Supplementary Methods

OUTLINE

I) Plasmid construction
- IA) Construction of Anchor plasmids
- IB) Construction of gRNA plasmids
  - Individual gRNA Plasmids without soluble CRY2
  - Multiplexed gRNA Plasmids without soluble CRY2
  - Multiplexed gRNA Plasmids with soluble CRY2
- IC) CRY2olig and derived plasmids

II) Tissue culture and cell preparations
- Mouse embryonic stem cell and induced pluripotent cell culture
- Primary mouse embryonic fibroblast culture
- Mitomycin-C inactivation
- Gelatin-coating plates
- Transfection condition
- Blue light illumination to cells
- Fixation for ChIP and 5C

III) Construction and validation of the light box
- Overall design
- Arduino code
- Functional validation

IV) RNA extraction, First strand and qRT-PCR
- RNA extraction
- Reverse transcription
- qPCR
- Standard curve preparation

V) Chromatin Immunoprecipitation (ChIP) – qPCR
- Antibody-bead binding
- ChIP
- qPCR

VI) *in situ* 3C and 5C
- *in situ* 3C
- 5C primer design
- 5C library preparation
- 5C data analysis
- Parsing 5C monomers and mapping

VII) Public sequencing data analysis

VIII) Immunofluorescence staining

IX) RNA FISH

X) Statistics

XI) Fusion protein sequences

XII) Life Science Reporting Summary

IA) CONSTRUCTION OF ANCHOR PLASMIDS

Anchor plasmid backbone assembly

We cloned the LADL Anchor and related control plasmids into a minimally sized backbone for optimal transfections. First, we created a minimal backbone containing the transcriptional terminator bGH along with appropriate restriction enzyme sites. Second, we cloned the individual Anchor plasmids into this backbone. To create the minimal backbone, we digested pUC19 plasmid with ZraI and PciI, gel extracted the 1,809 kb fragment containing the Ampicillin promoter and ORF, and treated with Alkaline Phosphatase at 37 °C for 60 min. The bGH polyA signal was PCR amplified using primers MRP175, 176 (**Supplementary Table 1**) and the template AddGene #62987 plasmid[19]. MRP175 and MRP176 primers also incorporated requisite restriction for PciI, SnaBI, EcoRI and ZraI sites for use in downstream applications. Thus, the 282 bp PCR product was digested with ZraI and PciI and ligated with the pUC19-derived vector backbone from above to get the Anchor backbone plasmid (Plasmid S13.1, **Supplementary Figure 2a**). The anchor plasmids (**Supplementary Figures 2b, c**) were cloned into this minimal backbone derived from pUC19 as described below.

LADL Anchor (dCas9-CIBN) plasmid

We next built the LADL anchor plasmid (**Supplementary Figure 2b**) by ligating multiple fragments in a single-step Gibson assembly. We first had to ensure we were using the appropriate PCR templates for the individual fragments. We had access to Cas9n (Addgene #62987 plasmid)[19] as a PCR template, which would need to be mutated at H840A before using it as a template for the dCas9 construct. We performed site-directed mutagenesis using the Quikchange II XL mutagenesis kit (Agilent, #200521), and mutated the H840A amino acid in Cas9n (Addgene #62987 plasmid)[19]. We verified the resultant dCas9 sequence with Sanger sequencing and used it as a PCR template for further cloning. Primers MRP171 and MRP 172 were used for the site-directed mutagenesis to mutate the H840A in the Cas9n construct (**Supplementary Table 1**).

We next PCR amplified the individual inserts (EF1a, 3XFLAG-dCas9, GS-CIBN and 2A-Puro) using the high fidelity Q5 polymerase (NEB) (**Supplementary Table 2**). We verified a single band of expected size on an agarose gel, treated with DpnII, purified with a Qiagen PCR clean-up kit, and quantified using the Nanodrop. The inserts were ligated into the SnaBI, EcoRI digested Anchor backbone plasmid S13.1 (**Supplementary Figure 2a**) using the NEB Gibson Assembly mix (100ng vector backbone, 0.3 pmol of total DNA fragments for 4 inserts ligated for 60 min at 50 °C in a thermocycler). Finally, the Kozak sequence was added upstream of the start site of 3XFLAG by digesting an intermediate plasmid with BamHI, ClaI and ligating the annealed and phosphorylated dsDNA oligo (MRP207 and MRP208; **Supplementary Table 1**) to get the "LADL Anchor" plasmid (**Supplementary Figure 2b**).

Empty anchor control plasmid

As a negative control for the LADL Anchor, we created a vector containing the EF1a promoter-Puromyocin using the same backbone plasmid S13.1 (see **Supplementary Figure 2a**) using Gibson assembly. The individual inserts (EF1a and Puromycin) were PCR amplified (primers detailed in **Supplementary Table 2**) using the high fidelity Q5 polymerase (NEB), verified to give a single band of the expected size on an agarose gel, DpnI treated, cleaned-up using the Qiagen PCR clean-up kit, and quantified using Nanodrop. These were then cloned into the SnaBI, EcoRI digested Addgene #58771 plasmid[20] used as a backbone using Gibson

Assembly. Positive clones were screened using diagnostic digests and verified by Sanger sequencing to give the "Empty anchor control plasmid" (**Supplementary Figure 2c**).

IB) CONSTRUCTION OF gRNA PLASMIDS

Overview

To achieve multiplexing of four gRNAs in a single plasmid, we adopted and modified the system developed by the Yamamoto lab, where single gRNAs are cloned into individual plasmids first and then combined together using Golden Gate Assembly[21]. Our multiplexed four gRNAs plasmids have two versions: without or with soluble CRY2 (plasmids in **Supplementary Figure 2i and 2l)**. The multiplexed gRNA plasmid without soluble CRY2 was created first and sequence verified to contain the multiplexed gRNAs in the expected order (**Supplementary Figure 2i).** Subsequently, the soluble CRY2 expression construct was inserted into this multiplexed gRNA plasmids **(Supplementary Figure 2l)**. In the current study, we designed 2 gRNAs per engineered loop anchor (2 gRNA x 2 loop anchors). Published CRISPRa and CRISPRi studies[13, 22] oftentimes use multiple guides, and, although these studies have a completely different goal, it did influence our decision. We have only tried 2 guides on each loop anchor and cannot predict whether loop efficiency would be altered with more or less guides. All gRNA primer sequences are provided in **Supplementary Table 3**.

Individual gRNA plasmids without soluble CRY2

The Addgene #58768 plasmid was digested with SnaBI and EcoRI to excise the Cas9 open reading frame and ligated with the annealed and phosphorylated dsDNA oligo (MRP173, MRP174; **Supplementary Table 1**) to get the Ampicillin resistant S12.1 gRNA multiplex backbone (BB) plasmid (**Supplementary Figure 2d**). For Step 1 of multiplexing, single gRNAs were cloned into one of the following plasmids: S12.1 (Ampicillin resistant) (**Supplementary Figure 2e)**, B1 (Addgene # 58778)[21] (**Supplementary Figure 2f)**, B2 (Addgene # 58779)[21] (**Supplementary Figure 2g)** or B3 (Addgene # 58780)[21] (**Supplementary Figure 2h).** The gRNA sequences and the plasmids they were cloned into are listed in **Supplementary Table 3 and 4, respectively**. Positive clones were screened with a diagnostic digest and verified by Sanger sequencing using the U6 promoter primer (GAGGGCCTATTTCCCATGATTCC).

Multiplexed gRNA plasmids without soluble CRY2

For Step 2 of multiplexing, plasmids containing single gRNAs were mixed together and multiplexed using the NEB Golden Gate Assembly mix[21]. Specifically, we used 75 ng of the gRNA 129 plasmid clone (**Supplementary Figure 2e)** and 114 ng of gRNA 135 plasmid, gRNA 115 plasmid, gRNA 117 plasmid each (**Supplementary Figure 2f, 2g, and 2h respectively)**. For optimal efficiency, we performed the Golden Gate assembly using the following cycling parameters: (37°C, 5 min → 16°C, 5 min) x 30 cycles → 55°C, 5 min. This procedure consistently gave us >90% efficiency in multiplexing four gRNAs at a time. Sanger sequencing for the multiplexed plasmids was performed using each individual gRNA as the sequencing primer (therefore not itself present in the Sanger trace) and checking for the presence of the adjacent gRNA sequence. The sequence verified multiplexed gRNA plasmid without soluble CRY2 was named "Empty bridge control" (**Supplementary Figure 2i**) and the individual gRNAs present in this plasmid are listed in **Supplementary Table 5.** All gRNA plasmids were

transformed into NEB Stable Competent Cells (NEB, C3040I) to minimize recombination between repetitive U6 promoters present in the multiplexed plasmid.

Multiplexed gRNA plasmids with soluble CRY2

For greater modularity, we built a separate plasmid as the source of soluble CRY2 that could be inserted into any gRNA expressing plasmid. We created the soluble CRY2 cassette in the plasmid S13.1 **(Supplementary Figure 2a)**. The individual inserts (EF1a, CRY2PHR, and 2A_mCherry) were PCR amplified from the templates listed in **Supplementary Table 2** using the high fidelity Q5 polymerase (NEB), verified to give a single band of the expected size on an agarose gel, DpnI treated, cleaned-up using the Qiagen PCR clean-up kit, and quantified using Nanodrop. The three PCR products were then cloned into the EcoRI+SnaBI digested S13.1 plasmid (**Supplementary Figure 2a**) using Gibson Assembly. Positive clones were screened using diagnostic digests and verified by Sanger sequencing to give the plasmid called "Empty target control plasmid" (**Supplementary Figure 2k**).

To demonstrate the modularity above, we used the Empty bridge control (**Supplementary Figure 2i**) as a backbone to create a multiplexed plasmid that also contains the soluble CRY2 transgene (termed the LADL Bridge+Target plasmid) (**Supplementary Figure 2l**). To build this vector, we digested the fragment containing the EF1alpha promoter and the CRY2-HA-2A-mCherry transgene from the Empty target control plasmid (**Supplementary Figure 2k**) with SnaBI+EcoRI and gel extracted the band. The fragment was then ligated into the multiplexed gRNA Empty bridge control plasmid (**Supplementary Figure 2i**) digested with SnaBI+EcoRI using NEB Quick Ligase. Positive clones were screened using diagnostic digests and verified by Sanger sequencing to give the plasmid called "LADL Bridge+Target" (**Supplementary Figure 2l**). The individual gRNAs present in the LADL Bridge+Target plasmid are listed in **Supplementary Table 6.**

One-sided guide control plasmid

We included an additional control containing two gRNAs that target the *Zfp462* promoter, but without the two gRNAs that target the *Klf4* SE. Initially, CRY2 was cloned into S12.1 using the EcoRI and SnaBI sites, and then gRNA 115 was cloned into S12.1. gRNA 117 was cloned into B1 (Addgene # 58778)[21]. We multiplexed gRNA 115 plasmid and gRNA 117 plasmid as well as B2 (Addgene # 58779)[21] and B3 (Addgene # 58780)[21] together to give the "One-sided guide control" plasmid (**Supplementary Figure 2m and Supplementary Table 6**).

IC) CRY2olig AND DERIVED PLASMIDS

The CRY2olig plasmid (Addgene #60032) used in the functional validation of the light box was a gift from Dr. Chandra Tucker[20]. We used this plasmid as a template to amplify mCherry. First, we mutated the two BbsI sites with two synonymous point mutations to ensure they would not be cut by BbsI during gRNA cloning. Thus, our "CRY2olig mut 2-1 plasmid" (**Supplementary Figure 2j)** was derived from the Addgene #60032 plasmid by sequentially mutating the two BbsI sites using the NEB Q5 Site-Directed Mutagenesis kit. At Site 1 nucleotide 729 was changed from a C to an A using primers AM_43 and AM_44. At Site 2 nucleotide 2574 changed from a G to an A using primers AM_45 and AM_46. The sequences of all primers used for cloning this vector are given in **Supplementary Table 1**.

II) TISSUE CULTURE AND CELL PREPARATIONS

Mouse embryonic stem (ES) cell culture

Murine v6.5 Embryonic Stem (ES) cells (v6.5; genotype 129SvJae x C57BL/6; male) were purchased from Novus Biologicals. Mouse ES cells were cultured in the following medium: DMEM (Corning, 10013CV) supplemented with 15 % Hyclone FBS (Thermo Fisher, SH3007003E), 1x MEM non-essential amino acid (Thermo Fisher, 11140076), 2 mM L-Glutamine (Thermo Fisher, 25030164), 100 U/ml Penicillin-Streptomycin (Thermo Fisher, 15140163), 1x 2-mercaptoethanol (EMD Millipore, ES-007-E), 10^3 U/ml Leukemia Inhibitory Factor (EMD Millipore, ESG1107) and maintained on Mitomycin-C (Fisher Scientific, BP2531-2) inactivated mouse embryonic fibroblast (pMEF) feeders at 37 °C and 5 % CO2, as previously described[14, 15]. Prior to transfection and puromycin selection, mouse ES cells were passaged once on gelatin-coated feeder-free plates in order to minimize pMEF contamination.

Mouse embryonic fibroblast (MEF) culture

MEFs were cultured at 37 °C and 5 % CO_2 in DMEM (Corning, 10013CV) supplemented with 10 % FBS (Atlanta Biologicals, S11550), 1x MEM non-essential amino acid (Thermo Fisher, 11140076), 2 mM L-Glutamine (Thermo Fisher, 25030164), 100 U/ml Penicillin-Streptomycin (Thermo Fisher, 15140163). At ~90 % confluency, MEFs were inactivated in 10 μg/ml Mitomycin-C (Fisher Scientific, BP2531-2) in culture media at 37 °C and 5 % CO_2 for 2 hours. 1.5 X 10^6 inactivated MEFs were plated on a 10 cm gelatin-coated plate to be used as a feeder layer for mouse ES cell culture.

Gelatin-coating plates

All plates for mouse ES cells and MEF cultures were coated with EmbryoMax® 0.1% Gelatin Solution (EMD Millipore, ES-006-B) for ~20 min at room temperature and dried before plating cells.

Transfection conditions

We seeded v6.5 mouse ES cells at 2.4 x10^4 cells/cm^2 on gelatin-coated feeder free plates. At 24 hours post-seeding, we co-transfected with 1.5 fmole/cm^2 of the puro-resistant LADL Anchor plasmid and LADL Bridge+Target (CRY2+gRNAs) plasmid for 24 hours in dark using Lipofectamine2000 (Thermo Fisher, 11-668-019) according to the manufacturer's protocol. All plasmids to be transfected were maxi-prepped with Qiagen Endofree Maxiprep kit (Qiagen, 12362) before transfection. At 24 hours post transfection, cells were selected in puromycin-selection media (3.5 μg/ml of puromycin in mouse ES cell culture media) for 36 hours. Mouse ES cells were either exposed to blue light or cultured in dark during puromycin-selection before harvesting as outlined in **Figure 1d.** Transfection efficiency of the two plasmids was evaluated by visually assessing the number of mCherry positive cells that survive puromycin selection. The optimal DNA mass and ratio of the two plasmids to be co-transfected were determined (**Supplementary Figure 4)**.

Blue light illumination to cells

LADL-engineered cells were stimulated using blue light (470 nm) with an intensity of ~1.5 or ~5 mW/cm^2 at 1 second pulse every 14.925 seconds or 0.067 Hz[13].

Fixation for ChIP and 5C

We crosslinked the LADL-engineered mouse ES cells after puromycin selection for ChIP and 5C experiments as previously described[14, 15, 17]. In brief, we washed puromycin-selected ES cells three times with 1X PBS to get rid of dead, un-transfected cells. The transfected cells that were still adhered to the plates were crosslinked with 1 % (v/v) formaldehyde in DMEM (Corning, 10013CV) at room temperature for 10 min, followed by quenching in 125 mM glycine at room temperature for 5 min and at 4 °C for an additional 15 min before cell collection. The mouse ES cells were ~50-70 % confluent at the time of fixation[14].

III) CONSTRUCTION OF THE LIGHT BOX

Overall design

We constructed a light box with a large enough footprint to conduct experiments on the cell numbers required for ChIP and 5C. A significant design change from previously published methods was required to illuminate 6-well plates[13, 20]. The main challenge we overcame was getting the same light intensity and blinking parameters across all the LEDs in the circuit (**Supplementary Figure 3b)**. First, a lightbox was built to illuminate cells at ~1.5 mW/cm^2. One 5 meter blue LED strip was cut into 6 smaller strips of 24 LEDs each (12 V DC weatherproof IP66 LED Tape Light 226 lumens/ft with 5050SMD 470 nm LEDs, WFLS-X3, superbrightleds.com). The 6 smaller LED strips were connected to each other in series with interconnects (2 Contact 10 mm Flexible Light Strip Interconnects, WFLS10-2CH, superbrightleds.com) and aligned parallel on the base of an acrylic box ~1 inch apart from each other. The box is a ¼" custom built black acrylic laser cut box measuring 36 x 48 x 8 cm. A ¼" clear acrylic lid was laser cut to the same dimensions. The LED strip was powered via a 12 V power supply (Mean Well LED Switching Power Supply LPV Series Single Output LED Power Supply 60 W 12 V DC, LPV-60-12, superbrightleds.com). The LED blinking at 0.067 Hz (1 second on, 13.925 seconds off) was controlled by a MOSFET (IRF520 Power MOSFET, SiHF520, Vishay Siliconix) and an Arduino (Arduino Uno Rev3, A000066, Arduino). We inserted a 10kΩ resistor between the MOSFET gate and the ground to prevent gate breakdown. Next, a lightbox was built to illuminate cells at ~5 mW/cm^2. 12 blue LEDs were arranged in 4 parallel lanes of 3 LEDs in series (470nm Rebel LED on a SinkPAD-II 10mm Square Base - 74lm @ 700mA, SP-05-B6, Luxeon Star LEDs). We soldered the LEDs together and secured them in a 8 cm x 12 cm x 8 cm plastic box. The LED strip was powered via a 12 V 2000 mA power supply (3-12 V Selectable Output Variable DC Supply, 9902 PS, MPJA). The LED blinking was controlled in the same way as in the lower intensity lightbox.

Arduino Code

```
void setup() {
// put your setup code here, to run once:
  pinMode(13, OUTPUT);

  digitalWrite(13, HIGH);   // Turn on the LED
  delay(60000);             // Wait for one minute

  digitalWrite(13, LOW);    // Turn off the LED
  delay(1000);              // Wait for one second
}
```

```
void loop() {
// put your main code here, to run repeatedly:
 digitalWrite(13, HIGH);   // Turn on the LED
 delay(1000);              // Wait for one second

 digitalWrite(13, LOW);    // Turn off the LED
 delay(13925);             // Wait for 14.925 seconds
}
```

Functional validation

We tested the light box functionality using transfected cells with 115 fmoles of mCherry-conjugated CRY2 (CRY2olig_mCherry; **Supplementary Figure 3a**) in 6-well plates[20]. The CRY2 oligomers that were assembled in response to blue light illumination were readily visualized as punctate signals with a fluorescence microscope[20]. Mouse embryonic stem cells were seeded for 24 hours and transfected for 24 hours. Transfected mouse ES cells with CRY2olig_mCherry were first focused using white light passing through a red film (intensity 10 % laser power) to ensure minimal exposure to ambient light. Then, the cells were imaged before and after blue light exposure using the Texas Red filter (Excitation: 560/40; Emission: 630/75) in order to observe association and dissociation kinetics of the clusters, respectively. Images of the same embryonic stem cell colony were taken at the following time points: before exposure to 470 nm blue light, after 4 minutes of blue light exposure (CRY2 clustering observed) and 26 minutes after blue light was turned off (CRY2 clusters dissociate). (**Supplementary Figure 3c-d**).

IV) qRT-PCR

RNA extraction

We harvested ~100,000 puromycin-selected mouse ES cells for RNA extraction using *mir*Vana™ miRNA Isolation kit (Thermo Fisher, AM1560) according to manufacturer's instruction.

Reverse transcription

We treated the extracted RNA with TURBO DNaseI (Thermo Fisher, AM2239) and quantified it using Qubit RNA BR assay (Thermo Fisher, Q10210). We used 100 ng of RNA to prepare cDNA using SuperScript® First-Strand Synthesis System for qRT-PCR (Thermo Fisher, 11904018) according to manufacturer's instructions.

qPCR

We mixed 1 µl of cDNA with 10 mM forward and 10 mM reverse primers in 1X Power SYBR® Green PCR Master Mix (Thermo Fisher, 4368706), and ran on quantitative PCR using SYBR Green standard curve method of StepOnePlus™ Real-Time PCR System (Thermo Fisher, 4376600). PCR cycles start with 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 65 °C for 45 sec. We validated the primer pair specificity by looking at single peaks from melting curve analysis at the end of each qPCR run.

Standard curve preparation

We designed qRT-PCR primers using Primer III (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and computationally validated their specificity using BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat) and NCBI Primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi). We mixed 10 mM of forward and reverse primers (**Supplementary Table 7**) with 1 µl of a cDNA in 1x Taq Master mix (NEB, M0270) and then amplified by 40 PCR cycles according to manufacturer's instruction. After confirming unique primer amplicons from each primer pair, PCR products were purified using DNA Clean & Concentrator™-5 (Zymo Research, D4013) according to manufacturer's protocol, followed by measuring their concentrations using Qubit dsDNA HS assay (Thermo Fisher, Q32851). We prepared serial dilutions of the purified products from 1 fmole/µl to 0.00001 fmole/µl.

V) CHROMATIN IMMUNOPRECIPITATION (ChIP) - qPCR

Antibody-bead binding

We performed chromatin immunoprecipitation (ChIP) for the LADL Anchor (dCas9-CiBN) on the crosslinked cell pellets, as previously described[23]. To immunoprecipitate the LADL Anchor, we used 5 µg anti-FLAG antibody (Sigma, F1804-200UG), and for pre-clearing step in ChIP, we used 100 µg IgG (Sigma, I8140-10MG). Both antibodies were pre-bound to 20 µl protein A (Thermo Fisher, 15918014) and 20 µl protein G (Thermo Fisher, 15920010) agarose beads in PBS at 4 °C for overnight with a rotation at 10 rpm. Next day, we washed both antibody-beads were in 1 ml ice-chilled PBS twice before used in ChIP.

ChIP

First, we lysed the crosslinked cell pellets in cell lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2 % (v/v) Nonidet-P40, 10 % (v/v) Protease Inhibitor Cocktail (Sigma, P8340-5ML) and 0.1 mM PMSF (Roche, 8553S)), and dounce homogenized 30 times using pestle A at room temperature. The nuclei fraction of the lysate was spun down at 2,500 g and 4 °C for 5min, and solubilized in 900 µl sonication buffer (50 mM Tris pH8.0, 10 mM EDTA, 0.5 % (w/v) SDS, 0.1 mM PMSF). To shear the DNA into 300-500 bp, we sonicated the samples using QSonica (Qsonica, Q800R2) at 100 % amplitude for 30 min at 4 °C with a cycle of 30 sec on and 30 sec off, and diluted in IP dilution buffer (20 mM Tris pH8.0, 2 mM EDTA, 150 mM NaCl, 1 % (v/v) TritonX-100, 0.1 mM PMSF). We precleared non-specific DNA fragments from the sheared lysates using pre-bound IgG-beads at 4 °C for 2 hours with rotation at 10 rpm, and centrifuged at 2,000 rpm for 5 min at 4 °C. We aliquoted 200 µl of the supernatant to a separate tube to reserve the DNA as input. To immunoprecipitate dCas9-CIBN, we mixed the remaining supernatent with pre-bound anti-FLAG antibody-beads at 4 °C overnight with rotation at 10 rpm.

After spinning the lysate with anti-FLAG antibody-beads at 2,000 rpm at 4 °C for 5 min, we washed the beads in different washing buffers as following order: one wash with IP wash buffer I (20 mM Tris pH8.0, 2 mM EDTA, 50 mM NaCl, 1% (v/v) TritonX-100, 0.1% (w/v) SDS, 0.1 mM PMSF), two washes with High Salt buffer (20 mM Tris pH8.0, 2 mM EDTA, 500 mM NaCl, 1 % (v/v) TritonX-100, 0.01 % (w/v) SDS, 0.1 mM PMSF), one wash with IP wash buffer II (10 mM Tris pH8.0, 1 mM EDTA, 250 mM LiCl, 1 % (v/v) NP40, 1 % (w/v) sodium deoxycholate, 0.1 mM PMSF) and, lastly, two washes in 1x TE. Each of the above washes was performed at 4 °C with a rotation at 10 rpm for 5 min each. Finally, protein-DNA complexes were eluted from beads by vortexing in Elution buffer (100 mM sodium bicarbonate, 1% (w/v) SDS) at room temperature for 1 min. We reverse crosslinked the eluent and the input DNA

aliquots at 65 °C overnight, and added 1x TE to bring the final volume up to 400 µl before digesting proteins using 20U of Proteinase K (NEB, P8107S) at 65 °C for 2 hours. We extracted and purified the DNA from the samples using phenol:chloroform:isoamyl alcohol (Fisher Scientific, BP1752I100) and ethanol precipitation method using 30 µg glycogen (Ambion, AM9510) and 80 mM NaCl. We resolved the DNA precipitates in 20 µl of 1x TE before subsequent analysis.

qPCR

To compare the LADL Anchor enrichment in the ChIP DNA, it is essential to use the equal masses of DNA for all the samples for qPCR. We measured the ChIP and input DNA concentrations using Qubit dsDNA HS assay (Thermo Fisher, Q32851). 20 pg of input and eluent DNAs were mixed with 10 mM of forward and reverse primers, 1x Power SYBR® Green PCR Master Mix (Thermo Fisher, 4368706), and quantitative PCR was performed using StepOne™ Real-Time PCR System (Thermo Fisher, 4376357) according to Standard SYBR Green protocol[14].

For PCR cycles, the PCR reaction was melted at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 65 °C for 45 sec using SYBR Green standard curve method of StepOnePlus™ Real-Time PCR System (Thermo Fisher, 4376600). We confirmed the primer pair specificity by looking at the single peaks of the melting curves in the end of each PCR run. Primer sequences used in ChIP-qPCR are described in **Supplementary Table 9**.

VI) *IN SITU* 3C AND 5C

In situ 3C

We created 3C libraries using the *in situ* 3C method with minor modifications[2, 18]. We lysed the crosslinked cell pellets in 250 µl of cell lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2 % (v/v) Nonidet-P40) supplemented with 50 µl of Protease Inhibitor Cocktail (Sigma, P8340-5ML)), and incubated in ice for 15 min. The nuclei in lysates were spun down at 2,500 g at 4 °C for 5 min, and washed in 500 µl of cell lysis buffer. We permeabilized the nuclei in 0.5 % (w/v) SDS at 62 °C for 10 min, followed by quenching in 1.13 % (v/v) Triton-X100 (final concentration) at 37 °C for 15 min. We digested genomic DNA with 100 U HindIII (NEB, R0104s) in 1x NEBuffer2 (NEB, B7002S) at 37 °C overnight. Next day, after HindIII inactivation at 62 °C for 20 min, we ligated the digested genomic DNA fragments in the nuclei with 2,000 U T4 DNA ligase (NEB, M0202S) in ligase buffer (0.83 % Triton-X100, 0.1 mg/ml BSA in 1x T4 DNA ligase buffer (NEB, B0202S)) at 16 °C for 2 hours. We spun down the nuclei at 2,500 g at 4 °C for 5 min, and lysed in nuclear lysis buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 % (w/v) SDS). We reverse crosslinked the DNA in the lysates at 65 °C initially for 4 hours in 20 U Proteinase K (NEB, P8107S), and subsequently overnight with additional 20 U of Proteinase K (NEB, P8107S). To purify DNA from residue proteins and RNA, we treated the samples with 50 mg RNaseA at 37 °C for 30 min (Roche, 10109169001) and performed phenol:chloroform extraction (Fisher Scientific, BP1752I100) and ethanol precipitation methods. After dissolving the DNA pellets in 500 µl TE, we centrifuged the samples on Amicon column filters (Millipore, MFC5030BKS) at 14,000 g for 10 min at room temperature. To wash out the salts in the samples, we washed the column filters with 500 µl TE at 14,000 g for 10 min at room temperature twice, and inverted the column filters and centrifuged at 1,000 g for 4 min at room temperature to elute the DNA. The 3C libraries were kept at -20°C until 5C was performed.

Chromosome-Conformation-Capture-Carbon-Copy (5C) primer design

All 5C primers were designed according to a double alternating design with the My5C primer design software (http://my5c.umassmed.edu/my5Cprimers/5C.php)[18, 24, 25]. Details of 5C primer sequences are described in **Supplementary Table 10 and 11**[18].

5C library preparation

5C was performed as previously described[15]. We mixed 370 ng of replicate 1 3C library **(Figure 3-4, Supplementary Figure 6-11)**, or 500 ng from replicate 2 3C library (**Supplementary Figure 7-10**), or 200 ng from replicate 5 3C library (**Supplementary Figure 7-10**), or 590 ng from replicates 3-4 3C libraries (**Supplementary Figure 7-10**) with salmon sperm DNA (Thermo Fisher, 15632-011) to ensure a final DNA mass of 1.5 μg. We mixed the DNA with 1 fmol of each 5C primer (**Supplementary Table 10 and 11**) in 1x NEBuffer4 (NEB, B7004S). We denatured the DNA in the 5C reaction at 95 °C for 5 min and annealed 5C primers at 55 °C for 16 hours. We then nick ligated annealed 5C primers using 10 U Taq ligase (NEB, M0208L) for 1 hour at 55°C, followed by inactivation at 75 °C for 10 min. We used 30 PCR cycles to amplify the 5C ligation product at an annealing temperature of 55 °C using 0.5 U of Phusion High-Fidelity DNA polymerase (NEB, M0530L), 120 μmoles universal T3/T7 primers, and 25 mM dNTP (Promega, U1330) in 1x Phusion HF Buffer (NEB, M0530L) according to the manufacturer's protocol. Before Illumina adaptor ligation, we purified 5C libraries using AgenCourt Ampure XP beads (Beckman Coulter, A63881) as described by the manufacturer.

To prepare sequencing libraries, we A-tailed 100 ng of purified 5C library before ligating Illumina sequencing adapters using the NEBNext Ultra DNA library prep kit (NEB, E7370S) for replicates 1-3 and using the NEBNext Ultra II DNA Library Prep Kit (NEB, E7645S) for replicates 4-5. We used NEBNext Multiplex oligos I and II (NEB, E7335S and E7550S) for all replicates according to manufacturer's instructions. The linkered 5C libraries were size-selected at 230 bp using Agencourt Ampure XP beads (Beckman Coulter, A63881) before amplification by 9 cycles of PCR for replicates 1-3, and 5 cycles for replicates 4-5 according to the manufacturer's protocol. We further purified sequencing libraries using AgenCourt Ampure XP beads (Beckman Coulter, A63881) and assessed their quality using the Agilent DNA 1000 reagent kit (Agilent, 5067-1504) on the Agilent Bioanalyzer 2100 (Agilent, 5067-4626). Each library was quantitated using Library Quantification Kit – Illumina/ABI Prism® (Kapa Biosystems, KK4835) before pooling and pair-ended Illumina sequencing on the NextSeq500 instrument (Illumina). Replicates 1-2 were sequenced with 37 bp paired end reads, and replicates 3-5 were sequenced with 75 bp paired end reads.

5C data analysis

We analyzed 5C data as detailed in[15] with minor modifications. The 37 bp pair-ended sequencing reads for replicates 1 and 2 were directly mapped to a pseudo-genome consisting of 5C primer sequences with Bowtie using parameters --tryhard and -m 2 and --trim5 6 (**Supplementary Table 10 and 11**). A summary of the mapped reads for replicate 1 and 2 is described in **Supplementary Table 12.** 5C primer pairs were counted as previously described[14, 15, 17]. Outlier values were removed if they are greater than the sum of 30 surrounding pixels by 32-fold. Raw counts were quantile normalized, binned into 4 kb resolution matrices, and balanced using the ICED algorithm[26]. We evaluated looping interactions by modeling the TAD and distance-dependence signal using the upper half of the donut filter for short range

interactions under 100 kb distance, and the full donut filter for longer range interaction greater than 100 kb using parameters p=10 and $w=40^2$. We modeled the distance- and TAD-corrected interaction frequency data (*i.e*. Observed/Expected) with a parameterized log-logistic distribution as described in [15]. Interaction scores were computed as $-10\log_2(\text{pvalue})$.

Parsing 5C monomers and mapping

The 75 bp end of pair-ended sequencing reads from replicates 3, 4, and 5 were independently mapped to the pseudo-genome consisting of 5C primer sequences. The length of these reads required additional processing. First, only the reads containing the HindIII recognition sequence (AAGCTT) were split into two sub-reads corresponding to the 20 bp of sequence immediately 3' and immediately 5' of the HindIII cut site, respectively. To assign specific 5C primer-primer ligations to each 75 bp end, subreads were mapped to the pseudogenome consisting of 5C primers using Bowtie with parameters --tryhard and -m 2. To identify the 5C "monomers" that have only one 5C ligation junction, we compared the primer-primer ligations between the paired reads. If both paired reads have the same primer-primer ligation junction, we classified them as a "monomer" and constructed counts files for downstream analysis as described above. A summary of the mapped reads for replicates 3-5 is described in **Supplementary Table 12.**

VII) PUBLIC DATA ANALYSIS

A list of all publicly available sequencing data sets that were used in this study is described in **Supplementary Table 8**. Sequencing reads were downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/) and mapped to NCBI Build 37 (UCSC mm9) using the Bowtie with parameters --tryhard and -m 2 for ChIP-seq, and Bowtie2 with parameters -X2000 --no-mixed --no-discordant for ATAC-seq. Only the sequencing reads that were uniquely mapped to the genome were analyzed in this study. Summary of mapped reads corresponding to the publicly available data is also described in **Supplementary Table 8**.

VIII) IMMUNOFLUORESCENCE STAINING (IF)

We fixed the LADL-engineered mouse ES cells in 4 % PFA in PBS for 15 - 20 mins at room temperature, and washed three times with 1x PBS. The fixed cells were stored at 4 °C until IF was performed. We incubated the fixed cells in blocking solution (10 % (v/v) Normal Donkey Serum (Jackson ImmunoResearch, 017-000-121), 0.1 % (v/v) TritonX100 in PBS) with gentle nutation for 1hr at room temperature. We next probed the cells with Rabbit α-Oct3/4 (Thermo Fisher, SC-9081) at 1:200 dilution in Blocking solution with gentle nutation overnight at 4 °C. The next day, we washed cells three times in 0.1 % (v/v) Tween20 in PBS for 10 min each to remove excess primary antibodies, and probed with Goat Anti-rabbit Alexa Fluor 488 (Thermo Fisher, A-11006) at 1:500 dilution in Blocking solution for 2 hours at room temperature in the dark. To remove excess secondary antibodies, we washed cells twice in 0.1 % Tween20 in PBS for 10 mins each and twice in PBS for 10 mins each. Finally, we mounted cells onto slide glass with ProLong Gold antifade reagent with DAPI (Thermo Fisher, P36935) before imaging on a Leica DMi8/LAS X Microscope.

IX) RNA FISH

We designed oligonucleotides for RNA FISH using the Stellaris probe design software available online (https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-

designer). Pools of 32 oligonucleotides were labeled with Atto674N (atto-tec) for *Klf4* and *Zfp462* exons and Atto700 for *Klf4* and *Zfp462* introns. We trypsinized cells and fixed in 3.7% formaldehyde and performed RNA FISH as previously described[27]. After blue light illumination at 5 mW/cm^2 for 24 hours, *Zfp462* or *Klf4* transcripts in LADL-engineered mouse embryonic stem cells and in three other controls (LADL+dark, Empty target control+dark, Empty bridge control+dark) were hybridized with 32 exon- and 32 intron-specific fluorescently labeled oligonucleotides before acquiring images for quantitative analysis. For each field of view, forty z-section images spaced at 0.3 um were acquired on a Nikon Ti-E widefield microscope using a 100x 1.4 NA objective and a cooled CCD camera. We used custom image processing scripts written in MATLAB to count mRNA and identify transcription sites. This software is available for download at https://bitbucket.org/arjunrajlaboratory/rajlabimagetools/wiki/Home. The estimates of nascent transcript numbers in **Figure 5c** and **Supplementary Figure 13b and d** were calculated by dividing the intensity of exon probe signal at the transcription site by the median intensity of all exon probe signals (primarily from mRNA) in the data set. Fluorescence-labeled oligonucleotide sequences for RNA FISH are given in **Supplementary Table 13**.

X) STATISTICS

The sample numbers corresponding to the individual experiments are included in the figure. **Figure 1e**, 2 independent experiments. **Figure 1f** is a representative image of 3 independent experiments. **Supplementary Figure 3d**, representative images of 2 independent experiments. **Supplementary Figure 4**, representative images of 2 independent experiments. **Supplementary Figure 5**, representative images of >10 independent experiments. **Figure 2d-f**, 1 experiment. **Supplementary Figures 7f** and **9f** include boxplots showing central tendency = median, box minima = 25th percentile, box maxima = 75th percentile, notches = 95% confidence interval, whiskers = 1.5x interquartile range. Figure 4c and **Supplementary Figure 7-10**, 5 independent experiments performed with LADL BL and LADL Dark (n=5), Empty Target Dark (n=3), Empty Anchor BL (n=1), Empty Anchor Dark (n=1), One-sided guide control BL (n=1), One-sided guide control Dark (n=1) where n = number of independent experiments. **Figure 4c** and **Supplementary Figures 8d** and **10c** strip charts show the median (red line). **Figure 4c** and **Supplementary Figure 8d**, p-values were computed using an unpaired, two-sided Mann Whitney U test. **Figure 5a** shows representative images of 3 independent experiments. **Figure 5b-d** and **Supplementary Figure 13**, 3 independent experiments, and p-values were computed using an unpaired one-tailed Mann Whitney U test with a null hypothesis that *Zfp462* levels in the LADL+BL condition are equal to the negative control conditions and an alternative hypothesis that *Zfp462* levels in the LADL+BL condition are greater than the negative control conditions. **Figure 5b-c** and **Supplementary Figure 13a-d**, p-values were computed using one-tailed Mann Whitney U test with a null hypothesis that *Klf4* levels in the LADL+BL condition are equal to the negative control conditions and an alternative hypothesis that *Klf4* levels in the LADL+BL condition are less than the negative control conditions. **Figure 5b-c** and **Supplementary Figure 13a-d** strip charts show the mean (red line). Sample sizes (n) represent **(Figure 5b, d**, and **Supplementary Figure 13a, c, e, f)** the number of cells, or **(Figure 5c**, and **Supplementary Figures 13 b, d)** the number of active transcription alleles.

XI) FUSION PROTEIN SEQUENCES

➔ **dCas9-CIBN**

Key highlighting the DNA sequence corresponding to the following:
FLAG
dCas9
GS linker
CIBN
2A
Puro

MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAADKKYSIGLAIGTNSVG
WAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRK
NRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYH
LRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFE
ENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDL
AEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLS
ASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIK
PILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNR
EKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTN
FDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTN
RKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILE
DIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSG
KTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKG
ILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQI
LKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDAIVPQSFLKDDSID
NKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSE
LDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDF
QFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQE
IGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLS
MPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVV
AKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELE
NGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKH
YLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKY
FDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDKRPAATKKAGQAKKKKSA
GGGGSGGGGSGGGGSDINGAIGGDLLLNFPDMSVLERQRAHLKYLNPTFDSPLAGFFAD
SSMITGGEMDSYLSTAGLNLPMMYGETTVEGDSRLSISPETTLGTGNFKKRKFDTETKD
CNEKKKKMTMNRDDLVEEGEEEKSKITEQNNGSTKSIKKMKHKAKKEENNFSNDSSKV
TKELEKTDYIHVVLFKLGSGEGRGSLLTCGDVEENPGPMTEYKPTVRLATRDDVPRAVR
TLAAAFADYPATRHTVDPDRHIERVTELQELFLTRVGLDIGKVWVADDGAAVAVWTTP
ESVEAGAVFAEIGPRMAELSGSRLAAQQQMEGLLAPHRPKEPAWFLATVGVSPDHQGK
GLGSAVVLPGVEAAERAGVPAFLETSAPRNLPFYERLGFTVTADVEVPEGPRTWCMTR
KPGA*

→ CRY2-HA-2A-mCherry
Key highlighting the DNA sequence corresponding to the following:
CRY2 (technically CRY2PHR)
HA
2A

mCherry

MKRPAATKKAGQAKKKKKMDKKTIVWFRRDLRIEDNPALAAAAHEGSVFPVFIWCPE
EEGQFYPGRASRWWMKQSLAHLSQSLKALGSDLTLIKTHNTISAILDCIRVTGATKVVF
NHLYDPVSLVRDHTVKEKLVERGISVQSYNGDLLYEPWEIYCEKGKPFTSFNSYWKKC
LDMSIESVMLPPPWRLMPITAAAEAIWACSIEELGLENEAEKPSNALLTRAWSPGWSN
ADKLLNEFIEKQLIDYAKNSKKVVGNSTSLLSPYLHFGEISVRHVFQCARMKQIIWARD
KNSEGEESADLFLRGIGLREYSRYICFNFPFTHEQSLLSHLRFFPWDADVDKFKAWRQG
RTGYPLVDAGMRELWATGWMHNRIRVIVSSFAVKFLLLPWKWGMKYFWDTLLDAD
LECDILGWQYISGSIPDGHELDRLDNPALQGAKYDPEGEYIRQWLPELARLPTEWIHHP
WDAPLTVLKASGVELGTNYAKPIVDIDTARELLAKAISRTREAQIMIGAAPASPKKKRK
VEASGSGRAVY<mark>YPYDVPDYA</mark>LFKL<mark>GSGEGRGSLLTCGDVEENPGP</mark>VSKGEEDNMAIIK
EFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMY
GSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLR
GTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTY
KAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK*

XII) LIFE SCIENCE REPORTING SUMMARY

Further information regarding experimental design, antibodies, cell lines and statistics may be found in the Life Sciences Reporting Summary.

Data Availability

5C data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE115963. Custom code for full reproducibility of all analyses is available upon request.

Methods only References

19. Konermann, S. et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**, 583-588 (2015).
20. Taslimi, A. et al. An optimized optogenetic clustering tool for probing protein interaction and function. *Nat Commun* **5**, 4925 (2014).
21. Sakuma, T., Nishikawa, A., Kume, S., Chayama, K. & Yamamoto, T. Multiplex genome engineering in human cells using all-in-one CRISPR/Cas9 vector system. *Sci Rep* **4**, 5400 (2014).
22. Cheng, A.W. et al. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Research* **23**, 1163 (2013).
23. Hsu, S.C. et al. The BET Protein BRD2 Cooperates with CTCF to Enforce Transcriptional and Architectural Boundaries. *Mol Cell* **66**, 102-116 e107 (2017).
24. Lajoie, B.R., van Berkum, N.L., Sanyal, A. & Dekker, J. My5C: web tools for chromosome conformation capture studies. *Nat Methods* **6**, 690-691 (2009).
25. Hnisz, D. et al. Activation of proto-oncogenes by disruption of chromosome neighborhoods. *Science* **351**, 1454-1458 (2016).
26. Imakaev, M. et al. Iterative correction of Hi-C data reveals hallmarks of chromosome organization. *Nat Methods* **9**, 999-1003 (2012).
27. Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A. & Tyagi, S. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat Methods* **5**, 877-879 (2008).