprecipitation, and genome-wide sequencing (Kempfer and Pombo, 2020). Second, the RED or dRED chimaera can be targeted directly to an R-loop-harboring locus for R-loop modulation in *cis* (Figure 1) (Khosraviani et al., 2021). The protocol described here is used for LasR targeting to highly repetitive ribosomal DNA repeats (rDNA) (Abraham et al., 2020; Khosraviani et al., 2021). While we have applied this system to achieve human R-loop control, we expect that analogous systems would be equally helpful to the study of R-loops in other species amenable to RNase H1-dependent RNA-DNA control and CRISPR-based genome editing. In addition, this system is very flexible, allowing for the easy substitution of the RNase H1 moiety of RED or dRED with any other R-loop-modulatory factors of interest.

Designing sgRNAs

Timing: 30–60 min

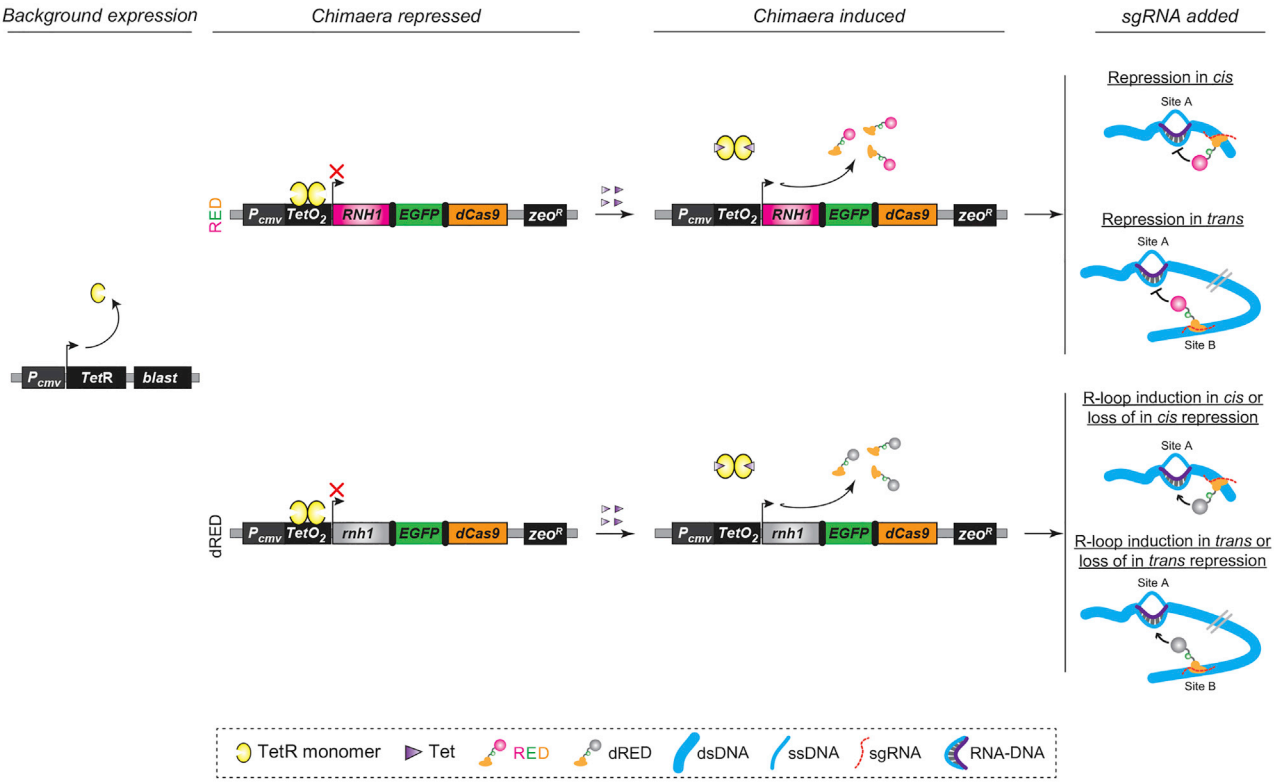

Figure 1. Schematic of the RED/dRED expression system

Expression of the RNaseH1-EGFP-dCas9 (RED) or dRNaseH1-EGFP-dCas9 (dRED) chimaeras is controlled in a tetracycline (Tet)-dependent manner. Experiments are conducted in Tet Repressor (TetR)-expressing cells. Chimeric gene expression is repressed via binding of TetR homodimers to two Tet Operator 2 ($TetO_2$) sequences; only one of which is illustrated for simplicity. Once added, Tet binds to the TetR homodimers, changing their conformation, and releasing them from $TetO_2$ sequences. This release allows the P_{cmv} promoter to drive the expression of the RED or dRED chimaera. By using sgRNAs, the experimenter can enrich the chimeric proteins at a given genetic locus for R-loop modulation in *cis* at the same locus or in *trans* at another spatially proximal locus.

The sgRNAs targeting a locus of interest can be determined using publicly available algorithms such as that provided by Thermo Fisher. Alternatively, the sgRNAs can be custom designed according to specific needs. A pool of three sgRNAs can be used to ensure efficient targeting of RED/dRED to the locus of interest (Abraham et al., 2020). Ideally, the three sgRNAs should be within a 200 bp window around the locus of interest while collectively targeting both DNA strands. When selecting the sgRNA sequences for the pool, it is desirable to select them based on the lowest number of off-target binding sites. Also, loci of interest located within open chromatin domains will be more amenable to dCas9-based targeting of the chimeric protein than loci within silent chromatin domains (Verkuijl and Rots, 2019).

To apply the LasR system for R-loop modulation in *cis*, first test single or pools of sgRNAs targeting the R-loop and its flanking regions. The LasR system can also be applied in *cis* as previously used to modulate R-loops at the single-copy human β-actin locus (Khosraviani et al., 2021).

One can also design sgRNAs complementary to a locus that is spatially proximal to the R-loop-harboring locus to apply the LasR system in *trans*. To identify loci that spatially interact with an R-loop-harboring locus of interest for R-loop modulation in *trans*, consult public databases of high-throughput chromosome conformation capture data such as the Hi-C Data Browser. Alternatively, verify that your R-loop harboring locus overlaps or neighbors CTCF and/or Cohesin ChIP-seq signals in publicly available data at ENCODE or UCSC Genome Browser. In this case, test sgRNA pools targeting a site that harbors

the closest CTCF and Cohesin peaks to the R-loop-harboring site that the experimenter wishes to study. We also refer the reader elsewhere for an overview of methods that can reveal the spatial proximity of DNA loci and might aid in the application of the LasR in *trans* if needed (Kempfer and Pombo, 2020). We have applied the LasR system in *trans* to achieve R-loop modulation at a specific intergenic region of the human rDNA repeats (Abraham et al., 2020).

1. One can either custom design sgRNAs using preferred criteria or employ a commercial service such as the ThermoFisher TrueDesign Genome Editor accessible via this link or IDT CRISPR Genome editing via this link.

   Note: The main advantage of obtaining such commercially available sgRNAs is that some are validated to target the locus of interest.

2. The 20 nucleotide targeting sequences can be ordered through Invitrogen™ TrueGuide™ gRNA Ordering Tool webpage available through this link. In this protocol, we use the TrueGuide 1-piece Synthetic sgRNAs.
3. Order the negative non-targeting sgRNAs, Invitrogen/Thermo Fisher (Cat. #A35526).
4. Resuspend the sgRNAs in TE buffer (RNase/DNase Free) according to manufacturer's instructions (100 pmol/μL), split the sgRNA solution into aliquots, and store them at −20°C. Prepare a solution with a working sgRNA concentration of 10 pmol/μL by diluting with nuclease-free ddH_2O. The working sgRNA solution can be stored at −20°C.

Required controls

To control for tetracycline-dependent induction of RED/dRED expression, include uninduced controls where cells are only treated with the vehicle solution. Negative controls for RNase H1 expression/activity include transfection with the empty vector or dRED, which does not resolve R-loops. To control for the preferential targeting of RED/dRED to the locus of interest and not unrelated genetic loci, also perform qPCR tests for unrelated R-loop-forming canonical loci (Chen et al., 2017; Malig et al., 2020; Skourti-Stathaki et al., 2014). Of note, the total amount of sgRNA transfected must remain consistent between each of the experimental and control conditions.

RED/dRED titration and expression

Timing: 3–4 days

The ideal level of RED/dRED protein expression necessary to achieve effective dCas9-dependent targeting to the locus of interest will vary between cell types and experimental setups. Therefore, before beginning this protocol, titration experiments need to be performed to determine the ideal induction conditions. Additionally, in experimental settings comparing RED and dRED, titration is necessary to ensure similar expression levels for the chimeric proteins.

5. Following 1–2 cell culture passages, harvest the cells and transfer the cell suspension to a 50 mL conical tube.
6. Take a 20 μL aliquot of the cell suspension and mix 1:1 with Trypan blue. Count cells using a hemocytometer.
7. Seed 7×10^5 cells per well of a 6-well tissue culture plate containing pre-warmed fresh complete medium. Place plates at 37°C and 5% CO_2 until the cell culture reaches ~70% confluency.

   Note: Alter the target cell density to maximize transfection efficiency and minimize potential cell toxicity.

8. Once the cells reach ~70% confluency, remove the cell culture medium and replace it with 1.5 mL of tetracycline-containing complete medium (Tet^+ complete medium). For the tetracycline

titration, set up the 6-well tissue culture plate with the following tetracycline final concentrations (using a 1 mg/mL stock concentration):

Reagent	Volume per well of a 6-well cell culture plate					
Complete medium	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL
Tetracycline	N/A	0.075 μL	0.15 μL	0.75 μL	1.5 μL	3 μL
Final Concentration	0 μg/μL	0.05 μg/mL	0.1 μg/mL	0.5 μg/mL	1 μg/mL	2 μg/mL

9. Prepare the following master mix (tube A) for plasmid transfection. Unless otherwise indicated, the volumes listed in steps 9–15 are per well of a 6-well plate; scale up the master mix volumes to suit experimental needs.

Reagent	Final concentration	Amount
1× Opti-MEM	1×	125 μL
Lipofectamine 3000	N/A	3.75 μL

IMPORTANT We suggest using 3.75 μL of Lipofectamine 3000 per well as a starting point. Further optimization with regards to the volume of Lipofectamine 3000 used may be required. Please refer to the manufacturer's protocol available through this link as needed.

10. Prepare the following master mix (tube B) for plasmid transfection.

Reagent	Final concentration	Amount
1× Opti-MEM	1×	125 μL
Plasmid DNA	8 μg/mL	1 μg (i.e., use 1 μL of 1 μg/μL stock plasmid)
P3000	N/A	2 μL

Note: 1 μg is recommended as a starting point. This step is only to assess the delivery and induction of the RED and dRED plasmids.

11. Pipette the master mix solution up and down—transfer 750 μL of mix A to mix B (for the totality of a 6-well plate).
12. Incubate the combined solution at 21°C–25°C for 15 min.
13. Add 250 μL of the combined mixture in drops to each well of a 6-well plate.
14. Incubate the cells at 37°C and 5% CO_2 until ready to harvest (~36–40 h).

IMPORTANT The optimum incubation time can be modified to optimize protein expression and minimize cell toxicity as needed (see troubleshooting #1). Toxicity can be assessed by visually inspecting the cells under a light microscope.

15. To analyze the expression of RED/dRED and endogenous RNase H1, follow Western blotting protocol (see troubleshooting #2).

Note: The expected Western blot band for RED/dRED is ~230 kDa for the chimeric proteins, while endogenous RNase H1 proteins are ~27 kDa in size (see Figure 2).

Subcellular localization of RED/dRED

Timing: 1–2 days

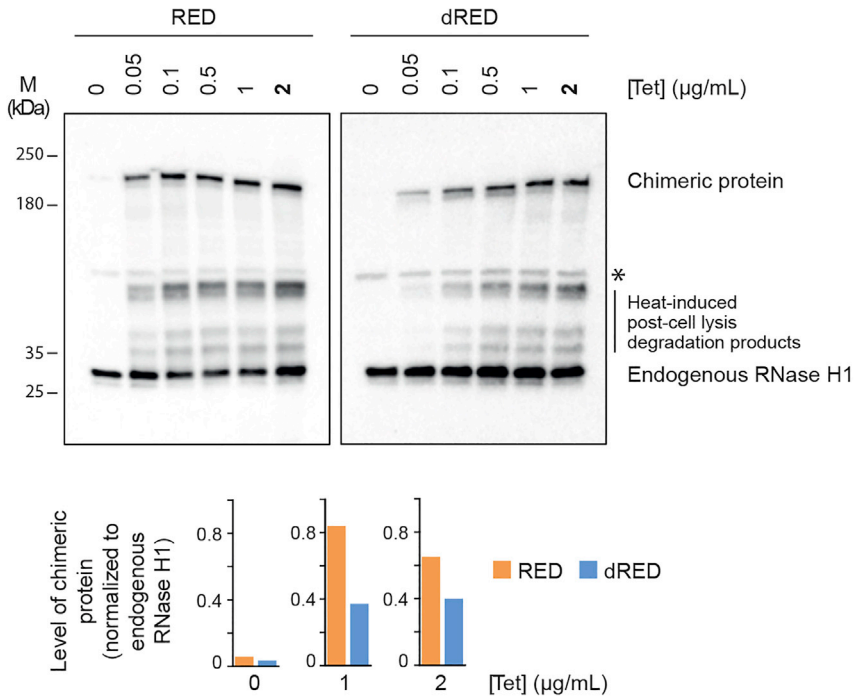

Figure 2. Representative results related to the confirmation of RED/dRED expression

Representative Western blot (N = 1) using an anti-RNase H1 antibody and showing the impact of changing tetracycline (Tet) concentration on the expression of RED and dRED chimeric proteins (230 kDa). Endogenous RNase H1 is also indicated (27 kDa). Such results may differ between cell types and experimental conditions, highlighting the importance of these titration experiments. Uncropped gels with key quantifications are shown. *Non-specific background band.

Live-cell microscopy can be used to visualize the EGFP moiety of RED and dRED. One can also assess the relative localization of the EGFP-tagged RED or dRED to other proteins or markers of cellular structures in live cells by co-transfecting plasmids encoding such markers or treating cells with molecular dyes. Immunofluorescence can also be used for antibody-based colocalization studies in fixed cells. In addition, live cells transfected to integrate the LasR system can be subjected to various treatments before being harvested for immunofluorescence microscopy.

Confirm RED/dRED enrichment

Timing: 4–5 days

Once the expression and nuclear localization of RED/dRED have been confirmed, sgRNA-dependent targeting of RED/dRED to the locus of interest can be confirmed using chromatin immunoprecipitation (ChIP). Anti-GFP antibody should be used to confirm the enrichment of RED/dRED at the locus of interest. See Figure 3 for example of ChIP results.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-RNase H1 antibody (1:1000)	Proteintech	Cat# 15606-1-AP; RRID: AB_2238624
Anti-rabbit IgG HRP-linked whole antibody (1:5000)	GE Healthcare	Cat# NA934-1ML; RRID: AB_2722659

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-GFP antibody (1:1000)	Abcam	Cat# ab290; RRID: AB_303395
Anti-IgG mouse monoclonal antibody (10 μg)	Sigma-Aldrich/Millipore	Cat# 12-371; RRID: AB_145840
Anti-RNA-DNA hybrid S9.6 antibody (10 μg)	Purified in house from hybridoma cells (ATCC Cat# HB-8730)	N/A
Chemicals, peptides, and recombinant proteins		
Tetracycline	Sigma-Aldrich/Millipore	Cat# T7660-25G
Tetracycline-free fetal bovine serum	WISENT	Cat# 081-150
Penicillin/Streptomycin	WISENT	Cat# 450-201-EL
Lipofectamine RNAiMAX	Thermo Fisher Scientific	Cat# 13778150
Lipofectamine 3000	Thermo Fisher Scientific	Cat# L3000015
Opti-MEM reduced serum medium	Thermo Fisher Scientific	Cat# 31985062
TrueGuide synthetic guide RNA (sgRNA)	Thermo Fisher Scientific	Link
Negative non-targeting sgRNA	Thermo Fisher Scientific	Cat. #A35526
Ponceau S	Sigma-Aldrich/Millipore	Cat# P3504-10G
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich/Millipore	Cat# D9542-1MG
DAKO fluorescence mounting medium	Agilent	Cat# S302380-2
Poly-L-Lysine	Sigma-Aldrich/Millipore	Cat# P1524-100MG
Complete protease inhibitor cocktail tablets, EDTA free	Roche Diagnostics	Cat# 11873580001
Dynabeads Protein G	Thermo Fisher Scientific	Cat# 10004D
RNase A	Thermo Fisher Scientific	Cat# EN0531
Proteinase K	Roche Diagnostics	Cat# 03115828001
Spermidine	BioShop	Cat# SPR070
SensiFast No-Rox SYBR	Bioline	Cat# BIO-98050
HindIII (20000 U/mL)	NEB	Cat# R0104S
EcoRI (10 U/ul)	NEB	Cat# ER0271
BsrGI (10000 U/mL)	NEB	Cat# R0575S
XbaI (20000 U/mL)	NEB	Cat# R0145S
SspI (5000 U/mL)	NEB	Cat# R0132L
Recombinant RNase H1	NEB	Cat# MO209S
Critical commercial assays		
Gel/PCR DNA Fragment Extraction kit	Geneaid	Cat# DF300
Experimental models: Cell lines		
TREx™-293 cells	Thermo Fisher Scientific	Cat# R71007
Oligonucleotides		
See Table S1 for sgRNA sequences		
See Table S2 for qPCR primer sequences		
Recombinant DNA		
pcDNA4/TO-RNaseH1-EGFP-dCas9	Abraham et al. (2020)	AddGene ID 139835
pcDNA4/TO-dRNaseH1-EGFP-dCas9	Abraham et al. (2020)	AddGene ID 139836
Software and algorithms		
sgRNA design algorithm	Thermo Fisher Scientific; IDT	Link Link
BioRad CFX Manager (version 3.1)	Bio-Rad	Cat# 184500; Link
NIS-Elements (version 4.10.00 Build 831)	Nikon Instruments Inc.	Link
GraphPad Prism (version 7.0e)	GraphPad	Link
Photoshop CS6 version 13.0 × 64 and Creative Cloud	Adobe	Link
Illustrator CS6 version 16.0.4 and Creative Cloud	Adobe	Link
Other		
Gel imager	ProteinSimple	AlphaImager HP System
BioRad Imager	Bio-Rad	Model #: Universal Hood III
Biosafety cabinet	Baker	Model# SG-403
Digital sonifier	Branson	Model# 250
1.5 mL DNA low-binding microfuge tubes	Eppendorf	Cat# 022431021
DynaMag™-2 Magnet	Thermo Fisher Scientific	Cat# 12321D

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nikon C2+ Confocal microscope	Nikon, Model C2+ replaced with AX/AXR	Link
Real-time PCR system	Bio-Rad	Model# CFX96, CFX384
Nanodrop 2000 spectrophotometer	Thermo Fisher Scientific	Cat# ND-2000C
Hemocytometer	Hausser Scientific	Cat# 3120
Cell counter	DeNovix CellDrop Cell Counter	Link

MATERIALS AND EQUIPMENT

Stock solutions and aliquots preparation

Reagent	Stock solution concentration	Preparation procedure
Tetracycline	1 mg/mL	Resuspend 1 mg of tetracycline in 1 mL of RNase-DNase-free sterile water. Store at −20°C for up to 3 months.
Anti-RNase H1 solution	~165 mg/mL	Dilute 2000× with blocking solution. Store at 4°C for up to 3 uses.
Anti-rabbit IgG HRP solution	1 mg/mL	Dilute 4000× with blocking solution. Store at 4°C for up to 3 uses.
Poly-L-Lysine solution	0.1 mg/mL	Dissolve 1 mg of stock powder in 1 mL sterile ddH_2O. Warm up the solution in 37°C bath, vortex, filter with a 0.22 mm syringe filter, and dilute with sterile ddH_2O to a final concentration of 0.1 mg/mL. Store at 4°C for up to 1 month.
Protease inhibitor cocktail solution	1 tablet/mL	Dissolve 1 tablet in 1 mL sterile ddH_2O. Store at −20°C for up to 3 months
Bromophenol blue	1%	Dissolve 0.1 g in 10 mL of sterile ddH_2O. Store in the dark at 21°C–25°C.
sgRNA	100 pmol/μL	Briefly spin down the tube. Resuspend the sgRNAs in RNase/DNase free TE buffer. Split the sgRNA solution into aliquots and store them at −20°C. Prepare a solution with a working sgRNA concentration of 10 pmol/μL by diluting with nuclease-free ddH_2O. The working sgRNA solution can be stored at −20°C.

TREx™-293 cells culturing medium

Reagent	Final concentration	Amount
DMEM	N/A	445 mL
Tetracycline-free FBS	10%	50 mL
100× penicillin/streptomycin	Pen: 1× (1 unit/mL) Strep: 1× (0.1 mg/mL)	5 mL
Total	**N/A**	**500 mL**

Note: The medium can be stored at 4°C for up to a month.

3× SDS loading buffer

Reagent	Final concentration	Amount
1 M Tris-HCl pH 6.8	188 mM	1.88 mL
10% SDS	3% (w/v)	3 mL
Glycerol	30% (v/v)	3 mL
1% bromophenol blue	0.03%	0.3 mL
β-mercaptoethanol	15%	1.5 mL
ddH_2O	N/A	0.32 mL
Total	**N/A**	**10 mL**

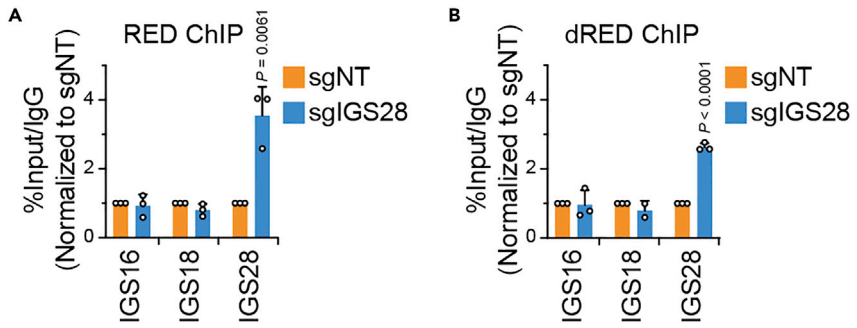

Figure 3. Representative results related to ChIP experiments employing the LasR system

(A and B) The short guide RNAs for the rDNA IGS28 site (sgIGS28) enriched the tetracycline-induced RED (A) and dRED (B) at the same locus in anti-GFP ChIP, using IgG as background control. Enrichments are normalized to non-targeting control signal (sgNT). HEK293T cells were used; data are shown as mean ± S.D.; two-tailed *t*-test; $N = 3$ biologically independent experiments.

⚠ CRITICAL: Work with β-mercaptoethanol under the fumehood with proper PPE.

Note: This buffer can be stored at −20°C for up to six months.

TE buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl pH 8.0	10 mM	500 µL
0.5 M EDTA pH 8.0	1 mM	100 µL
ddH_2O	N/A	49.4 mL
Total	**N/A**	**50 mL**

Note: This buffer can be stored at 21°C–25°C for up to six months.

DRIP restriction enzyme cocktail		
Reagent	Final concentration	Amount
100 mM Spermidine	1 mM	3.5 µL
10× NEB Buffer 2.1	1×	35 µL
HindIII (20,000 U/mL)	100 U	5 µL
EcoRI (10 U/µL)	100 U	10 µL
BsrGI (10,000 U/mL)	100 U	10 µL
XbaI (20,000 U/mL)	100 U	5 µL
SspI (5,000 U/mL)	10 U	2 µL
Genomic DNA	N/A	200 µL
ddH_2O	N/A	79.5 µL
Total	**N/A**	**350 µL**

DRIP 10× binding buffer		
Reagent	Final concentration	Amount
1 M $NaPO_4$ pH 7.0	100 mM	5 mL
5 M NaCl	1.4 M	14 mL
10% Triton X-100	0.5% (v/v)	2.5 mL
ddH_2O	N/A	28.5 mL
Total		**50 mL**

Note: Dilute with ddH_2O to 1× before use. This buffer can be stored at 21°C–25°C for up to six months.

DRIP elution buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl pH 8.0	50 mM	2.5 mL
0.5 M EDTA pH 8.0	10 mM	1 mL
10% SDS	0.5% (w/v)	2.5 mL
ddH_2O	N/A	44 mL
Total	**N/A**	**50 mL**

Note: This buffer can be stored at 21°C–25°C for up to six months.

STEP-BY-STEP METHOD DETAILS

Cell culture and co-transfection

Timing: 5–7 days

HEK293 TREx™ cells expressing TetR are initially cultured in a tetracycline-free growth medium. The cells are seeded at a density to yield a ~70% confluent cell culture on the day of transfection. This protocol is also compatible with other cell lines expressing TetR.

1. Culture TREx™-293 cells in complete medium consisting of 10 mL of DMEM supplemented with 10% (v/v) tetracycline-free FBS and 1× penicillin-streptomycin (at final concentration of 1 unit/mL of penicillin, 0.1 mg/mL streptomycin) 37°C and 5% CO_2 in 100 mm tissue culture plates.
2. Passage the cells when they reach 80%–90% confluency by removing the culture medium and washing the cells once with pre-warmed (37°C) 1× PBS.
   a. Aspirate the PBS and add 1 mL of 0.025% (w/v) 1× trypsin-EDTA at 37°C.
   b. Wait for 1 min to allow for cellular dissociation from the cell culture plate.
   c. Add 9 mL of pre-warmed (37°C) complete medium to stop the reaction.
   d. Pipette well to suspend the cells and transfer 2 mL of the cell suspension to a new 100 mm tissue culture plate containing 8 mL of fresh pre-warmed complete medium.

   Note: Passage cells every 2–3 days at a splitting ratio of 1:5 – 1:10 to prevent fully confluent cultures and maintain cell viability.

3. If cells are ready to be set up for transfection, then count the cells and seed according to experiment. 60 mm plates are used below for the DNA-RNA immunoprecipitation (DRIP) as more cells are required for this assay.
   a. Take a 20 µL aliquot of the cell suspension and mix it 1:1 with Trypan blue before counting cells using a hemocytometer.
   b. To have cells at ~70% confluence in a 60 mm plate, seed 2.0 ×10^6 cells per 60 mm tissue culture plate containing pre-warmed and fresh complete medium. Place the plates at 37°C and 5% CO_2 for next day transfection.
4. On the day of transfection, prepare Tet^+ complete medium by adding 10 µL of the 1 mg/mL tetracycline stock solution to 10 mL of pre-warmed complete medium in a 50 mL conical tube (Figure 4A).

   Note: The final concentration of tetracycline in the Tet^+ complete medium can be modified as needed to reflect the optimal RED/dRED induction conditions revealed by tetracycline titration in steps 10–20 (before you begin).

5. Prepare two master mixes for plasmid transfections using the following volumes per 60 mm cell culture dish. Mix thoroughly by pipetting up and down (Figure 4B).

Tube A		
Reagent	Final concentration	Amount (per 60 mm plate)
1× Opti-MEM	1×	250 μL
Lipofectamine 3000	N/A	9 μL

Tube B		
Reagent	Final concentration	Amount (per 60 mm plate)
1× Opti-MEM	1×	250 μL
DNA Plasmid	12 μg/mL	X (3 μg recommended as a starting point)
P3000	N/A	6 μL

6. Add Tube A to Tube B to create mix A and mix well by pipetting.
7. Incubate for 15 min at 21°C–25°C.
8. Add 500 μL of the combined mixture to cells dropwise.
9. Prepare the sgRNA transfection mix and Lipofectamine RNAiMAX mix by combining the following for each 60 mm cell culture dish:

Tube A		
Reagent	Final concentration	Amount (per 60 mm plate)
1× Opti-MEM	1×	375 μL
RNAiMAX	N/A	21 μL

Tube B		
Reagent	Final concentration	Amount (per 60 mm plate)
1× Opti-MEM	1×	375 μL
sgRNA	10 pmol/μL	1.5 μL

⚠ CRITICAL: Include a non-targeting sgRNA control.

Note: Volumes listed are for one 60 mm plate. Scale up master mix according to experimental needs.

Note: Test the number of sgRNAs required for successful targeting by including conditions in which cells are transfected with different pools of sgRNAs and with each of the sgRNA used in such pools individually. The total concentration of sgRNAs per experimental condition should remain constant. The sgRNAs used here are in Table S1.

10. Incubate Tube A for 1 min at 21°C–25°C.

Note: If using a pool of sgRNAs, add 1.5 μL of each sgRNAs to the same Tube B. In this case the volume of the non-targeting sgRNA control should be equivalent to the total volume of sgRNAs in the pool. So, for 3 sgRNAs in a pool, the volume of the non-targeting sgRNA control would be 4.5 μL (3 × 1.5 μL).

11. Add 375 μL of Tube A to each sgRNA mix tube (Tube B) to make mix B and incubate for 5 min at 21°C–25°C.

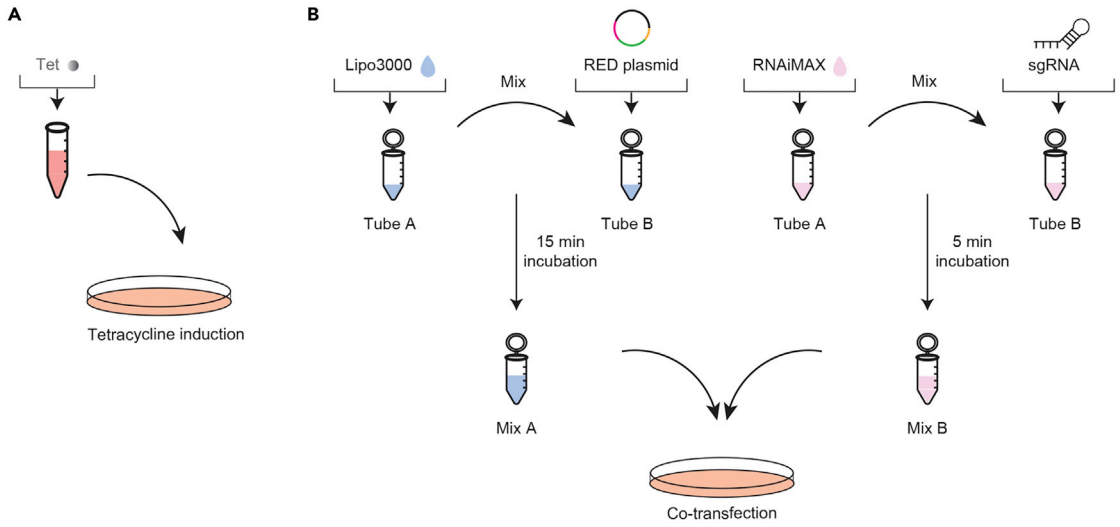

Figure 4. Overview of the co-transfection of cells with RED/dRED plasmid and sgRNAs

(A) Change the media for the cells by adding Tet^+ media.

(B) For mixture A, mix Tube A (containing Lipofectamine 3000 and Opti-Mem) with Tube B (containing Plasmid DNA, P3000, and Opti-Mem), and incubate for 15 min at 21°C–25°C. For mixture B, mix Tube A (containing RNAiMAX and Opti-Mem) with Tube B (containing sgRNAs and Opti-Mem), and incubate for 5 min at 21°C–25°C. After incubation, add both Mix A and Mix B to the cells.

12. Gradually drip 750 μL of the combined mix onto the cells before incubating them for 36 h at 37°C and 5% CO_2 (see troubleshooting #1).

Note: Test different incubation lengths to achieve optimal RED/dRED protein expression with minimal cell toxicity.

DNA-RNA immunoprecipitation (DRIP) to measure LasR-based R-loop modulation

Timing: 4–5 days

We describe a DNA-RNA immunoprecipitation (DRIP) protocol (Figure 5) that uses both commercially available and reagents generated in-house. Specifically, we use an anti-RNA-DNA hybrid (S9.6) antibody purified in-house from mouse hybridoma cells (ATCC, HB-8730). In this protocol, we digest genomic DNA with a cocktail of restriction enzymes and confirm digestion using agarose gel electrophoresis. The input DNA and DNA from IPs are purified using a commercially available Gel/PCR DNA Fragment Extraction Kit and used in qPCR. If assessing R-loop modulation in *trans*, as in the case of the rDNA shown in this protocol, include qPCR primers for both the targeted locus (i.e., IGS28) and the R-loop-containing locus modulated in *trans* (i.e., IGS18) (Abraham et al., 2020). If assessing R-loop modulation in *cis*, as in the case of the *β-ACTIN* locus shown elsewhere (Khosraviani et al., 2021), include the qPCR primers that generate PCR amplicons that are within the vicinity of the sgRNA sites as summarized further below.

13. Cell culture and RED/dRED induction conditions can be performed as presented herein previously. Remove the cell culture medium and wash cells twice with 5 mL of ice-cold 1× PBS.

⚠ CRITICAL: Wash three times if the culture medium contains toxic drugs. Dispose of the toxic liquid waste according to the biosafety guidelines.

14. Add 5 mL 1× PBS to each plate. Gently scrape the cells into a 15 mL conical tube.
15. Pellet the cells gently by centrifuging at 270 × *g* for 5 min at 21°C–25°C.

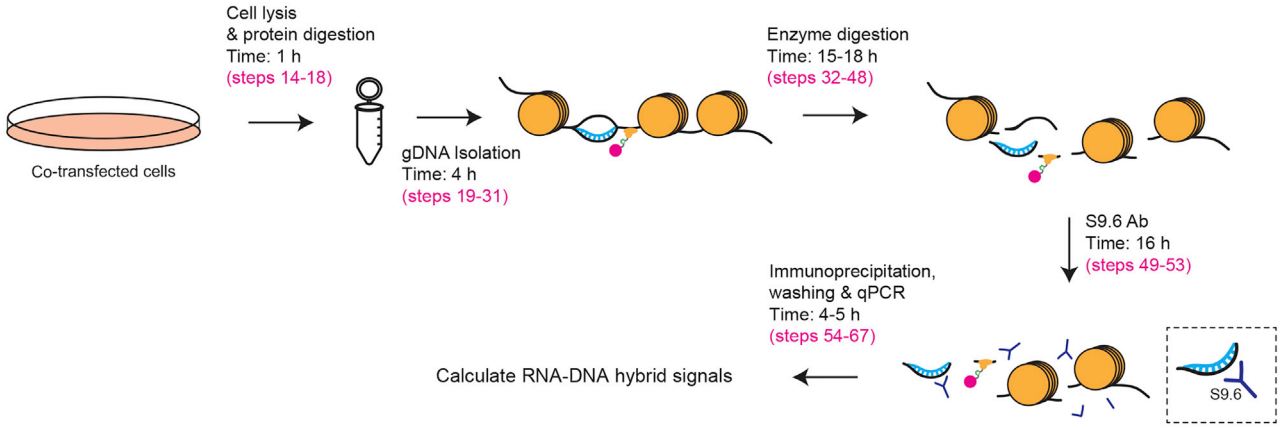

Figure 5. Overview of DNA-RNA immunoprecipitation (DRIP) using the S9.6 antibody

Co-transfected cells are first lysed, and the proteins are digested (steps 14–18). Genomic DNA (gDNA) is isolated (steps 19–31) followed by a 15–18 h digestion with restriction enzymes (steps 32–48) to obtain DNA fragments of 200–500 bp. The digested DNA is incubated for 15–18 h with the S9.6 antibody (Ab) that detects the RNA-DNA hybrid moiety of R-loops (steps 49–53). The immunocomplexes are precipitated and washed, DNA fragments are purified, and qPCR is run to measure RNA-DNA signals (steps 54–67).

16. Resuspend the cells in 1.6 mL of TE.
17. Add 41.5 μL of 20% SDS and 5 μL of 20 mg/mL proteinase K. To resuspend the cells, gently pipette up and down with a 1,000 μL micropipette tip, which one can cut to widen the tip orifice and minimize cellular stress during pipetting.
18. Incubate with constant rotation for 15–18 h at 37°C.
19. Add 1.6-mL of phenol-chloroform to each tube.

⚠ CRITICAL: Perform this step under the fume hood and wear PPE.

20. Gently invert to mix the tube, then centrifuge it at 450 × *g* for 5 min at 21°C–25°C.
21. Use a widened 1,000 μL micropipette tip (as in step 129) to carefully transfer the top aqueous layer (~1.5 mL) to a new conical tube.
22. Add 1.4 mL of phenol-chloroform and repeat major steps 20 and 21.
23. While samples are being centrifuged, add 80 μL of 3 M NaOAc pH 5.2 and 1,920 μL of 100% EtOH to a new 15 mL conical tube.
24. Transfer 800 μL of the aqueous phase from step 22 to the tube from step 23.
25. Gently mix by inverting the tube until a 'whitish' fiber-like precipitate appears.

⚠ CRITICAL: Do not centrifuge to pellet the DNA as this can promote RNA contamination and interfere with immunoprecipitation.

26. Spool the DNA fiber with a 100 μL micropipette tip and transfer the DNA fiber into a 1.5 mL tube.
27. Wash the DNA fiber five times each with 1,000 μL of 70% EtOH and gentle inverting of the tube to allow the fibers to settle.

⚠ CRITICAL: Do not centrifuge to pellet the DNA as this can promote RNA contamination and interfere with immunoprecipitation.

28. After the final wash, carefully remove as much of the EtOH as possible.
29. Dry the genomic DNA (gDNA) pellets for 2–3 h at 21°C–25°C.
30. Carefully add 200 μL of TE to each tube.
31. Using a cut 200 μL micropipette tip, very gently resuspend the gDNA pellet.

⚠ CRITICAL: The mixture will be viscous, and the DNA fibers will be visible. Do not pipette excessively or vortex.

32. For each sample, mix the following in a fresh 1.5 mL tube on ice:

DRIP restriction enzyme cocktail		
Reagent	Final concentration	Amount
100 mM Spermidine	1 mM	3.5 μL
10× NEB Buffer 2.1	1×	35 μL
HindIII (20,000 U/mL)	100 U	5 μL
EcoRI (10 U/μL)	100 U	10 μL
BsrGI (10,000 U/mL)	100 U	10 μL
XbaI (20,000 U/mL)	100 U	5 μL
SspI (5,000 U/mL)	10 U	2 μL
Genomic DNA	N/A	200 μL
ddH_2O	N/A	79.5 μL
Total	**N/A**	**350 μL**

Note: Buffers and restriction enzymes used here should be relevant to the experiment or genome of interest.

Note: Genomic DNA should be added last.

Note: This cocktail should be freshly prepared on ice before use.

33. Mix by pipetting up and down with a cut 200 μL micropipette tip.
34. Incubate with constant rotation for 15–18 h at 37°C.
35. Using a 1% agarose gel with Red Safe staining, run 9 μL of the digested DNA at 125 V for 25 min at 21°C–25°C to confirm DNA digestion. The resulting DNA sizes should appear as a smear peaking in intensity at ~200–500 bp in size (Figure 6A; see troubleshooting #3–4).
36. Add 40 μL of 3 M NaOAc pH 5.2 to the digested DNA.
37. Add 400 μL of phenol-chloroform and mix well.

⚠ CRITICAL: Perform this step under the fume hood.

38. Centrifuge at 16,100 × *g* for 5 min at 4°C.
39. Transfer 300 μL of the top aqueous phase to a new tube.
40. Repeat steps 37–39.
41. Add 2.4 volumes (~600 μL) of cold 100% EtOH.
42. Precipitate DNA by incubating for 15 min at −20°C.
43. Centrifuge at 16,100 × *g* for 30 min at 4°C to pellet the DNA.
44. Remove the supernatant and add 1 mL of cold 70% EtOH.
45. Centrifuge at 16,100 × *g* for 5 min at 4°C.
46. Air dry for 30 min under the fume hood.
47. Resuspend the pellet in 50 μL of TE.
48. Measure the concentration (OD_{260}) of the DNA using NanoDrop2000 or similar method. The concentration should be ~400–1,000 ng/μL (see troubleshooting #5).
49. Add 4.4 μg of digested DNA for each IP in a new 1.5 mL low retention tube and top-up to a total of 100 μL with nuclease-free ddH_2O.

Note: As an anti-RNA-DNA hybrid specificity control, treatment with recombinant RNase H1 in vitro can be performed by incubating 4.4 μg of digested DNA with 20 μL (100 U) of RNase H1, 10 μL of 10× RNase H1 buffer (supplied with recombinant RNase H1), and topping up to 100 μL with nuclease-free water. Samples can then be incubated for 15–18 h at 37°C before proceeding with the immunoprecipitation (Abraham et al., 2020).

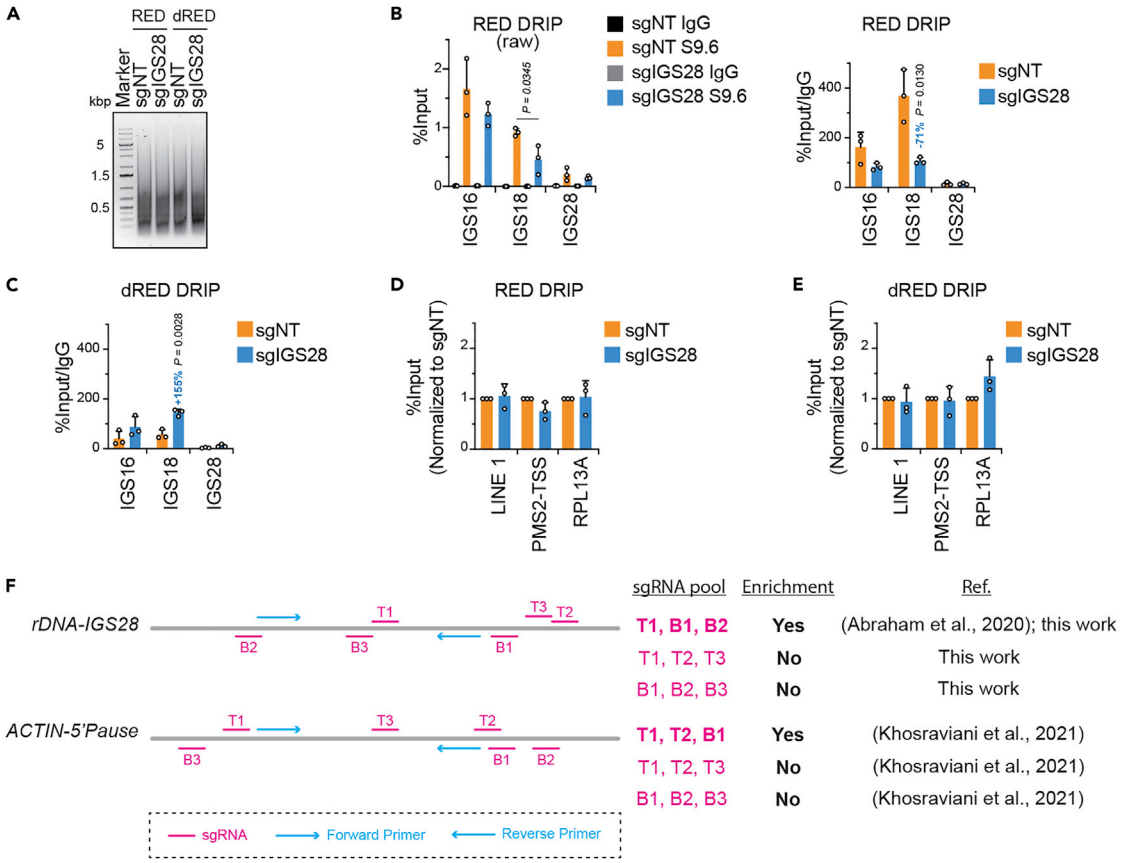

Figure 6. Representative results related to DRIP experiments employing the LasR system

(A) Representative agarose gel electrophoresis illustrating typical chromatin fragments obtained following the digestion of DNA with restriction enzymes in preparation for chromatin pulldown experiments.

(B and C) DRIP analysis shows that using RED (B) or dRED (C) together with sgIGS28 respectively decreased and increased R-loop levels in *trans* at the spatially proximal IGS18 site. RED results in (B) are shown before (left, %Input) and after (right, %Input/IgG) normalization to IgG signals to control for background variability especially within highly repetitive DNA loci.

(D and E) DRIP analysis shows that using RED (D) or dRED (E) together with sgIGS28 does not alter the levels of R-loops at unrelated genetic loci.

(F) Schematic summarizing the pools of sgRNAs tested so far with this LasR system protocol when applied in *trans* at rDNA as shown previously (Abraham et al., 2020) and herein or used in *cis* at the 5′Pause site of the single-copy *β-ACTIN* locus as shown elsewhere (Khosraviani et al., 2021). Testing different pools of sgRNAs targeting both DNA strands allowed for the combination of three sgRNAs that specifically target to the locus of interest and enriched the RED and dRED chimaeras in ChIP at the targeted loci. (A–F) HEK293T cells were used; data are shown as mean ± S.D.; two-tailed *t*-test (A–C) or two-tailed ANOVA with Tuckey's multiple comparisons test (D and E); $N = 3$ biologically independent experiments.

Note: Perform two independent IPs for each sample by using the S9.6 antibody to pull down DNA-RNA hybrids or anti-IgG as mock pulldown control.

50. Add 350 μL of TE buffer and 50 μL of 10× binding buffer to each sample.
51. Set aside 50 μL of each sample to serve as input.

Note: Note that this is 10% of input, and this information will be used to quantify enrichments as percent input.

52. Add 10 μg of the S9.6 antibody to each IP tube. For the mock IP controls, add 10 μg of the mouse anti-IgG antibody instead.
53. Rotate for 15–18 h at 4°C.

54. Per each IP sample, wash 50 μL of Dynabeads or similar three times with 1 mL of the 1× binding buffer by rotating for 10 min at 21°C–25°C for each wash in a new set of tubes. Pellet the beads on the DynaMag™-2 Magnet rack or centrifuge if applicable/preferred, and remove the 1× binding buffer.

Note: Using less than 50 μL of Dynabeads can significantly lower the percent input.

55. Add the antibody-DNA mix from step 52 directly into the tubes prepared in step 54.
56. Rotate for 2 h at 4°C.
57. Pellet the beads and discard the supernatant.
58. Wash the beads three times with 1 mL of the 1× binding buffer by rotating for 10 min at 21°C–25°C.
59. Resuspend the beads in 250 μL of DRIP elution buffer.
60. Add 200 μL of DRIP elution buffer to each input (from step 51).
61. Add 7 μL of proteinase K to a final concentration of 0.5 mg/mL to each sample and mix well.
62. Incubate for 45 min at 55°C.
63. Pellet the beads in the IP samples and transfer the eluate to a new low retention tube.
64. Purify the DNA samples using Geneaid Gel/PCR DNA fragment extraction kit according to the manufacturer's protocol here, with the exception to elute the samples with 250 μL.
65. Run quantitative real-time PCR (qRT-PCR) using SensiFast No-ROX SYBR according to manufacturer's protocol here (see troubleshooting #6–7). The primers used here are listed in Table S2.
66. Perform the following to calculate the %Input/IgG for each sample:

Adjusted Input (%Input) = Log (Your dilution factor, 2).

Corrected Ct = 100 × 2^(Adjusted Input – Ct IP).

Data can be presented relative to the negative background control (IgG):

%Input/IgG = corrected Ct for IP/Corrected Ct for IgG.

67. Compare the DRIP-qPCR results to the representative data shown here (Figures 6B–6E).

EXPECTED OUTCOMES

As shown in other reports (Abraham et al., 2020; Khosraviani et al., 2021; Li et al., 2020) and in the representative data here (Figures 3 and 6), locus associated R-loop modulation is achievable with careful design and controls. The LasR system can be used to accomplish R-loop modulation in *cis* or trans (Abraham et al., 2020; Khosraviani et al., 2021). The tetracycline-dependent titration of RED and dRED protein expression should reveal the ideal chimeric protein induction conditions (Figure 2). In addition, when compared to the combination of RED/dRED with non-targeting sgRNA controls, the combination of RED/dRED with locus-targeting sgRNAs can be expected to preferentially enrich the chimeric proteins at the targeted locus over other genetic loci. Moreover, targeting RED/dRED to the given locus of interest may alter R-loop levels at the associated locus or at other genetic loci that may be proximal in nuclear space due to chromatin looping (Abraham et al., 2020). For instance, the intergenic spacers (IGS) of ribosomal RNA genes (rDNA) can serve as a positive control in future experimental design. At this locus, targeting RED/dRED to the 28 kb site of the rDNA IGS (IGS28) alters R-loop levels at the spatially proximal IGS18 site instead of affecting R-loops at the IGS28 site to which it is targeted (Figure 3) (Abraham et al., 2020; Shiue et al., 2014; Zentner et al., 2011). As expected, targeting RED and dRED to IGS28 using sgIGS28 significantly lowered (-71%) and increased (+155%) RNA-DNA hybrid levels at IGS18, respectively (Figures 6B and 6C). This modulation of IGS18 R-loop levels occurred without affecting known R-loops at *LINE1*, *PMS2-TSS*, or *RPL13A* control loci (Figures 6D and 6E). Similarly, this LasR system has been used

to achieve R-loop modulation in *cis* at the *β-ACTIN* locus (Khosraviani et al., 2021). Together, we anticipate that this LasR system and herein described protocol will provide researchers with a vastly improved ability to ascribe observed phenotypes to targeted changes in relative R-loop levels at loci of interest.

LIMITATIONS

Like any experimental tool, one must consider certain caveats when using the LasR system. First, all of the limitations associated with CRISPR- and dCas9-based technologies apply to this system (Anzalone et al., 2020). For example, CRISPR-dCas9-based systems require 5′NGG PAMs (Gilbert et al., 2013; Jinek et al., 2012; Wiedenheft et al., 2012). In addition, poorly understood and rare dCas9-dependent chromosome rearrangement events may occur (Anzalone et al., 2020). However, confounding effects related to such rare events can be alleviated through the recommended transient expression of RED and dRED proteins. This approach preserves the diversity of the cell population while avoiding rare phenotypes associated with stable transfection and clone selection. However, if stable transfection is preferred, the potential off-target effects of a constitutive CRISPR-dCas9 system must be carefully evaluated, such as via genome-wide sequencing (Barski et al., 2007; Johnson et al., 2007; Mikkelsen et al., 2007; Sanz and Chedin, 2019; Solomon et al., 1988). Additionally, the sgRNA design may be modified to alter the residence time of dCas9 onto chromosomes, thereby minimizing potentially rare replication interference effects should they be applicable to the studied locus (Boyle et al., 2017). Second, RED and dRED may target chromosomal R-loops outside of the locus of interest in a manner that is independent of sgRNAs and dCas9. For example, the RNase H1 moiety of the RED protein will have a background affinity for genetic loci harboring naturally elevated R-loop levels, which may differ across experimental conditions and cell types. In this case, the use of non-targeting sgRNA controls and sgRNAs targeting unrelated genetic loci ensures that the natural affinity of the chimeric proteins to unrelated genetic loci does not trigger the same phenotypic changes seen using sgRNAs targeting the locus of interest. Similarly, since the targeting of dCas9 to specific genetic loci mostly requires the use of a pool of sgRNAs (Abraham et al., 2020), another critical control consists of testing whether RED/dRED targeting and related R-loop-modulatory effects are lost when the sgRNAs are used individually instead of as a pool. Thus, the non-targeting sgRNA negative controls and the individual sgRNA negative controls ensure that phenotypic changes do not reflect unforeseen effects related to any moiety of the chimeric proteins or the individual sgRNAs used. One can combine the LasR system with genome-wide sequencing to evaluate global RED/dRED enrichments and effects on R-loops as described elsewhere (Barski et al., 2007; Johnson et al., 2007; Mikkelsen et al., 2007; Sanz and Chedin, 2019; Solomon et al., 1988). However, this paper aims to supply a detailed, accessible protocol to accurately evaluate the impact of the preferential enrichment of RED/dRED at specific loci on a phenotype of interest. Third, different R-loops can exhibit varying sensitivities to RNase H1 activity (Crossley et al., 2020). Due to this phenomenon, use of the LasR system at these loci may not confer strong R-loop repression. So, in such cases, it may prove necessary to replace the RNase H1 moiety of the chimeric protein with another R-loop-modulatory protein, such as Senataxin. Fourth, transient transfections can trigger cell toxicity depending on the cell type used and the experimental conditions. Therefore, one may need to adopt various approaches to introduce the LasR system components into cells. For example, one may consider different transfection reagents or electroporation.

TROUBLESHOOTING

Problem 1

Observe a lot of cell death following co-transfection with RED/dRED and sgRNAs (see before you begin step 14 and major step 12).

Potential solution

This may be due to the toxicity of the transfection reagent, such as Lipofectamine 300, tetracycline, or both. There are three solutions for overcoming this problem. (1) Reduce the amount of

Lipofectamine 3000 (refer to the manufacturer's protocol here if needed), (2) reduce the amount of tetracycline, or (3) reduce the post-transfection incubation time. We recommend testing 12 h and 24 h incubation times.

Problem 2

Don't observe a band for the RED/dRED chimeric protein in the immunoblot (see before you begin step 15).

Potential solution

There are three possible solutions to this problem. (1) The number of cells seeded can be increased to increase the amount of protein loaded. (2) The amount of tetracycline used and/or DNA plasmid transfected can be increased to further induce the expression of the RED/dRED chimeric protein in the cell. We recommend an upper limit of 2 μg/mL for tetracycline and plasmid amounts from 1 to 5 μg. (3) The concentration of the primary antibody used in Western blotting can be increased.

Problem 3

The DNA runs too high on the agarose gel (see major step 35).

Potential solution

This can result from either incomplete restriction enzyme digestion or low quality DNA. There are four possible solutions to this problem. (1) Increase the units of the restriction enzymes, (2) ensure the gDNA is free from contaminants, (3) repeat phenol/chloroform extraction (major steps 19–21), or (4) wash the gDNA fibers additional times with 70% EtOH (major step 27).

Problem 4

Have low DNA yield (see major step 35).

Potential solution

Increase the number of cells by seeding more than one plate or using a larger cell culture dish, and pool the cells together during protein digestion and gDNA isolation steps. Scale up the reagents for protein digestion and gDNA extraction accordingly.

Problem 5

Observe low quality DNA (see major step 48).

Potential solution

Repeat the phenol/chloroform extraction (major steps 37–39) to remove contaminants from the gDNA. Alternatively, genomic DNA extraction kits, such as GenElute™ Mammalian Genomic DNA (Sigma, Cat# G1N10-1KT), can be used.

Problem 6

The Ct value from the qPCR amplification is too low (see major step 65).

Potential solution

This can result from samples that are too concentrated. Therefore, dilute the samples further to overcome this problem. Keep in mind that the difference of one Ct value corresponds to a 2-fold difference.

Problem 7

The Ct value from the qPCR amplification is too high (see major step 65).

Potential solution

This can result from samples that are too dilute. So, elute the samples in major step 64 with a smaller volume of TE.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Karim Mekhail (karim.mekhail@utoronto.ca).

Materials availability

The RED/dRED-expressing plasmids used in this protocol have been deposited at Addgene (RED ID: 139835; dRED ID: 139836).

Data and code availability

The published article includes all the datasets generated or analyzed during this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101734.

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AUTHOR CONTRIBUTIONS

K.J.A. and K.M. conceived the project. N.K., J.N.Y.C., and K.M. wrote, and K.J.A. edited the manuscript. N.K. and J.N.Y.C. performed experiments, and N.K. analyzed all data. K.M. supervised the research and obtained the operating funds.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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