Jun 11, 2024

CRISPRi of regulatory elements upon degron operation (CRUDO)

This protocol is a draft, published without a DOI.

Philine Guckelberger[1,2,3], Benjamin R. Doughty[4], Glen Munson[5], Charles P. Fulco[3], Drew T. Bergman[3], Judhajeet Ray[5], Evelyn Jagoda[5], Chad J. Munger[4,6], Andreas R. Gschwind[4,6], Lars M. Steinmetz[4,7,8], Eric S. Lander[3,9,10], Jesse Engreitz[4,5,6,11,12]

[1]Department of Genome Regulation, Max Planck Institute for Molecular Genetics, Berlin, Germany;
[2]Department of Biology, Chemistry and Pharmacy, Freie Universität Berlin, Berlin, Germany;
[3]Broad Institute of MIT and Harvard, Cambridge, MA, USA;
[4]Department of Genetics, Stanford University School of Medicine, Stanford, CA, USA;
[5]The Novo Nordisk Foundation Center for Genomic Mechanisms of Disease, Broad Institute of MIT and Harvard, Cambridge, MA, USA;
[6]Basic Sciences and Engineering Initiative, Betty Irene Moore Children's Heart Center, Lucile Packard Children's Hospital, Stanford, CA, USA;
[7]Stanford Genome Technology Center, Palo Alto, CA, USA;
[8]European Molecular Biology Laboratory (EMBL), Genome Biology Unit, Heidelberg, Germany;
[9]Department of Systems Biology, Harvard Medical School, Boston, MA, US;
[10]Department of Biology, MIT, Cambridge, MA, USA;
[11]Gene Regulation Observatory, Broad Institute of MIT and Harvard, Cambridge, MA, USA;
[12]Stanford Cardiovascular Institute, Stanford University, Stanford, CA, USA

Philine Guckelberger
MPIMG

Protocol Citation: Philine Guckelberger, Benjamin R. Doughty, Glen Munson, Charles P. Fulco, Drew T. Bergman, Judhajeet Ray, Evelyn Jagoda, Chad J. Munger, Andreas R. Gschwind, Lars M. Steinmetz, Eric S. Lander, Jesse Engreitz 2024. CRISPRi of regulatory elements upon degron operation (CRUDO). **protocols.io https://protocols.io/view/crispri-of-regulatory-elements-upon-degron-operati-dfbk3ikw**

Protocol status: Working
We use this protocol and it's working

Created: June 08, 2024

Last Modified: June 11, 2024

Protocol Integer ID: 101452

Funders Acknowledgement:
PhD Fellowship
Grant ID: Boehringer Ingelheim Fonds
National Science Foundation Graduate Research Fellowship
Grant ID: DGE-1656518
Stanford Interdisciplinary Graduate Fellowship
Grant ID: Stanford Bio-X
NIH
Grant ID: R01HG011664
NHGRI Impact of Genomic Variation on Function Consortium
Grant ID: UM1HG009375
ENCODE Consortium
Grant ID: UM1HG009375
NHLBI
Grant ID: R01HL159176
NHGRI IGVF Consortium
Grant ID: UM1HG011972
Novo Nordisk Foundation
Grant ID: NNF21SA0072102
NHGRI Genomic Innovator Award
Grant ID: R35HG011324
Stanford University
Grant ID: Gordon and Betty Moore and the BASE Research Initiative

Abstract

Enhancers are key drivers of gene regulation thought to act via 3D physical interactions with the promoters of their target genes. However, genome-wide depletions of architectural proteins such as cohesin result in only limited changes in gene expression, despite a loss of contact domains and loops[1-4]. Consequently, the role of cohesin and 3D contacts in enhancer function remains debated[5-7]. Here, we developed CRISPRi of regulatory elements upon degron operation (CRUDO), a novel approach to measure how changes in contact frequency impact enhancer effects on target genes by perturbing enhancers with CRISPRi and measuring gene expression in the presence or absence of cohesin. We systematically perturbed all 1,039 candidate enhancers near five cohesin-dependent genes and identified 34 enhancer-gene regulatory interactions. Of 26 regulatory interactions with sufficient statistical power to evaluate cohesin dependence, 18 show cohesin-dependent effects. A decrease in enhancer-promoter contact frequency upon removal of cohesin is frequently accompanied by a decrease in the regulatory effect of the enhancer on gene expression, consistent with a contact-based model for enhancer function[8-11]. However, changes in contact frequency and regulatory effects on gene expression vary as a function of distance, with distal enhancers (e.g., >50Kb) experiencing much larger changes than proximal ones (e.g., <50Kb). Because most enhancers are located close to their target genes, these observations can explain how only a small subset of genes — those with strong distal enhancers — are sensitive to cohesin. Together, our results illuminate how 3D contacts, influenced by both cohesin and genomic distance, tune enhancer effects on gene expression.

Note: The list of authors for this protocol does not include all authors of the accompanying manuscript, only those who played a role in developing and executing the CRUDO method.

Before start

Our goal was to create a method for assessing the impact of changes in 3D contact on enhancer function. To achieve this, we developed CRISPRi of regulatory elements upon degron operation (CRUDO), a technique that combines dCas9-KRAB-mediated perturbations with targeted protein degradation using an auxin-inducible degron system. CRUDO offers versatility, allowing us to address various fundamental questions in gene regulation by employing different factors and target enhancer-promoter pairs for perturbation. In our specific case, we targeted RAD21, a subunit of the cohesin complex crucial for maintaining 3D genome architecture. However, CRDUO can easily be adapted according to a specific research questions.

Designing a CRUDO experiment

1 **General considerations for selection of acellular model for CRUDO.**

CRUDO is a versatile approach that is adaptable to address a range of questions concerning gene regulation. Consequently, the choice of a cellular model predominantly depends on the specific research question at hand. For instance, if investigating a particular disease or trait, selecting a cellular model relevant to the context becomes crucial. Conversely, if addressing amore fundamental research question, leveraging an existing cellular model may be beneficial, avoiding the need to create a new auxin-inducible or CRISPRi cell line. In general, when applying CRUDO, opting for a cell line receptive to engineering can significantly enhance efficiency, particularly for tasks such as virus delivery and perturbation strength.

2 **General considerations for selection of a gene targets for CRUDO.**

To optimize experimental outcomes when applying CRUDO, we recommend selecting target genes with relatively high expression levels (e.g., RNA-seq TPM >20). This is especially crucial if you anticipate perturbations to decrease target gene expression, ensuring good sensitivity and accuracy in measurements. Additionally, the duration of perturbation and protein degradation influences the choice of target genes. Since we measure mature mRNAs, there's a lag in observing effects, which may require longer perturbation and protein degradation periods for precise effect size measurements. However, logistical constraints or biological factors may limit extended durations. In such cases, selecting genes with short mRNA half-lives minimizes temporal delays in effect size measurement. In our case, we were restricted to a 6-hour cohesin degradation window, prompting the selection of genes exhibiting a reduction in expression after 6 hours of auxin treatment by at least -0.25 and -0.5 log2 fold change in RNA-seq and PRO-seq data. The reduction in RNA-seq indicates that the mRNA half-lives of these genes are short enough to identify changes after 6h of treatment. Depending on research objectives, other criteria may also be relevant. In our context, as we aimed to understand how changes in 3D contacts impact gene regulation, accordingly we chose genes showing decreased expression (as measured by PRO-seq) upon cohesin removal. Furthermore, we selected genes with diverse regulatory landscapes based on ABC-predicted enhancers spanning various linear genomic distances.

3 **General considerations for selection of a CRUDO read-out.**

Similarly, the choice of read-out for CRUDO experiments depends on the specific research question. We have so far combined CRUDO with two different read-outs: RNA fluorescence in situ hybridization and cell sorting (FlowFISH[11]) or targeted Perturb-seq (TAP-seq[12]).

A key advantage of CRUDO-FlowFISH (CRUDO-FF) is the precise measurement of each candidate element's effect on gene expression. This is achieved by analyzing thousands of single cells per gRNA, many gRNAs per element, and hundreds to thousands of negative control gRNAs in a single pooled experiment. Robust measurements are ensured by increasing both the number of gRNAs per element and the number of cells analyzed for each perturbation. Because CRUDO-FF uses cell sorting, the number of analyzed cells can be significantly higher, while sequencing costs are minimized as only the gRNAs from each

sorting bin need to be sequenced. However, CRUDO-FF is limited to measuring one gene at a time. FlowFISH is recommended as the CRUDO readout due to its high sensitivity, particularly when analyzing changes in gene expression for a limited number of target genes (ideally, not more than five), and when screening numerous elements to benefit from the large number of cells that can be screened. For CRUDO-FF we use the PrimeFlow probesets and kit from Thermo Fisher Scientific (#88–18005).

Alternatively, CRUDO can be combined with TAP-seq (CRUDO-TAP) by performing single-cell RNA sequencing and amplifying the targets of interest. This targeted approach enhances measurement sensitivity and decreases sequencing costs compared to conventional whole transcriptome single-cell RNA-sequencing techniques. CRUDO-TAP is recommended when only perturbing a few elements (ideally less than 30) while aiming to understand their impact on many different genes. For CRUDO-TAP, the TAP-seq primer designing pipeline[12] should be used to design primers for cDNA amplification of the genes of interest, typically including genes expressed within 1Mb of any gRNA in the library pool, housekeeping genes, and other genes specific to the screen.

Culturing of CRUDO cells (HEK293T and HCT-116)

4 **Introduction to culturing of CRUDO cells.**

The HCT-116 cell line, HCT-116-RAD21-mAID-mClover[1,13], serves as our cellular model and is puromycin, hygromycin, and neomycin resistant because these markers are present on the auxin-inducible cohesin degradation plasmids. Consequently, we relied on alternative selection markers whenever feasible. For most cell lines lacking these resistant markers, other constructs may function as suitable alternatives.

5 **Cell type-specific media.**

Culture HEK293T cells (Takara, #632180) in DMEM media (Thermo Fisher Scientific, #11995065) supplemented with 10% HIFBS (Thermo Fisher Scientific, #10082147). Culture HCT-116 cells in McCoy's 5A media (Thermo Fisher Scientific, #16600082) supplemented with 2mM L-glutamine (Thermo Fisher Scientific, 25030081), 100U/ml Penicillin with 100µg/ml Streptomycin (Thermo Fisher Scientific, #15140122), and 10% HIFBS.

6 **Maintenance of cells.**

Maintain a confluency of 20-80% by splitting the cells every two days. Store the cells in an incubator at 37°C and 5% CO2.

7 **Harvesting and passaging of cells.**

Harvest or passage the cells by aspirating the media, washing the adherent cells with PBS (Thermo Fisher Scientific, #10010023), and incubating the cells with trypsin (Thermo Fisher Scientific, #25200114) for 2 minutes. To quench the reaction, add fresh media on top and centrifuge the cells at 400g for 5 minutes. Resuspend the cells in fresh media for further use. Count the cells using a Countess cell counter. To do this, take 10µl of cells and mix with 10µl of trypan blue solution, then add 10µl of this mix to one chamber on a Countess slide.

Lentivirus preparation

8 **Plating HEK293T cells for virus production.**

To begin virus production, harvest, count, and plate 2.5 million HEK293T cells on a 10cm dish (VWR, #10062880) as described in the "Culturing of CRUDO Cells" section.

9 **Transfect HEK293T cells for virus production.**

24-hours post-plating, transfect the HEK293T cells with the following transfection reagents:

- 7.2µg of our transfer plasmid (expressing dCAS9-KRAB-BFP or gRNAs, and a selectable marker between viral LTR sequences)
- 5.4µg of psPAX2 (a lentiviral co-packaging plasmid, Addgene, #12260)
- 2.16µg of pMD2.G (a lentiviral co-packaging plasmid, Addgene, #12259)
- 1,150µl Opti-MEM (Thermo Fisher Scientific, #31985070)
- 35µl XtremeGene9 (MilliporeSigma, #6365787001) in 10ml of DMEM with10% HIFBS media.

Combine the transfection reagents and incubate at room temperature for 15 minutes before adding the solution dropwise to the cells in HEK296T media. After 16 hours, exchange the media for fresh HEK296T media.

10 **Harvest virus produced by HEK293T cells.**

At 24 hours post-transfection, harvest the first batch of viral supernatant. Then add fresh HEK293T media to the cells. Store the viral supernatant at 4°C and harvest the viral supernatant again at 48 hours post-transfection.

11 **Filter and concentrate the viral supernatant.**

Combine the harvested virus supernatant for each construct, filter the supernatant using a vacuum-driven filtration system (MilliporeSigma, #SE1M003M00), and concentrate the virus 20X with centrifugal filters (MilliporeSigma #UFC910024).

12 **Store the viral supernatant.**

Store the filtered and concentrated viral supernatant at 4°C unless the time before use exceeds two weeks. In that case, divide the virus into 1ml aliquots and snap freeze at -80°C.

13 **Transducing HCT-116 cells.**

Transduce the HCT-116 cells in a 24-well plate with a cell density of 250,000 cells per well. For transduction, supplement the HCT-116 media with 10µg/ml polybrene (MilliporeSigma #TR1003G). Add the virus to the wells and infect the cells by centrifuging at 1,200g and 32°C for 45 minutes.

14 **Blasticidin selection of HCT-116 cells.**

24-hours post-transduction, remove the media containing the virus from the cells and exchange it for fresh media supplemented with 7.5µg/ml Blasticidin S HCl (Thermo Fisher Scientific, #A1113903) to start the 5-day selection.

Virus titration

15 **Selecting a target MOI.**

Depending on your transduction experiment, different multiplicities of infection (MOIs) may be required. For example, when transducing gRNA libraries, it is generally preferred to avoid multiple integrations within cells. Conversely, when aiming to generate stable Cas9-expressing cell lines, multiple integrations and a diverse population of cells with distinct integrations can be advantageous to prevent silencing of these constructs during passaging. We use the Poisson distribution to estimate the MOI that best suits the infection efficiency. For gRNA libraries, we typically aim for an MOI of 0.1, as this is estimated to result in only 0.5% of cells with multiple integrations. For the generation of stable cell lines, our target MOI is usually 1, where it is estimated that 63% of cells will have one or more integrations.

16 **Transduction for viral titration.**

For viral titration, follow the transduction protocol as outlined in step 13, "Transducing HCT-116 cells," but double the cell number to transduce in a 6-well plate. Test various doses of filtered and concentrated viral supernatant to determine the concentration of virus required for your target MOI. Typically, we select six virus doses, ranging up to 1 milliliter, but this may vary (i.e. in microliters: 0, 10, 30, 60, 80, 250, 750).

Infect one 6-well plate with the virus of interest using the chosen doses and two 6-well plates (in duplicate) with a fluorescent virus (for example, Addgene, #85556), using the same virus doses. Additionally, include control wells, such as those containing only virus and no selection.

17 **Start selection of viral titration.**

24-hours post-transduction, start the selection process on the plate containing the virus of interest and on one of the GFP virus plates.

18 **Quantify infection efficiency on the non-selected GFP Plate.**

To quantify infection efficiency, use a flow cytometer (such as an Accuri or any other Cytoflex) and analyze the non-selecting GFP-virus plate. Measure the percentage of GFP-positive cells across the different wells infected with various concentrations of the GFP virus. Identify the well that most closely matches the target infection efficiency. For example, for an MOI of 0.1, select the well exhibiting approximately 10% GFP-positive cells (e.g., the well with 30µl of GFP virus).

19 **Choose the dose of your virus of interest for your target MOI.**

Five days post-selection, compare the GFP-selected well determined from the flow check (i.e., the 30µl well) to the wells containing the virus of interest. Select the virus of interest well that closely resembles the GFP-selected well. Confirm this choice by cell counting. For instance, if 30µl of unselected GFP virus resulted in a 10% infection efficiency and the 30µl well of the selected GFP virus closely resembled the 10µl well of the selected virus of interest, we opted for 10µl of the virus of interest for the actual experiment.

Generating an inducible CRISPRi cell line

20 **Introduction to generating an inducible CRISPRi cell line.**

To generate an HCT-116 CRISPRi inducible cell line we transduce HCT-116 cells as outlined in step 13, "Transducing HCT-116 cells".

21 **Integrating a tetracycline activator in HCT-116 cells.**

To introduce the tetracycline activator, transfect HCT116 cells with a plasmid expressing rtTA coupled to a neomycin resistance cassette (Takara, #631363). Select the infected cells by supplementing the media with 200µg/ml G418 Sulfate (Thermo Fisher Scientific, #10131035).

22 **Integrating the tetracycline operator-controlled CRISPRi machinery.**

To integrate the inducible CRISPRi machinery, proceed by transducing the rtTA-positive cells with a construct expressing KRAB-dCas9 linked to BFP under a TRE3G promoter (Addgene, #85449). Select these cells using fluorescent cell sorting (FACS) for BFP. To activate BFP expression, treat the cells with 1µg/ml doxycycline (Thermo Fisher Scientific, #BP26531) via the rtTA site. As a control for negative BFP expression during cell sorting, also treat untransduced HCT-116 cells with 1µg/ml doxycycline.

Note: As our HCT-116 cell line is inherently neomycin-resistant, we omitted the selection from step 21 and solely relied on sorting based on doxycycline-induced expression. This selection method requires both the tetracycline activator and tetracycline operator-controlled CRISPRi machinery to be successfully integrated. But for most cell lines that do not have a neomycin resistance this selection step will increase efficiency.

23 **FACS sort for stable CRISPRi expression.**

As transgenes are often subject to silencing, stable CRISPRi expression can be maintained by performing a repeated BFP-based FACS sort. After selection, allow the cells to grow and recover for a week. Then, activate BFP expression using 1µg/ml doxycycline (Thermo Fisher Scientific, #BP26531) and conduct FACS sorting for the top 10% of BFP-expressing cells. Following sorting, allow the cells to grow for another week before repeating the process.

Validating the CRISPRi cell line

24 **Introduction to validating the CRISPRi cell line.**

We express gRNAs using the sgOpti (Addgene, #85681) with a modified gRNA scaffold for a TAP-seq read-out and the CROP-opti vector (Addgene, #106280) for a FlowFISH read-out. All customized plasmid maps are available in the IGVF portal and can be provided upon request.

25 **Generating a blasticidin-resistant gRNA expression vector.**

To adapt the CROP-opti vector (Addgene, #106280) or sgOpti (Addgene, #85681) to express a Blasticidin resistance cassette (in place of the original Puromycin resistance cassette remove the Puromycin resistance cassette by digesting the CROP-opti vector with BsiWI (New England Biolabs) and MluI (New England Biolabs) and the sgOpti vector with BamHI (New England Biolabs) and MluI (New England Biolabs). PCR-amplify the Blasticidin resistance gene from a different vector that has a Blasticidin resistance cassette (i.e. lenti-dCas-VP64_Blast vector Addgene, #61425) and CROP-Opti or sgOpti specific homology

arms for cloning. Follow HiFi DNA Assembly Cloning Kit (NEB) recommendations for assembling the Blasticidin-resistant vectors and chemically transform the assembled product into competent cells (New England Biolabs, #C2987H). Grow the bacteria on LB plates at 30°C overnight. Select single colonies to inoculate in liquid culture overnight, prepared the DNA (Machery Nagel, NucleoBond Xtra Midi EF), and screen them for correct assembly by Sanger sequencing.

To capture guides directly with 10x beads for TAP-seq we added the We added the cs1 direct capture sequence[14] into the sgOpti-Blasticidin gRNA scaffold by digesting out the standard scaffold with NsiI-HF (NEB) and EcoRI-HF (NEB) and assemble the modified scaffold using the HiFi DNA Assembly Cloning Kit (NEB).

26 **Choosing and preparing gRNAs for validating the CRISPRi cell line.**

To validate the CRISRPi cell line test the knockdown efficiency using previously validated gRNAs targeting the TSS of the ubiquitous but not essential gene ENO1 and non-targeting negative control gRNAs.
ENO1 gRNA #1: CCGGCGAGATCTCCGTGCTC
EMO1 gRNA #2: GACAGTATCTGTGGGTACC
Non-targeting control gRNA #1: GGCGCTTACGCGCGGGCCG
Non-targeting control gRNA #2: CGCGCGCTAACTGGCGCTA
Non-targeting control gRNA #3: ATGTGTTGTAACCTCCACT
Non-targeting control gRNA #4: ATCGCGAGGACCCGTTCCGCC
For each gRNA, order an oligo with Gibson overhangs (i.e., from IDT, liquid, 100µM). For Gibson assembly, the oligo sequence is:
TATCTTGTGGAAAGGACGAAACACCGnGTTTAAGAGCTATGCTGGAAACAGCA; n=gRNA

27 **Preparing the CROP-opti backbone for cloning of CRISPRi knockdown lentiviral plasmids.**

To linearize the plasmid for integrating the gRNAs digest the CROP-opti blast plasmid with BsmBI (New England Biolabs) and purify the backbone with 0.7X AMPure XP beads.

28 **Generating single gRNA CRISPRi knockdown lentiviral plasmids.**

For cloning prepare the following mix and if needed supplement with H2O for a 10µl reaction and using a PCR-cycler incubate at 50°C for one hour:

- 2ng gRNA with homology overhangs
- 50ng linearized CROP-opti backbone
- 5µl 2X HiFi Mix (NEB)

Take 1µl of the cloning reaction and transform into 50µl of Dh5a (NEB competent bacteria) and streak out on ampicillin plates and incubate at 30°C (to prevent lentiviral recombination) overnight.

The next day pick colonies and grow them in 6 ml LB plus carbenicillin (a more stable form of ampicillin, 50µg/ml) at 30°C overnight. Miniprep the DNA (ideally endotoxin-free) and send for Sanger sequencing the gRNA insertion site (i.e. a primer in the U6 promoter: CGATTTCTTGGCTTTATATATCTTGTG).

29 **Generating single gRNA CRISPRi knockdown HCT-116 cell lines.**

Prepare virus for your positive and negative control gRNAs (refer to step 26, 'Choosing and preparing gRNAs for validating the CRISPRi cell line') following the steps outlined in the

'Lentivirus preparation' section. Transduce your dox-inducible CRSPRi cell line in triplicate following step 13, 'Transducing HCT-116 cells', and select your cells. If you are using a blasticidin gRNA expressing vector, select the cells as described in step 14, 'Blasticidin selection of HCT-116 cells'. After selection, allow your cells a few days to recover. Then, activate CRISPRi expression by treating them with 1µg/ml doxycycline (Thermo Fisher Scientific, #BP26531). After 24 hours of treatment, harvest your cells for RNA extraction by aspirating the media and adding RLT buffer, ensuring you have 100µl of RLT buffer for every 5-20K cells. Use immediately or snap freeze and store at -80°C.

30 **RNA extraction.**

To extract RNA from 5K-20K cells, begin by aliquoting 15µl per sample of MyONE SILANE (Thermo # 37002D) magnetic beads for the cleanup process. Place the tube containing SILANE beads on a magnetic rack, allowing 30 seconds for the beads to fully separate. Remove the supernatant, then take the tube off the rack and resuspend the beads once in Buffer RLT (Qiagen). Place the tube back on the magnet, wait for the beads to separate, and discard the supernatant. Resuspend the beads in 5µl RLT per sample and add to 90µl of resuspended cells in RLT. Depending on the sample number the cleanup can be performed in tubes, or a 96-well plate.

Next, add an equal volume of 100% isopropanol (i.e., 95µl), mix well by pipetting, and incubate at room temperature for 2 minutes. Place the tube on the magnetic rack for about 2 minutes until the beads are separated, then remove and discard the supernatant. Wash the beads twice by removing the tube from the magnetic rack, resuspending the beads in 150µl of 70% ethanol, and then removing the supernatant after placing them back on the rack.

Carefully remove all remaining 70% ethanol and dry the beads on the magnetic rack for up to 2 minutes. While the samples are drying, prepare the Elution/DNAse mix (volumes per sample):

- 16 µl H2O
- 2 µl TURBO DNase Buffer
- 2 µl TURBO DNase enzyme.

Add 20 µl of Elution/DNase mix to each sample, pipet to resuspend the beads, and transfer to a fresh 96-well plate. Incubate at 37°C for 15 minutes.

Clean up the samples by adding 70µl RLT to each sample, mixing, and then adding 90µl isopropanol. Incubate at room temperature for 2 minutes, then place the tube on the magnetic rack until the beads are separated. Remove the supernatant and wash the beads twice as before.

Finally, remove any remaining ethanol and let the beads dry for about 2 minutes. Elute the RNA by adding 25 µl H2O to each sample, pipetting to mix, and letting it sit for 2 minutes. Place the tube back on the magnet, remove the water and RNA from the magnet, and transfer to a new plate. The RNA can be used immediately or stored at -80°C.

31 **Reverse transcription.**

For the Reverse Transcription process, begin by aliquoting 12.2 µl of RNA, denature it at 75°C for 3 minutes, and immediately place it on ice.

Prepare the reverse transcription mix (volumes per sample):

- 1.2µl H2O

- 0.8µl 100µM Random Hexamers
- 2µl 10x AffinityScript Buffer
- 2µl DTT (100mM)
- 0.8µl 100mM dNTP mix (25mM/base)
- 1µl AffinityScript RT enzyme

Add 7.8µl of the reverse transcription mix to the 12.2µl of RNA, and mix thoroughly by pipetting. Incubate the mixture at 25°C for 10 minutes, 55°C for 1 hour, 72°C for 10 minutes, and finally at 4°C for 1 minute. Dilute the cDNA 1:10 by adding 180µL H2O. The cDNA can be used immediately or stored at -20°C.

32 **RNA expression analysis via qPCR.**

To assess target gene expression, perform a qPCR using SYBR Green I Master Mix (Roche) with primers for gRNA target genes (i.e. ENO1) and GAPDH as a housekeeping control. We calculate differences using the ΔΔCT method.
ENO1 qPCR forward primer: GCCAAGTACAACCAGCTCCT
ENO1 qPCR reverse primer: GAGGGGTCTGTGTAGCCAAC
GAPDH qPCR forward primer: AGCACATCGCTCAGACAC
GAPDH qPCR reverse primer: GCCCAATACGACCAAATCC

For the qPCR setup, you will need 2x 19µl of the diluted cDNA for each of the genes we want to analyze. Begin by preparing the master mix for all samples with the following volumes per sample:

- 1µl of 50 µM Primer pair (25µM each)
- 20µl of Roche Sybr Green Master Mix 2x (Roche)

In a 96-well plate, start by adding 19µl of the 1:10 diluted cDNA of each sample into two separate wells, with each well dedicated to one primer pair. After that, add 21µl of the target gene mix to one of the wells and 21µl of the housekeeping control mix to the other. Mix both wells thoroughly by pipetting. Finally, for technical replicates, split each well of the 96-well plate into 3 reactions of 12µl each, transferring them into a 384-well plate for the qPCR analysis.

Generating a pooled gRNA library expressing CRISPRi cell line

33 **Designing a gRNA library.**

Design a gRNA library to target the TSSs of your target genes, candidate enhancers, and negative controls. Candidate enhancers are typically DNase-seq peak peaks and can be selected using ABC enhancer-gene pair predictions or other criteria. For each genomic target specify a region of 500bp centered around the DNase-seq peak. Design the gRNAs to be tiled across each element by making a list of every possible target site with an NGG PAM sequence. Rank the target sites by calculating a specificity score based on potential off-target effects as previously described[15]. Remove target sites with a score <20 and include up to 10-30 gRNAs per genomic target site (for FlowFISH screens, less for TAP-seq screens, i.e. 2-4). Add approximately 20% non-binding negative control sequences to your gRNA pool. And for cloning purposes add a ‘G’ to the 5’ end of every target sequence, unless already present.

34 **Amplifying the gRNA library pool for cloning.**

Order a PCR-tagged custom oligo pool (Agilent) corresponding to the gRNA library design including homology overhangs as described in step 26, 'Choosing and preparing gRNAs for validating the CRISPRi cell line'. Resuspended the gRNA oligo pool in water at a 100 µM concentration. For PCR amplification dilute an aliquot to 1ng/µl.

PCR amplification primers:

CRISPR pool amp forward primer: GGCTTTATATATCTTGTGGAAAGGACGAAACACCG

CRISPR pool amp reverse primer: CTTATTTAAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAAC

Start by preparing the PCR mix:

- 25µl 2X NEBNext Master Mix
- 5µl Primer Mix (5 µM each)
- 2µl oligo pool (1ng/µl)
- 18µl H2O

PCR amplify in two stages: 98°C 30 seconds; 98°C 15 seconds, 62°C 15 secs, 72°C 15 seconds (4 cycles); 98°C 10 seconds, 72°C 15 seconds, 72°C 5 secs (10-20 cycles); 72°C 120 seconds; 4°C hold.

Try multiple numbers of cycles to find the cycle number that gives you a good amount of product and uses up most of the primer, while avoiding primer dimer or larger products, and overamplification. Purify the final amplification product with 1.5X AMPure XP beads.

35 **Cloning the gRNA library pool.**

Linearize the CROP-Opti or sgOpti backbone as described in step 27, 'Preparing the CROP-opti backbone for cloning of CRISPRi knockdown lentiviral plasmids'.

For cloning prepare the following mix and if needed supplement with H2O for a 30µl reaction and using a PCR-cycler incubate at 50°C for one hour:

- 70ng purified gRNA library pool
- 500ng linearized backbone
- 15µl 2X HiFi DNA Assembly Cloning Kit (NEB)

Add a no-insert control for estimating background activity.

Purify the assembled product with 0.7X AMPure XP beads and elute in 15µl H2O. Use electroporation to transform the full 15µl of assembled product into 25µl of electrocompetent bacteria (Endura, #60242). Electroporate using the following settings:

- 10 uF
- 600 Ohms
- 1800 Volts

Transfer to the reaction to a tube with 950µl of SOC media and incubate at 30°C for one hour. Take a 10µ aliquot (1% of the electroporation reaction) and add to 90 µl SOC medium, mix, and serial dilute 5 times. For the serial dilution use 10µl of each prior mix into 90µl SOC medium, for 1:1K, 1:10K, 1:100K, 1:1M, 1:10M, 1:100M dilutions from the expanded electroporation). Streak out each of these dilutions onto an ampicillin LB plate and grow overnight at 30°C. Expand the remaining transformed cells in 100ml LB plus carbenicillin (50µg/ml) at 30°C overnight.

The next day, estimate the number of colonies from your serial dilution plates (i.e. 10 colonies on your 1:1M dilution plate corresponds to a total of 10 million transformed cells). Aim for a complexity of at least 100- 500 colonies per gRNA in your library pool. The number of colonies on your no-insert control should not exceed 5% of your library pool count. Purify the overnight cultures using an Endotoxin-Free Plasmid Midi Kit (Machery Nagel, NucleoBond Xtra Midi EF). Sequence gRNAs to verify the library complexity.

36 **Sequencing cloned gRNA library pools.**

To determine even gRNA distribution in your cloned gRNA library pool, start by amplifying the purified library cloning product using sequencing primers that include an Illumina adapter sequence and an index sequence for multiplexing.
Sequencing forward primer:
AATGATACGGCGACCACCGAGATCTACAC nnnnCGATTTCTTGGCTTTATATATCTTGTG
Sequencing reverse primer:
CAAGCAGAAGACGGCATACGAGATnnnnnnnn ACAGTCGAGGCTGATCAGC
Prepare the PCR mix:

- 20µl 2X Q5 Hot Start Master Mix (NEB)
- 3µl Primer Mix (25 µM each)
- 1µl purified library (1ng/µl)
- 16µl H2O

PCR amplify in two stages: 98°C 30 seconds; 98°C 15 secs, 64°C 15 seconds, 72°C 15 seconds (4 cycles); 98°C 15 secs, 72°C 15 secs, 72°C 5 secs (16 cycles); 72°C 120 seconds; 4°C hold. Run an aliquot of a gel to check the approximately 200bp-sized amplicon. Purify the PCR product twice with 1X AMPure XP beads and elute the final product in 15µl H2O.

Perform MiSeq sequencing aiming for 100-1,000 reads per gRNA in your pool and spike in the following custom primers:
Port 18 - Custom Read 1 primer: CGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG
Port 19 - Custom Index 1 primer: AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCG
The gRNA sequence read-out and dual indexing strategy requires 21bp for Read 1, 8bp for Index 1, and 4bp for Index 2. Paired-end sequencing is not required.

Generate a text file containing each of your guide IDs and sequences on separate lines. Then, use Bowtie[16] to generate "genome" files, treating the guide sequences as the genome. Subsequently, run Bowtie to identify exact matches for each guide and count the number of unique matches obtained for each guide. Calculate the library pool 'skew', which represents the difference in count frequency between the top and bottom 10th percentiles of guides. Aim for a skew of less than 5.

Note: This sequencing strategy is compatible with MiSeq, HiSeq, and NovaSeq runs. However, for a NextSeq run, an additional custom Index 2 primer is necessary. This requirement stems from the NextSeq workflow, which requires a custom Index 2 primer when using a custom Read 1 primer. Similarly, if using a custom Read 2 or custom Index 1

primer, a custom Index 2 primer is also needed. The custom Index 2 primer should be the reverse complement of the custom Read 1 primer.
Custom Index 2 primer:
CGGTGTTTCGTCCTTTCCACAAGATATATAAAGCCAAGAAATCG

37 **Generating guide library expressing CRISPRi cell lines.**

Prepare a lentivirus library following the 'Lentivirus preparation' section and titrate this library according to the 'Virus titration' section. Transduce the doxycycline-inducible CRISPRi HCT-116 cell line according to step 13, 'Transducing HCT-116 cells' with the gRNA library aiming for an MOI of 0.1 and a coverage of 500 transduced cells for each gRNA (i.e. for 1,000 gRNAs start with a population of 5,000,000 cells for the transduction). After selection combine all transduced cells from individual wells to one large plate/flask and maintain them in 1/3 of the selection antibiotic concentration, (i.e. 2.5µg/ml Blasticidin) and at a 500X coverage of the gRNAs at all times to maintain library complexity and gRNA distribution (i.e. for 1,000 gRNAs this should be at least 500,000 cells post-selection).

Performing the CRUDO screen

38 **Induce CRISPRi machinery and target protein degradation.**

To conduct the CRUDO screens, expand the library pool of transduced cells to 50 million for each target gene. This number accounts for potential losses during harvesting, ensuring robust coverage. However, if your library pool exceeds 15,000 gRNAs, increase the number of cells to maintain a coverage of at least 500X for subsequent steps. Divide the 50 million cells into four flasks, each containing 10 million cells, with duplicates for each condition (replicate numbers can be adjusted as needed).
In each flask, activate CRISPRi expression by treating with 1µg/ml doxycycline (Thermo Fisher Scientific, #BP26531). After 18 hours of doxycycline treatment, add 500µM IAA (MilliporeSigma, #I37505GA) to two flasks per target gene to activate the targeted degradation of your protein of interest.
Following 24 hours of treatment, harvest the cells according to step 7, 'Harvesting and passaging of cells' procedure, and proceed with your chosen read-out.

39 **Performing CRUDO-FlowFISH.**

For a FlowFISH read-out of your CRUDO screen follow the steps as previously described[11,17].
Briefly for each sample take 10 million of the harvested cells and stain each sample with the PrimeFlow RNA Assay Kit (Thermo Fisher Scientific, #88–18005 for the respective target gene with an Alexa Fluor 647 (AF647, 'Type 1') probeset and against for RPL13A to have a positive control housekeeping gene with Alexa Fluor 488 (AF488, 'Type 4'). Use an Astrios EQ Sorter (i.e. Beckman Coulter, #B25982 or similar) and sort the cells into six bins based on the fluorescence intensity of the respective target gene. Normalize for staining efficiency. Then on the collected cells reverse-crosslink and extract the gDNA (i.e. 24 samples per gene: 2 conditions, 2 replicates, and 6 sorting bins). Finally, sequence the guide RNA integrations according to step 32, 'Sequencing cloned gRNA library pools'. Code for processing CRISPRi FlowFISH data is available at https://github.com/EngreitzLab/crispri-flowfish.

40 **Performing CRUDO-TAPSeq.**

For a TAP-seq read-out of your CRUDO screen adapt the previously described[12] steps as follows:

Briefly, resuspend the harvested cells in PBS with 1% BSA (ThermoFisher #J65097.A1). Perform 10x encapsulation and barcoding following manufacturer's instructions (10x Chromium Next GEM Single Cell 3' GEM Kit v3.1) and load 16,000 cells per lane. The number of lanes required per sample depends on the number of gRNAs in your pool aim for 1,000 recovered cells for each gRNA. For instance, if your gRNA pool consists of 100 gRNAs you will want to recover 10,000 cells, which requires one 10x lane loaded with 16,000 cells (check the manufacturer's recommendations for cell recovery and cell loading numbers). Once the cDNA is obtained amplify your target gene set and gRNA library. First amplify the cDNA using a mix of Partial R1, Partial R1N, Partial TSO, and a mix of the TAP-seq outer primers. Then perform a size selection using AmpureXP beads to separate the gDNA and mRNA amplicons. Continue to amplify the gRNA amplicons using the Feature SI Primers 3 primers. Amplify the mRNA pool using Read 1 and a mix of the TAP-seq inner primers. After indexing sequenced the libraries aiming for 20,000 reads per cell for the mRNA and 2,000 reads per cell for the gRNA libraries. Code for processing TAP-seq data is available at https://github.com/argschwind/TAPseq_workflow.

Protocol references

1. Rao, S. S. P. et al. Cohesin loss eliminates all loop domains. Cell 171, 305-320.e24 (2017).
2. Nora, E. P. et al. Targeted degradation of CTCF decouples local insulation of chromosome domains from genomic compartmentalization. Cell 169, 930-944.e22 (2017).
3. Schwarzer, W. et al. Two independent modes of chromatin organization revealed by cohesin removal. Nature 551, 51–56 (2017).
4. Wutz, G. et al. Topologically associating domains and chromatin loops depend on cohesin and are regulated by CTCF, WAPL, and PDS5 proteins. EMBO J. 36, 3573–3599 (2017).
5. Oudelaar, A. M. & Higgs, D. R. Publisher Correction: The relationship between genome structure and function. Nat. Rev. Genet. 22, 808 (2021).
6. Robson, M. I., Ringel, A. R. & Mundlos, S. Regulatory landscaping: How enhancer-promoter communication is sculpted in 3D. Mol. Cell 74, 1110–1122 (2019).
7. Beagan, J. A. & Phillips-Cremins, J. E. On the existence and functionality of topologically associating domains. Nat. Genet. 52, 8–16 (2020).
8. Zuin, J. et al. Nonlinear control of transcription through enhancer-promoter interactions. Nature 604, 571–577 (2022).
9. Kubo, N. et al. Promoter-proximal CTCF binding promotes distal enhancer-dependent gene activation. Nat. Struct. Mol. Biol. 28, 152–161 (2021).
10. Kane, L. et al. Cohesin is required for long-range enhancer action at the Shh locus. Nat. Struct. Mol. Biol. 29, 891–897 (2022).
11. Fulco, C. P. et al. Activity-by-contact model of enhancer-promoter regulation from thousands of CRISPR perturbations. Nat. Genet. 51, 1664–1669 (2019).
12. Schraivogel, D. et al. Targeted Perturb-seq enables genome-scale genetic screens in single cells. Nat. Methods 17, 629–635 (2020).
13. Natsume, T., Kiyomitsu, T., Saga, Y. & Kanemaki, M. T. Rapid protein depletion in human cells by auxin-inducible degron tagging with short homology donors. Cell Rep. 15, 210–218 (2016).
14. Replogle, J. M. et al. Combinatorial single-cell CRISPR screens by direct guide RNA capture and targeted sequencing. Nat. Biotechnol. 38, 954–961 (2020).
15. Hsu, P. D. et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. 31, 827–832 (2013).
16. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25 (2009).
17. Zhou, R. FlowFISH with PrimeFlow v1. (2023) doi:10.17504/protocols.io.36wgq3q4olk5/v1.