

1   **Defining Essential Enhancer for Pluripotent stem cells using Features Oriented**  
2   **CRISPR-Cas9 Screen**

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39 **ABSTRACT**

40 Cis Regulatory Elements (CREs) regulate the expression of the genes in their  
41 genomic neighborhoods and influence cellular processes such as cell-fate  
42 maintenance and differentiation. To date, there remain major gaps in the functional  
43 characterization of CREs and the identification of its target genes in the cellular native  
44 environment. In this study, we performed a Features Oriented CRISPR Utilized  
45 Systematic (FOCUS) screen of OCT4-bound CREs using CRISPR/Cas9 to identify  
46 functional enhancers important for pluripotency maintenance in mouse ES cells. From  
47 the initial 235 candidates tested, 16 CREs were identified to be essential stem cell  
48 enhancers. Using RNA-seq and genomic 4C-seq, we further uncovered a complex  
49 network of candidate CREs and their downstream target genes, which supports the  
50 growth and self-renewal of mESCs. Notably, an essential enhancer, CRE111, and its  
51 target, *Lrrc31*, form the important switch to modulate the LIF-JAK1-STAT3 signaling  
52 pathway.

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54 **KEY WORDS**

55 CRISPR screen, OCT4-Bound, Essential cis-regulatory elements, LRRC31,  
56 JAK-STAT3, Super-enhancer, Pluripotency

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58 **INTRODUCTION**

59 Cis-regulatory elements(CREs) are regions of non-coding DNA that regulate the

60 expression of their target genes. 11% of the mouse genome was predicted to be  
61 non-redundant cis-regulatory elements (Shen et al., 2012). CREs were found to play  
62 roles in governing cell identity by regulating cell-type specific transcriptomic profiles  
63 (Buecker et al., 2014; Shen et al., 2012). Over the years, detailed identification and  
64 characterization of cell-type specific CREs were made possible through collaborative  
65 efforts such ENCODE and the Roadmap epigenomic project. Its hallmark includes the  
66 ability to regulate gene expression independent of their orientation and distance away  
67 from the target genes. Furthermore, a single CRE may regulate the expression of  
68 several genes at any one time or target different downstream genes in different cell  
69 types (Shlyueva et al., 2014). Notably, the major gaps in our knowledge of the  
70 cis-regulatory elements are the functional characterizations and the identifications of  
71 their target genes in the cell type of interest.

72 Many genetic approaches, such as reporter assays and STARR-seq (Arnold et al.,  
73 2013), were developed to address this. However, these methods relied heavily on the  
74 functional readout of the enhancer fragment outside of their native genomic  
75 architecture, which led to inaccurate representations of their endogenous activity. To  
76 fully address the contribution of cis-regulatory elements to biological systems within  
77 their genomic environment, it is imperative to disrupt their activities *in situ* within  
78 their genomic environment.

79 Pluripotency is the ability of stem cells to differentiate into all other cell types that  
80 constitute the entire organism. In the past few decades, many studies have defined the

81 essential genes involved in maintaining pluripotency. Among them, *Oct4* was  
82 identified as a master transcription factor for the regulation of pluripotency and  
83 self-renewal in embryonic stem cells. The genomic binding profile of OCT4 protein  
84 in both the mESCs and hESCs has been elucidated (Chen et al., 2008; Loh et al.,  
85 2006; Boyer et al., 2005). Interestingly 30% of OCT4 bound sites were mapped to the  
86 distal regions (10-100kb) of the nearest gene, or to the gene deserts (>100kb to the  
87 nearest gene) (Loh et al., 2006). Recently several studies have been published on the  
88 characterization of CREs centered around the genomic regions of the *Oct4* gene (Diao  
89 et al., 2016; Diao et al., 2017).

90 Here, we describe a Features Oriented CRISPR Utilized Systematic (FOCUS) screen  
91 for essential OCT4-bound sites in mouse ES cells. From the screen, we identified  
92 seventeen high-confidence cis-regulatory elements which are critical for the  
93 maintenance of ES cells. Using a system approach, integrating genomic and  
94 functional analyses, such as ChIP-seq, ATAC-Seq, RNA-Seq, HiC-Seq, 4C-Seq,  
95 genetic knock-down, proteomics and rescue experiments, we further defined the target  
96 genes for these CREs and uncovered several novel regulators including the  
97 LRRC31-JAK-STAT3 axis, which plays a critical role in governing the proper signal  
98 transduction in ES cells.

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103 **RESULTS**

104 **FOCUS identified essential cis-regulatory elements for pluripotency**  
105 **maintenance**

106 In order to functionally dissect the Oct4 bound cis-regulatory elements (CREs) that  
107 are essential for pluripotency maintenance in mouse ES cells, a Features Oriented  
108 CRISPR Utilized Systematic (FOCUS) screen of OCT4-bound CREs was conducted.  
109 To build the FOCUS library (**Figure 1A, Figure S1A**), we first collated mESC  
110 ATAC-seq data to ascertain the accessible chromatin regions(Li et al., 2017). Out of  
111 27,513 sites identified, 6,392 demonstrated high confidence OCT4-binding based on  
112 published ChIP-Seq datasets (**Table S1**) (Chen et al, 2008). Using FIMO (Grant et al.,  
113 2011), we next determined intergenic OCT4-bound CREs, which contain the *Oct4*  
114 motif. Finally, we shorted listed putative targets for the primary screen after taking  
115 into account the presence of PAM motif (NGG) in or near the CREs.

116 sgRNAs were designed for 235 CREs based on their targetability by the  
117 CRISPR/Cas9 genome editing system, as assessed by the CRISPR sgRNA designer  
118 tool (**Table S2**) (Doench et al, 2016). Two non-targeting sgRNAs (NT1 and NT2)  
119 were used as negative controls in the FOCUS screen, whereas sgRNA against the

120 Oct4 distal enhancer (DE) was used as a positive control (**Figure 1B**). Two  
121 independent read-outs, namely the Oct4 immunofluorescence signal and the *Oct4*-DE  
122 mCherry reporter signal, were used. We validated the specificity of the *Oct4*-DE  
123 mCherry reporter by comparing its activities in E14 mESCs, differentiated E14 and  
124 MEF cells (**Figure S1B, S1C and S1D**). To eliminate the bias introduced by the  
125 number of cells in our screens, we first assessed the correlation between cell number  
126 and OCT4 immunofluorescence signal in mESCs (**Figure S1E**). To this end, the  
127 correlation was used to normalize OCT4 immunofluorescence signal derived from  
128 both the primary and secondary screens. For each of the 235 cis-regulatory elements,  
129 z-score was calculated from 4 biological replicates (**Figure 1C**). Based on a targeted  
130 error rate of 0.05, a cut-off threshold of absolute value  $>2$  SD from the mean of the  
131 non-targeting controls was used to determine candidate CREs which resulted in the  
132 reduction of OCT4 signal when they were knocked out (KO). Expectedly, the control  
133 sgRNA targeting the *Oct4* DE was the top hit from the screen with an average z-score  
134 of -4 (**Figure 1C, Table S3**). The observation that the average z-scores of both  
135 non-targeting sgRNAs fell within -2 to +2 further increased the confidence of our  
136 FOCUS screen (**Figure 1C**). To exclude the error introduced during the imaging  
137 analysis, random images of hits were extracted to confirm the reduction of OCT4  
138 immunofluorescence signal (**Figure 1D**). To further test the robustness of our screen,  
139 the result was independently normalized to the two non-targeting sgRNAs, and the  
140 correlation between these two sets of analyses was calculated. We observed a Pearson

141 correlation coefficient of 0.9 (**Figure S1F**). We next examined the *Oct4* DE mCherry  
142 signal for the KO CREs. We detected good correlation between OCT4  
143 immunofluorescence and *Oct4* DE mCherry signal for both the hit and non-hit CREs  
144 (**Figure 1E**). To confirm their knock-out, the targeting efficiency of 10 randomly  
145 picked hit CRE sgRNAs were examined using the SURVEYOR assay (**Figure S1G**).  
146 For three CREs, we evaluated the percentage of mutated *Oct4* motif in the  
147 sgRNA-transfected mESCs using DNA sequencing. Around 38% to 47% of the  
148 sgRNA-transfected mESCs showed DNA deletion at the targeted *Oct4* motif (Figure  
149 S1H). Further, to exclude off-target effects of the sgRNAs, a secondary screen was  
150 performed using independent sgRNAs targeting the 19 hit CREs identified from the  
151 primary screen. Of note, 16 candidate CREs showed consistent phenotypes from the  
152 two batches of sgRNA KO (**Figure 1F**).

153 **Knockout of candidate CRE hits affects both the maintenance and establishment**  
154 **of pluripotent stem cells**

155 A detailed evaluation of the deleterious effects of knocking out the candidate CREs  
156 was then undertaken. First, to examine the consequence of our CREs on self-renewal  
157 of mESCs, we assayed colony formation in cells in which candidate CREs were  
158 knocked out. For seven CREs, a marked decrease in the ability of the cells to form  
159 ES-like colonies was observed, based on a final colony count after alkaline  
160 phosphatase (AP) staining (**Figure 2A**). We detected similar phenotypic outcome

161 when CREs were knocked out in ES-D3 cells using a different set of sgRNAs (**Figure**  
162 **S2A**). Next, the expression of a set of pluripotency and differentiation genes was  
163 determined by qRT-PCR upon knockout of candidate CREs in ES-E14 cells. As  
164 expected, the *Oct4* expression level decreased significantly when candidate CREs  
165 were knocked out (**Figure 2B**). Interestingly, we did not observe comparable  
166 downregulation of *Nanog* and *Sox2* across the different CREs KO. For most CREs, a  
167 universal increase in the expression of differentiation markers was detected with no  
168 tendency towards any particular lineage. Notably, we observed a specific upregulation  
169 of *Gata6* for KO CRE 4, suggesting that individual CREs may regulate pluripotency  
170 through varied pathways. We then induced differentiation towards the ectoderm  
171 lineage in ES-E14 cells by treatment with retinoic acid, followed by the measurement  
172 of the expression of several marker genes (*Pax6*, *Gbx2*, *Foxj3*, *Mcm7* and *Sox1*)  
173 (Zhang et al., 2015). Notably, knockout of the candidate CREs led to an increase in  
174 the expression of ectoderm markers, suggesting that the loss of function of our  
175 candidate CREs could destabilize the pluripotency state of ES cells and prime the  
176 mES cells for directed differentiation (**Figure 2C, S2B**).

177 Next, a somatic cell reprogramming assay was performed by infecting MEFs with the  
178 sgRNAs to knock out candidate CREs, before reprogramming them to induced  
179 pluripotency via the OSKM retroviral system (Takahashi and Yamanaka, 2006).  
180 Decreased reprogramming efficiency was seen for most candidate CREs as compared  
181 to the non-targeting control, when assessed by colony counting based on AP staining

182 10 days post-infection (d.p.i). (**Figure 2D, S2C**). In order to ascertain that the final  
183 phenotypic effects were independent of any proliferation defects, a cell proliferation  
184 assay was performed for these CREs KO MEFs. Only slight increase in cell  
185 proliferation rate was observed in most of the CREs KO MEFs compared to the KO  
186 NT. This suggests that the reduced reprogramming efficiency was indeed specific and  
187 not due to changes in cell proliferation (**Figure S2D**). We then examined the  
188 chromatin state of candidate CREs based on ATAC-seq datasets (Fang et al., 2018).  
189 For the hit CREs which affected the reprogramming process, we observed a gradual  
190 opening of its chromatin regions from Day 0 to Day 12 of reprogramming (**Figure**  
191 **2E, S2E and S2F**). Together, the data suggests that the candidate CREs identified  
192 from the FOCUS screen play important roles in both the maintenance and  
193 establishment of pluripotent stem cells.

194 **Candidate CREs display enhancer activity in mESCs**

195 We next investigated the profile of histone markers (H3K4me, H3K4me3, H3K27ac,  
196 H3K9ac, H3K9me3 and H3K27me3) on our hit CREs using published ENCODE  
197 datasets(ENCODE Project Consortium, 2012). A significant enrichment of active  
198 enhancer histone marks (H3K4me1 and H3K27ac) was detected in our candidate  
199 CREs (**Figure 3A, 3B, S3A, S3D and S3E**). Apart from histone modifications, a  
200 significant enrichment was also observed for pluripotency-associated transcription  
201 factors (OCT4, SOX2 and NANOG) and active enhancer-related transcription factors

202 (MED1 and P300) (**Figure 3C, S3B, S3D and S3E**) (Chen et al., 2008). The  
203 enrichment of transcription factors' motifs was further compared between the hit  
204 CREs and non-hit CREs. Notably, a specific enrichment of pluripotency-related  
205 transcription factor motifs (*Brn1*, *Zic3*, *Sfl* and *Arnt*) was detected in the hit CREs  
206 which reinforced the significances of multiple transcription factors co-binding on  
207 functional enhancers (**Figure 3D, S3C**) (Forristal et al., 2010; Fuellen and  
208 Struckmann, 2010; Gu et al., 2005; Lim et al., 2007; Kim et al., 2018). Of note, when  
209 *Zic3* or *Brn1* motif were mutated, the enhancer activity of CRE132 was further  
210 depleted, indicating the importance of these identified motifs (**Figure 3E**).

211 We next examined active enhancer histone marks, namely H3K4me and H3K27ac, in  
212 four candidate CREs disrupted by sgRNAs (**Figure 3F**). We observed that the  
213 enrichment of these histone marks, as well as OCT4 binding (**Figure S3H**), was  
214 significantly reduced. This suggests that the binding of OCT4 is essential for the  
215 maintenance of the active histone marks on enhancer regions. Notably the decrease  
216 was specific to the locus where individual CREs were knocked out (**Figure 3F, S3F**  
217 and **S3G**). Furthermore, we functionally assessed the enhancer activity of the CREs  
218 using a luciferase reporter assay. Consistent with the enrichment of active histone  
219 marks and transcription factors, our hit CREs displayed a significantly elevated  
220 luciferase signal in E14 mES cells as compared to the control NIH3T3 cells.  
221 Remarkably, the luciferase activities decreased when ES-E14 cells were induced to  
222 differentiate via the introduction of retinoic acid. This strongly suggests that the

223 enhancer activities of our candidate CREs are specific to the pluripotent mESCs  
224 (**Figure 3G, S3I**). Taken together, our data indicates that a majority of the CRE hits  
225 have important functional roles in pluripotent cells as active enhancers.

226 **Knockout of candidate CREs elicited overlapping and distinct transcriptomic**  
227 **effects**

228 To further evaluate the function of candidate CREs in ES cells, RNA-seq libraries  
229 were generated for each CRE knockout in ES-E14. All the RNA-seq libraries were of  
230 good quality (**Figure S4A, S4B**). As expected, *Oct4* expression was downregulated in  
231 all CREs KO RNA-seq libraries (**Figure S4C**). Gene ontology (GO) analysis was  
232 performed on both the up-regulated and down-regulated genes when candidate CREs  
233 were knocked out. Genes involved in cell differentiation, endodermal cell lineage and  
234 multicellular organism development were enriched in the up-regulated genes. We also  
235 detected enrichment of genes implicated in the activation of MAPK activity and  
236 positive regulation of ERK1 and ERK2 cascade, supporting the notion that the  
237 knockout of hit CREs could prime ES cells for differentiation process (**Figure 4A**)  
238 (Lanner and Rossant, 2010). Amongst the down-regulated genes, a significant  
239 enrichment for genes involved in stem cell population maintenance was observed  
240 (**Figure 4A**). Apart from that, we also detected an enrichment for the WNT signalling  
241 pathway which has been reported to be involved in the maintenance of pluripotency in  
242 mESCs (Miyabayashi et al., 2007; Sokol et al., 2011). Using CTEN analysis, we

243 uncovered the enrichment of different lineage-associated genes when candidate CREs  
244 were knocked out (**Figure 4B**).

245 Next, we performed Pearson correlation analysis and principal component analysis  
246 (PCA) on these RNA-seq libraries (**Figure 4C, 4D, S4E**). Surprisingly, CRE111 and  
247 CRE 210 appeared as two distinct clusters compared to the other CREs. KEGG  
248 pathway analysis of the genes downregulated in CRE111 and CRE210 showed an  
249 enrichment for signalling pathways involved in pluripotency maintenance (**Figure**  
250 **S4D**). To test the similarity between CRE111, CRE210 and *Oct4* DE, dysregulated  
251 genes were compared between each KO CRE RNA-seq library and the KO *Oct4* DE  
252 RNA-seq library (**Figure 4E, S4F**). Indeed, KO CRE210 and CRE111 seemed to  
253 elicit transcriptomic profiles more similar to the KO *Oct4* DE. Through combinatorial  
254 knockout of CRE111 and CRE210, we observed a further reduction in *Oct4*  
255 expression and increase in differentiation gene expression compared to single  
256 knockout, suggesting these two CREs could affect pluripotency through different  
257 mechanism (Figure 4F, S4H). When we compared CRE111 KO RNA-seq library with  
258 the *Oct4* KD microarray library, a significant overlap was also observed (**Figure S4G**)  
259 (Loh et al., 2006).

260 **Candidate CREs regulate novel and known pluripotency genes**

261 Next, to investigate the 3D genomic regions interacting with the hit CREs, we  
262 performed 4C-seq on seven CREs which showed the strongest phenotype in our

263 functional assay (**Figure 2**). Out of these obtained high quality 4C-seq libraries for 4  
264 CREs, we managed to get 4C-seq libraries with good quality (**Figure 5A, S5A, S5B**  
265 **and Table S5**), we then integrated the 4C-seq targets with KO CRE RNA-seq  
266 libraries to validate the functional enhancer-promoter interactions (**Figure 5B, Figure**  
267 **S5E**). For two CREs, CRE111 and CRE132, their interaction to putative target genes  
268 based on 4C-seq were validated using a detailed 3C assay (**Figure S5D**). Of the four  
269 CREs we examined for 3D interactions, most of the interacting regions localized  
270 between 5kb and 50kb to the nearest transcription start site (TSS) (**Figure S5C**).

271 For the remaining CREs, we performed an integrative analysis based on the published  
272 Hi-C dataset (Bonev et al., 2017) and our KO CRE RNA-seq libraries to predict the  
273 interacting gene targets (**Figure 5C, S5F and S5G**). Consistent with previous reports  
274 that topologically associating domains (TADs) are the boundaries for  
275 enhancer-promoter interactions, most of the interactions of candidate CREs happened  
276 within a TAD (**Figure 5A, 5C and S5F**) (Dixon et al., 2012). Interestingly, for  
277 CRE210, we identified *Zfp322a* (Ma et al., 2014), a known pluripotent gene, as its  
278 putative target. This further support the notion that our candidate hit CREs are critical  
279 to pluripotency maintenance (**Figure 5C, 5D and S5E**). Interestingly, potential  
280 interacting target genes of our candidate CREs formed a tight protein-protein network  
281 with known pluripotency regulators (**Figure 5E**). Furthermore, when we knocked  
282 down the target genes using siRNA, we observed significant decrease in the  
283 expression of pluripotent markers such as *Oct4* and *Sox2*, and a differentiation

284 morphology as compared to siNT control (**Figure 5E, 5F**).

285 ***Lrrc31* regulates the maintenance of pluripotency in mES cells through the**  
286 **JAK-STAT3 signaling pathway**

287 We next focused on one of the hit CREs, CRE 111, to further elucidate its detailed  
288 underlying mechanism in pluripotency maintenance. Firstly, to understand the  
289 functional significance of the interaction between CRE111 and its putative target gene,  
290 *Lrrc31*, we examined its over expression upon CRE111 knockout. Indeed, we  
291 observed partial rescue of the CRE111 KO dysregulated genes (**Figure 5H**). Hence,  
292 CRE111 may function through its activation of *Lrrc31* to govern stem cell  
293 pluripotency.

294 To address the role by which *Lrrc31* effects pluripotency in mESCs, we first knocked  
295 down *Lrrc31* using either siRNA or shRNA. Both assays lead to a differentiation  
296 morphology when *Lrrc31* was depleted (**Figure 6A**). As seen in the CRE 111  
297 knock-out mESCs, *Oct4* expression was similarly decreased in *Lrrc31* depleted cells  
298 (**Figure S6A**), while the expression of differentiation genes was markedly  
299 upregulated (**Figure 6B**). Apart from knocking down *Lrrc31*, we also generated  
300 *Lrrc31* KO clones using CRISPR (**Figure S6D**). Compared to NT clone, *Lrrc31* KO  
301 leads to a significant reduction of pluripotent genes' expression and self-renewal  
302 capacity of mESCs (**Figure 6C, S6E**). We next evaluated the role played by *Lrrc31* in  
303 mouse somatic cell reprogramming. Using siRNA, we depleted *Lrrc31* at different

304 time points during reprogramming and observed a specific reduction of  
305 reprogramming efficiency when *Lrrc31* was knocked down at day 9 post viral  
306 infection (**Figure 6D, S6C**). Conversely, we observed drastic upregulation of *Lrrc31*  
307 in successfully reprogrammed cells compared to non-reprogrammed cells from our  
308 previously published RNA-seq datasets (**Figure S6F**) (Fang et al, 2018). Taken  
309 together, this data suggests that *Lrrc31* is an important effector for the acquisition of  
310 pluripotency at the late stage of reprogramming.

311 To further elucidate its mechanistic role, we performed RNA-seq on *Lrrc31*  
312 knock-down mESCs. GO analysis on the upregulated genes showed enrichment for  
313 receptor binding function (**Figure S6I**). Consistent with qRT-PCR (**Figure 6B**), genes  
314 related to cell differentiation function were also enriched. A significant overlap  
315 between the *Lrrc31* knockdown and CRE111 knockout RNA-seq libraries was  
316 similarly observed, further supporting the notion that CRE111 controls pluripotency  
317 by regulating downstream *Lrrc31* (**Figure S6G**).

318 LRRC31 contains nine leucine-rich repeat domains, which was previously reported to  
319 function as a protein recognition motif (Kobe et al, 2001). We next overexpressed  
320 LRRC31-FLAG in mESCs and performed a mass spectrometry analysis to detect its  
321 binding partners (**Figure 6E**). A cytoplasmic localization was observed for LRRC31  
322 in mESCs (**Figure S6B**). 18 proteins were identified as high confidence hits from two  
323 biologically independent samples performed at different proteomics facilities (**Table**

324 **S6**). Among them, JAK1 was one of the top hits. Of interest, JAK1 has previously  
325 been reported to phosphorylate STAT3 in mESCs (Do et al., 2013; Onishi and  
326 Zandstra, 2015). Hence, we investigated the level of STAT3 phosphorylation upon  
327 *Lrrc31* knock down. A marked reduction in phosphorylated STAT3 was observed  
328 when *Lrrc31* was depleted (**Figure 6F**). To further assess the effect of *Lrrc31* on  
329 STAT3 function, STAT3 ChIP was performed on the *Lrrc31* knockdown cells. *Lrrc31*  
330 knockdown resulted in a significantly decreased binding of STAT3 on its target genes,  
331 including pluripotency factors such as *Oct4* and *Esrrb* (**Figure 6H, S6J, S6K**).  
332 Consequentially, decreased expression of STAT3 target genes in *Lrrc31* knockdown  
333 ES-E14 was observed (**Figure S6A**). Furthermore, a rescue of both the STAT3  
334 binding and the expression of its target gene, *Esrrb*, was revealed when a dominant  
335 active STAT3 mutant was expressed in *Lrrc31* depleted ES-E14 (**Figure 6H, 6I**).  
336 Similar rescue was also observed for the *Lrrc31* KD-specific differentially expressed  
337 genes when dominant active STAT3 was overexpressed in *Lrrc31* KD mESC (**Figure**  
338 **S6L**). Interestingly, CRE111 was also bound by phospho-STAT3 and the binding was  
339 reduced in *Lrrc31* knockdown, suggesting a positive feedback regulation of *Lrrc31*  
340 expression (**Figure 6G**). This was further validated using luciferase assay of CRE111  
341 when *Lrrc31* or *Stat3* was depleted in ES-E14 (**Figure 6J**).

342 Next, we hypothesized that the down-regulation of *Lrrc31* in F9 mouse embryonal  
343 carcinoma and hESCs, where LIF/JAK1/STAT3 signalling pathway is not essential,  
344 would not affect their respective cell state (Dahéron et al., 2004; Kawazoe et al.,

345 2009). As expected, both knock down of *Lrrc31* and knock out of CRE111 in F9 cells  
346 did not affect the expression of pluripotency genes, such as *Oct4* (**Figure S6M and**  
347 **S6N**). Similarly, knock down of *Lrrc31* in hESCs elicited the same effects (**Figure**  
348 **S6O**). Taken together, our data suggests that *Lrrc31* functions as a novel regulator for  
349 pluripotency maintenance in mESCs by effecting the phosphorylation of STAT3 in  
350 mESCs.

351 ***Lrrc31* regulated the binding of STAT3 to the super-enhancers**

352 To further elucidate the regulatory relations between *Lrrc31* and STAT3, we  
353 performed STAT3 ChIP-seq in mESCs when *Lrrc31* was knocked down by shRNAs.  
354 An enrichment of STAT3 motif was detected indicating the good quality of our  
355 STAT3 ChIP-seq libraries (**Figure 7A**). By overlapping the differential STAT3  
356 binding peaks of two *Lrrc31* shRNA constructs, 4267 binding sites were chosen as the  
357 bona fide *Lrrc31*-regulated STAT3 binding sites (**Figure 7B**). GO analysis revealed  
358 that pluripotency-related signaling, such as TGF-beta and Wnt pathways, were  
359 regulated by *Lrrc31* (**Figure 7C**).

360 Next, we clustered STAT3 ChIP-seq libraries with published transcription factors and  
361 histone modification ChIP-seq libraries (**Figure 7D, 7E**). Out of the four clusters,  
362 cluster 2 and cluster 4 showed quite a similar pattern with the decreased STAT3  
363 binding and enrichment of active histone modifications, H3K4me3 and H3K27ac,  
364 SMC1 and OCT4 (**Figure 7E**). For both clusters, we detected the enrichment of

365 pluripotent related genes. Interestingly, for cluster 3, we observed a strong enrichment  
366 for CTCF and the deprivation of active histone modifications, which is confirmed by  
367 the motif enrichment analysis (**Figure S7A**). To test whether the binding of STAT3 is  
368 essential for the binding of CTCF at the cluster 3 sites, we performed CTCF  
369 ChIP-qRT-PCR after the knock-down of *Lrrc31*, *Stat3* or control NT in mESCs.  
370 Interestingly, a reduction of the CTCF's binding of cluster 3 sites was detected when  
371 either *Stat3* or *Lrrc31* was knocked down (**Figure 7F**).

372 Apart from CTCF, we also observed an enrichment of both MED1 and H3K27ac,  
373 markers for super-enhancer, on the cluster 2 and cluster 4 sites (**Figure 7E**). Using  
374 published super-enhancer dataset, we detected an enrichment of STAT3 on the  
375 super-enhancers of mESCs and the enrichment was reduced when *Lrrc31* was  
376 knocked down (**Figure 7G, 7H, S7B and S7D**). GO analysis on the STAT3-bound  
377 super-enhancers' target genes revealed an enrichment of genes associated with stem  
378 cell population maintenance (**Figure S7C**). Moreover, these target genes' expression  
379 was significantly reduced in *Lrrc31* knock-down mESCs (**Figure 7I**). Together, our  
380 data suggested that, *Lrrc31* actuates the binding of STAT3 on the super-enhancers of  
381 mESCs through its regulation of STAT3 phosphorylation (**Figure 7J**).

## 382 DISCUSSION

383 Several recently-published studies sought to characterize and identify important CREs  
384 which may be involved in biological processes of interest (Diao et al., 2017; Fulco et

385 al., 2016; Korkmaz et al., 2016). These screens utilized a pooled strategy involving  
386 the application of large number of CRE specific sgRNAs. Nonetheless, pooled  
387 screens have been reported to result in high false discovery rates, in part due to the  
388 introduction of biases at different stages of the screen. Whereas, our FOCUS screen  
389 pre-selected 235 CREs based on ATAC-seq and OCT4 ChIP-seq data in mESCs.  
390 Furthermore, each CRE was knocked out individually to assess its function in  
391 pluripotency. As a result, of the 19 CREs identified as putative hits from our primary  
392 screen, 84% could be validated in the secondary screen. This indicates the robustness  
393 and reliability of our method. To date, our study is the first systematic CRISPR screen  
394 to identify novel CREs, localized to the intergenic genomic regions, involved in the  
395 regulation of pluripotency.

396 Of note, we detected a large number of the candidate CREs which displayed enhancer  
397 activity in mESCs. This further highlights the key role that enhancer elements play in  
398 the maintenance of cell identity. Lending support to the observation was the  
399 enrichment of pluripotency transcription factors and proteins relating to enhancer  
400 function (eg. P300 and MED1) at our essential stem cell CREs. It is noteworthy that  
401 from the FOCUS screen, 5 candidate CREs did not exhibit characteristics typical of  
402 active enhancers, suggesting that they might regulate pluripotency in a different  
403 manner.

404 CRE 111, an essential stem cell enhancer was found to target and regulate *Lrrc31*.

405 Using protein mass spectrometry and ChIP, we further observed that Lrrc31 functions  
406 through JAK1 to modulate the phosphorylation of STAT3. It is worth noting that the  
407 expression level of LRRC31 is maintained at basal level in mESCs and the  
408 overexpression of LRRC31 shows strong cytotoxicity in mESCs (data not shown).  
409 This suggests that LRRC31 expression in mESCs is fine-tuned within an optimal  
410 range for its normal cellular function.

411 As with all screens, our study has its drawbacks, including the limited number of  
412 targetable CREs. In our strategy, CRE KO is achieved by mutating the Oct4 motif  
413 inside the CRE using CRISPR Cas9. Based on published literature (Cong et al.,  
414 2013), Cas9 usually will generate indel from 1bp to 20bp through NHEJ. Thus,  
415 making the sgRNAs targeting within the 14bp Oct4 Motif is extremely important for  
416 the efficiency of CRE KO. But, due to the PAM domain restriction, a large number of  
417 CRE (364 CREs) with Oct4 motif do not have a targetable site within the motif. Thus,  
418 by using modulated Cas9 fusion proteins, such as dCas9-LSD1 or dCas9-KRAB, we  
419 can mitigate the restrictions and to increase the coverage of FOCUS screen. Similarly,  
420 use of Cas variants with alternative PAM domain, would enable the targeting of more  
421 CREs with less sequence restriction (Kearns et al., 2015).

422  
423 In summary, the FOCUS screen described in our study represents the first systematic  
424 dissection of functional OCT4-bound CREs which are essential for pluripotency  
425 maintenance. It illuminates a previously undescribed layer of regulatory mechanisms,

426 one of which includes CREs and its target genes, in the overall pluripotency circuitry.

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560 **Figure 1. FOCUS screen identified cis-regulatory elements essential for**  
561 **pluripotency maintenance**

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563 (A) Schematic of FOCUS screen. sgRNAs targeting accessible OCT4 binding  
564 cis-regulatory elements in mESCs were designed and transfected into mESCs for  
565 the FOCUS screen.

566 (B) An image of the FOCUS screen. sgRNA targeting the Oct4 Distal Enhancer was  
567 used as positive control (red square). KO NT1 and KO NT2 were used as negative  
568 controls (white squares). OCT4 immunofluorescence signal and nuclei staining  
569 are shown in green and blue respectively.

570 (C) Distribution plot of the FOCUS screen. 19 out of 225 cis-regulatory elements with  
571 Z scores of <-2 (red dotted line) were identified as candidates potentially  
572 important for pluripotency maintenance (red dots). Non-targeting controls are  
573 labelled as blue and green dots. Remaining cis-regulatory elements are marked in  
574 grey. Z-score of 4 replicates are shown in the same line for each cis-regulatory  
575 element.

576 (D) Representative images of OCT4 (green) and DNA (blue) for selected hits  
577 identified from the FOCUS screen.

578 (E) Primary screen of 225 cis-regulatory elements with two independent assays,  
579 OCT4 (green) and Oct4-DE *mcherry* reporter (red). Each row represents the  
580 knockout of a single cis-regulatory element. Each column represents individual  
581 replicates.

582 (F) Secondary screen of 19 candidate CRE hits. Rep 1 and 2 are two sgRNAs  
583 targeting different locations of the same cis-regulatory element. Z-score was  
584 calculated with a reference to KO NT2. The bar chart shows mean  $\pm$  SD of 3

585 biological replicates.

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605 **Figure 2. Knockout of candidate CRE hits affects both the maintenance and**  
606 **establishment of pluripotent stem cells.**

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608 (A) Colony formation assay showing that the knock-out of candidate CREs  
609 compromised self-renewal of mESCs. Left: Schematic of colony formation assay.  
610 Middle: Normalized AP+ colony number of CREs (black) knockout. KO NTs  
611 (grey) were used as negative controls, while KO Oct4 DE and KO Sox2 Enh (red)  
612 were used as positive controls. The red dotted line shows the level of normalized  
613 AP+ colony number of KO NT2. The bar chart shows mean  $\pm$  SD of 3 biological  
614 replicates. Right: Representative images of each candidate CRE knockout.

615 (B) qRT-PCR showing decreased *Oct4* expression and increased lineage-specific gene  
616 expression at Day 3 post knockout of candidate CREs. Each row represents the  
617 expression of a gene. Each column represents the knockout of an individual  
618 candidate CRE. Data shown was normalized to E14 transfected with KO NT  
619 sgRNA.

620 (C) qRT-PCR showing increased expression of differentiation genes when candidate  
621 hit CREs were knocked out during RA-induced differentiation. Cells transfected  
622 with KO NT sgRNA was used as control (grey) and all data was normalized to it.  
623 The bar chart shows mean  $\pm$  SD of 3 replicates. Student's t-test was used for  
624 statistical analysis. \* represents p-value  $<0.05$ .

625 (D) Knockout of candidate CREs affected the establishment of pluripotency during  
626 somatic cell reprogramming. Schematic of the experiment (top). Reprogramming

627 efficiency was assayed at 10 d.p.i by AP staining (bottom). KO NT (grey) was  
628 used as a negative control, while KO Oct4 DE (red) was used as a positive control.  
629 The bar chart shows mean  $\pm$  SD of 3 biological replicates.

630 (E) Average enrichment plot of ATAC-seq signal of candidate CREs during somatic  
631 cell reprogramming. Different time points are shown in different colours.

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647 **Figure 3. Candidate CREs display typical enhancer activity in mESCs.**

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649 (A) Average enrichment plot of histone marks on hit CREs. Different histone marks  
650 are indicated in different colours.

651 (B) Enrichment of H3K4me1 (left) and H3K27ac (right) on hit CREs (black)  
652 compared to a negative control region (grey) by ChIP-qPCR. Oct4 distal enhancer  
653 was used as positive control (dark red). The red dotted line shows the background  
654 enrichment of the negative control region. Data are representative of at least 3  
655 independent experiments. The bar chart shows mean  $\pm$  SD.

656 (C) Average enrichment plot of pluripotency transcription factors on hit CREs.  
657 Different transcription factors are indicated in different colours.

658 (D) Transcription factor motifs enriched at candidate CRE sites.

659 (E) Luciferase activity of reporter plasmids containing CRE132 with mutated *Zic3*  
660 and *Brn1* motif. The bar chart shows mean  $\pm$  SD of 3 independent experiments.

661 (F) Knockout of *Oct4* motif decreased the enrichment of H3K4me1 (left) and  
662 H3K27ac (right) active histone marks on candidate CREs. Each row represents  
663 the relative enrichment of the histone modifications on five candidate CREs when  
664 one CRE was knocked out using sgRNA. The relative enrichment of histone  
665 marks is shown in a color scale ranging from blue (decreased binding) to red (no  
666 change as compared to control).

667 (G) Luciferase activity of reporter plasmids containing a fragment of candidate CREs  
668 in E14 (black), RA treated E14 (dark grey) and 3T3 (light grey) (right). Schematic

669 of the experiment was shown on the left. The bar chart shows mean  $\pm$  SD of 3  
670 independent experiments.

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689 **Figure 4. Knockout of candidate CREs elicited overlapping and distinct**  
690 **transcriptomic effects.**

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- 692 A) Gene Ontology analysis of differentially expressed genes in the CRE KO libraries.  
693 X-axis represents the  $-\text{Log10}(p\text{-Value})$  and Y-axis represents GO term.
- 694 B) Lineage enrichment analysis of the CREs KO RNA-seq libraries. Each row  
695 represents a single lineage. Each column represents individual CRE KO RNA-seq  
696 library.  $-\text{Log10}$  (Benjamini-Hochberg adjusted P) is represented as colour from  
697 red (enriched) to yellow (most enriched)
- 698 C) Pearson correlation analysis shows that CREs 111 and 210 clustered distinctly.  
699 The correlation score is represented as colour from yellow (low) to red (high)
- 700 D) Principal component analysis (PCA) of the CRE KO RNA-seq libraries. CRE111  
701 clustered distinctively from the other CREs. KO NTs are shown in grey, CREs are  
702 shown in blue.
- 703 E) Stacked column plot showing the percentage of overlapped genes which were  
704 differentially expressed between CRE KO and *Oct4* DE RNA-seq libraries.
- 705 F) Combinatorial KO of CRE111 and CRE210 further decreased the expression of  
706 *Oct4* compared to single KO. Cells transfected with KO NT sgRNA was used as  
707 control (grey) and all data was normalized to it. The bar chart shows mean  $\pm$  SD  
708 of 3 replicates. Student's t-test was used for statistical analysis.

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715 **Figure 5. Candidate CREs regulate novel and known pluripotency genes.**

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717 A) Integrative genomic view of Hi-C, ChIP-seq and RNA-seq data of CRE 111. Top  
718 panel: Hi-C interactions within the same TAD of CRE111. Middle panel:  
719 ChIP-seq UCSC browser of pluripotency and architectural proteins, as well as  
720 active enhancer marks. Bottom panel: RNA-seq UCSC browser of KO CRE111  
721 and KO NT. The location of CRE111 is highlighted using red line. Putative target  
722 of CRE111 is indicated using grey shades.

723 B) The bar chart showing the expression change of genes located within the same  
724 TAD as CRE111 when CRE111 was knocked out. The y-axis represents the  
725 -Log2(Fold change). The genes with decreased expression are shown as the blue  
726 bars. The genes with increased expression are shown as the red bars.

727 C) Integrative genomic view of Hi-C, ChIP-seq and RNA-seq data of CRE 210. Top  
728 panel: Hi-C interactions within the same TAD of CRE210. Middle panel:  
729 ChIP-seq UCSC browser of pluripotency and architectural proteins, as well as  
730 active enhancer marks. Bottom panel: RNA-seq UCSC browser of KO CRE210  
731 and KO NT. The location of CRE210 is highlighted using red line. Putative target  
732 of CRE210 is indicated using grey shades.

733 D) The bar chart showing the expression change of genes located within the same  
734 TAD as CRE210 when CRE210 was knocked out. The y-axis represents the  
735 -Log2(Fold change). The genes with decreased expression are shown as the blue  
736 bars. The genes with increased expression are shown as the red bars.

737 E) Target genes of CREs form a tight network with known pluripotency regulators.  
738 Target genes are shown as red circles. Known pluripotency regulators are shown  
739 as grey circles.

740 F) qRT-PCR showing that knockdown of CRE target genes (black) decreased the  
741 expression of *Oct4* (top panel) and *Sox2* (bottom panel) significantly as compared  
742 to siNT control (grey). The bar chart shows mean  $\pm$  SD of 3 replicates.

743 G) Brightfield images showing morphology changes in E14 cells induced by  
744 knockdown of CRE target genes.

745 H) The interaction of CRE 111 and *Lrrc31* in E14 cells was confirmed by the 3C  
746 assay. X-axis shows 3C fragments corresponding to actual genomic locations.  
747 Y-axis shows relative interaction frequency between E14 and the BAC control.  
748 The corresponding location of each fragment in the genome is indicated using red  
749 dotted line. The bar chart shows mean  $\pm$  SD of 3 replicates.

750 I) qRT-PCR showing gene expression in the rescue of the expression of upregulated  
751 (red) or downregulated (blue) genes in CRE 111 KO, by the overexpression of  
752 *LRRC31* in E14 cells. Red dotted line represents the relative expression of genes  
753 in WT E14 mESCs. The bar chart shows mean  $\pm$  SD of 3 replicates.

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755 **Figure 6. *Lrrc31* regulates the maintenance of pluripotency in mES cells through**  
756 **the JAK-STAT3 signaling pathway.**

757

758 A) Brightfield images showing the effects of knocking down *Lrrc31* using both  
759 siRNA and shRNA. Note the drastic change in morphology of the E14 cells.

760 B) qRT-PCR showing that *Lrrc31* knock-down resulted in reduced expression of  
761 pluripotency genes and corresponding increase in expression of differentiation  
762 genes. Two constructs of *Lrrc31* shRNA are shown as dark and light blue bars,  
763 while the vector control is shown in grey. The bar chart shows mean  $\pm$  SD of 3  
764 independent experiments.

765 C) qRT-PCR showing that knockout of *Lrrc31* using sgRNA reduced the expression  
766 of pluripotency genes. Two clones of *Lrrc31* -/- are shown as orange and yellow  
767 bars, while the *Lrrc31* +/+ is shown in grey. The bar chart shows mean  $\pm$  SD of 3  
768 independent experiments.

769 D) Knockdown of *Lrrc31* at different time points of somatic cell reprogramming  
770 process. Top panel: Schematic of the *Lrrc31* knockdown during somatic cell  
771 reprogramming. Middle panel: Reprogramming efficiency (AP+ colony numbers)  
772 was assayed at 12 d.p.i. *Lrrc31* knockdown (black) at 9 d.p.i gave rise to reduced  
773 reprogramming efficiency as compared to the siNT control (grey). The bar chart  
774 shows mean  $\pm$  SD of 3 biological replicates. Bottom panel: Representative images  
775 of each treatment group.

776 E) Identification of LRRC31 interacting partners. Schematic of mass spectrometry  
777 experiment using LRRC31-FLAG pull down (left). Potential interacting partners  
778 of LRRC31 (right). Differentially enriched bands were highlighted with black  
779 arrows. The 75 KDa band represents the bait, LRRC31-FLAG protein. The  
780 100KDa band represents potential interacting partners of LRRC31. The protein ID  
781 is listed in the box. High confidence hits are highlighted in red.

782 F) Western blot shows that *Lrrc31* knockdown caused the corresponding decrease in  
783 STAT3 phosphorylation. Band intensity was quantified and normalized to ACTIN.

784 G) ChIP qRT-PCR showing that the knockdown of *Lrrc31* decreased the binding of  
785 phospho-STAT3 on its targets in E14 cells. The shRNA of *Lrrc31* is shown as  
786 black bars. The empty vector of pSUPER was used as control (light grey). shRNA  
787 targeting *Stat3* was used as positive control (dark red). The bar chart shows mean  
788  $\pm$  SD of 3 independent experiments.

789 H) Rescue of STAT3 binding on its target genomic regions by STAT3 dominant  
790 active mutant overexpression in *Lrrc31* knockdown ES-E14. The STAT3  
791 dominant active mutant overexpression in *Lrrc31* knockdown ES-E14 is shown as  
792 black bars. The *Lrrc31* knockdown only is shown as white bars. The empty vector  
793 of pSUPER with STAT3 dominant active mutant overexpression was used as  
794 control (light grey). The bar chart shows mean  $\pm$  SD of 3 replicates.

795 I) Rescue of *Esrrb* expression by STAT3 dominant active mutant overexpression in  
796 *Lrrc31* knockdown ES-E14. The bar chart shows mean  $\pm$  SD of 3 replicates.

797 J) Relative luciferase activity of CRE111 reporter plasmids in sh*Lrrc31* transfected  
798 E14 (black), sh*Stat3* transfected E14 (red) and shEV transfected E14 (grey). The  
799 bar chart shows mean ± SD of 3 replicates.  
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823 **Figure 7. Lrrc31 regulated the binding of STAT3 to the super-enhancers.**

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825 A) Transcription factor motifs enriched at STAT3 binding peaks.

826 B) The Venn diagram showing the overlap of differentially STAT3 binding sites

827 between two Lrrc31 shRNA constructs.

828 C) Gene ontology analysis of differentially STAT3 binding sites when Lrrc31 was

829 knocked down using shRNA. X-axis represents the  $-\text{Log10}(p\text{-Value})$  and Y-axis

830 represents GO term.

831 D) Pearson correlation analysis shows the correlation of STAT3 binding peak

832 between sh*Lrrc31* and sh*STAT3*. The correlation score is represented as colour

833 from yellow (low) to red (high)

834 E) Enrichment of several histone marks and transcription factors at the genomic

835 regions of STAT3 binding sites. The heatmaps are clustered according to the

836 enrichment profile of all the ChIP-seq libraries indicated in the figure. The top

837 panel represent the average enrichment plot of the indicated ChIP-seq library.

838 F) ChIP qRT-PCR showing that both *Lrrc31* knock-down and *Stat3* knock-down

839 reduced the binding of CTCF on the cluster 3 STAT3 binding sites. The shRNA of

840 *Lrrc31* is shown as red bars. The empty vector of pSUPER was used as control

841 (light grey). shRNA targeting *Stat3* was shown as black bars. The bar chart shows

842 mean  $\pm$  SD of 3 independent experiments.

843 G) Average enrichment plot showing the reduced binding of STAT3 on the

844 super-enhancer when *Lrrc31* was knocked down. The y-axis represents average

845 normalized number of fragments at the corresponding genomic regions indicated

846 in the x-axis.

847 H) UCSC screenshot of binding profile of STAT3 on Sox2 super-enhancer (indicated

848 by the binding of MED1 and H3K27ac) when *Stat3* or *Lrrc31* was knocked down.

849 I) Box plot showing the expression level of super-enhancer target genes when

850 *Lrrc31* was knocked down by shRNA. Student's t-test was used for statistical

851 analysis. \*\*\*\* represents p-value  $<0.0001$ .

852 K) In WT mESCs (top), OCT4 binds onto CRE 111 to maintain the expression of

853 Lrrc31. LRRC31 interacts with JAK1 to phosphorylate STAT3 upon the binding

854 of LIF on LIFR/Gp130. Phosphorylated STAT3 is then translocated into the

855 nucleus and binds onto both pluripotent genes' promoter, such as *Oct4* and *Esrrb*,

856 and super-enhancers to activate their expression. Knocking out CRE111 using

857 CRISPR (bottom) decreases the expression of Lrrc31. The loss of LRRC31 affects

858 the phosphorylation of STAT3 through JAK1. Decreased level of phosphorylated

859 STAT3 further diminishes the expression of downstream pluripotency genes.

860

861

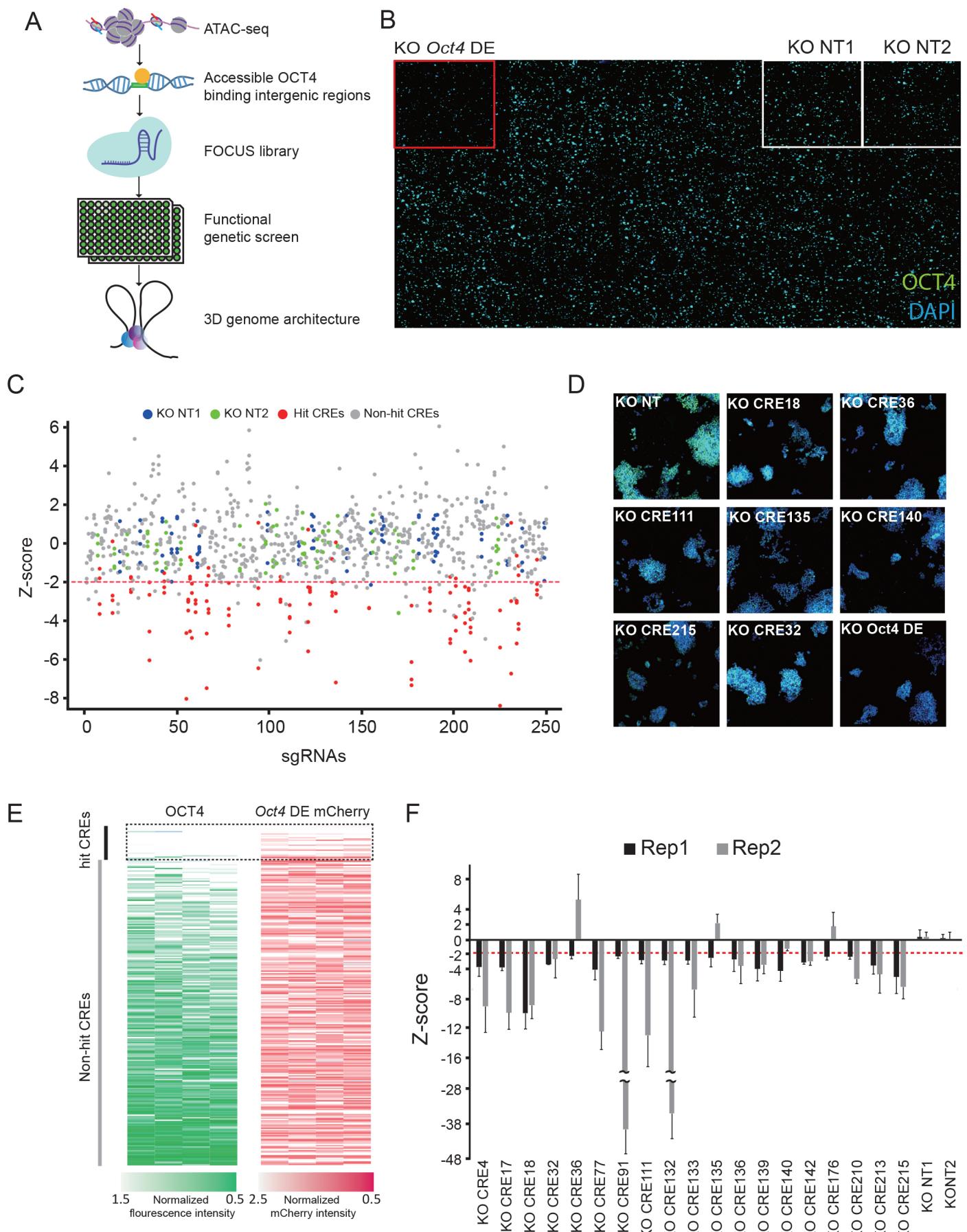
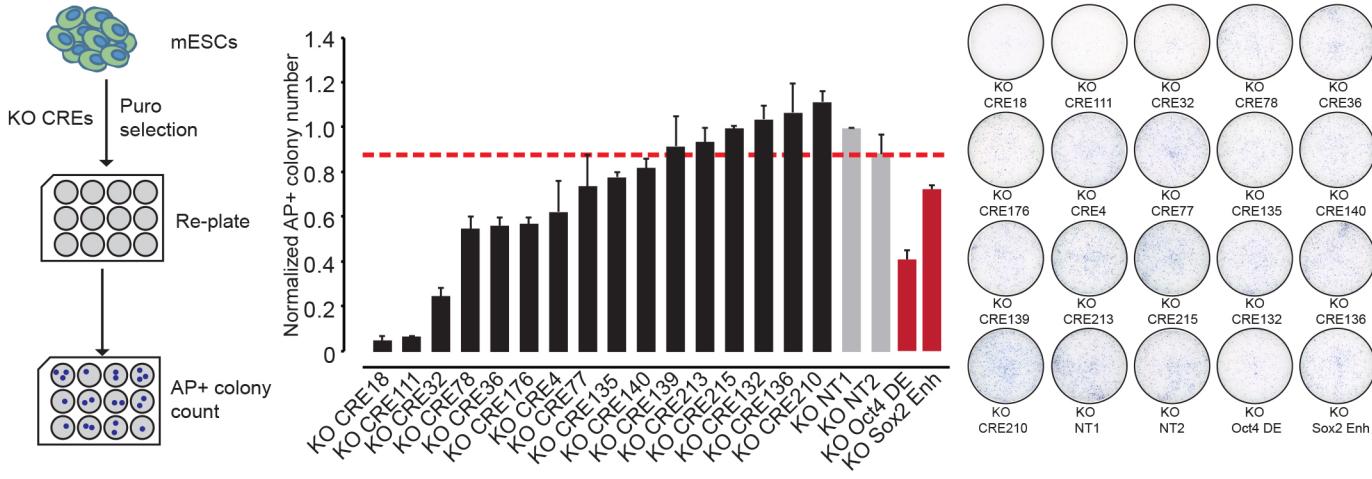
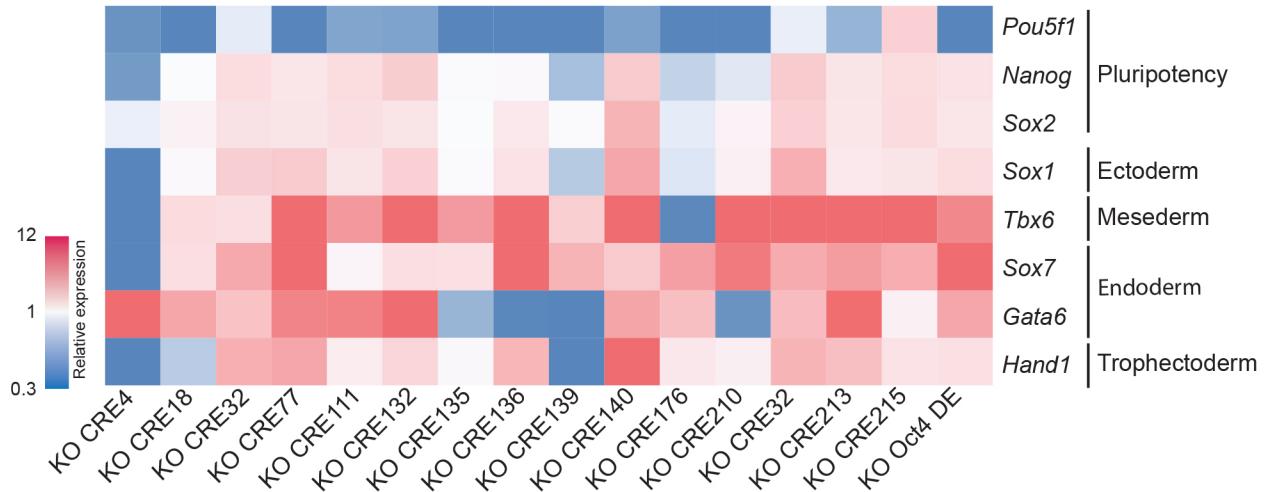


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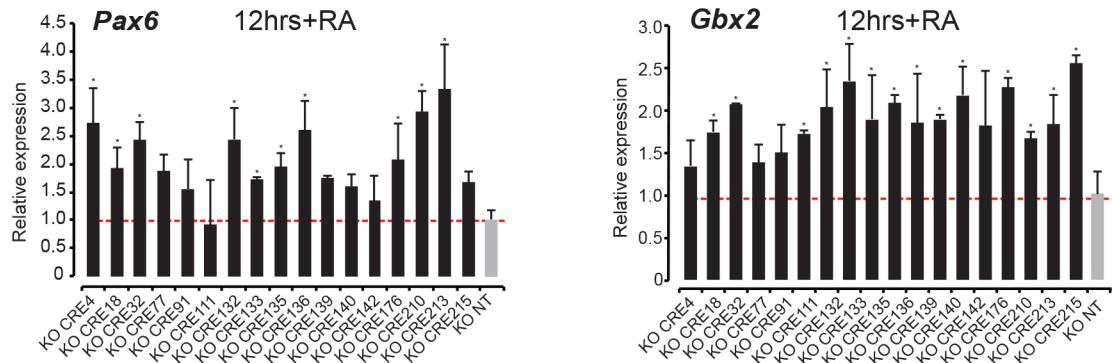
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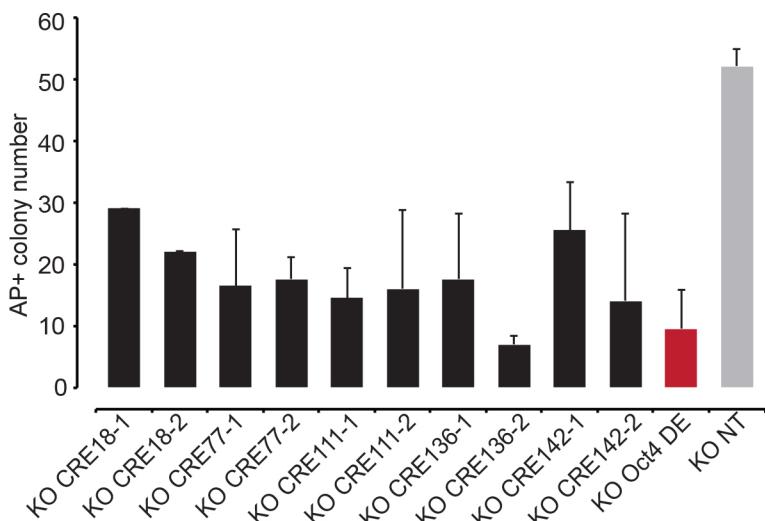
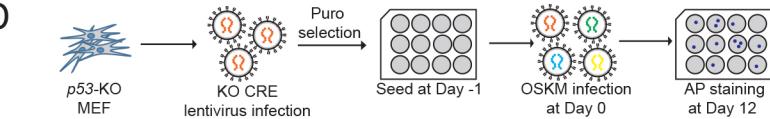
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C



D



E

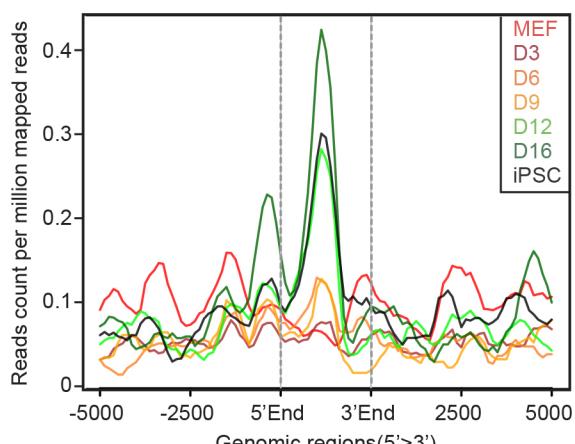


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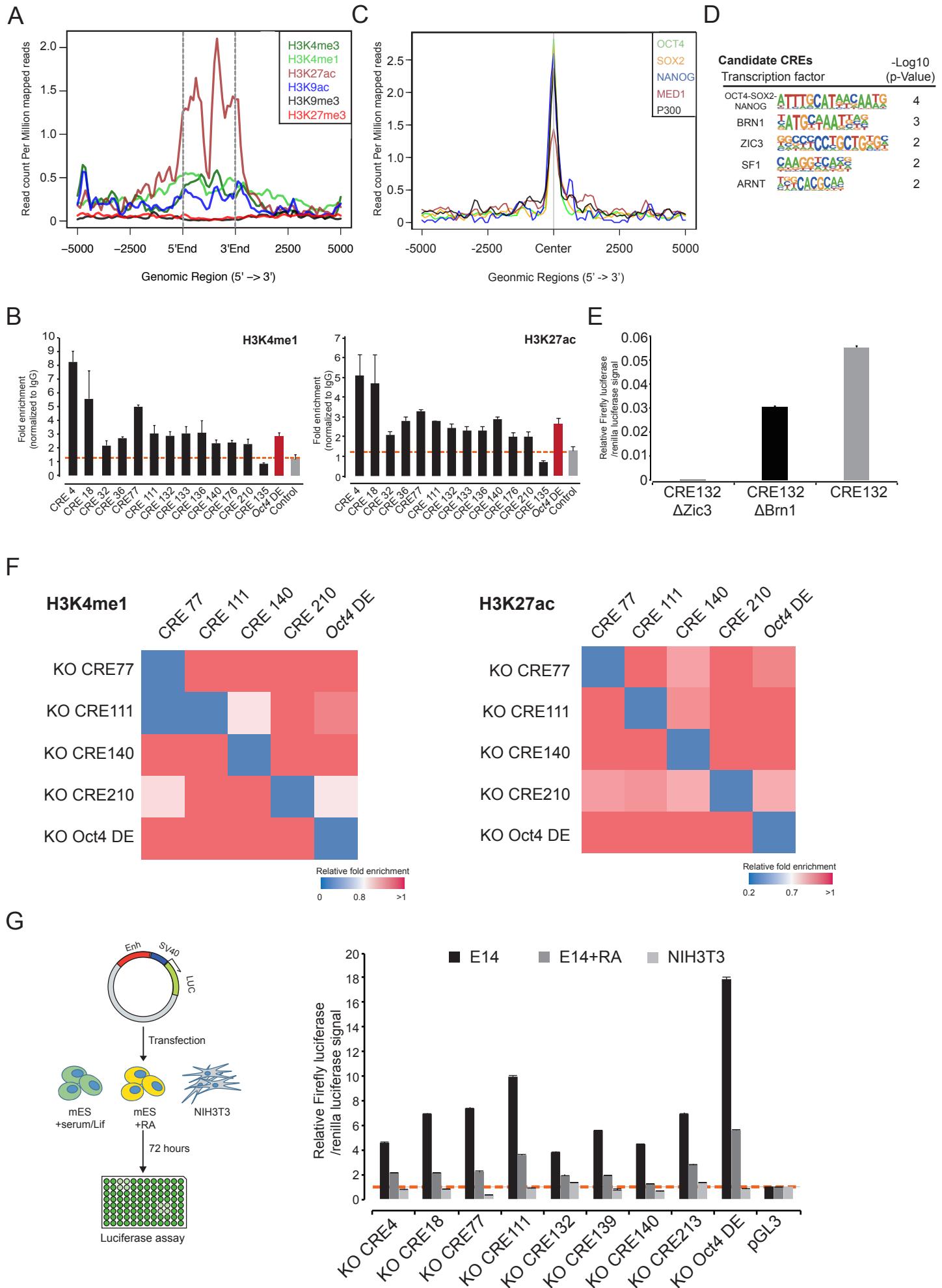
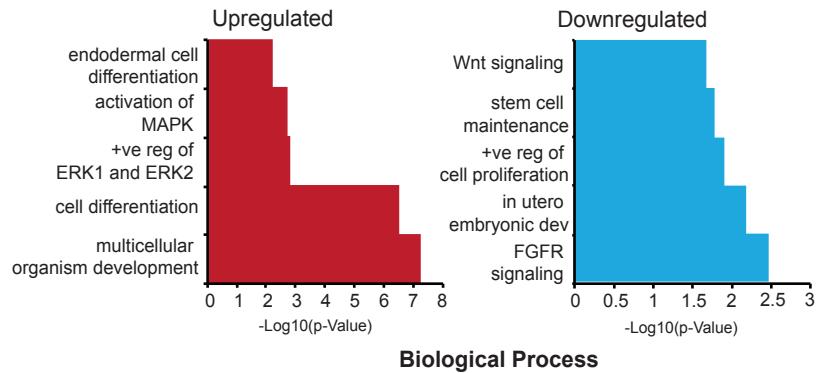
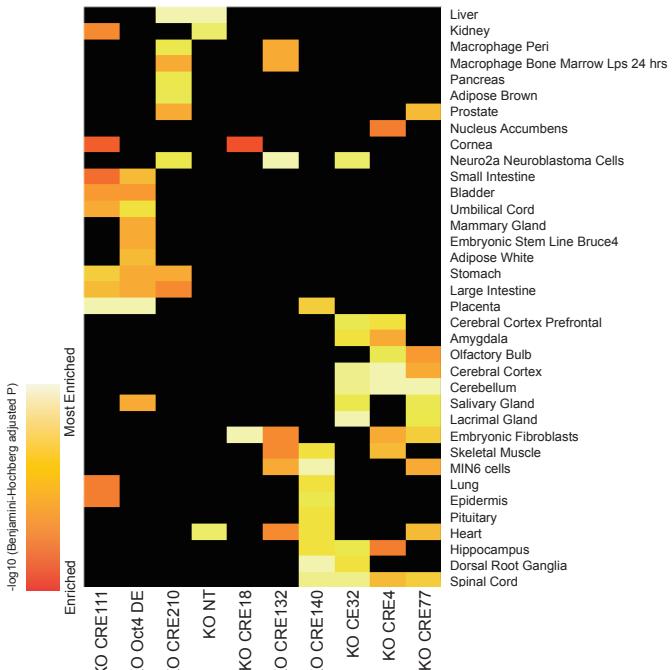


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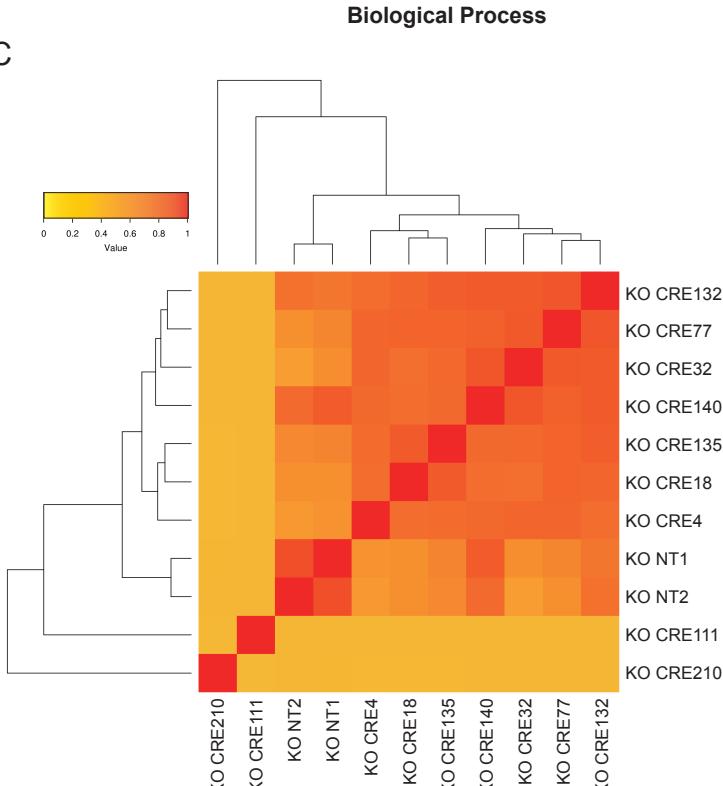
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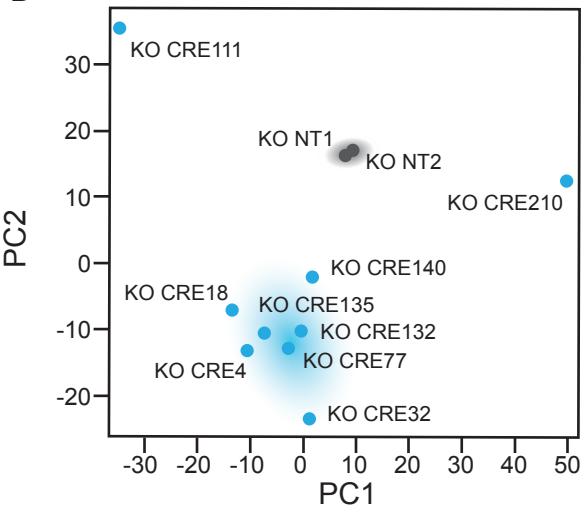
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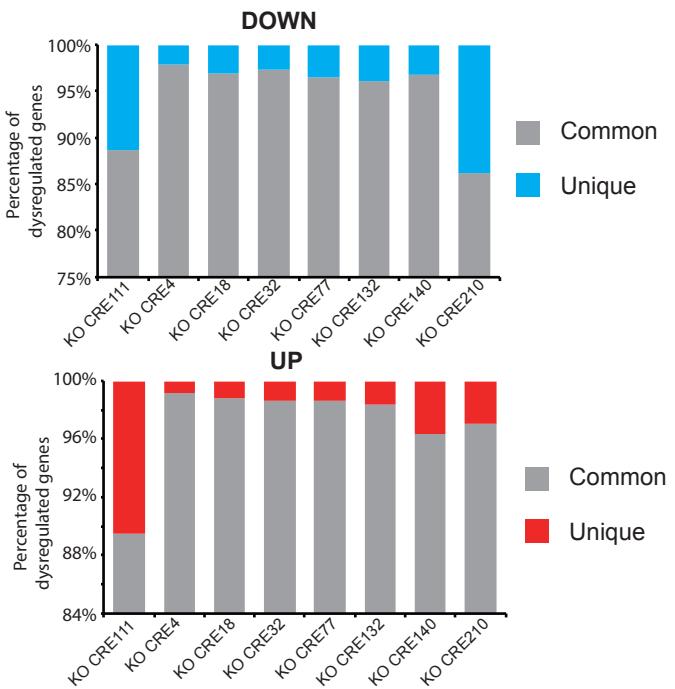
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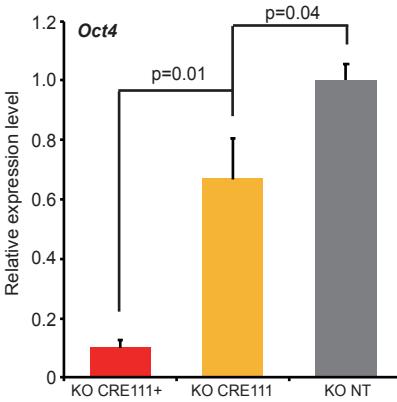
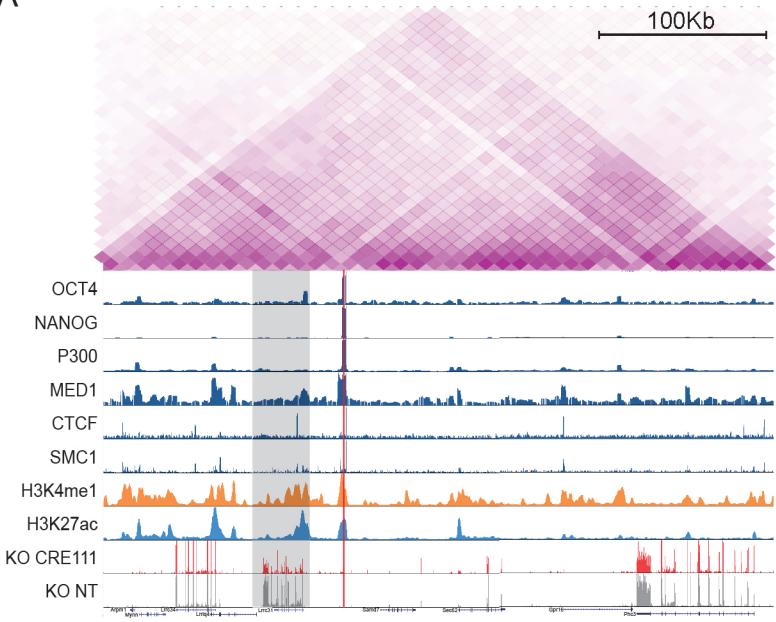
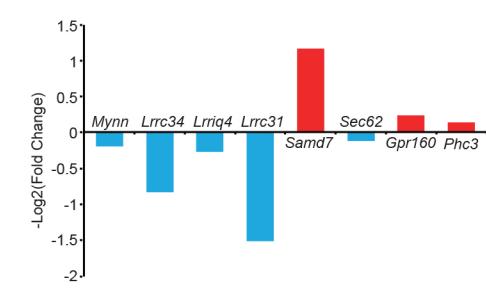


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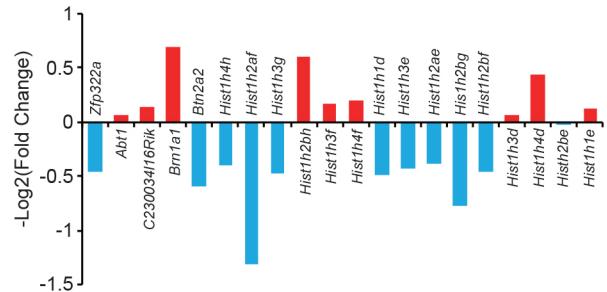
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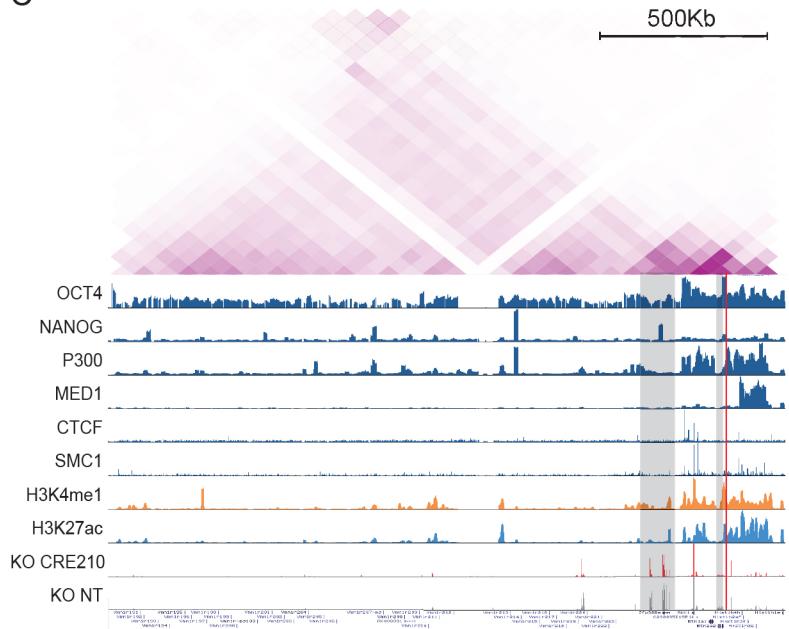
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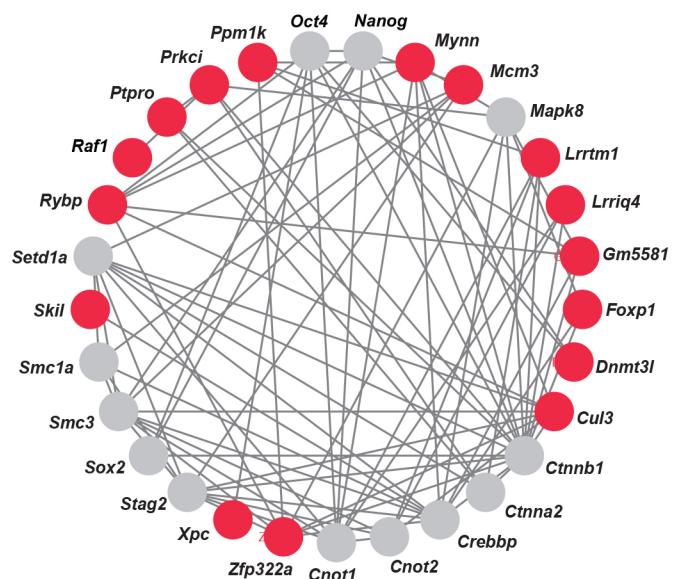
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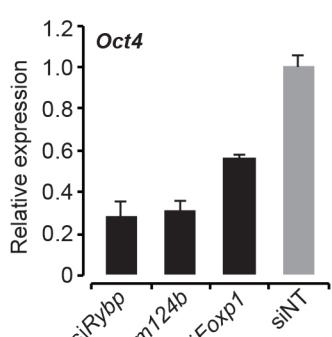
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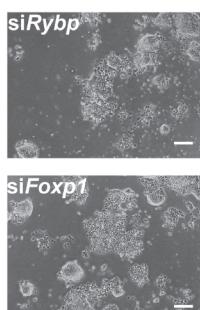
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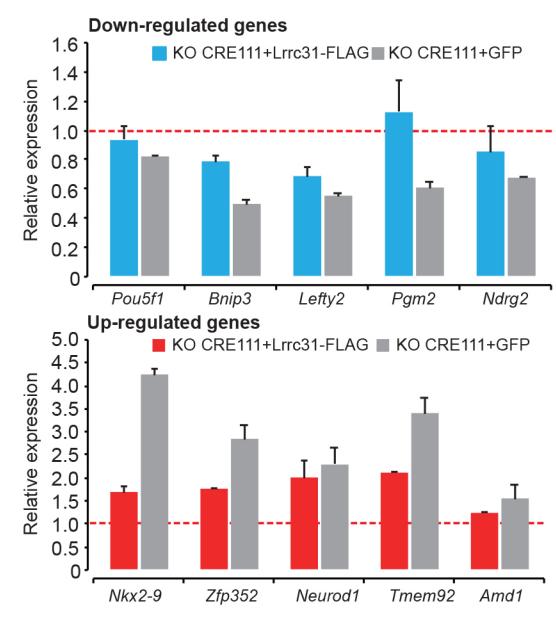


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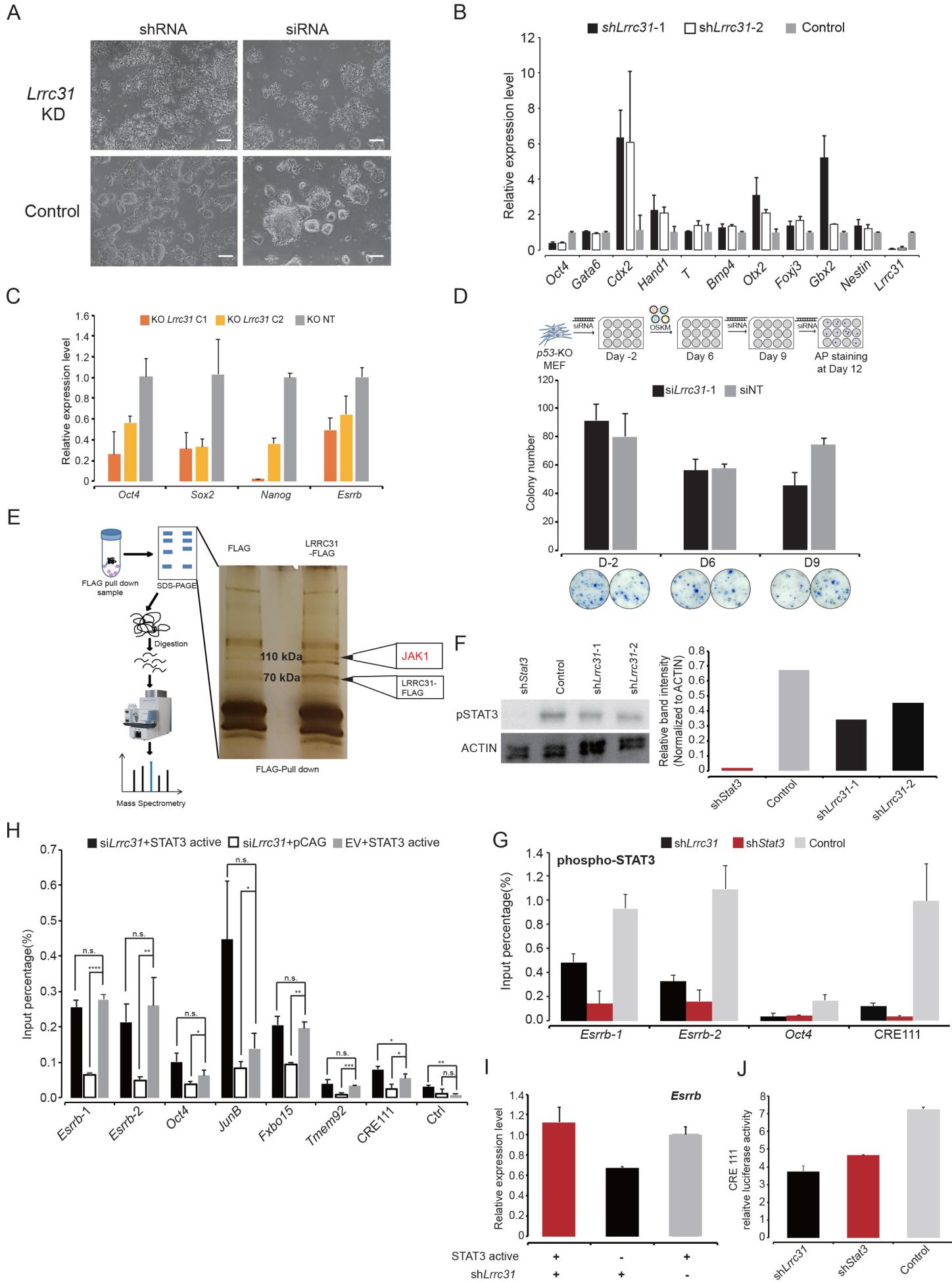


Figure 6

