

1           **Transcriptional repression by FACT is linked to regulation of**  
2                   **chromatin accessibility at the promoter of ES cells**

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20 **The conserved and essential histone chaperone FACT (Facilitates Chromatin**  
21 **Transcription) reorganizes nucleosomes during DNA transcription, replication and repair**  
22 **and ensures both, efficient elongation of RNA Pol II and nucleosome integrity. In**  
23 **mammalian cells, FACT is a heterodimer, consisting of SSRP1 and SUPT16. Here, we**  
24 **show that in contrast to yeast, FACT accumulates at the transcription start site of genes**  
25 **reminiscent of RNA Polymerase II profile. Depletion of FACT in mouse embryonic stem**  
26 **cells leads to up-regulation of pro-proliferative genes and key pluripotency factors**  
27 **concomitant with hyper-proliferation of mES cells. Using MNase-, ATAC-, and Nascent**  
28 **Elongating Transcript Sequencing (NET-seq) we show that up-regulation of genes**  
29 **coincides with loss of nucleosomes upstream of the TSS and concomitant increase in**  
30 **antisense transcription, indicating that FACT impacts the promoter architecture to**  
31 **regulate expression of these genes. Finally, we demonstrate a role for FACT in cell fate**  
32 **determination and show that FACT depletion primes ES cells for the neuronal lineage.**

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## 42 **Introduction**

43 The basic functional unit of chromatin is the nucleosome consisting of around 147 bp of DNA  
44 wrapped around an octamer of histone proteins – two copies each of histones H2A, H2B, H3  
45 and H4. *In vitro*, chromatinized DNA templates are refractory to transcription suggesting that the  
46 nucleosome might provide a barrier for the elongating RNA polymerase. Using elegant  
47 biochemical fractionation assays coupled to *in vitro* transcription assays, FACT was initially  
48 characterised as a factor that alleviated the repressive nature of chromatin *in vitro* (Orphanides,  
49 Wu, Lane, Hampsey, & Reinberg, 1999). Meanwhile, it has been demonstrated that FACT can  
50 cooperate with all RNA polymerases in the cell and ensure both, efficient transcription  
51 elongation and nucleosome integrity. Both FACT subunits are highly conserved across all  
52 eukaryotes with the exception of an HMG-like domain present in SSRP1 but absent in the yeast  
53 homolog Pob3. In yeast, an HMG domain protein named Nhp6 has been proposed to provide  
54 the DNA binding capacity of FACT (Formosa et al., 2001).

55 The molecular basis for FACT activity has long remained elusive. However, recent biochemical  
56 and structural studies are starting to elucidate how FACT engages nucleosomes (Hondele et al.,  
57 2013; Hsieh et al., 2013; Kemble, McCullough, Whitby, Formosa, & Hill, 2015; Winkler & Luger,  
58 2011). Via its several domains, FACT binds to multiple surfaces on the nucleosome octamer  
59 and acts by shielding histone-DNA interactions. Initially, it was proposed that FACT would evict  
60 an H2A/B dimer from the nucleosome in front of the polymerase and then reinstate nucleosome  
61 integrity in its wake. However, other data suggests that this dimer replacement is not part of  
62 FACT function as it leaves the histone composition of the nucleosome intact (Formosa, 2012).  
63 Based on recent biochemical data (Hsieh et al., 2013), a model emerges in which RNA Pol II  
64 enters the nucleosome and partially uncoils the nucleosomal DNA. At the same time, FACT  
65 binds to the proximal and distal H2A/H2B dimer and these FACT–dimer interactions facilitate  
66 nucleosome survival.

67 Although the genetics and biochemistry of FACT are relatively well understood, it is not known  
68 whether cell-type dedicated functions are conferred by this histone chaperone. Interestingly,  
69 genome-wide expression analyses across cell and tissue types implicate a role of FACT in  
70 maintaining an undifferentiated state. Depletion of FACT subunits leads to growth reduction in  
71 transformed, but not in immortalized cells (Garcia et al., 2013), indicating that FACT is essential  
72 for tumour growth, but not for proliferation of untransformed cells. Finally, FACT regulates the  
73 expression of Wnt-target genes during osteoblast differentiation in mesenchymal stem cells and  
74 its deletion leads to a differentiation skew (Hossan et al., 2016). Taken together, these data  
75 suggested a more specific role for the FACT complex in undifferentiated cells as previously  
76 assumed.

77 Recent studies have demonstrated that RNA Pol II can transcribe in both sense and anti-sense  
78 directions near many mRNA genes (Kwak, Fuda, Core, & Lis, 2013; Mayer et al., 2015). At  
79 these so-called bidirectional promoters, RNA Pol II initiates transcription and undergoes  
80 promoter-proximal pausing in both the sense (at the protein-coding TSS) and anti-sense  
81 orientation (Kwak et al., 2013; Mayer et al., 2015). Divergent transcription is often found at  
82 mammalian promoters that are rich in CpG content, but lack key core promoter elements such  
83 as the TATA motif (Scruggs et al., 2015). A broader nucleosome free region (NFR) in the  
84 promoter region is often accompanied by divergent transcription, and can lead to binding of  
85 more transcription factors resulting in higher gene activity (Scruggs et al., 2015).

86 Here, we have confirmed an indispensable role of FACT in undifferentiated cells based on the  
87 expression levels of both FACT subunits and, thus chose mouse embryonic stem cells as a  
88 model to investigate how FACT might shape the transcriptome and maintain an undifferentiated  
89 state. To achieve this, we performed CHIP- and RNA-seq to identify genes bound and regulated  
90 by FACT. To address at a mechanistic level how FACT might regulate transcription in ES cells,  
91 we combined this analysis with MNase digestion of chromatin coupled to deep sequencing  
92 (MNase-seq), Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), and

93 Nascent Elongating Transcript Sequencing (NET-seq). Using these approaches, we have  
94 identified a specific gene cluster comprising of genes involved in embryogenesis/ neuronal  
95 development that are up-regulated upon FACT depletion. In addition, we observed a  
96 concomitant increase in chromatin accessibility around the transcription start site, suggesting  
97 that maintenance of nucleosomes at this position by FACT is part of the mechanism how FACT  
98 impacts on the regulation of these genes. Finally, our data support a role of FACT in the  
99 maintenance of a pluripotent state by showing that its depletion leads to faster differentiation  
100 into the neuronal lineage.

101

## 102 **Results**

### 103 **Occupancy of FACT correlates with marks of active gene expression**

104 High expression of FACT has been associated with stem or less-differentiated cells (Garcia et  
105 al., 2011). Indeed, we were able to confirm that low FACT levels correlate with highly  
106 differentiated cell lines as opposed to stem and cancer cells (**Supplementary Fig. 1A**). In  
107 addition, differentiation of murine ES cells into terminally differentiated cardiomyocytes  
108 (Wamstad et al., 2012) reveals that FACT levels diminish throughout the course of  
109 differentiation (**Supplementary Fig. 1B**). Thus, we chose to explore how FACT contributes to  
110 the transcriptome of undifferentiated cells using mouse ES cells. Initially, we applied to mESCs  
111 a chromatin immunoprecipitation and sequencing (ChIP-seq) assay to identify potential DNA  
112 binding regions for both FACT subunits. Subsequently, we examined FACT co-enrichment with  
113 several other transcription factors, histone marks, and chromatin remodellers over the gene  
114 body area of all uniquely annotated protein-coding genes ( $n = 11,305$ ). High correlation scores  
115 were observed between SSRP1, SUPT16, H3K4me3, H3K27ac, and Pol II variants (Pol II S5ph,  
116 Pol II S2ph) confirming the role of FACT in active gene expression (**Fig. 1A & Supplementary**

117 **Fig. 1E).** A good correlation was also observed between both FACT subunits and Chd1, in line  
118 with data demonstrating physical interaction and co-localization in mammalian cells (Kelley,  
119 Stokes, & Perry, 1999). However, only a moderate correlation was observed between FACT  
120 and H3K36me3 on a genome wide level despite the fact that H3K36me3 directly recruits FACT  
121 to actively transcribed genes (Carvalho et al., 2013). We suspect that the strong enrichment of  
122 FACT subunits around the TSS might mask this potential correlation. Nevertheless, FACT  
123 subunits also co-localize to the gene body of actively transcribed genes and enrich towards the  
124 TES, similarly to H3K36me3 (**Supplementary Fig. 1C,D**). Pearson's correlation among FACT  
125 and active marks remained elevated when we focused on promoter and enhancer regions ( $n =$   
126 19,461) (**Fig. 1B**). Both subunits displayed very similar binding pattern to each other over the  
127 transcription start site (TSS) of all the annotated genes and were tightly linked to H3K4me3  
128 levels (**Fig. 1C**).

129

### 130 **Regulation of gene expression by FACT**

131 To investigate how FACT orchestrates transcriptional regulation in mESCs, we depleted SSRP1  
132 levels using short hairpin RNAs (shRNA – **Supplementary Fig. 2A**). Importantly, this also led to  
133 a simultaneous depletion of SUPT16 levels as assessed by Mass spectrometry (Supplementary  
134 Table 7). This interdependence of the two FACT subunits has been observed before (Garcia et  
135 al., 2013). Surprisingly, we observed an increase in mESC proliferation following *Ssrp1* knock-  
136 down (KD) as measured by proliferation rate via MTT (metabolic activity measurement) cell  
137 proliferation assays using independent shRNAs (**Fig. 2A & Supplementary Fig. 2B**). This is in  
138 contrast to previously published data from tumour cell lines, in which proliferation rates  
139 decrease and also from terminally differentiated cells, where FACT depletion has no effect on  
140 proliferation (Garcia et al., 2013). Subsequently, we sequenced the whole transcriptome (RNA-

141 seq). In total, we characterised 3,003 differentially expressed genes; 1,655 down-regulated and  
142 1,348 up-regulated (**Fig. 2B**). Down-regulated genes were over-represented for pathways  
143 involved in development, while up-regulated genes were involved in metabolic processes and  
144 positive regulation of proliferation (**Fig. 2C**), indicating that the change in the transcriptome  
145 accounts for the faster proliferation rates. These results suggest that FACT impacts  
146 developmental processes and negatively controls cell proliferation in mES cells by controlling  
147 gene expression patterns. A low correlation (Pearson's  $R = 0.11$ ) was observed between the  
148 coverage of SSRP1 (ChIP-seq) and the gene fold change (RNA-seq) of those genes in the  
149 *Ssrp1* KD (**Fig. 2D**), indicating that FACT binding alone is not a predictor for gene expression  
150 changes. Taking these findings together, FACT can work directly as an enhancer or repressor  
151 of transcription in mES cells.

152 Given the high correlation of FACT with H3K4me3 (Fig. 1A) and to understand how the  
153 transcriptional changes might be linked to differences in recruitment of transcriptional regulators  
154 we performed an IP for H3K4me3 followed by Mass spectrometry both in Control and SSRP1-  
155 depleted ES cells (**Supplementary Fig. 3A, Supplementary Table 5**). We observed an  
156 increased binding of Oct4 and Sox2 to H3K4me3 in the *Ssrp1* KD state, in line with the  
157 observation that FACT depletion impacts developmental processes. Interestingly, we observed  
158 reduced binding of many splicing factors on H3K4me3 in the absence of FACT  
159 (**Supplementary Fig. 3A**). Differential splicing analysis between Control and *Ssrp1* KD  
160 conditions confirmed in total 356 Exon skipping/inclusion and 97 Intronic retention events  
161 following FACT depletion (**Supplementary Fig. 3B,C**). Interestingly, a fraction of the differential  
162 gene isoforms generated in the *Ssrp1* KD group is over-represented in limbic system and  
163 dendrite development pathways (**Supplementary Fig. 3D**), suggesting that genes involved in  
164 neuronal development might be influenced by FACT.

165

## 166 **Depletion of FACT induces very specific changes in chromatin accessibility**

167 Since FACT is responsible for the remodelling of nucleosomes in front of RNA polymerase and  
168 the re-establishment of nucleosome integrity in its wake (Formosa, 2012), we speculated  
169 whether some of the observed transcriptional alterations could be connected to changes in  
170 nucleosome occupancy upon depletion of FACT. Mononucleosome-sized DNA fragments upon  
171 treatment with MNase (135-170 bp) were purified from Control and *Ssrp1*-depleted conditions,  
172 and sequenced (**Supplementary Fig. 4A,B**). Nucleosome occupancy was plotted for four  
173 different gene classes according to the presence of SSRP1 in the control group (ChIP-Seq) and  
174 their relative gene Fold Change (RNA-seq) in the *Ssrp1* KD state. Overall, we observed little  
175 changes in nucleosome occupancy genome-wide (**Fig. 3A**). Genes that are down-regulated in  
176 the *Ssrp1* KD (“Down-regulated” class) and bound by FACT exhibit a global mononucleosomal  
177 shift by a few nucleotides right after the +1 nucleosome. Up-regulated genes showed a loss of  
178 nucleosome occupancy in the gene body area regardless of FACT-bound status (Non-SSRP1  
179 and SSRP1 targets) (**Fig. 3A,B**), potentially reflecting the higher transcription rate through these  
180 genes. However, specifically in up-regulated genes bound by FACT (“Up-regulated” class), we  
181 observed a significant loss of nucleosomes upstream of the TSS (**Fig. 3A,B**). This difference in  
182 nucleosome occupancy at the promoter region is highly reproducible among the different  
183 replicates (**Supplementary Fig. 4C**). Splitting the up-regulated genes by the amount of  
184 H3K4me3 levels (k-means clustering) as a proxy for gene-expression levels, also revealed that  
185 the loss of nucleosomes at the promoter is more profound over the promoters of lowly  
186 expressed/ repressed genes (Control state) (**Supplementary Fig. 4C**). The observed  
187 nucleosome depleted regions (NDRs) were different between up- and down-regulated genes.  
188 Such architectural differences have been previously attributed to different levels of GC  
189 frequency. Indeed, GC frequency over SSRP1 targets was higher and broader in the “Down-



190 regulated" class corroborating a more open chromatin state (Fenouil et al., 2012) (**Fig. 3A &**  
191 **Supplementary Fig. 4D**).

192 To confirm this difference in chromatin accessibility using an additional approach, we performed  
193 ATAC-seq in Control and *Ssrp1*-depleted ES cells (**Fig. 3C**). In line with the observations of the  
194 MNase-seq experiments, we observed a statistically significant increase ( $P < 10^{-10}$ ) in chromatin  
195 accessibility in the absence of FACT upstream of the promoter region of FACT-bound, up-  
196 regulated genes (**Fig. 3C,D,E**). In combination with the RNA-seq data, this reduction in  
197 nucleosome occupancy (and subsequently increase in chromatin accessibility) at the TSS  
198 suggests that FACT might act as a repressor by enabling a more closed chromatin conformation  
199 state at promoter regions.

200

201 **Gain in chromatin accessibility upon FACT depletion upstream of the TSS coincides with**  
202 **antisense transcription**

203 Over the last decade, it has become apparent that promoters can drive expression of sense and  
204 antisense RNAs, with proximally paused RNA Pol II on both strands (Jonkers, Kwak, & Lis,  
205 2014; Seila et al., 2008). *In vitro*, FACT has been demonstrated to facilitate transcription through  
206 chromatinized templates (Orphanides et al., 1999) and reduces pausing of the elongating  
207 polymerase when it encounters nucleosomes (Hsieh et al., 2013). In yeast, depletion of Spt16  
208 leads to upregulation of antisense transcription from gene-internal cryptic promoters (Feng et  
209 al., 2016). Thus, in order to understand how the observed changes in chromatin accessibility  
210 would impact transcription initiation and to get more mechanistic insight into how FACT might  
211 dampen expression of genes in mES cells, we performed NET-seq (Mayer & Churchman, 2016)  
212 (**Supplementary Fig. 5A**), a method that allows quantitative, strand-specific and nucleotide  
213 resolution mapping of RNA Pol II.

214 Initially, we sought to determine whether nascent transcription positively correlates with mRNA  
215 levels. A higher correlation of nascent RNA – mRNA expression and a significantly higher slope  
216 ( $P < 10^{-5}$ ) was observed over SSRP1-target regions in the Control state suggesting higher levels  
217 of Pol II pausing and mRNA levels in the presence of FACT (**Fig. 4A**). Nevertheless, in the  
218 *Ssrp1* KD state the SSRP1-bound regions maintained a higher slope, suggesting that pausing  
219 and elongation speed of RNA Pol II are not controlled entirely by FACT (**Supplementary Fig.**  
220 **5B**). To confirm this, we measured the travelling ratio of RNA Pol II over down-regulated and  
221 up-regulated genes. Indeed, “up-regulated” SSRP1-bound genes show a lower travelling ratio  
222 overall, but no significant difference was observed among this group of genes following FACT  
223 depletion (Control to *Ssrp1* KD comparison), indicating that FACT is not involved into the  
224 release of Pol II towards successive elongation in mammalian cells (**Fig. 4B**).

225 Next, we assessed RNA Pol II pausing and directionality over up-regulated genes. NET-seq  
226 density plots identified that FACT targets displayed higher levels of promoter-proximal RNA Pol  
227 II in the sense strand, but not in the antisense strand, compared to SSRP1-unbound promoters  
228 (**Fig. 4C,D**). Upon knock-down of FACT, SSRP1 targets displayed an increase ( $P < 10^{-6}$ ) in  
229 divergent transcription compared to the non-SSRP1 targets (**Fig. 4E**). This occurred precisely at  
230 locations where nucleosomes were depleted upon knock-down of FACT (**Fig. 4F**). No change in  
231 antisense transcription was observed for Down-regulated (**Supplementary Fig. 6A,B**) or  
232 Unchanged (**Supplementary Fig. 6C,D**) genes suggesting that the presence of FACT over a  
233 specific gene class (Up-regulated genes) decreases the rate of antisense transcription by  
234 maintaining higher nucleosome density upstream of the TSS.

235 A correlation between loss of nucleosomes upstream of the TSS, increase in antisense and  
236 sense transcription has recently been reported to occur in mammalian cells (Scruggs et al.,  
237 2015). Furthermore, this study showed that antisense transcription can lead to a more open  
238 chromatin structure enabling increased binding of transcription factors, which is favourable for

239 sense transcription. Taken together, our data suggest that the repressive function of FACT is  
240 linked to nucleosome deposition at the promoter and obstruction of anti-sense transcription.

241

## 242 **ES cells differentiate more efficiently into the neuronal lineage upon FACT depletion**

243 Finally, we wanted to investigate, whether the transcriptional changes induced by depletion of  
244 FACT have physiological consequences. We tested this by differentiating mES cells into the  
245 neuronal lineage. The rationale for this approach stems from previous studies that pinpoint a  
246 specific role for FACT in neurons (Neumüller et al., 2011; Vied et al., 2014) as well as from the  
247 gene ontology enrichment for neuronal terms that we obtained from mRNA isoform analysis  
248 (**Supplementary Fig. 3E**). We induced differentiation of ES cells towards a neuronal lineage via  
249 embryoid body formation and treatment with retinoic acid (Bibel, Richter, Lacroix, & Barde,  
250 2007). We created early stage Neural Precursor Cells (NPCs – 3 days into the differentiation  
251 process) and interrogated the whole transcriptome via RNA-seq. We identified that in early  
252 stage NPCs, expression of key neurogenesis markers (*Pax6*, *Nes*, *Tubb3*) increases whereas  
253 FACT mRNA levels and key pluripotency factors are unchanged but still maintained at a high  
254 level (**Fig. 5A**). A quarter of the up-regulated genes in ES cells after *Ssrp1* KD overlaps with the  
255 up-regulated genes instigated by neuronal differentiation ( $P < 10^{-13}$ , Fisher's exact test; **Fig. 5B**)  
256 and are over-represented in pathways involved in neuronal development. Similarly to our  
257 previous observations,  $\beta$ 3-Tubulin (*Tubb3*) (SSRP1-bound gene), as an example for  
258 neurogenesis genes up-regulated upon FACT depletion, shows higher chromatin accessibility  
259 levels at the promoter region upon knock-down of FACT. This opening of the promoter is  
260 accompanied by an increase in anti-sense transcription (**Fig. 5C**).

261 We then depleted *Ssrp1* levels at the onset of neuronal differentiation and performed  
262 immunofluorescence for neurogenesis ( $\beta$ 3-Tubulin) and dendritic (MAP2) markers at the same

263 time point as the RNA-seq experiment. *SSRP1 KD* caused a substantial increase in the  
264 expression of those markers, indicating that loss of FACT function primes ES cells for the  
265 neuronal lineage and enhances early neuronal differentiation (**Fig. 5D**).

266

## 267 ***Discussion***

268 In this study we have addressed the role of the histone chaperone FACT in mouse ES cells. In  
269 contrast to the genomic profile identified for *S. cerevisiae* FACT, where the protein occupancy is  
270 depleted at the transcription start site and accumulates in the gene body (True et al., 2016), the  
271 genomic profile of mammalian FACT over active genes is reminiscent of a profile of the Ser5  
272 phosphorylated form of RNA Pol II. This recruitment to the transcription start site might reflect  
273 binding of FACT to RNA Pol II. A similar profile for SSRP1 has been reported recently in  
274 HT1080 cells (Garcia et al., 2013).

275 In general, FACT depletion does not lead to gross alterations of the nucleosomal landscape as  
276 measured by MNase- and ATAC-seq. In particular, genes down-regulated upon FACT depletion  
277 only show a slight shift of nucleosomes, similar to what has been observed in yeast upon FACT  
278 inactivation (Feng et al., 2016). It is tempting to speculate that the reason for down-regulation  
279 lies in the originally described function of FACT to help passage of RNA Pol II through  
280 chromatin (Orphanides et al., 1999) and its depletion makes this process less efficient. FACT-  
281 bound genes that are up-regulated upon *Ssrp1* depletion show a significant alteration in  
282 nucleosomal occupancy around the transcriptional start site (TSS). FACT depletion leads to loss  
283 of nucleosomes and increased rates of bi-directional nascent transcription suggesting that these  
284 genes are usually dampened or repressed (in case of silent genes) by the maintenance of  
285 nucleosomes at these sites. The loss of nucleosomal occupancy upon depletion of FACT goes  
286 hand-in-hand with an increase in antisense transcription. Based on the data presented here, we

287 cannot determine if the loss of nucleosomes precedes upregulation of antisense transcription or  
288 vice versa. Also, it is not clear, whether this is driven by FACT alone, or in combination with  
289 RNA polymerase and/or chromatin remodellers. However, it is clear that this observed effect is  
290 very specific to SSRP1-bound genes, in which the histone chaperone operates as a repressor,  
291 suggesting that FACT is required to maintain the observed high level of nucleosome occupancy  
292 and to inhibit antisense transcription. One should note however, that this gene class shows low  
293 levels of antisense transcription (**Fig. 4C,D**). Therefore, one plausible model would be that  
294 FACT is required on these promoters to reinstate nucleosomes after initiation of antisense  
295 transcription. Depletion of FACT would lead to loss of this function and loss of nucleosomes,  
296 which in turn would drive higher levels of antisense transcription. It is of interest to note that  
297 FACT depletion in *S. cerevisiae* by using a thermosensitive allele of *spt16* also leads to  
298 upregulation of sense/antisense transcription. However, this occurs at cryptic promoters within  
299 the coding region of the gene due to a defect in re-establishing chromatin structure after  
300 passage of the elongating polymerase (Feng et al., 2016). Given the differences of FACT  
301 occupancy between mammals (this study and Garcia et al., 2013) and yeast (True et al., 2016),  
302 this might reflect evolutionary differences between mammalian and yeast FACT.

303 This scenario described for mammalian FACT would lead to a wider NFR and allow more  
304 efficient recruitment of TFs and RNA polymerase. In addition, the torque generated by two  
305 divergently elongating RNAPII molecules can create sufficient negative supercoiling density in  
306 the DNA between the two promoters, which is known to increase RNAPII transcription efficiency  
307 (Seila, Core, Lis, & Sharp, 2009). Taken together, we have shown that FACT can function both  
308 as an enhancer and a repressor of transcription. The repressive function of FACT correlates  
309 well with nucleosomal occupancy at the TSS and suppression of antisense transcription. In ES  
310 cells, genes repressed by FACT in this way encode for proteins involved in embryogenesis,

311 particularly in early neuronal differentiation, which is accelerated when FACT is depleted (**Fig.**  
312 **6**).

313 FACT expression correlates with the differentiation state of the cell, being highest in  
314 undifferentiated and lowest in terminally differentiated cells. This cannot be simply explained by  
315 differences in proliferation rates as e.g. NIH-3T3 also exhibit low levels of FACT expression, but  
316 proliferate comparably to mouse ES cells. These observations suggest that FACT assists to  
317 maintain a chromatin/ transcription state that allows self-renewal. Indeed, depletion of FACT  
318 leads to an imbalance of the ES cell transcriptome. On the one hand, pro-proliferative genes are  
319 up-regulated and lowly expressed developmental factors are further down-regulated leading in  
320 the hyper-proliferation of ES cells. Moreover, the FACT-depleted gene signature has a large  
321 overlap with gene expression changes observed upon differentiation into the neuronal lineage.  
322 Interestingly, a comparison of expression patterns in the early developing mouse brain identified  
323 a set of only 13 genes, including *Ssrp1* with high correlation of expression in the proliferating  
324 cells of the VZ (Ventricular Zone) of the neocortex at early stages of development (Vied et al.,  
325 2014). This is a transient embryonic layer of tissue containing neural stem cells (Rakic, 2009)  
326 and a place for neurogenesis during development dependent on the Notch pathway (Rash, Lim,  
327 Breunig, & Vaccarino, 2011). Similarly to our study, hyperproliferation in a stem cell  
328 compartment upon FACT depletion has been observed before. *Drosophila* neuroblasts  
329 hyperproliferate upon deletion of *SSRP1* suggesting that it is involved in the regulation of  
330 balancing neuroblast self-renewal and differentiation (Neumüller et al., 2011). A very recent  
331 report also highlights the role of FACT in assisting cell fate maintenance. Using a genetic screen  
332 in *C. elegans*, all FACT subunits were identified as barriers for cellular reprogramming of germ  
333 cells into the neuronal lineage (Kolundzic et al., 2017). Comparable to our results, the authors  
334 did not observe major chromatin architecture alterations, but observed larger colonies during  
335 reprogramming assays in the absence of FACT, indicative of higher proliferation rates. In

336 agreement with these reports, our data demonstrate that FACT-depleted ES cells differentiate  
337 much more efficiently into early neuronal precursors. Taken together, the data suggest a role for  
338 FACT activity during neuronal differentiation and the proper levels of FACT might assist in  
339 balancing proliferation speed and timing of differentiation processes.

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341

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### 353 ***Author Contributions***

354 C.M. and P.T. designed the study, C.M. performed all experiments and analysed data, C.M. and  
355 P.T. interpreted results and wrote the manuscript.

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358 ***Conflict of Interest***

359 The authors declare no conflict of interest.

360

361 **Deposition of sequencing data**

362 Data have been deposited in Gene Expression Omnibus (GEO) under accession numbers GSE

363 90906 (ChIP-seq, RNA-seq, chrRNA-seq, MNase-seq, ATAC-seq, and NET-seq).

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## 377 **Materials and Methods**

378 **Cell culture.** The E14 cell line (mESCs) was cultured at 37 °C, 7.5% CO<sub>2</sub>, on 0.1% gelatin  
379 coated plates, in DMEM + GlutaMax™ (Gibco) with 15% fetal bovine serum (Gibco), MEM non-  
380 essential amino acids (Gibco), penicillin/streptomycin (Gibco), 550 μM 2-mercaptoethanol  
381 (Gibco), and 10 ng/ml of leukaemia inhibitory factor (LIF) (eBioscience). HEK293T, N2a, MEFs,  
382 NIH3T3, and B16 cell lines were cultured at 37 °C, 5% CO<sub>2</sub> in DMEM + GlutaMax™ (Gibco) with  
383 10% fetal bovine serum (Gibco), and penicillin/streptomycin (Gibco). Early Neuronal Precursor  
384 Cells (NPCs) were generated as previously described (Bibel et al., 2007). Briefly, embryoid  
385 bodies were created with the hanging drop technique and were further treated with 1 μM retinoic  
386 acid (RA) for 4 days. RA-treated embryoid bodies were trypsinised and cultured in DMEM +  
387 GlutaMax™ (Gibco) with 15% fetal bovine serum without LIF for 3 days.

388 **Depletion of SSRP1 from mESCs via shRNA and RNA preparation.** E14 were transfected  
389 with lentiviral vectors containing either a scramble Control or *Ssrp1* shRNAs (MISSION®  
390 shRNA, Sigma) with the following sequences:

<b><u>Scramble</u></b>	
<b><u>Control</u></b>	CCGGGCGCGATAGCGCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCTTTTT
<b><u>shRNA 1</u></b>	
<b><u>(Ssrp1)</u></b>	CCGGCCTACCTTTCTACACCTGCATCTCGAGATGCAGGTGTAGAAAGGTAGGTTTTTG
<b><u>shRNA 2</u></b>	
<b><u>(Ssrp1)</u></b>	CCGGGCGTACATGCTGTGGCTTAATCTCGAGATTAAGCCACAGCATGTACGCTTTTTTG
<b><u>shRNA 3</u></b>	
<b><u>(Ssrp1)</u></b>	CCGGGCAGAGGAGTTTGACAGCAATCTCGAGATTGCTGTCAAACCTCCTGCTTTTTTG
<b><u>shRNA 4</u></b>	
<b><u>(Ssrp1)</u></b>	CCGGCCGTCAGGGTATCATCTTTAACTCGAGTTAAAGATGATACCCTGACGGTTTTTG

391

392 A combination of two different *Ssrp1* shRNAs was used (1&2, 3&4) at a time and depletion was  
393 quantified via western blotting using a monoclonal anti-*Ssrp1* antibody (Biolegends). Anti-alpha

394 Tubulin was used as a reference control. The 1&2 combination was used for subsequent  
395 experiments as it yielded higher depletion of SSRP1 levels (Supplementary Figure 2a,b).  
396 Forty-eight hours (48h) after transfection, puromycin (2 µg /ml) selection was applied for an  
397 additional 24h period, before cell collection and RNA preparation. Total RNA was obtained via  
398 phenol-chloroform extraction (QIAzol Lysis Reagent – QIAGEN) followed by purification via  
399 Quick-RNA™ MicroPrep (Zymo Research). Library preparation and ribosomal depletion were  
400 performed via the NEBNext Directional RNA Ultra Kit (NEB) and the RiboZero Kit (Illumina)  
401 according to the manufacturer's instructions, respectively. Four different biological replicates  
402 (Control or SSRP1-depleted mESCs) were prepared and processed for transcriptome analysis.

403

404 **MTT proliferation assay.** 48h after transfection, different cell densities ( $3 \times 10^4$ ,  $2 \times 10^4$ ,  $1 \times 10^4$ )  
405 were seeded on 96-well plates (Sarstedt) along with puromycin (2 µg /ml). Twenty-four hours  
406 later, the CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega) was used  
407 according to the manufacturer's instructions in order to assess the rate of cell proliferation  
408 between the two conditions (Control, *Ssrp1* KD). Statistical analysis was performed using a two-  
409 tailed *t-test*.

410

411 **Transcriptome analysis in SSRP1-depleted mESCs.** Sequenced reads were aligned to the  
412 mm10 genome via STAR (v 2.4.1b) (Dobin et al., 2013). Gene and exon counts were obtained  
413 from featureCounts of the Rsubread package (R/Bioconductor). Only reads with CPM (counts  
414 per million) > 1 were kept for subsequent analysis. Counts were normalised using the internal  
415 TMM normalisation in edgeR (Robinson, McCarthy, & Smyth, 2009) and differential expression  
416 was performed using the limma (Ritchie et al., 2015) package. All of the RNA-seq data  
417 presented in this manuscript have been normalised to the total library size. Significant genes

418 with an absolute logFC > 1 and Adjusted P.Value < 0.01 were considered as differentially  
419 expressed (Supplementary Table 1). The “Unchanged” gene class (n=2,179) was obtained from  
420 genes with an Adjusted P.Value > 0.05. The diffSplice function implemented in limma was used  
421 to identify differentially spliced exons between the two conditions (Supplementary Table 2).  
422 Significant exons with an FDR < 0.001 were considered as differentially spliced. Retention  
423 Introns were identified using the MISO (Katz, Wang, Airoidi, & Burge, 2010) (Mixture of  
424 Isoforms) probabilistic framework (Supplementary Table 3).

425

426 **Retention intron events.** We verified the presence of retained introns in the *Ssrp1* KD by  
427 randomly selecting ten intron retention events. The FastStart SYBR Green Master (Roche) was  
428 used along with the following primers to amplify via PCR the retained intragenic regions;

<u>Gene name</u>	<u>Forward primer</u>	<u>Reverse primer</u>
<i>Men1</i>	ATTTCCCAGCAGGCTTCAGG	GGGATGACACGGTTGACAGC
<i>Dvl1</i>	CCTGGGACTACCTCCAGACA	CCTTCATGATGGATCCAATGTA
<i>Map4k2</i>	GCTGCAGTCAGTCCAGGAGG	TCCTGTTGCTTCAGAGTAGCC
<i>Ctsa</i>	GCAATACTCCGGCTACCTCA	TGGGGACTCGATATACAGCA
<i>Pol2ri</i>	CGAAATCGGGAGTGAGTAGC	GGTGGGAAGAAGGAACGATCA
<i>Wipf2</i>	TAGAGATGAGCAGCGGAATC	TCGAGAGCTGGGGACTTGCA
<i>Fuz</i>	GACCCAGTGTGTGGACTGTG	GACAAAGGCTGTGCCAGTGG
<i>Rfx5</i>	CACCAGTTGCCCTCTCTGAA	CAATTCTCTTCCTCCCATGC
<i>Fhod1</i>	CACCAGGGAGCAGAGATGAT	CCATCAACATTGGCCTAACC
<i>Tcirg1</i>	AGCGACAGCACTCACTCCTT	CAACACCCCTGCTTCCAGGC

429

430 Amplified products were run on a 1.5% Agarose gel and visualized under UV. Band  
431 quantification was performed with ImageJ.

432 **Chromatin Immunoprecipitation (ChIP) of FACT subunits.** ChIP was performed in ~20  
433 million ES cells, per assay, as described previously (Tessarz et al., 2014) with a few  
434 modifications. Briefly, cells were crosslinked with 1% formaldehyde for 20 min followed by  
435 quenching for 5 min with the addition of glycine to a final concentration of 0.125 M. After  
436 washing with PBS buffer, cells were collected and lysed in Cell Lysis buffer (5 mM Tris pH8.0,  
437 85 mM KCl, 0.5% NP40 ) with proteinase inhibitors (10 µl/mL Phenylmethylsulfonyl fluoride  
438 (PMSF), 1 µl/mL Leupeptin and 1 µl/mL Pepstatin). Pellets were spun for 5 min at 5000 rpm at  
439 4°C. Nuclei were lysed in Nuclei Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris HCl) and  
440 samples were sonicated for 12 min. Samples were centrifuged for 20 min at 13,000 rpm at 4°C  
441 and the supernatant was diluted in IP buffer (0.01% SDS, 1.1% Triton-X-100, 1.2 mM EDTA,  
442 16.7 mM Tris HCl, 167 mM NaCl) and the appropriate antibody was added and left overnight  
443 with rotation at 4°C. Anti-Ssrp1 and anti-Supt16 antibodies were purchased from Biolegends  
444 (#609702) and Cell Signalling (#12191), respectively. Anti-AP-2γ (Tfap2c) antibody was  
445 purchased from Santa Cruz (#sc-12762). Two biological replicates were prepared for each  
446 FACT subunit, using independent cell cultures and chromatin precipitations. Protein A/G  
447 Dynabeads (Invitrogen) were added for 1h and after extensive washes, samples were eluted in  
448 Elution Buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). 20 µL of 5 M NaCl were added and samples were  
449 reverse-crosslinked at 65°C for 4h. Following phenol-chloroform extraction and ethanol  
450 precipitation, DNA was incubated at 37°C for 4h with RNase (Sigma).

451 **ChIP-seq library preparation, sequencing, and peak-calling.** Approximately 10-20 ng of  
452 ChIP material was used for library preparation. End-repair and adaptor ligation was prepared as  
453 described previously with a few modifications (Tessarz et al., 2014). Double sided size  
454 selections (~200 – 650bp) were performed using the MagSI-NGS Dynabeads (MagnaMedics,

455 #MD61021) according to the manufacturer's instructions. Purified adapter-ligated ChIP material  
456 was run on a high sensitivity DNA chip on a 2200 TapeStation (Agilent) to assess size  
457 distribution and adaptor contamination.

458 Samples were single-end deep-sequenced and reads were aligned to the mm10 genome using  
459 Bowtie2 (v 2.2.6)(Langmead & Salzberg, 2012). Peak-calling was performed using PePr (v 1.1)  
460 (Zhang, Lin, Johnson, Rozek, & Sartor, 2014) with peaks displaying an FDR < 10<sup>-5</sup> considered  
461 as statistically significant (Supplementary Table 4). Peak annotation was performed via the  
462 chipenrich (Welch et al., 2014) R package with the following parameters (locusdef =  
463 "nearest\_gene", method = "broadenrich").

464

465 **ChIP-seq normalisation and metagene analysis.** All the ChIP-seq BAM files were converted  
466 to bigwig (10 bp bin) and normalised to x1 sequencing depth using Deeptools (v 2.4) (Ramirez  
467 et al., 2016). Blacklisted mm9 co-ordinates were converted to mm10 using the LiftOver tool from  
468 UCSC and were further removed from the analysis. Average binding profiles were visualised  
469 using R (v 3.3.0). Heatmaps were generated via Deeptools. For the average profiles in  
470 **Supplementary Fig. 1C,D**, RPKM values from Control ES RNA-seq data were divided into four  
471 different quantiles and the average profile for each FACT subunit was generated for each  
472 quantile. The Pearson's correlation plot in **Figure 1A** was generated using all unique annotated  
473 mm10 RefSeq genes ( $n = 11,305$ ) from UCSC (blacklisted regions were removed).

474

475 **MNase-seq following SSRP1 depletion in mESCs.** ES cells were cultured and transfected  
476 with shRNA vectors as described above. Biological replicates were obtained from two  
477 independent transfection experiments for each shRNA vector. Briefly, ~5 million cells were  
478 crosslinked with 1% formaldehyde for 20 min followed by quenching for 5 min with the addition

479 of glycine to a final concentration of 0.125 M. After washing with PBS buffer, cells were  
480 collected and lysed in Cell Lysis buffer (5 mM Tris pH8.0, 85 mM KCl, 0.5% NP40 ) with  
481 proteinase inhibitors (10 µl/mL Phenylmethylsulfonyl fluoride (PMSF), 1 µl/mL Leupeptin and 1  
482 µl/mL Pepstatin). Nuclei were gathered by centrifugation (5000 rpm for 2 min) and were treated  
483 with 10 Kunitz Units/10<sup>6</sup> cells of micrococcal nuclease (NEB, #M0247S) for 5 min at 37°C in 40  
484 µl of Micrococcal Nuclease Buffer (NEB, #M0247S). The reaction was stopped with the addition  
485 of 60 µl 50 mM EDTA, 25 µl 5 M NaCl, 15 µl 20% NP-40 and incubated on a rotator for 1h at  
486 room temperature to release soluble nucleosomes. Samples were centrifuged for 5 min at  
487 10,000 g and supernatant was transferred to a new tube. This centrifugation step is important to  
488 obtain highly soluble nucleosomes and remove nucleosome-protein complexes, which can raise  
489 bias in subsequent data interpretation (Carone et al., 2014) (**Supplementary Fig. 7**). Samples  
490 were reverse-crosslinked by incubating overnight at 65°C with 0.5% SDS and proteinase K.  
491 Following phenol-chloroform extraction and ethanol precipitation, DNA was incubated at 37°C  
492 for 4h with RNase (Sigma). All samples were run in a 2% agarose gel and fragments <200 bp  
493 were extracted and purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-  
494 Nagel) according to the manufacturer's instructions.

495 Purified DNA (500 ng) was used for library preparation as described above. The only difference  
496 was the PCR amplification step where we used the same conditions as mentioned in (Henikoff  
497 et al., 2011) but with only three amplification cycles. Libraries were verified using a 2200  
498 TapeStation and were paired-end deep-sequenced (~250 million reads per sample). For quality  
499 checks and reproducibility, please refer to **Supplementary Fig. 7**.

500

501 **MNase-seq normalisation and metagene analysis.** All the MNase-seq BAM files were  
502 converted to bigwig, binned (1 bp), smoothed (20-bp window), and normalised to x1 sequencing  
503 depth using Deeptools (v 2.4). Moreover, they were split into two different categories according

504 to fragment length; <80 bp Transcription factor (TF)-sized fragments and 135-170 bp  
505 mononucleosome fragments). Average nucleosome occupancy profiles were visualised using R  
506 (v 3.3.0). For the **Supplementary Fig. 7D,E** the mm10 annotated exon list for  
507 mononucleosomal profiling was obtained from UCSC.

508 **ATAC-seq following SSRP1 depletion in mESCs.** ES cells were cultured and transfected with  
509 shRNA vectors as described above. Biological replicates were obtained from two independent  
510 transfection experiments for each shRNA vector. ATAC-seq was performed on 50,000 cells as  
511 previously described (Buenrostro, Giresi, Zaba, Chang, & Greenleaf, 2013). All samples were  
512 PCR amplified for 9 cycles were paired-end sequenced on an Illumina Hi-Seq 2500 platform.

513 **ATAC-seq normalisation and metagene analysis.** Sequenced paired mates were mapped on  
514 mm10 genome build using Bowtie2 with the following parameters:  $-X$  2000. Reads  
515 corresponding to nucleosome free regions were selected via a random forest approach using  
516 the “ATACseqQC” R package. All the ATAC-seq BAM files were converted to bigwig, binned (1  
517 bp), and normalised to x1 sequencing depth using Deeptools (v 2.4). Duplicated reads were  
518 removed. Chromatin accessibility profiles were visualised using R (v 3.3.0).

519 **Mass spectrometry sample preparation and analysis.** Nuclei were isolated from ~5 million  
520 ES cells under hypotonic conditions and samples were incubated overnight at 4°C with an anti-  
521 H3K4me3 antibody (Active Motif, #39159) in the presence of low-salt Binding buffer (150 mM  
522 NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40), protease inhibitors, and Protein G Dynabeads  
523 (Invitrogen). The following day, after several rounds of bead washing with Binding Buffer,  
524 samples were incubated overnight at 37°C with Tris pH 8.8 and 300 ng Trypsin Gold (Promega).  
525 In total, four samples were prepared for each condition (Control, *Ssrp1* KD). For the full protein  
526 interactome of both FACT subunits, nuclei were extracted as described above, and anti-*Ssrp1*  
527 and anti-Supt16 antibodies were used. Peptides were desalted using StageTips(Rappsilber,

528 Ishihama, & Mann, 2003) and dried. The peptides were resuspended in 0.1% formic acid and  
529 analyzed using liquid chromatography - mass spectrometry (LC-MS/MS).

530

531 **LC-MS/MS analysis.** For mass spectrometric analysis, peptides were separated online on a 25  
532 cm 75  $\mu\text{m}$  ID PicoFrit analytical column (New Objective) packed with 1.9  $\mu\text{m}$  ReproSil-Pur  
533 media (Dr. Maisch) using an EASY-nLC 1000 (Thermo Fisher Scientific). The column was  
534 maintained at 50°C. Buffer A and B were 0.1% formic acid in water and 0.1% formic acid in  
535 acetonitrile respectively. Peptides were separated on a segmented gradient from 5% to 25%  
536 buffer B for 45 min, from 25% to 35% buffer B for 8 min, and from 35% to 45% buffer B for 4  
537 min, at 200nl / min. Eluting peptides were analyzed on a QExactive HF mass spectrometer  
538 (Thermo Fisher Scientific). Peptide precursor mass to charge ratio ( $m/z$ ) measurements (MS1)  
539 were carried out at 60000 resolution in the 300 to 1500  $m/z$  range. The top ten most intense  
540 precursors with charge state from 2 to 7 only, were selected for HCD fragmentation using 27%  
541 collision energy. The  $m/z$  of the peptide fragments (MS2) were measured at 15000 resolution,  
542 using an AGC target of 1e6 and 80 ms maximum injection time. Upon fragmentation, precursors  
543 were put on an exclusion list for 45 seconds.

544

545 **LC-MS/MS data analysis.** The raw data were analysed with MaxQuant (Jurgen Cox & Mann,  
546 2008) (v 1.5.2.8) using the integrated Andromeda search engine (Jürgen Cox et al., 2011).  
547 Fragmentation spectra were searched against the canonical and isoform sequences of the  
548 mouse reference proteome (proteome ID UP000000589, downloaded August 2015) from  
549 UniProt. The database was automatically complemented with sequences of contaminating  
550 proteins by MaxQuant. For the data analysis, methionine oxidation and protein N-terminal  
551 acetylation were set as variable modifications. The digestion parameters were set to “specific”



552 and “Trypsin/P,” allowing for cleavage after lysine and arginine, also when followed by proline.  
553 The minimum number of peptides and razor peptides for protein identification was 1; the  
554 minimum number of unique peptides was 0. Protein identification was performed at a peptide  
555 spectrum matches and protein false discovery rate of 0.01. The “second peptide” option was on  
556 in order to identify co-fragmented peptides. Successful identifications were transferred between  
557 the different raw files using the “Match between runs” option, using a match time window of 0.7  
558 min. Label-free quantification (LFQ) (Jurgen Cox, Hein, Lubner, & Paron, 2014) was performed  
559 using an LFQ minimum ratio count of 2.

560

561 **Identification of co-enriched proteins.** Analysis of the label-free quantification results was  
562 done using the Perseus computation platform (Tyanova et al., 2016) (v 1.5.0.0) and R. For the  
563 analysis, LFQ intensity values were loaded in Perseus and all identified proteins marked as  
564 “Reverse”, “Only identified by site”, and “Potential contaminant” were removed. Upon log2  
565 transformation of the LFQ intensity values, all proteins that contained less than four missing  
566 values in one of the groups (control or *Ssrp1* KD) were removed. Missing values in the resulting  
567 subset of proteins were imputed with a width of 0.3 and down shift of 1.8. Next, the imputed  
568 LFQ intensities were loaded into R where a two side testing for enrichment was performed using  
569 limma (Kammers, Cole, Tiengwe, & Ruczinski, 2015; Ritchie et al., 2015). Proteins with an  
570 adjusted p-value of less than 0.05 were designated as significantly enriched in the control or  
571 knockdown (H3K4me3 IP) (Supplementary Table 5). The complete list of differential protein  
572 expression between Control and *Ssrp1* KD can be found in Supplementary Table 7.

573 **NET-seq library preparation.** ES cells were cultured and transfected with shRNA vectors as  
574 described above. Biological replicates were obtained from two independent transfection  
575 experiments for each shRNA vector. NET-seq libraries were prepared as previously described

576 (Mayer & Churchman, 2016) with a few modifications. Briefly, chromatin associated nascent  
577 RNA was extracted through cell fractionation in the presence of  $\alpha$ -amanitin, protease and  
578 RNAase inhibitors. > 90% recovery of ligated RNA and cDNA was achieved from 15 % TBE-  
579 Urea (Invitrogen) and 10% TBE-Urea (Invitrogen), respectively, by adding RNA recovery buffer  
580 (Zymo Research, R1070-1-10) to the excised gel slices and further incubating at 70°C (1500  
581 rpm) for 15 min. Gel slurry was transferred through a Zymo-Spin IV Column (Zymo Research,  
582 C1007-50) and further precipitated for subsequent library preparation steps. cDNA containing  
583 the 3' end sequences of a subset of mature and heavily sequenced snRNAs, snoRNAs, and  
584 rRNAs, were specifically depleted using biotinylated DNA oligos (Supplementary Table 6).  
585 Oligo-depleted circularised cDNA was amplified via PCR (5 cycles) and double stranded DNA  
586 was run on a 4% low melt agarose gel. The final NET-seq library running at ~150 bp was  
587 extracted and further purified using the ZymoClean Gel DNA recovery kit (Zymo Research).  
588 Sample purity and concentration was assessed in a 2200 TapeStation and further deep  
589 sequenced in a HiSeq 2500 Illumina Platform (~400 million reads per replicate).

590 **NET-seq analysis.** All the NET-seq fastq files were processed using custom Python scripts  
591 (<https://github.com/BradnerLab/netseq>) to remove PCR duplicates and reads arising from RT  
592 bias. Reads mapping exactly to the last nucleotide of each intron and exon (Splicing  
593 intermediates) were further removed from the analysis. The final NET-seq BAM files were  
594 converted to bigwig (1 bp bin), separated by strand, and normalized to x1 sequencing depth  
595 using Deeptools (v 2.4) with an “-Offset 1” in order to record the position of the 5' end of the  
596 sequencing read. NET-seq tags sharing the same or opposite orientation with the TSS were  
597 assigned as ‘sense’ and ‘anti-sense’ tags, respectively. Promoter-proximal regions were  
598 carefully selected for analysis to ensure that there is minimal contamination from transcription  
599 arising from other transcription units. Genes overlapping within a region of 2.5 kb upstream of  
600 the TSS were removed from the analysis. For the NET-seq metaplots, genes underwent several

601 rounds of k-means clustering in order to filter regions; in a 2kb window around the TSS, rows  
602 displaying very high Pol II occupancy within a <100 bp region were removed from the analysis  
603 as they represent non-annotated short non-coding RNAs. Average Pol II occupancy profiles  
604 were visualised using R (v 3.3.0). In **Figure 4B** the Proximal Promoter region was defined as -  
605 30 bp and +250 bp around the TSS. For **Figure 4A,B**, gene body coverage was retrieved by  
606 averaging all regions (FACT-bound and non-FACT-bound) +300 bp downstream of TSS  
607 (Transcription Start site) and -200 bp upstream of TES (Transcription End Site). Comparison of  
608 the two linear regressions was performed by calculating the z-score via

$$z = \frac{\beta_1 - \beta_2}{\sqrt{s_{\beta_1}^2 + s_{\beta_2}^2}}$$

609 where  $\beta$  and  $s_{\beta}$  represent the 'slope' and the 'standard error of the slope', respectively. *P value*  
610 was calculated from the respective confidence level yielded by the z score.

611

612

613 **Immunofluorescence and confocal microscopy.** Early Neuronal Precursor Cells (NPCs)  
614 were generated and *Ssrp1* levels were knocked-down as described above. Cells were fixed with  
615 100% Ethanol for 10 min and processed for immunofluorescence. Permeabilisation and  
616 blocking was performed for 1 h at room temperature with 1% BSA and 0.1% NP-40 in PBS.  
617 Incubation with primary antibodies was carried at room temperature for 2 hours by using rabbit  
618 anti- $\beta$ 3-Tubulin (1:300; Cell Signaling) and mouse anti-MAP2 (1:300; Millipore.). After washing  
619 in blocking buffer, the secondary antibodies anti-rabbit and anti-mouse Alexa Fluor 568  
620 (1:1,000; Life Technologies.) were applied for 2 h at room temperature. Slides were extensively  
621 washed in PBS and nuclei were counterstained with DAPI before mounting. Fluorescence

622 images were acquired using a laser-scanning confocal microscope (TCS SP5-X; Leica),  
623 equipped with a white light laser, a 405-diode UV laser, and a 40× objective lens.

624

625 **Gene Ontology Analysis.** All GO terms were retrieved from the metascape online platform  
626 (<http://metascape.org/>).

627

628 **Accession numbers and references of publicly available datasets.** H3K4me3, H3K27me3,  
629 Pol II S5ph, H3K4me1, H3K27Ac, CTCF (ENCODE Consortium – E14 cell line); Chd1, Chd2,  
630 (de Dieuleveult et al., 2016) : GSE64825; p53 (Li et al., 2012): GSE26360; Pol II S2ph (Brookes  
631 et al., 2016): GSM850470.

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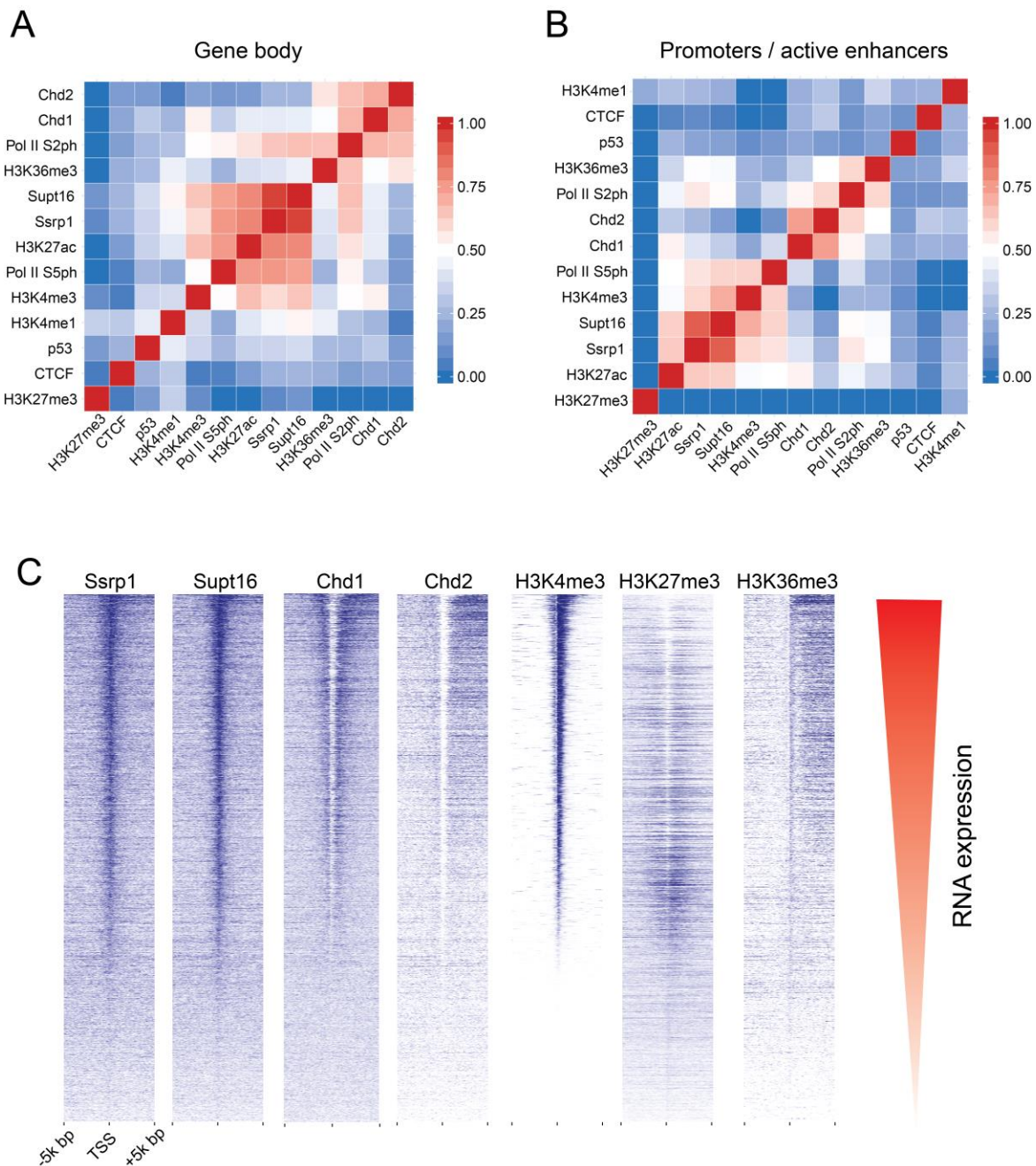
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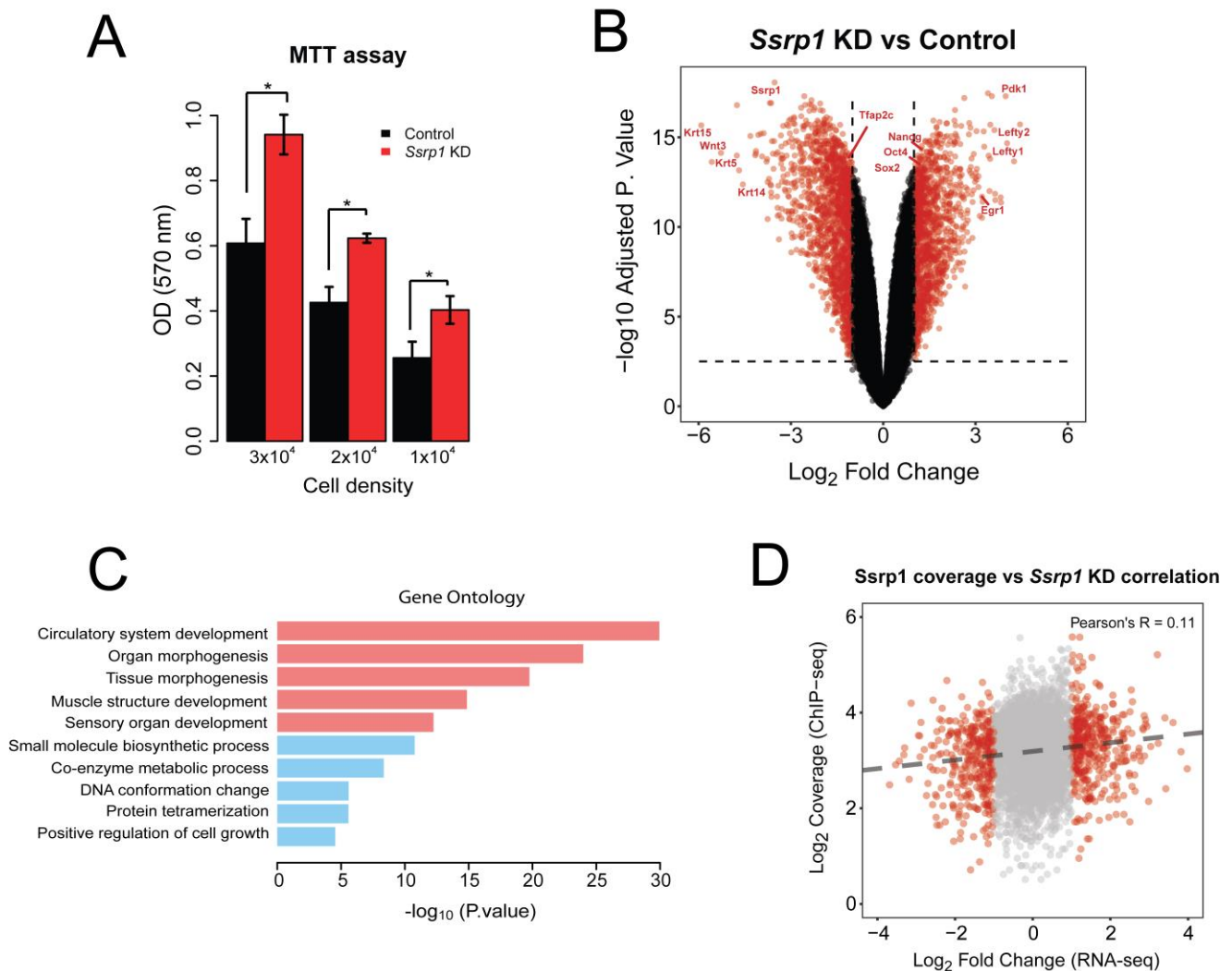
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809 **Figure 1: Correlated occupancies across FACT-bound regions.** **A**, Heatmap representing  
 810 Pearson's correlation between FACT subunits (SSRP1, SUPT16), and other factors over the  
 811 gene body area of all uniquely annotated protein-coding genes ( $n = 11,305$ ). **B**, Same as (**A**) but  
 812 for promoter/active enhancer regions ( $n = 19,461$ ) characterised by high H3K27ac and/or Pol II  
 813 density. **C**, Distribution of FACT and other factors (ChIP-seq tags indicated in blue) over the TSS  
 814 of 11,305 unique RefSeq genes, sorted by H3K4me3 levels. Coinciding RNA expression levels  
 815 are shown in red.



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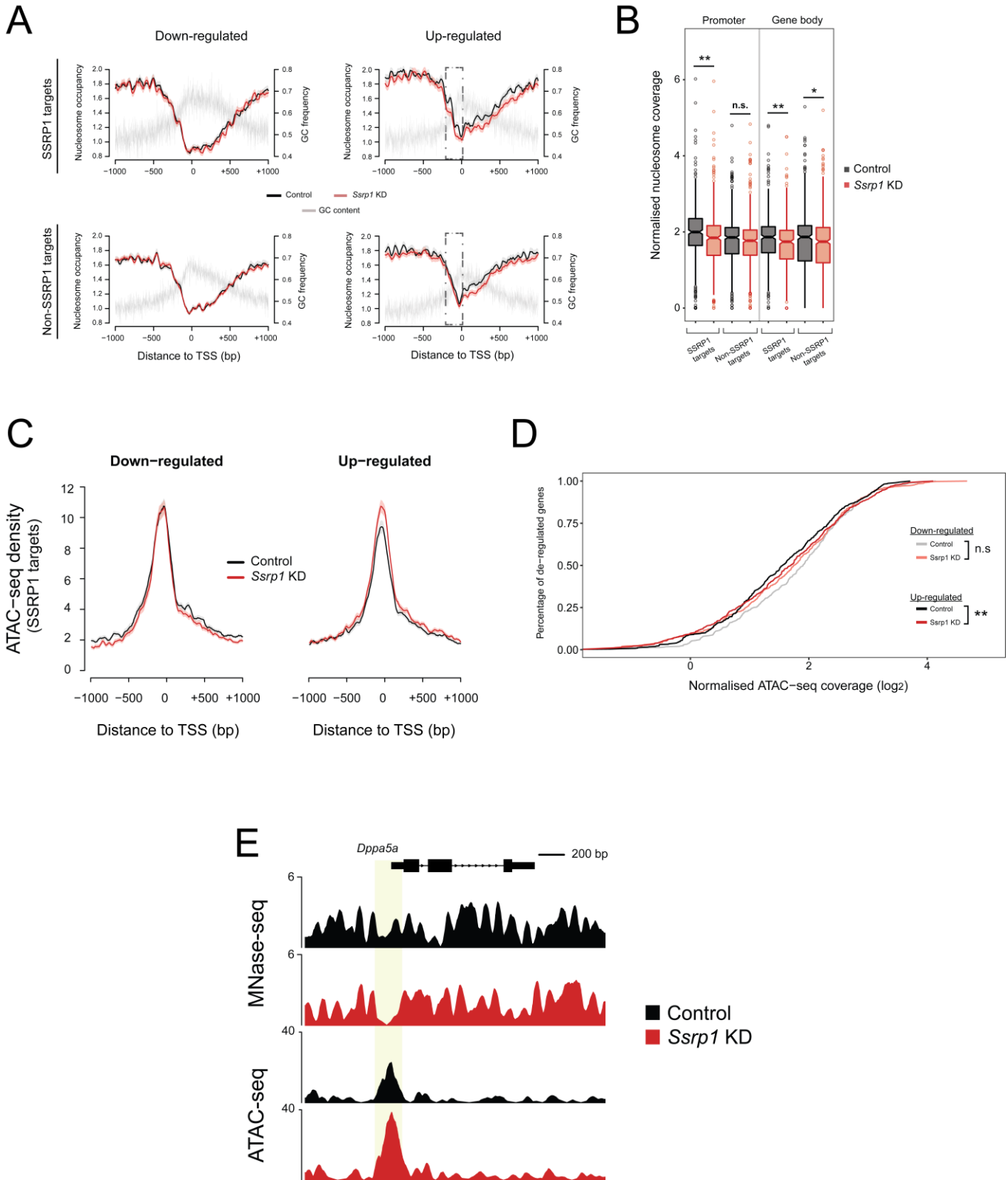
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818 **Figure 2: Regulation of gene expression by FACT.** **A**, MTT assay assessing cell metabolic  
 819 activity in mESCs at different cell densities following depletion of FACT levels. Values are mean  
 820 and SE of three independent transfection experiments are displayed. Significance was  
 821 calculated via a two-tailed *t*-test ( $P < 0.05$ ). **B**, Volcano plot of differentially expressed genes  
 822 between the Control and KD group. Values with logFC > 1 or logFC < -1 and Adjusted P.value <  
 823 0.01 are highlighted in red. **C**, Gene ontology analysis of all differentially expressed genes (Red:  
 824 pathways for down-regulated genes, Blue: pathways for up-regulated genes). **D**, Scatterplot of  
 825 log (SSRP1 coverage) (ChIP-seq) over logFC (RNA-seq).

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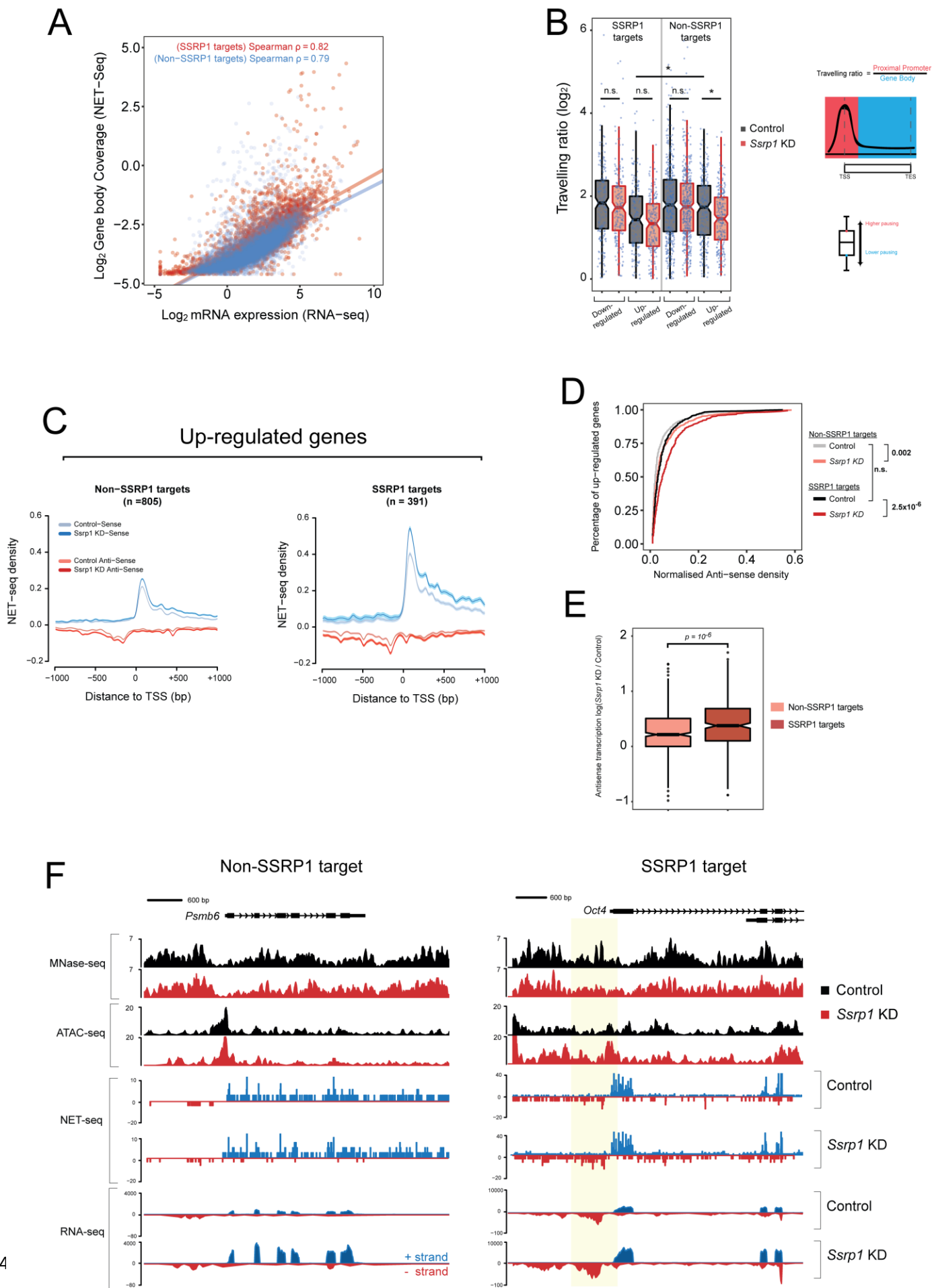
831 **Figure 3: Regulation of gene expression by FACT through chromatin accessibility. A,**  
832 Nucleosome occupancy of all de-regulated genes. Datasets are split by their FACT occupancy  
833 status (SSRP1 and Non-SSRP1 targets) and their relative transcriptional direction (“Down-  
834 regulated”, “Up-regulated”) following SSRP1 depletion. Solid lines indicate the mean values,  
835 whereas the shading represents the SE of the mean. **B.** Boxplots measuring the nucleosome  
836 occupancy ( $\log_2$ ) over promoters and gene body area of Up-regulated genes (\*\*  $p < 0.001$ , \*  $p <$   
837  $0.05$ , n.s. =not significant). The assessed promoter region is shown in dashed boxes indicated  
838 in **(A)**. Significance was calculated using the Welch Two-Sample t-test. **C.** Metaplot of open  
839 chromatin assessed by ATAC-seq among Down-regulated and Up-regulated genes both in  
840 Control and *Ssrp1* KD conditions. **D,** Cumulative distribution of ATAC-seq density for genes and  
841 conditions displayed in **(C)**. Significance was calculated using the Welch Two-Sample t-test (\*\*  $p$   
842  $< 10^{-9}$ , n.s. =not significant. **E,** Interrogation of nucleosome occupancy (MNase-seq) and  
843 chromatin accessibility (ATAC-seq) over the *Dppa5a* gene promoter for Control and *Ssrp1* KD  
844 conditions. Changes in nucleosome occupancy and chromatin accessibility are highlighted in  
845 yellow.

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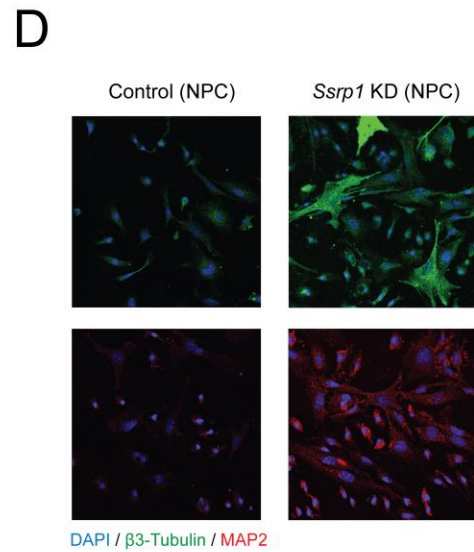
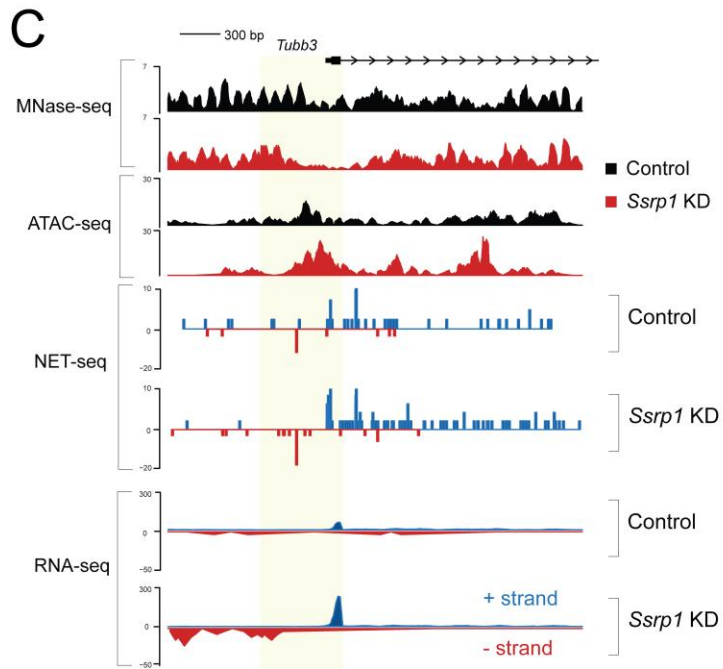
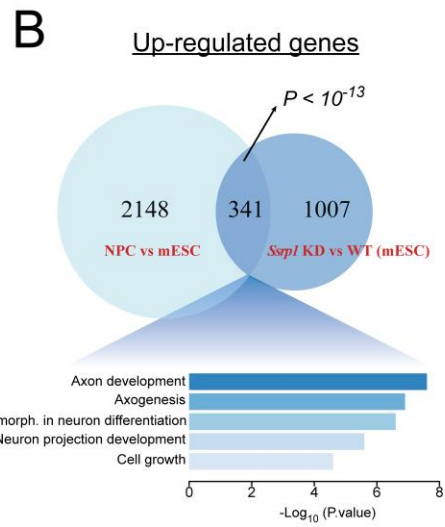
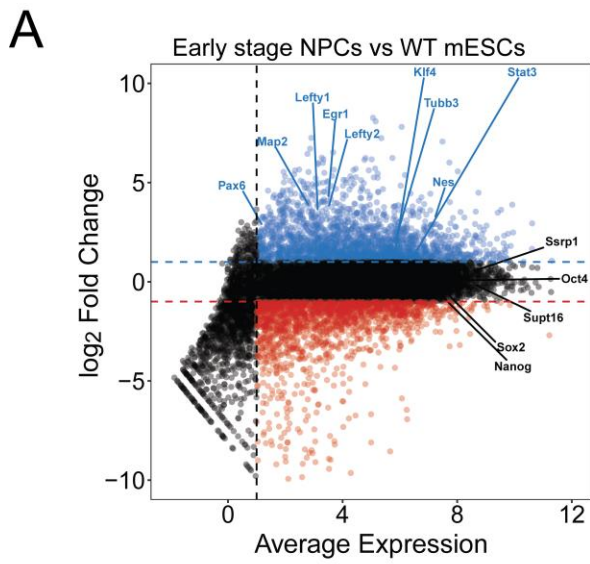




850 **Figure 4: Regulation of RNA Pol II directionality by FACT. A,** Scatterplots of log gene body  
851 coverage (NET-seq) versus log mRNA expression (RNA-seq) for SSRP1 (n=4,576) and Non-  
852 SSRP1 (n=8,844) target regions in the Control state (Z-score = 5.3,  $P < 10^{-5}$ ). **B,** Measure of  
853 Pol II pause/release. Travelling ratio is defined as NET-seq density of proximal promoter versus  
854 gene body area. The log transformed travelling ratio for each gene class is displayed with  
855 boxplots. The Welch Two-sided t-test was used to calculate significance between Control and  
856 *Ssrp1* KD (\*  $p < 0.05$ , n.s. =not significant). **C,** NET-seq density plots (Control and *Ssrp1* KD  
857 group) of Up-regulated genes split by FACT-bound status (Non-SSRP1 and SSRP1 targets).  
858 Solid lines indicate mean values, whereas the shading represents the 95% confidence interval.  
859 **D,** Cumulative distribution of anti-sense transcription (NET-seq) in a window 1000 bp upstream  
860 of the TSS. The Welch Two-sided t-test was used to calculate significance between Control and  
861 *Ssrp1* KD among Non-SSRP1 and SSRP1 targets. **E,** Boxplots assessing fold change (*Ssrp1*  
862 KD vs Control) in anti-sense transcription (NET-seq) in a window 1000 bp upstream of the TSS.  
863 The Welch Two-sided t-test was used to calculate significance between Non-SSRP1 and  
864 SSRP1 targets. **F,** Nucleosome occupancy (MNase-seq), open chromatin (ATAC-seq), and  
865 transcriptional activity (NET-seq/ RNA-seq) over an SSRP1 (*Oct4*) and non-SSRP1 (*Psemb6*)  
866 target gene between Control and *Ssrp1* KD conditions. Nucleosomal loss and increase in  
867 antisense transcription at the *Oct4* promoter is highlighted in yellow.

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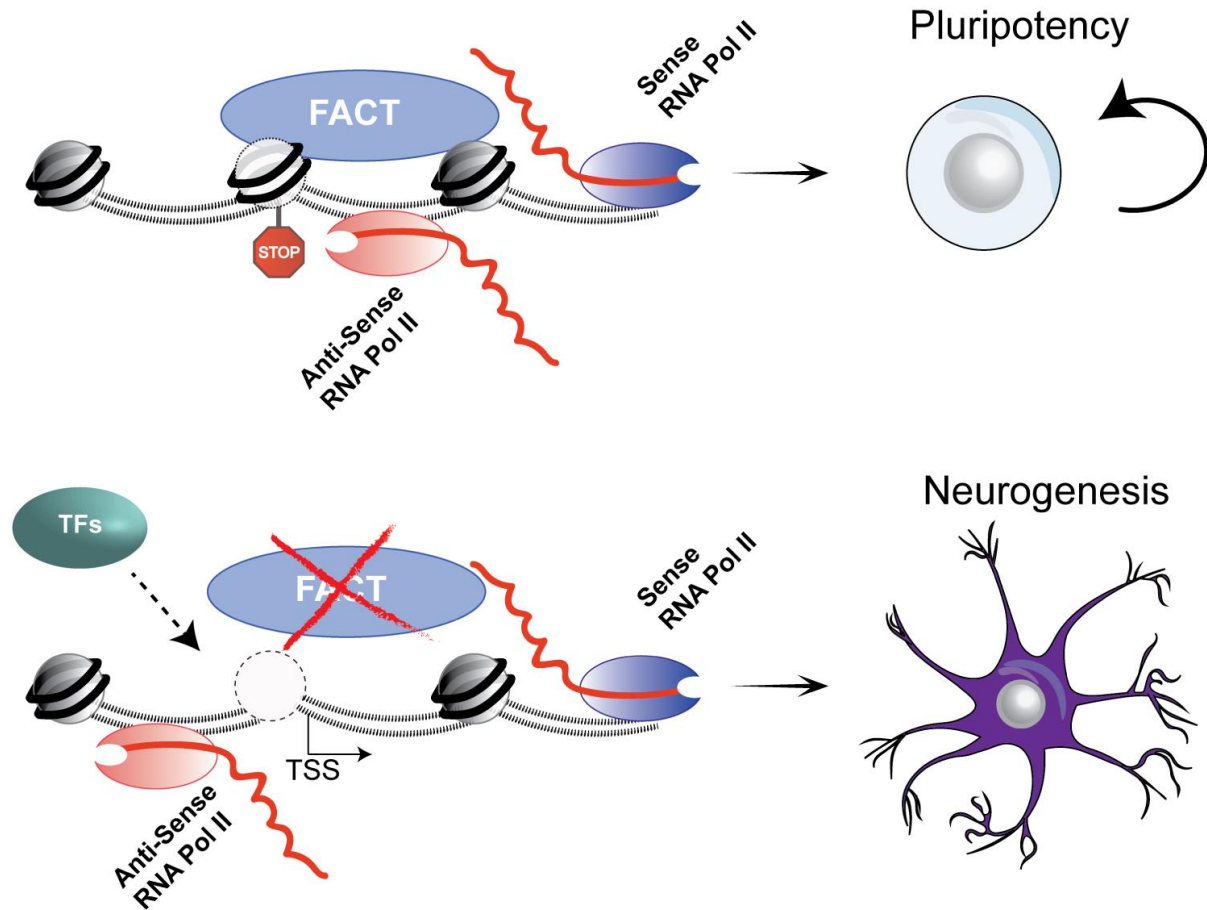
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871 **Figure 5: FACT regulates neurogenesis through Pol II / nucleosome dynamics. A**, MA plot  
872 depicting differential expression in NPCs versus WT ES cells. Up-regulated genes are  
873 highlighted in blue whereas down-regulated genes are highlighted in red. **B**, Venn diagram  
874 showing the overlap of up-regulated genes between NPC vs mESCs and Control vs *Ssrp1* KD  
875 mESCs. **C**, Interrogation of nucleosome occupancy (MNase-seq), chromatin accessibility  
876 (ATAC-seq), and transcriptional activity (NET-seq/ RNA-seq) over the *Tubb3* gene promoter for  
877 Control and *Ssrp1* KD conditions. Changes in nucleosome occupancy, chromatin accessibility,  
878 and Pol II occupancy are highlighted in yellow. **D**, Immunofluorescence (IF) analysis of early  
879 stage NPCs following *Ssrp1* depletion. (Blue) DAPI, nuclei; (Green)  $\beta$ 3-Tubulin (*Tubb3*),  
880 neurons; (Red) MAP2, dendrites.

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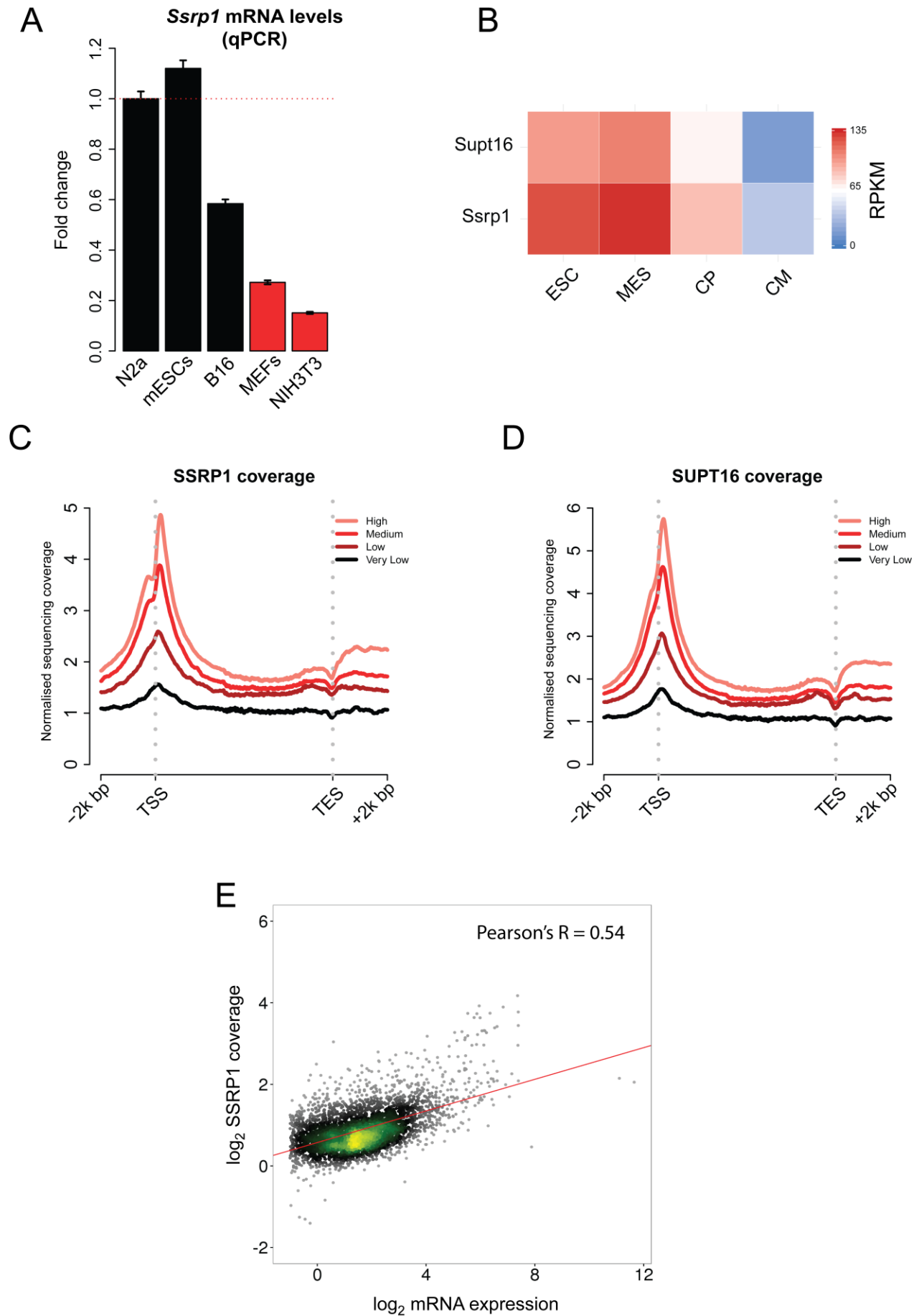
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884 Figure 6: Model of the suppressive role of FACT in gene expression and maintenance of  
885 pluripotency. Upper; FACT places a nucleosomal barrier at the promoter region of genes  
886 involved in embryogenesis/ neurogenesis that hinders divergent travelling of Pol II and enables  
887 a closed chromatin conformation state. Lower; In the absence of FACT, the nucleosomal barrier  
888 is alleviated, thus allowing bi-directional travelling of Pol II, recruitment of TFs, increased gene  
889 expression, and ultimately, activation of neurogenesis cues.

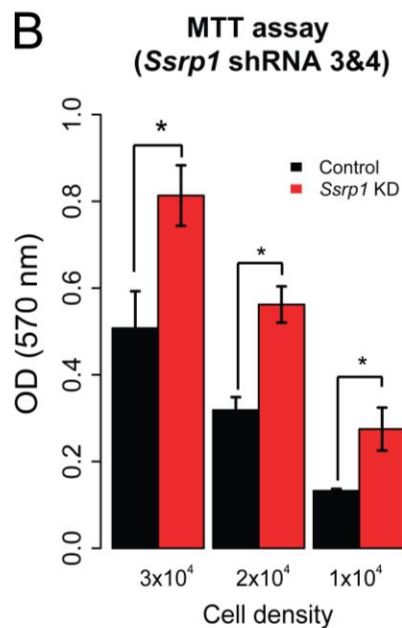
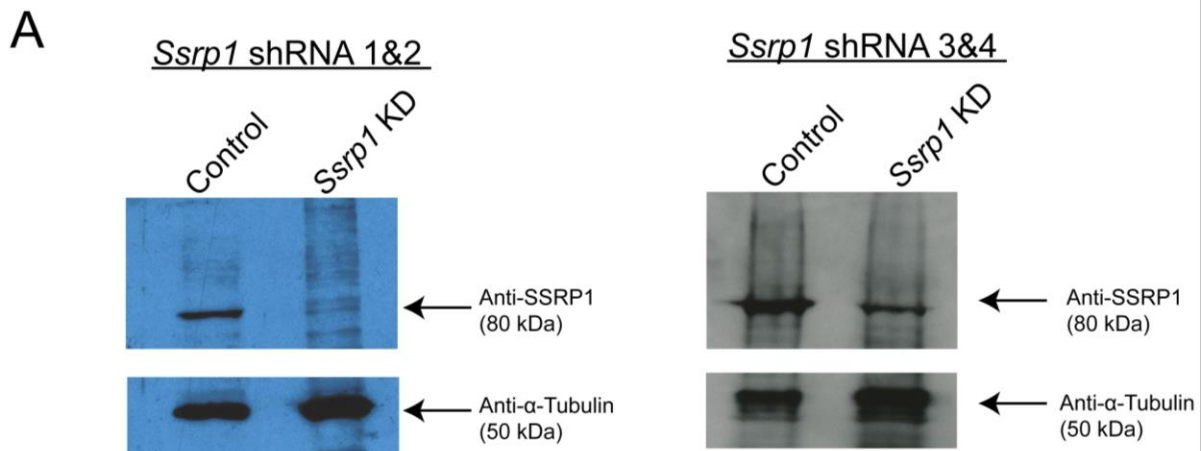
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892 **Supplementary Figure 1: FACT correlates with active gene expression. A**, qPCR of *Ssrp1*  
893 levels among stem (mESCs), cancer (N2a, B16), and differentiated cell lines (MEFs, NIH3T3).  
894 Mean and SE of three biological replicates are shown. Data were normalised to *Gapdh* mRNA  
895 levels. **B**, Heatmap assessing the mRNA levels (RPKM) of FACT (*Ssrp1*, *Supt16*) at different  
896 timepoints of differentiation of ES cells to cardiomyocytes (Wamstad et al., 2012) (ESC =  
897 Embryonic Stem Cells, MES = Mesodermal cells, CP = Cardiac Precursors, CM =  
898 cardiomyocytes). **C**, Distribution of SSRP1 relative to the TSS ( $\pm 2000$  bp) and the TES ( $\pm 2000$   
899 bp) for four different gene classes ranked by level of RNA abundance (High, Medium, Low, Very  
900 Low). **D**, Same as **(C)** but for SUPT16. **E**, Scatterplot of the  $\log_2$  SSRP1 coverage versus  $\log_2$   
901 mRNA expression.

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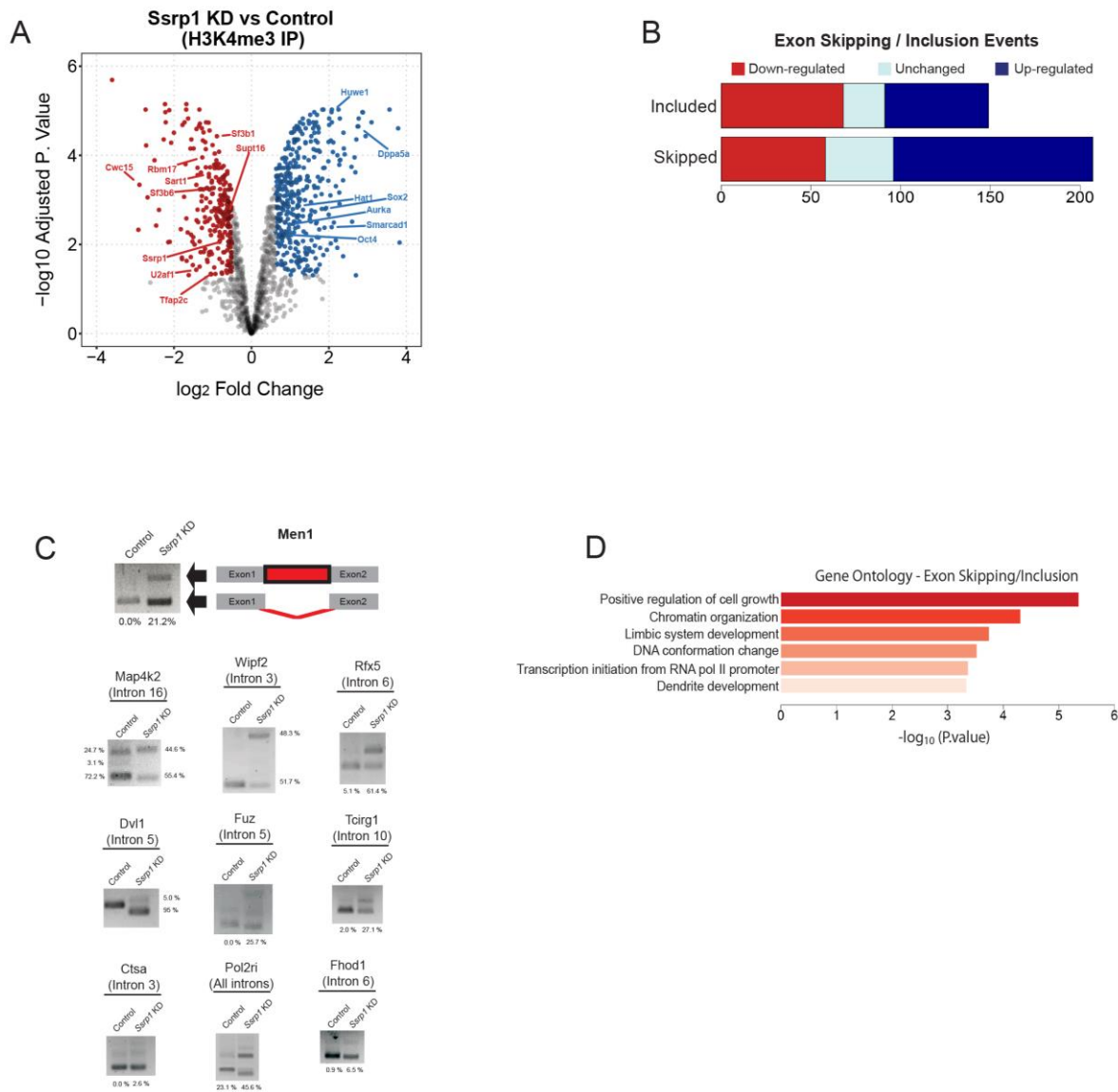


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905 **Supplementary Figure 2: FACT depletion promotes increased proliferation rate in**  
906 **mESCs.** **a**, Western blots after transfection of mESCs with different combinations of *Ssrp1*  
907 shRNA vectors (shRNA 1&2 or shRNA 3&4). Anti- $\alpha$ -Tubulin was used as a reference. **b**, MTT  
908 assay following transfection with *Ssrp1* shRNA 3&4 vectors. Values are mean and SE of three  
909 independent transfection experiments are displayed. Significance was calculated via a two-  
910 tailed *t*-test (\**P* < 0.05).

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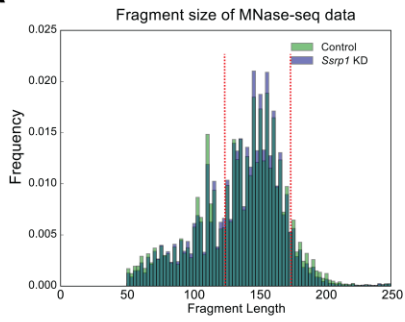




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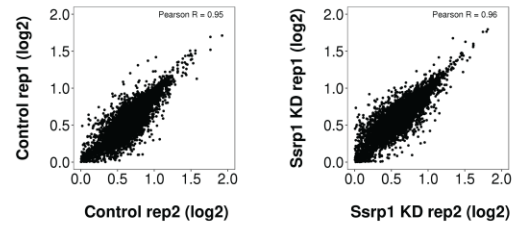
913 **Supplementary Figure 3: FACT facilitates alternative splicing of RNA transcripts.** **A**,  
 914 Volcano plot of depleted/enriched proteins at H3K4me3 following *Ssrp1* depletion. **B**, Average  
 915 distribution of SSRP1, SUPT16, and H3K4me3 aligned to the 5' Splice Site (5'SS) of all genes  
 916 grouped by first exon length. **C**, Barplots representing the number of included/skipped exons  
 917 categorized by their gene expression status (red: "Down-regulated", cyan: "Unchanged", blue:  
 918 "Up-regulated"). In total, we have identified 149 included and 207 skipped exon events in the  
 919 *Ssrp1* KD group. **D**, Graphical representation of an intronic retention event (*Men1*) in the KD  
 920 group. Also, analysis of intron inclusion events or isoform switches after FACT depletion.  
 921 Unspliced transcript percentage was measured according to band intensity. **E**, Gene ontology  
 922 analysis of transcripts (FACT-bound) that display alternative exon usage between the two  
 923 conditions

**A**

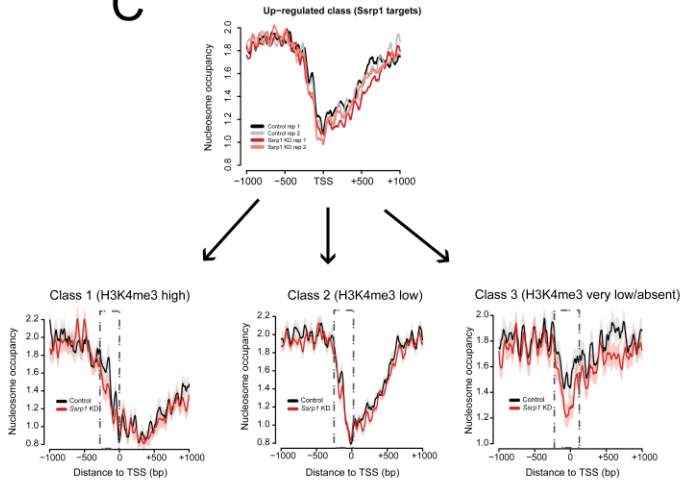


**B**

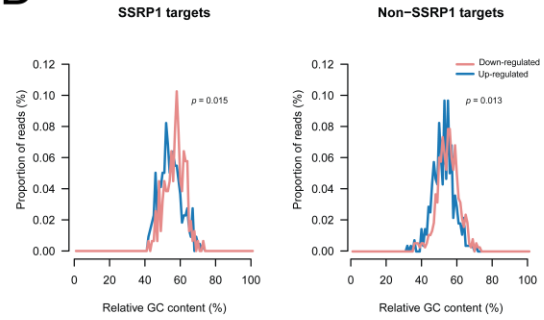
**MNase-seq correlation**



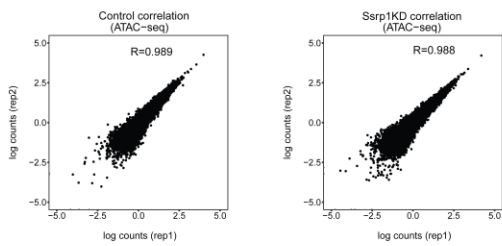
**C**



**D**



**E**



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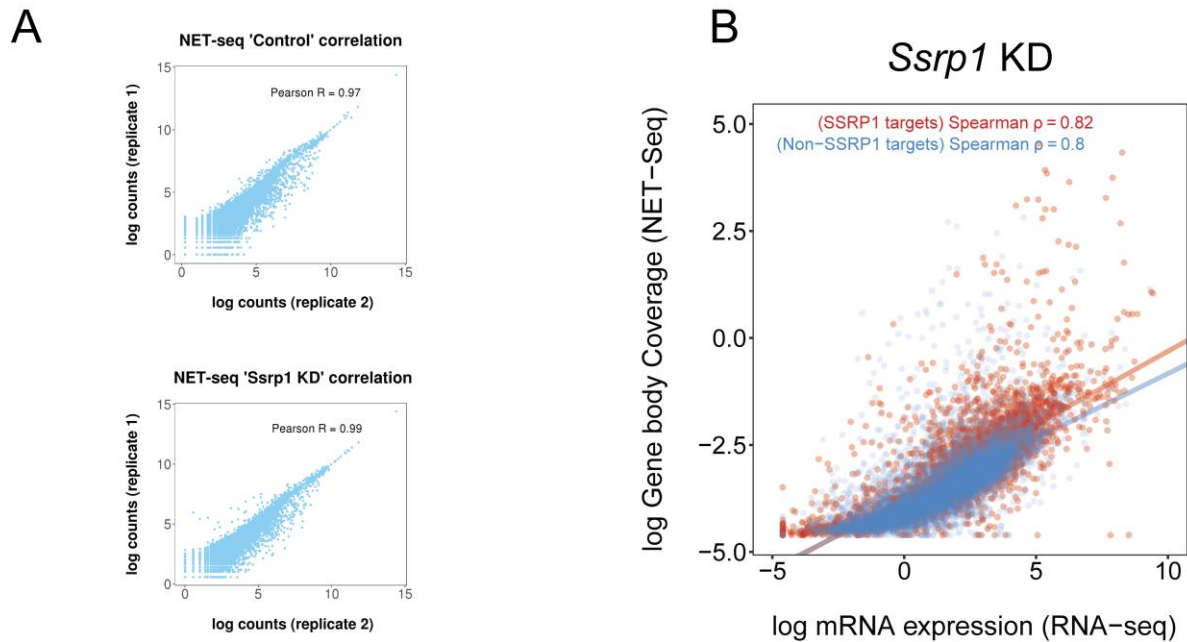
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930 **Supplementary Figure 4: Reproducibility assessment of MNase / ATAC-seq datasets. A,**  
931 Histogram showing the sequenced paired-end fragments lengths extracted from Control and  
932 *Ssrp1* depleted conditions. Fragments between 135-170 bp (indicated in red) have been  
933 computationally selected and used to plot mono-nucleosomal occupancy over promoter regions.  
934 **B,** Correlation scatterplots (MNase-seq) accessing replicate reproducibility in each condition.  
935 Pearson's correlation is indicated at the top of each plot. **C,** Nucleosome occupancy metaplots  
936 for each replicate for the composite metaplot in **Figure 4A** (Up-regulated; SSRP1 targets). This  
937 nucleosome occupancy at the promoter region derives from three distinctive gene clusters of  
938 diverse gene expression and H3K4me3 levels. **D,** GC content frequency of all "Up-regulated"  
939 and "Down-regulated" genes. Data are also split by FACT-bound dependency. **E,** Correlation  
940 scatterplots (ATAC-seq) accessing replicate reproducibility in each condition. Pearson's  
941 correlation is indicated at the top of each plot.

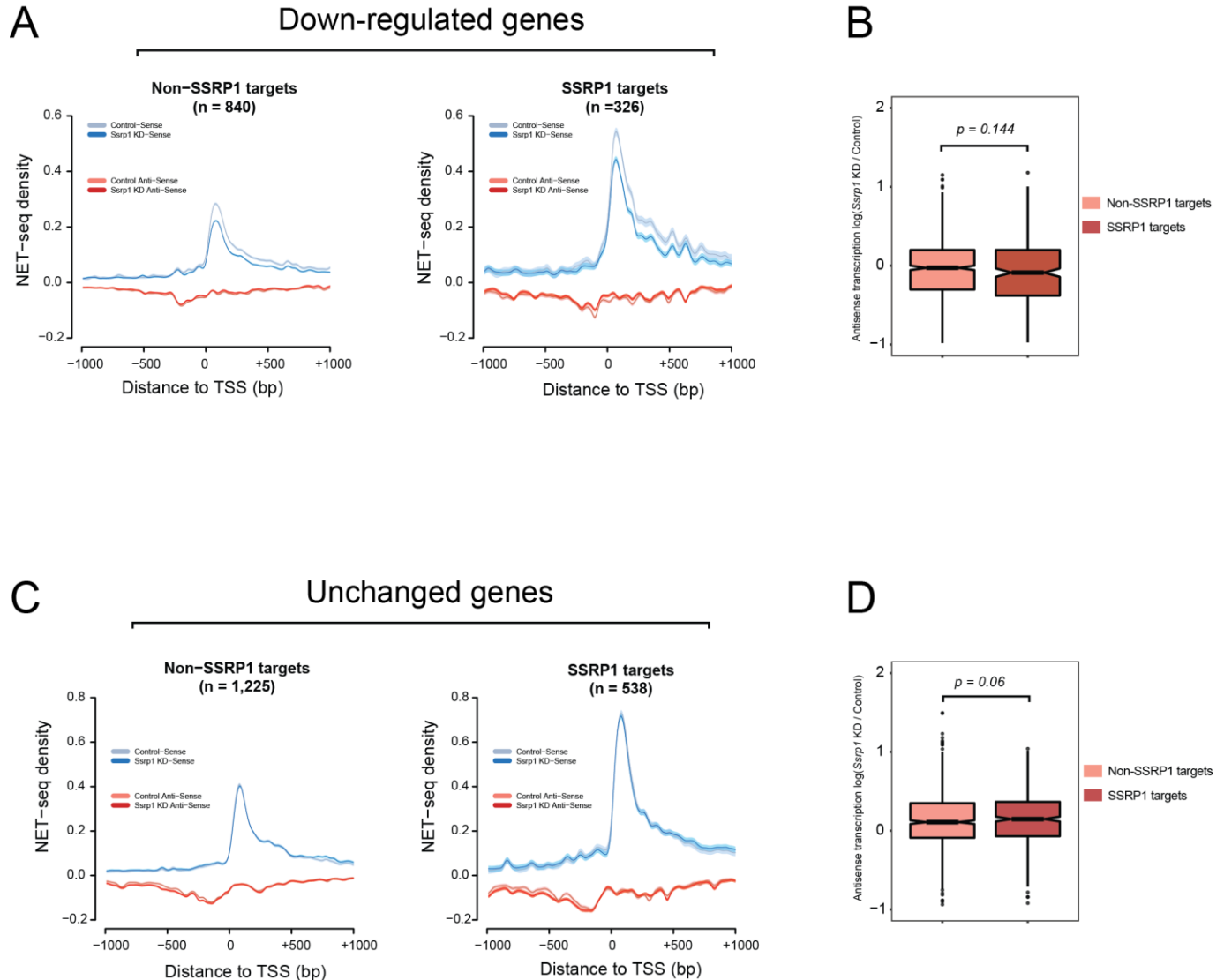
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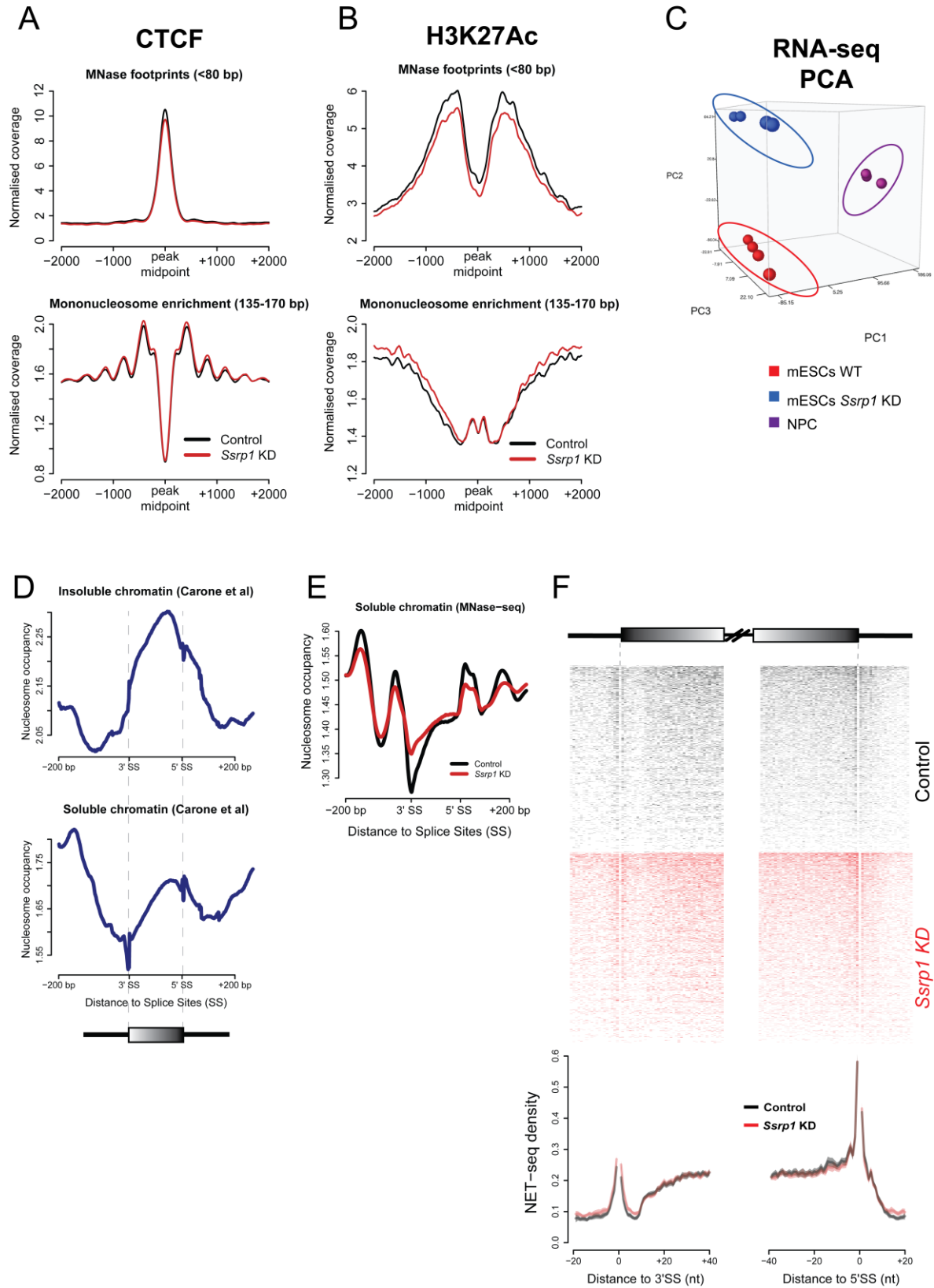
944 **Supplementary Figure 5: Assessment of NET-seq datasets.** **A**, Correlation scatterplots  
945 (NET-seq) assessing replicate reproducibility in each condition. Pearson's correlation is  
946 indicated at the top of each plot. **B**, Scatterplots of log gene body coverage (NET-seq) versus  
947 log mRNA expression (RNA-seq) for SSRP1 (n=4,576) and non-SSRP1 (n=8,844) target  
948 regions in the *Ssrp1* KD state (Z-score = 7.2,  $P < 1 \times 10^{-5}$ )

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951 **Supplementary Figure 6: Pol II pausing over other gene classes.** **A**, NET-seq density plots  
 952 (Control and *Ssrp1* KD group) of Down-regulated genes split by FACT-bound status (Non-  
 953 SSRP1 and SSRP1 targets). Solid lines indicate mean values, whereas the shading represents  
 954 the 95% confidence interval. **B**, Boxplots assessing fold change (*Ssrp1* KD vs Control) in anti-  
 955 sense transcription (NET-seq) in a window 1000 bp upstream of the TSS. The Welch's Two-  
 956 sided t-test was used to calculate significance between Non-SSRP1 and SSRP1 targets. **C**,  
 957 Same as **(A)** but for genes whose expression does not change (Unchanged genes) in the *Ssrp1*  
 958 KD condition. **D**, Same as **(B)** but for Unchanged genes.



960 **Supplementary Figure 7: Quality control assessing MNase-/NET-seq integrity. A,** CTCF  
961 peak midpoints were used as a reference and nucleosome occupancy for short (<80 bp) and  
962 long (135-170 bp) MNase footprints was plotted. **B,** Same as **(A)** but for H3K27Ac. Both  
963 nucleosome profiles over CTCF and H3K27Ac sites are consistent to previous studies (Carone  
964 et al., 2014; Teif et al., 2012). **C,** PCA plot showing clustering of different replicates (RNA-seq)  
965 presented in this manuscript. **D,** MNase-seq datasets of soluble (higher intron occupancy) and  
966 insoluble (higher exon occupancy) chromatin retrieved from Carone *et al.* **E,** Mean nucleosomal  
967 density (207,232 exons) of our insoluble MNase-seq dataset. The soluble nucleosome profile in  
968 **“C”** is highly consistent to our MNase treated samples for both conditions where nucleosomal  
969 occupancy on introns is similar or higher compared to the exons. Identical occupancy has also  
970 been observed by chemical mapping of nucleosomes (Voong et al., 2016). **f.** NET-seq  
971 heatmaps and density plots over 41,356 exons with the highest Pol II coverage. Solid lines on  
972 the NET-seq meta-exon plots indicate the mean values, whereas the shading represents the  
973 95% confidence interval.

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