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1   **TITLE:**
2   A Method to Study *de novo* Formation of Chromatin Domains
3
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18   **KEYWORDS:**
19   PRC2, chromatin domains, H3K27me2, H3K27me3, epigenetics, nucleation, spreading,
20   inducible/conditional rescue.
21
22   **SUMMARY:**
23   This method is designed to follow formation of PRC2-mediated chromatin domains in cell lines,
24   and the method can be adapted to many other systems.
25
26   **ABSTRACT:**
27   The organization and structure of chromatin domains are unique to individual cell lineages. Their
28   misregulation might lead to a loss in cellular identity and/or disease. Despite tremendous efforts,
29   our understanding of the formation and propagation of chromatin domains is still limited.
30   Chromatin domains have been studied under steady-state conditions, which are not conducive
31   to following the initial events during their establishment. Here, we present a method to inducibly
32   reconstruct chromatin domains and follow their re-formation as a function of time. Although,
33   first applied to the case of PRC2-mediated repressive chromatin domain formation, it could be
34   easily adapted to other chromatin domains. The modification of and/or the combination of this
35   method with genomics and imaging technologies will provide invaluable tools to study the
36   establishment of chromatin domains in great detail. We believe that this method will
37   revolutionize our understanding of how chromatin domains form and interact with each other.
38
39   **INTRODUCTION:**
40   Eukaryotic genomes are highly organized and changes in the chromatin accessibility directly
41   controls gene transcription[1]. The genome contains distinct types of chromatin domains, which
42   correlate with transcriptional activity and replication timing[2,3]. These chromatin domains range
43   in size from a few kilobases (kb) to more than 100 kb and are characterized by an enrichment in

44 distinct histone modifications[4]. The central questions are: how are these domains formed and
45 how are they propagated?
46
47 One of the most well-characterized chromatin domains is fostered through the activity of the
48 Polycomb repressive complex 2 (PRC2). PRC2 is a multi-subunit complex composed of a subset
49 of the Polycomb Group (PcG) of proteins[5,6], and catalyzes the mono-, di- and trimethylation of
50 lysine 27 of histone H3 (H3K27me1/me2/me3)[7–10]. H3K27me2/me3 are associated with a
51 repressive chromatin state, but the function of H3K27me1 is unclear[6,11]. One of the core
52 components of PRC2, embryonic ectoderm development (EED), binds to the end product of PRC2
53 catalysis, H3K27me3, through its aromatic cage and this feature results in the allosteric
54 stimulation of PRC2[12,13]. The PRC2 enzymatic activity is crucial for preserving cellular identity
55 during development as the inappropriate expression of certain developmental genes that are
56 contraindicated for a specific lineage, would be detrimental[5,6]. Hence, unraveling the
57 mechanisms by which PRC2 fosters the formation of repressive chromatin domains in mammals
58 is of fundamental importance to understanding cellular identity.
59
60 All of the past experimental systems designed to investigate chromatin domain formation
61 including PRC2-mediated chromatin domains, were performed under steady-state conditions,
62 which are unable to track the unfolding events of chromatin domain formation in cells. Here, we
63 present a detailed protocol to generate an inducible cellular system which monitors the initial
64 recruitment and propagation of chromatin domains. Specifically, we focus on tracking the
65 formation of PRC2-mediated repressive chromatin domains that comprise H3K27me2/3. This
66 system that can capture the mechanistic details of chromatin domain formation, could be
67 adapted to incorporate other chromatin domains, such as the widely studied domains comprising
68 either H2AK119ub or H3K9me. In combination with genomics and imaging technologies, this
69 approach has the potential to successfully address various, key questions in chromatin biology.
70
71 **PROTOCOL:**
72
73 **Generation of inducible EED rescue mESCs**
74
75 **1.   Cell culture**
76
77 1.1.   Use feeder-free C57BL/6 mouse embryonic stem cells (mESCs) possessing a stably
78 integrated CreERT2 transgene, which can translocate to the nucleus upon administration of 4-
79 Hydroxytamoxifen (4-OHT)[13].
80
81 1.2.   Grow mESCs in conventional ESC medium[14,15], supplemented with 1000 U/mL LIF, 1 µM
82 ERK inhibitor PD0325901 and 3 µM GSK3 inhibitor CHIR99021. For conventional ESC medium, use
83 knockout DMEM containing 15% fetal bovine serum (FBS), 2 mM L-glutamine, 1X
84 penicillin/streptomycin and 0.1 mM 2-mercaptoethanol.
85
86 1.3.   Use plates coated with 0.1% gelatin solution for culturing mESCs.
87

2. Generation of clonal EED knockout (KO) mESCs

2.1. Design guide RNAs (gRNAs) to delete Exon 10 and Exon 11 of endogenous copy of EED in mESCs using the CRISPR design tool in Benchling[16]. EED-KO-gRNA-1 targets the intron immediately upstream of exon 10 and EED-KO-gRNA-2 targets the intron immediately downstream of Exon 11. The simultaneous application of these gRNAs deletes both Exon 10 and Exon 11 by non-homologous end joining (NHEJ).

2.2. Clone gRNAs into pSpCas9(BB)-2A-GFP (PX458) using instructions in Ran et al., 2013[17].

2.3. In a 6-well plate format, transfect 2×10^5 mESCs with 1 µg of each EED-KO-gRNA-1 and EED-KO-gRNA-2 (see **Table of Materials**) using transfection reagent by following the manufacturer's instructions. Change the media 24 h after transfection.

2.4. Two days after the transfection, isolate GFP positive cells using Fluorescence-activated cell sorting (FACS). Expect the transfection efficiency to vary around 10-30 %. Sort around 5×10^5 cells to capture sufficient GFP positive cells for plating.

2.5. Plate the isolated GFP positive mESCs into 15 cm plates pre-coated with 0.1% gelatin (10-20 $\times 10^3$ cells per plate) for colony picking.

2.6. Grow the cells in ESC medium for about one week until single colonies are visible. Change the media every 2 days.

2.7. Pick a minimum of 48 colonies using 20 µL micropipette tip. Scrape over the colony while aspirating into the micropipette tip. Do not break up the colony into single cells. Transfer the colony into an

2.8. Pick a minimum of 48 colonies using 20 µL micropipette tip. Scrape over the colony while aspirating into the micropipette tip. Do not break up the colony into single cells. Transfer the colony into an accutase-containing (20 µL) 96 well plate.

2.9. Incubate the colonies for 10 min at 37 °C until all cells are dissociated.

2.10. Add 200 µL of ESC media into each well using multichannel pipette.

2.11. Mix well and plate the cells into two separate 96 well plates using multichannel pipette.

2.12. Use one of the 96 well plates for genotyping and keep the other growing until genotyping is concluded. Use DNA extraction solution to extract DNA from the 96 well plate by following the manufacturer's instructions.

2.13. Use genotyping primers **Gnt_EED-KO-up** and **Gnt_EED-KO_down**, which span the deleted site and perform genotyping PCR with Taq DNA polymerase following the manufacturer's

132   instructions.

133

134   NOTE: Other types of DNA polymerases can also be used for genotyping, however, Taq DNA
135   polymerase offers convenience as the PCR reaction can directly be loaded on a gel when its
136   colored reaction buffer is used.

137

138   2.12.1) Observe a DNA product of lower molecular weight in cells with a homozygous deletion
139   relative to the wild-type (WT) case.

140

141   NOTE: To generate PRC2 null cells, homozygous deletion of exons 10 and 11 is necessary to
142   destabilize and degrade EED, an essential subunit of core PRC2[13]. The CRISPR targeting efficiency
143   is around 10 %.

144

145   2.14.   Validate the deletion of EED exon 10 and 11 and the loss of EED protein by Sanger
146   sequencing and Western blotting, respectively.

147

148   2.15.   Confirm the depletion of EED and H3K27me2/me3 from chromatin in EED KO cells by
149   ChIP-seq using antibodies against H3K27me2/me3 and EED. Use the protocol given in Oksuz et
150   al., 2017[15] for ChIP-seq experiments.

151

152   **3.   Engineering the EED knockout mESCs to harbor Cre-ERT2 based inducible EED**
153   **expression**

154

155   3.1.   Design gRNA (EED-gRNA-inducible) to introduce a cut within the intron following exon 9
156   of EED using the CRISPR design tool in Benchling[16] (see **Table of Materials**).

157

158   3.2.   Clone gRNAs into pSpCas9(BB)-2A-GFP (PX458) using instructions in Ran et al., 2013[17].

159

160   3.3.   Design a donor template DNA that comprises EED cDNA sequence after exon 9 and a C-
161   terminal Flag-HA tag upstream of a T2A-GFP sequence, all in reverse orientation with respect to
162   the endogenous gene sequence.

163

164   3.3.1.   Flank the cassette with a splice-acceptor and a polyadenylation sequence nested between
165   heterologous inverted loxP sites (lox66 and lox71)[18].

166

167   3.3.2.   Include at least 500 bp of homology arms from each end. Split the donor template into 2
168   segments of gBlocks gene fragments (see gBlock-1, gBlock-2-WT "https://benchling.com/s/seq-
169   l2LLlWNEnLrfGXcbdCxl"   and   gBlock-2-cage-mutant   "https://benchling.com/s/seq-
170   n8eiZCB2XAkOuzzpv6qM") and assemble them into PCR Blunt vector using Gibson cloning
171   following the manufacturer's instructions.

172

173   NOTE: gblock-2 contains an aromatic residue (Y365) within the cage of EED that is important for
174   interaction with H3K27me3[12]. gBlock-2-Wt contains wild type residue, whereas gblock-2-cage-
175   mutant contains the cage-mutant of EED (Y365A), incapable of binding to H3K27me3. Inducible

176 WT EED rescue is denoted as **i-WT-r** and inducible cage-mutant EED rescue is denoted as **i-MT-r**
177 for convenience.

179 3.4.   In a 6-well plate format, transfect 2 x 10^5 mESCs with 1 µg of the gRNA (EED-gRNA-
180 inducible) and 1 µg of the Donor templates (i-WT-r or i-MT-r) using transfection reagent and
181 isolate GFP positive cells using FACS.

183 3.5.   Follow steps 2.3-2.11 to isolate individual colonies ready for genotyping for successful
184 targeting.

186 3.6.   Use genotyping primers **Inducible_Genotype-FW-1** and **Inducible_Genotype-REV-1**,
187 which span the inserted cassette and perform genotyping PCR using Taq DNA polymerase.

189 NOTE: The targeting efficiency for CRISPR is around 10 %.

191 3.6.1. Confirm correct integration of the cassette by PCR using **Inducible_Genotype-FW-2** and
192 **Inducible_Genotype-REV-2** primers, which are outside of the homology arms.

194 NOTE: Homozygous integration of the cassette is necessary to accomplish efficient rescue of EED.
195 Note that cells with homozygous integration will produce a single large DNA product as compared
196 to WT EED, which will produce a short product.

198 3.7.   Confirm the integration of the cassette by Sanger sequencing.

200 3.8.   Confirm flipping of the cassette and the expression of EED and other PRC2 core
201 components (e.g., EZH2 and SUZ12) upon 4-OHT administration by Western blotting.

203 3.9.   Confirm the expression of T2A-GFP and percentage of flip by flow cytometry. Note that
204 expression of GFP is indicative of flipping of the cassette and the expression of EED.

206 4.   **Following nucleation and spreading of PRC2 activity on chromatin**

208 4.1.   Confirm that i-WT-r and i-MT-r mESCs do not have leaky expression of GFP by flow
209 cytometry. In the case of leaky expression of GFP, isolate GFP-negative cells by FACS before
210 starting the experiment.

212 4.2.   Expand the i-WT-r and i-MT-r mESCs into five 15 cm plates (5x 10^6 cells per plate).

214 4.3.   Induce expression of WT or cage-mutant EED by administration of 0.5 µM 4-OHT for 0 h,
215 12 h, 24 h, 36 h and 8 days (one 15 cm plate per condition). Change the media after 12 h for
216 treatments longer than 12 h. Isolate the successfully recombined cells by FACS using GFP. Adjust
217 the time of the treatments such that all of the conditions are collected at once.

219 4.4.   Perform ChIP-seq for H3K27me2, H3K27me3 to investigate their temporal deposition to

220 <mark>chromatin in response to re-expression of WT or cage-mutant EED. Use ChIP-seq protocol</mark>
221 <mark>including library preparation detailed in Oksuz et al., 2017[15].</mark>

223 4.5.   Use spike-in control in each sample to allow for quantitative comparison among different
224 time points[19].

226 4.5.1) For spike-in control, use chromatin from *Drosophila melanogaster* (in a 1:50 ratio to the
227 mESC-derived chromatin) as well as *Drosophila* specific H2Av antibody (1 μL of H2Av antibody
228 per 4 μg of Drosophila chromatin according to manufacturer's instructions) in each sample.

230 4.5.2) Prepare the chromatin from *Drosophila* in a manner similar to that from mESCs using the
231 protocol detailed in Oksuz et al., 2017[15].

233 4.6.   Map the sequence reads for ChIP-seq to mm10 genome with Bowtie 2 using default
234 parameters[20].

236 4.7.   Normalize the mouse ChIP-seq reads to spike-in *Drosophila* read counts[21]. Calculate the
237 spike-in normalization factor using the following formula: 1×10^6/unique *Drosophila* read counts.
238 Expect to get around 1×10^6 *Drosophila* read counts and 20×10^6 mouse read counts per
239 experiment.

241 4.7.2) Do not include the *Drosophila* chromatin when sequencing the input samples.

243 4.8.   Use **genomecov** tool from bedtools to convert bam file into bedgraph[22]. Next, convert the
244 bedgraph into bigwig file using **bedGraphToBigWig** tool from USCS[23,24]. See the following script:

```
gen="path to mm10 genome"
chr="path to mm10 chromosome sizes"
inp_bam="path to input bam file "
multiply="calculate the spike-in normalization factor"
bedtools genomecov -bg -scale $multiply -ibam $inp_bam -g $gen > output.bedGraph
sort -k1,1 -k2,2n output.bedGraph > output_sorted.bedGraph
bedGraphToBigWig output_sorted.bedGraph $chr output.bw
```

253 4.9.   Visualize the ChIP-seq read densities by uploading the bigwig files on the USCS genome
254 browser[24].

256 **5.   Monitoring emergence and growth of the H3K27me3 foci in the mESCs nuclei**

258 5.1.   Plate 1×10^4 mESCs into 8-well chamber slides pre-coated with 0.1% gelatin.

260 5.2.   Next day, start performing consecutive 4-OHT (0.5 μM) inductions to collect the cells
261 expressing EED for indicated time points (0 h, 12 h, 24 h and 36 h). Change the media after 12 h
262 for treatments longer than 12 h.

263

264 5.3.   Fix the mESCs with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room
265 temperature (RT) for 10 min.
266
267 5.4.   Permeabilize the cells with PBS/0.25% Triton X-100 at RT for 30 min.
268
269 5.5.   Block the cells with PBS/5% donkey serum/0.1% Triton X-100 (blocking buffer) at RT for
270 30 min.
271
272 5.6.   Dilute H3K27me3 primary antibody 1:500 in the blocking buffer and add onto the cells.
273
274 5.7.   Incubate the cells with primary antibody overnight at 4 °C.
275
276 5.8.   Next day, perform 3 washes with PBS/0.1% Triton X-100.
277
278 5.9.   Dilute secondary antibody (Alexa fluor 595) 1:1000 in the blocking buffer and add onto
279 cells.
280
281 5.9.1) Perform all incubations in dark in the following steps as secondary antibodies conjugated
282 with Alexa Fluor are light sensitive.
283
284 5.10.   Incubate the secondary antibody for 2 h at RT.
285
286 5.11.   Perform 3 washes with PBS/0.1% Triton X-100.
287
288 5.12.   Dilute DAPI (1 mg/mL) 1:4000 with PBS/0.1% Triton X-100 and add onto cells.
289
290 5.13.   Incubate the cells with DAPI for 10 min at RT.
291
292 5.14.   Mount the cells with Aqua mount.
293
294 5.15.   Image the cells with confocal microscopy at 63X magnification.
295
296 5.16.   Process and pseudo-color the images using a distribution of ImageJ, Fiji[25].
297
298 **REPRESENTATIVE RESULTS:**
299
300 **A general scheme of the conditional rescue system**
301 **Figure 1** shows the targeting scheme to conditionally rescue EED KO cells with either WT or cage-
302 mutant (Y365A) EED that is expressed from the endogenous EED locus. After knocking out EED,
303 a core subunit of PRC2 that is essential for its stability and enzymatic activity, a cassette within
304 the intron following exon 9 of EED is introduced (**Figure 1**). The cassette consists of the remaining
305 3' cDNA sequence of EED, in reverse orientation with respect to the endogenous gene sequence.
306 The cassette is flanked by heterologous inverted loxP sites (lox66 and lox71)[18]. The cells are
307 propagated until the complete loss of H3K27me2/3 is observed. Upon activation of Cre

308   recombinase expression by the addition of 4-OHT, the cassette is inverted such that exon 9 is
309   spliced into the cassette using the splice acceptor sequence (**Figure 1**). With this system, EED KO
310   cells can now be rescued by either WT or cage-mutant versions of EED both of which have a C-
311   terminal Flag-HA tag. The downstream T2A-GFP provides a marker to select for cells that undergo
312   a successful inversion event. The polyA signal prevents transcription of downstream sequences.
313   With this system, the kinetics of PRC2 recruitment and the formation of H3K27me domains can
314   now be followed.

315

316   **Tracking the temporal deposition of PRC2-mediated H3K27me2/3**
317   To follow the temporal dynamics of PRC2-mediated chromatin domain establishment, the WT or
318   cage-mutant version of EED is re-expressed in the background of EED KO cells, upon 4-OHT
319   treatment (**Figure 2A,B**). To follow deposition of H3K27me2/3 marks, ChIP-seq of
320   H3K27me2/me3 are performed after re-expression of WT or cage-mutant EED for the indicated
321   time points. The emergence of H3K27me3 is observed at 12 h after WT EED expression at discrete
322   regions that are denoted as "nucleation sites" (**Figure 2C**), and then at regions distant from the
323   initial nucleation sites by 24 h[13]. Eventually, the distribution of H3K27me3 nearly approximates
324   that of the levels seen in WT parental cells at 36 h after WT EED expression. The temporally
325   established downstream sites of H3K27me3 are termed "spreading sites"[13]. This system can also
326   track the temporal deposition of H3K27me2, which appears to precede H3K27me3 deposition
327   (**Figure 2C,D**). Lastly, re-expression of cage-mutant EED exhibits different dynamics relative to
328   WT EED (**Figure 2C,D**). In this case, deposition of H3K27me3 is very inefficient and instead,
329   H3K27me2 becomes apparent and more concentrated at the nucleation sites, indicating that the
330   cage-mutant EED is unable to spread the modification to neighboring regions.

331

332   The emergence of H3K27me3 foci and their growth can also be visualized by microscopy (**Figure
333   2E**)[13]. Before the induction of WT EED expression, H3K27me3 staining is not apparent. However,
334   12 h of WT EED expression shows evidence of H3K27me3 foci formation. These foci increase in
335   number and size by 24 h, and eventually spread to large regions of the nucleus by 36 h of WT EED
336   expression. This system gives evidence of the *de novo* formation of PRC2-mediated chromatin
337   domains as monitored by ChIP-seq and immunofluorescence (**Figure 2C-E**)[13].

338

339   **FIGURE LEGENDS:**
340   **Figure 1: Targeting scheme to conditionally rescue EED KO mESCs either with WT or cage-mt
341   EED (Y365A)**. Deletion of exon 10 and 11 causes destabilization and degradation of EED and
342   global loss of H3K27me2/me3. A cassette in the intron between exon 9 and 10 is inserted in the
343   reverse direction as indicated. Addition of 4-OHT inverts the cassette such that endogenous exon
344   9 splices into the cage-mutant or wild type cDNA of the cassette allowing inducible re-expression
345   of WT or cage-mutant EED.This figure has been modified from Oksuz et al., 2018[13].

346

347   **Figure 2: Validations and representative applications of inducible EED rescue systems (i-WT-r
348   and i-MT-r).** (**A**). i-WT-r and i-MT-r systems are validated by Western blot using indicated
349   antibodies on whole extracts after 4-OHT treatment to induce expression of WT (i-WT-r) or cage-
350   mutant (i-MT-r) EED, at the time point indicated. (**B**). Flow cytometry analysis of GFP in i-WT-r
351   cells before (0 h) and after (36 h) 4-OHT treatment for confirming the efficiency of the expression

352 of T2A-GFP, which is indicative of successful flip of the inverted cassette. (**C**, **D**). ChIP-seq tracks
353 for H3K27me3 (**C**) and H3K27me2 (**D**) near the Emx1 gene in the WT, or in i-WT-r or in i-MT-r
354 cells, for the indicated times of 4-OHT treatment. Early and delayed sites for deposition of the
355 histone marks are indicated. (**E**). Immunofluorescence using H3K27me3 antibody at 0, 12, 24 and
356 36 h after rescue of WT EED expression in i-WT-r mESCs. Staining at 36 h is shown at lower
357 exposures. The rightmost panel is a zoomed-in image of the cell labeled with a red arrow. This
358 figure has been modified from Oksuz et al., 2018[13].
359
360 **DISCUSSION:**
361 A powerful approach towards understanding the mechanistic details during the formation of a
362 given chromatin domain, is to first disrupt the domain and then track its reconstruction in
363 progress within cells. The process can be paused at any time during the reconstruction to analyze
364 in detail the events in progress. Previous studies on chromatin domains were unable to resolve
365 such events as they were performed under steady-state conditions (e.g., comparing wild-type
366 and gene knockout). Here, we outline a system to assess the dynamic formation of chromatin
367 domains, as highlighted by the recruitment and spreading of PRC2-mediated repressive domains
368 in cells.
369
370 The most critical step for this inducible system is the accurate design of DNA constructs to re-
371 express the desired proteins (or RNA) after their respective deletion from the cells. Various
372 mutations, tags, and fluorescent markers could be introduced within these constructs, depending
373 on the downstream applications. For example, instead of using self-cleaving T2A peptide
374 immediately before GFP to disconnect it from the protein of interest, various fluorescent fusion
375 proteins could be generated for high resolution imaging and/or single-particle tracking
376 experiments to monitor the initial events of chromatin domain formation in living cells.
377
378 Although this method could be modified in many ways to provide insights into the formation of
379 chromatin domains, it does have some limitations. First, it requires a cell line that harbors the
380 Cre-ERT2 gene. Second, optimizations are necessary to determine the time points after 4-OHT
381 treatment. Third, this system is not reversible so as to monitor the events during deconstruction
382 of a chromatin domain. Degron-based methods could be used as an alternative to the method
383 described here[26,27]. These systems enable reversible and rapid degradation of proteins, but
384 usually require costly molecules for protein destabilization, such as auxin or shield[26,27].
385 Furthermore, these degron-based methods have limitations. They could only be used to re-
386 express the WT version of the protein after its degradation. In contrast, the system described
387 herein allow conditional expression of the mutant version of the protein of interest in addition
388 to its WT counterpart. Combining such a degron system with the method described here would
389 be a powerful means to reversibly modulate formation of chromatin domains. An alternative
390 method for following the formation of chromatin domains entails the use of specific inhibitors to
391 deplete a defined chromatin modification from the chromatin and then washout the inhibitor to
392 follow *de novo* deposition of the modification. For example, treatment with EZH2 inhibitor and
393 subsequent washout is used for tracking the re-formation of H3K27me domains[28]. However,
394 EZH2 inhibitor is not effective in completely depleting H3K27me, even 7 days after treatment. In
395 this case, the presence of pre-existing H3K27me might recruit and activate PRC2[12], thereby

396 complicating interpretation of the results. As well, the use of the inhibitor and washout strategy
397 is limited due to the absence of specific and potent inhibitors for many chromatin domains.
398
399 In addition to the applicability of this method to cellular systems, it can be used to generate
400 inducible mutations/tagging on desired proteins in animals. In some cases, mutating a protein or
401 inserting a tag could be lethal during development. This method bypasses this lethality and
402 provides a temporal and spatial control of the mutation/tagging of proteins by coupling with
403 tissue specific Cre-ERT2 mouse strains. These types of experiments would be valuable to
404 determine the effect of a mutation in a specific tissue and/or at a specific stage of development.
405 Importantly, it allows for the isolation of cells that undergo a successful recombination via the
406 reporter within the cassette. This facilitates biochemical analyses, such as affinity purification
407 from specific tissues, to isolate the tissue-specific interactome for a given protein. This system
408 could be geared to monitor dynamic changes in any given cellular process, and hence is not
409 limited to tracking chromatin domain formation.
410

416 **DISCLOSURES:**
417 D.R. is a co-founder of Constellation Pharmaceuticals and Fulcrum Therapeutics. Authors declare
418 that they have no competing interests.
419

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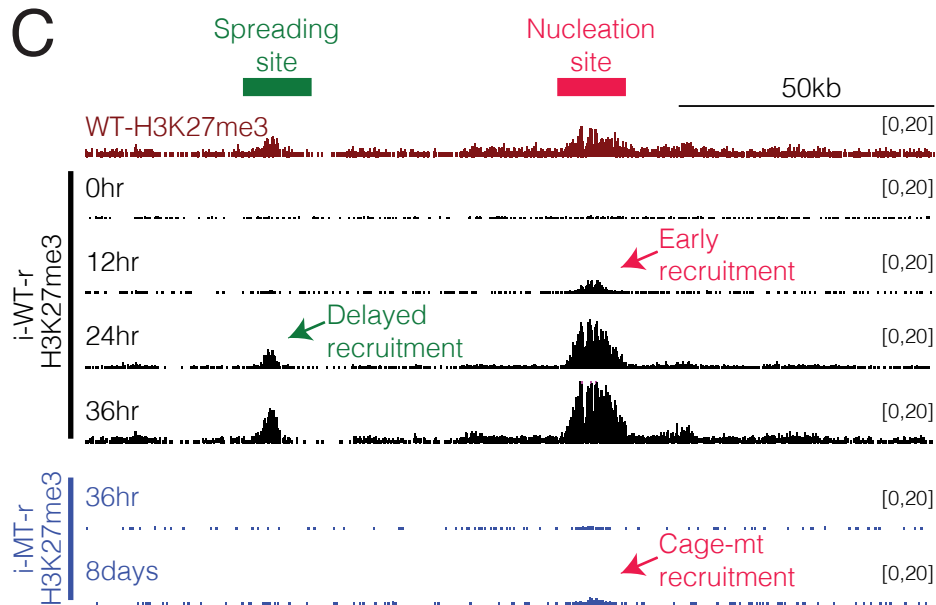490   10.1038/s41594-018-0036-6 (2018).
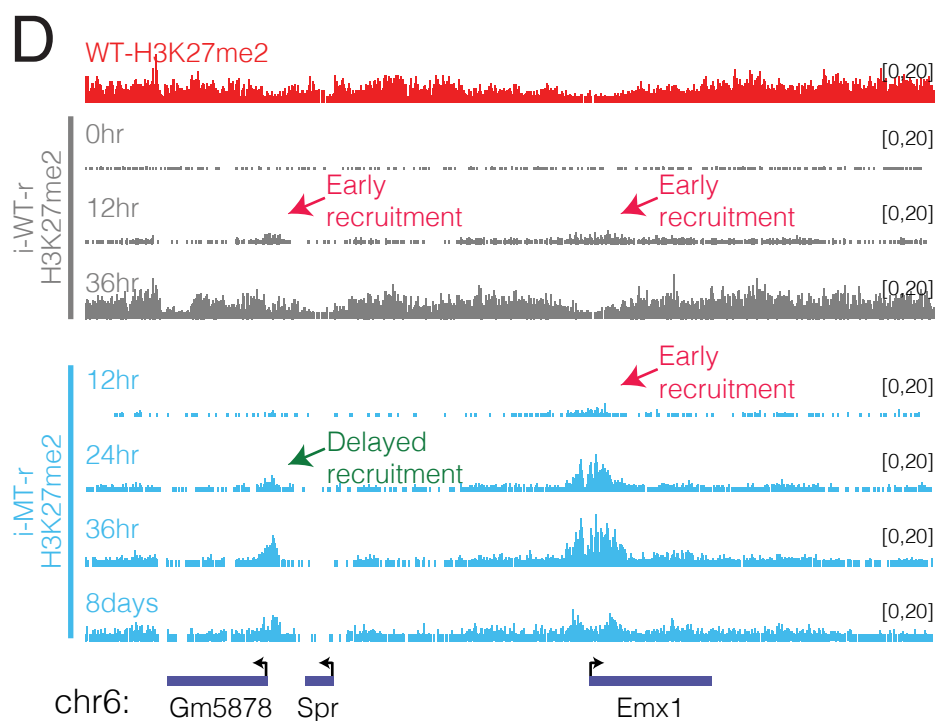491

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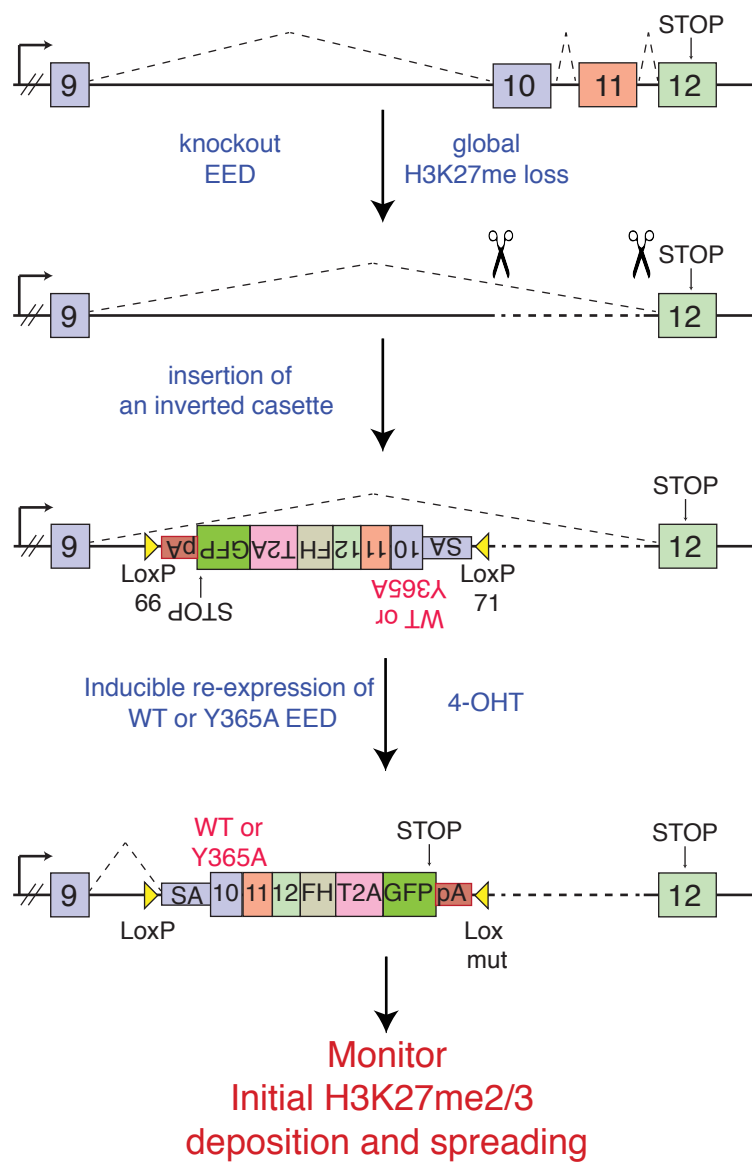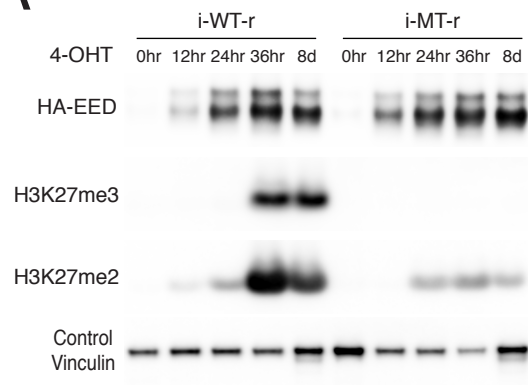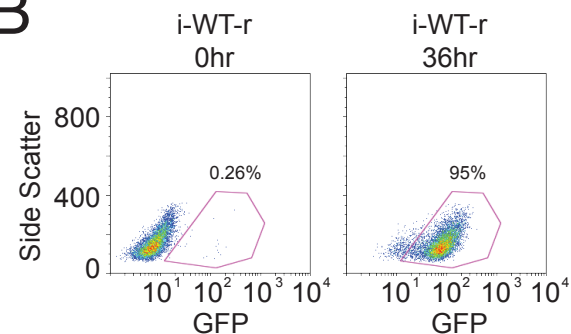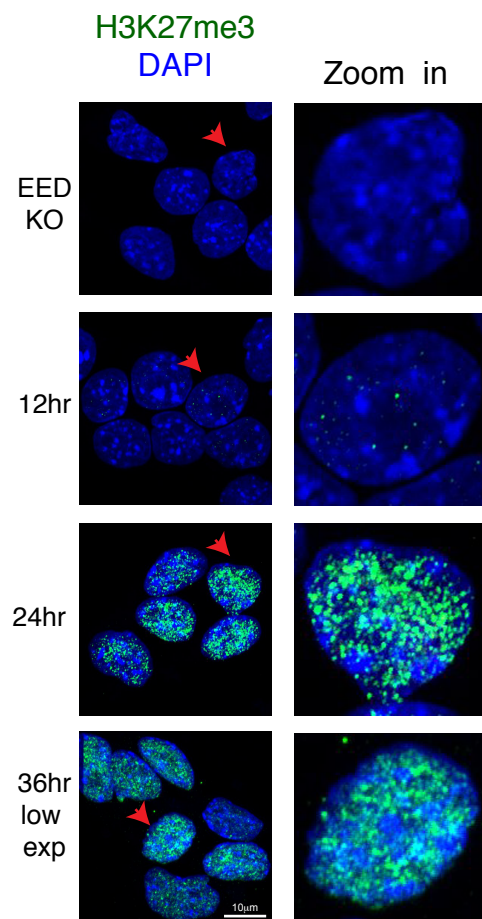
Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
(Z)-4-Hydroxytamoxifen (5 mg)	Sigma	H7904-5MG	For induction of EED expres
16% Paraformaldehyde aqueous solution (10x10 ml)	Electron Microscopy Sciences	15710	For immunofluorescence
2-mercaptoethanol	LifeTechnologies	21985-023	For mESCs culture
2% Gelatin Solution	Sigma	G1393-100ml	For mESCs culture
Accutase 500 ML	Innovative Cell Tech/FISHER	AT 104-500	For mESCs culture
Alexa Fluor 594 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson immunoresaerch	711-585-152	For immunofluorescence
Aqua-Mount Mounting Medium	FISHER/VWR	41799-008	For immunofluorescence
CHAMBER SLD TC PRMA 8-CHM 16 PK	Fisher Sci	177445PK	For immunofluorescence
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) - 10 mg	Life Tech	D1306	For immunofluorescence
ERK inhibitor, PD0325901	Stemgent	04-0006	For mESCs culture
ESGRO Recombinant Mouse LIF Protein	Millipore/Fisher	ESG1107	For mESCs culture
FBS Stem Cell Qualified	Atlanta	S10250	For mESCs culture
Gibson Assembly Master Mix	NEB	E2611L	For Donor template cloning
GSK3 inhibitor, CHIR99021	Stemgent	04-0004	For mESCs culture
H3K27me2 (D18C8) rabbit mAB	Cell Signaling	9728S	Antibody for ChIP-seq
H3K27me3	Cell Signaling	9733S	Antibody for ChIP-seq
Histone H2Av antibody (pAb)	Active motif	39715	Spike-in control for ChIP-se
Knockout DMEM	Invitrogen	10829-018	For mESCs culture
L-glutamine	Sigma	G7513	For mESCs culture
Lipofectamine 2000	LifeTech	11668019	For transfection
MangoTaq DNA Polymerase	Bioline	BIO-21079	For Genotyping PCR
Normal donkey serum (10 mL)	Jackson ImmunoResearch	017-000-121	For immunofluorescence
Penicillin-Streptomycin	Sigma/Roche	P0781	For mESCs culture
pSpCas9(BB)-2A-GFP (PX458)	Addgene	48138	For gRNA cloning
QuickExtract DNA Extraction Solution	Lucigen	QE0905T	For Genotyping PCR
Triton X-100	Sigma	T8787-250ML	
Zero Blunt PCR Cloning Kit	Thermo Fisher	K270020	For Donor template cloning

Primers/gBlocks	Sequence	Comments/Description
EED-KO-gRNA-1	ctctggctactgtcaactag	
EED-KO-gRNA-2	TAGGCTATGACGCAGCTCAG	gRNAs pairs to knockout EED in C57BL/6 ES(
EED-gRNA-inducible	atggcaccccgaaattagaa	
i-WT-r Donor	https://benchling.com/s/seq-l2LLlWNEnLrfGXcbdCx	gRNA and Donor to generate i-WT-r system
EED-gRNA-inducible	atggcaccccgaaattagaa	
i-MT-r Donor	https://benchling.com/s/seq-n8eiZCB2XAkOuzzpv6(	gRNA and Donor to generate i-MT-r system

Genotyping Primers	Sequence	Comments/Description
Gnt-EED-KO-FW-1	ctgtaggctgccatctgtga	
Gnt-EED-KO-REV-1	agccagggctacacagagaa	Wild type allele will produce a product of 1.!
Inducible_Genotype-FW-1	tgcagtgaaacaaatttggaa	When the casette is inserted, the primers w
Inducible_Genotype-REV-1	gagaggggtggcactgtaaa	produce a product of ~200 bp.
Inducible_Genotype-FW-2	ccccctctttctcctttct	When the casette is inserted, the primers w
Inducible_Genotype-REV-2	atgcctgggtgaatgaaaaa	produce a product of 1560 bp.

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Cs for i-WT-r and i-MT-r systems.

in the EED-KO background.

in the EED-KO background.

9 kb. Knockout allele will produce a product of 200 bp.

ill produce 1863 bp. The wild type allele will

ill produce 3200 bp. The wild type allele will

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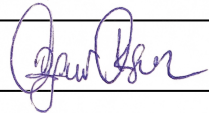
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