

1                   **Pax6 and KDM5C co-occupy a subset of developmentally**  
2                   **critical genes including Notch signaling regulators**  
3                   **in neural progenitors**

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41 **ABSTRACT**

42

43 **Pax6 is a key transcription factor in neural development. While generally viewed as a**  
44 **transcriptional activator, mechanisms underlying Pax6 function as a repressor is less well**  
45 **understood. Here we show that Pax6 acts as a direct repressor of transcription associated**  
46 **with a decrease in H3K4me3 levels. Genome wide analysis of the co-occupancy of the**  
47 **H3K4 demethylase KDM5C and Pax6 with H3K4me3-negative regions revealed 177 peaks**  
48 **on 131 genes. Specific analysis of these Pax6/KDM5C/H3K4me3- peaks unveiled a number**  
49 **of genes associated with Notch signaling, including *Dll1*, *Dll4*, and *Hes1*. RNA knockdown of**  
50 **PAX6/KDM5C in human neural progenitors resulted in increased *DLL4* gene expression,**  
51 **decreased *DLL1* expression, and no significant effect on HES1 mRNA levels, differences**  
52 **that could be due to gene-specific variations in the chromatin landscape. Our findings**  
53 **suggest that PAX6 and KDM5C co-regulate a subset of genes implicated in brain**  
54 **development, including members of the Notch signaling family.**

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58 The proper development of the mammalian cerebral cortex is strictly dependent on precise  
59 spatial and temporal intracellular and extracellular cues<sup>1,2</sup>. Genetic studies of psychiatric  
60 disorders have revealed that many of the factors mutated in such disease are regulators of  
61 neural development<sup>3</sup>. Further, a subset of these genes harboring critical mutations has been  
62 shown to be regulators of chromatin, i.e. epigenetic regulators<sup>4</sup>. Indeed, genetic defects in  
63 epigenetic regulators seem to be common in neurodevelopmental disorders such as Rett  
64 syndrome, Rubinstein-Taybi syndrome, and other states of mental retardation<sup>5,6</sup>.

65

66 It has further been shown that valproic acid (VPA), but not lithium, are teratogenic, and  
67 offspring of mothers taking VPA show higher frequency of autistic spectrum disease<sup>7</sup>. This is  
68 a highly relevant observation as VPA and lithium share many signaling mechanisms<sup>8</sup> but  
69 while VPA is in addition an inhibitor of histone deacetylase (HDAC) activity, lithium is not<sup>9,10</sup>.  
70 Altogether, these reports highlight the importance of appropriate function of histone  
71 modifying proteins in neural development.

72

73 We and others have previously demonstrated specific roles for HDACs, histone acetyl  
74 transferases (HATs), associated co-activators and co-repressors, as well as DNA methylation  
75 regulators in many events during neural development, including differentiation,  
76 proliferation, cell death, senescence, and autophagy<sup>11-17</sup>. These factors have in addition  
77 been linked to DNA-binding transcription factors involved in neurodevelopmental disorders  
78 and fundamental neurodevelopment, such as REST and MeCP2<sup>18-23</sup>.

79

80 During the past years, roles for factors regulating histone methylation has emerged<sup>24,25</sup>. One  
81 such histone demethylase that has previously been linked to psychiatric disease, especially  
82 X-linked mental retardation, is KDM5C (also known as SMCX, JARID1C)<sup>26</sup>. KDM5C is a  
83 demethylase of lysine 4 on histone H3 (H3K4). H3K4 trimethylation (H3K4me3) is strongly  
84 and directly associated with transcriptional activation<sup>27,28</sup> and KDM5C is therefore primarily  
85 regarded as an inhibitor of transcriptional activation. KDM5C has more recently been linked  
86 to other psychiatric disorders including autism, in line with its presumed general importance  
87 for proper brain development<sup>29</sup>. Jumonji domain-proteins similar to KDM5C interact with  
88 sequence-specific transcription factors<sup>18</sup>, but less is known regarding such KDM5C-recruiting  
89 factors.

90

91 PAX6 is a DNA-binding transcription factor that has been implicated in the fundamental  
92 regulation of the development of the cerebral cortex<sup>30,31</sup>. Mutations in PAX6 are most  
93 commonly associated with aberrations in eye development, but studies have demonstrated  
94 that mutations in PAX6 can also be associated with neurological and psychiatric conditions<sup>32</sup>.  
95 Although PAX6 has long been studied from a genetic point of view, relatively little is known  
96 of the mechanisms underlying its function. It has been shown to act as a transcriptional  
97 activator, and in this role associated with H3K4me3 methyl transferases<sup>33</sup>. However, there  
98 are further reports of PAX6 preferentially binding to methylated DNA<sup>34</sup>, and when Pax6 is  
99 ablated, the expression of a subset of genes has been shown to be increased<sup>35</sup>. Indeed, it has  
100 recently been reported that PAX6 can interact with class I HDACs, specifically HDAC1,  
101 suggesting that PAX6 may mediate direct repression of transcription<sup>36</sup>. The increased gene  
102 expression could however theoretically be explained by a lack of activation of a secondary  
103 repressor, the association with binding to methylated DNA could be mere association, and  
104 HDAC1 is not ubiquitously expressed in neural progenitors<sup>11</sup>. There is thus a need for  
105 increased understanding of putative mechanisms underlying Pax6 function as a  
106 transcriptional repressor.

107

108 The aim of this study was to analyze Pax6 occupancy patterns and compare these to  
109 H3K4me3 levels and binding of the H3K4 demethylase KDM5C. Combining chromatin  
110 immunoprecipitation (ChIP) assays with sequencing (ChIP-Seq) is a powerful method to  
111 identify genome-wide DNA binding sites for transcription factors and other proteins. To this  
112 end we have downloaded and analysed two published ChIP-seq data sets<sup>37,38</sup>. While we  
113 found a subset of Pax6 peaks coinciding with H3K4me3-positive regions, most Pax6 binding  
114 was detected in regions with low H3K4me3 (H3K4me3-). Indeed, transcriptional assays  
115 showed that PAX6 could act as a direct repressor of transcription, and this repression was  
116 associated with a small but reproducible decrease in H3K4me3 levels. Analysis of the  
117 occupancy of KDM5C at regions with high and low levels of H3K4me3 revealed that KDM5C  
118 occupied H3K4me3-positive loci at a majority of peaks, but as expected, a large number of  
119 the KDM5C-occupied regions were H3K4me3-. We therefore made a comparison of all three  
120 datasets and found 177 peaks on 131 genes where Pax6 and KDM5C overlapped with  
121 H3K4me3- regions. When analyzing the function of the Pax6/KDM5C-occupied, H3K4me3- -

122 genes, we found a number of genes associated with Notch signaling, including *Dll1*, *Dll4*, and  
123 *Hes1*. RNA knockdown of PAX6 and KDM5C in human iPS-derived neural progenitors (hNPs)  
124 resulted in an increase in DLL4 gene expression, whereas no significant effect of HES1 mRNA  
125 levels was detected, and DLL1 gene expression actually decreased. Our results suggest that  
126 PAX6 and KDM5C are required components for proper regulation of gene expression of  
127 Notch signaling factors.

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129

130 **RESULTS**

131

132 *Genome wide analysis reveals Pax6 occupancy on H3K4me3–positive and –negative regions*

133

134 Pax6 has been linked to transcriptional activation and shown to be associated with H3K4  
135 methyl transferases<sup>33</sup> whereas other reports have suggested that Pax6 may be linked to  
136 transcriptional repression<sup>34,36</sup>. Indeed, knockdown of Pax6 in neural progenitors (NPs) yields  
137 both increased and decreased gene expression (ref. 35 and data not shown). To investigate  
138 putative mechanisms underlying Pax6 function, we initiated an *in silico* study of genome  
139 wide Pax6 occupancy to compare with the H3K4me3 landscape in the rodent developing  
140 cortex using stringent bioinformatics analysis to interpret the results (**Supplementary file 1**).  
141 This initial analysis revealed that 9,352 ChIP-Seq peaks were positive for Pax6 binding (**Figure**  
142 **1a**) in E12.5 mouse forebrain tissue<sup>38</sup>. When analyzing the enrichment of peaks near genes  
143 using the Reactome pathway terms, the results were predominantly associated to biological  
144 processes of development, axon guidance, and Notch signaling (**Figure 1b**) (over represented  
145 p-value 4.01E-08). Notch receptor family, its ligands, and signaling intermediate factors are  
146 essential for correct control of proliferation and differentiation, as well as other cellular  
147 events, in neural and organism development<sup>39,40</sup>. We then compared the Pax6 ChIP-seq  
148 peaks to regions rich in H3K4me3, and this analysis unveiled 15,745 H3K4me3-positive  
149 peaks. A large proportion of those peaks was associated with cell cycle control (**Figure 1a,c**)  
150 (over represented p-value 4.16E-54). This is completely in accordance with H3K4me3 as an  
151 active mark of transcription as most cells in an E12.5 mouse forebrain are dividing.

152

153 When combining the two datasets, we found 1,735 overlapping peaks between Pax6 and  
154 H3K4me3 (**Figure 1a**). This confirms previous observations of Pax6 association with  
155 H3K4me3 methyl transferases<sup>33</sup>. The number of overlapping peaks was however lower than  
156 expected and the vast majority of Pax6-positive peaks were associated with H3K4me3-  
157 negative (H3K4me3-) regions (**Figure 1a, Supplementary file 1**).

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161

162 *PAX6 can act as a repressor in transcriptional assays in association with decreased H3K4me3*

163

164 The analysis of PAX6 occupancy in combination with H3K4me3-negative regions prompted  
165 us to investigate whether PAX6 could act directly as a repressor in transcriptional assays. We  
166 first constructed a Gal4-PAX6 fusion construct that could be used in a doxycycline (DOX)  
167 sensitive assay (DOX-on)<sup>41</sup> (**Supplementary figure 1**), and analyzed its activation of a UAS-  
168 promoter in a luciferase reporter context in stably transfected HEK293 cells. This assay  
169 revealed that PAX6 indeed can act as a direct transcriptional repressor (**Figure 1d**)  
170 ( $p=0.0008$ ), in accordance with recent observations<sup>36</sup>.

171

172 We next utilized the DOX-on system to investigate the effects on chromatin modifications on  
173 the stably transfected promoter (**Figure 1e**). We analyzed three of the most well-studied  
174 lysine modifications, i.e. H3K4me3, acetylation of lysine 9 on histone H3 (H3K9ac) and  
175 acetylation of lysine 27 on histone H3 (H3K27ac). Whereas H3K9ac did not decrease, we  
176 found a reproducible decrease of H3K27ac by activation of Gal4-PAX6 in this assay (**Figure**  
177 **1e**). This is in accordance with the recent observations linking PAX6 to class I HDACs such as  
178 HDAC1<sup>36</sup>.

179

180 Importantly, we found a small but reproducible Gal4-PAX6-mediated decrease in H3K4me3  
181 levels in this assay (**Figure 1e**). This result suggests that PAX6 may act as a transcriptional  
182 repressor at least in part by recruiting an H3K4me3 demethylase.

183

184

185 *KDM5C occupancy is found both on H3K4me3-positive and -negative genes*

186

187 As the results from the transcriptional assays suggested that Pax6 could be acting as a  
188 repressor of transcription at least in part by bringing in H3K4me3 demethylase activity, we  
189 pursued additional *in silico* analysis of KDM5C, the most prevalent H3K4 demethylase in the  
190 developing nervous system cells<sup>42</sup>. This analysis revealed 7,715 KDM5C peaks (**Figure 2a**) in  
191 neurons isolated from E16.5 mouse embryonic cortices and harvested after 10 days in vitro  
192 culture<sup>37</sup>, and further analysis of the most significant Reactome terms demonstrated that

193 the most pivotal class of genes occupied by KDM5C was associated with neuronal systems  
194 (over represented p-value 3.25E-12) (**Figure 2b**).

195

196 Surprisingly, we found that 4,350 KDM5C peaks overlapped with the H3K4me3-positive  
197 regions detected from the embryonic forebrain ChIP-Seq (**Figure 2a**). This is interesting due  
198 to the presumed H3K4 demethylase activity of KDM5C. Nevertheless, the analysis revealed  
199 that 3,365 KDM5C peaks were associated with low H3K4me3 levels (**Figure 2a**), in  
200 accordance with the enzymatic function of KDM5C. We concluded from this analysis that  
201 KDM5C can bind to both H3K4me3-positive and -negative regions.

202

203

204 *Pax6 and KDM5C co-occupies 177 peaks on 131 genes mostly associated with brain*  
205 *development and Notch signaling*

206

207 We next combined our analyses of Pax6 and KDM5C occupancies with H3K4me3 levels. This  
208 analysis revealed seven combinations of occupancies where it should be noted that the vast  
209 majority of Pax6 and KDM5C peaks did not overlap. Still 662 Pax6/KDM5C peaks were found  
210 to be present on H3K4me3-positive regions (**Figure 3a**). Most interesting in the context of  
211 the present study was however the genes that were co-occupied by Pax6 and KDM5C on  
212 regions with low H3K4me3. Here we discovered a subset of 177 peaks on 131 different  
213 genes that were co-occupied by Pax6 and KDM5C on H3K4me3- regions.

214

215 When analyzing the biological function associated with the overlapping peaks using the  
216 Reactome terms, we made the intriguing finding that a substantial part of these 131 genes  
217 were associated with Notch signaling pathway (**Figure 3b**) (over represented p-value 4.68E-  
218 06). In contrast, when analyzing the 662 peaks of Pax6 and KDM5C co-occupancy on  
219 H3K4me3-positive regions, the top Reactome terms were *per contra* “gene expression” and  
220 BMP signaling (**Figure 3c**) (over represented p-value 1.25E-05 and 1.03E-04). These results  
221 suggest that Notch signaling factors may be specifically regulated by Pax6 and KDM5C.

222

223



224 *Pax6 and KDM5C peaks show distinct patterns in relation to the H3K4me3 landscape on*  
225 *DLL1, DLL4, and HES1 genes*

226

227 We next analyzed the Pax6/KDM5C/H3K4me3- peaks in detail on specific genes involved in  
228 Notch signaling. DLL1 is a ligand of the Notch receptor, and its gene was found to contain  
229 multiple PAX6 peaks of which one overlapped with KDM5C (**Figure 4a**). The Pax6/KDM5C-  
230 overlapping peaks were flanked by high H3K4me3 levels, yet there was a small overlap of  
231 binding in a short region with low H3K4me3 levels (**Figure 4a**). DLL4 is another ligand of the  
232 Notch receptor. We could only detect one Pax6 peak in the vicinity of its gene but it  
233 overlapped with KDM5C in an H3K4-negative region of the gene, in contrast to a second  
234 KDM5C-peak that overlapped with an H3K4me3-positive site but no Pax6 binding (**Figure**  
235 **4b**).

236

237 Thirdly, we analyzed the Pax6/KDM5C/H3K4me3-landscape of the gene encoding the Notch-  
238 associated transcription factor HES1. This gene showed a marked overlap of Pax6/KDM5C-  
239 overlapping peaks in an H3K4me3-negative region, whereas downstream of this site, we  
240 found very high levels of H3K4me3 (**Figure 4c**). In summary, whereas we can confirm the  
241 overlap of Pax6 and KDM5C peaks on Notch signaling-related genes, the peaks showed  
242 variability in character and the gene regions displayed major variations in the H3K4me3  
243 landscape, hinting at possible differences in gene regulatory mechanisms.

244

245

246 *RNA knockdown of PAX6 and KDM5C in human iPS-derived neural progenitor cells result in*  
247 *differential gene expression levels of DLL1 and DLL4 but not HES1.*

248

249 Lastly, to investigate whether the detected Pax6/KDM5C peaks in neural progenitors on  
250 Notch signaling-associated genes were of relevance for regulation of gene expression, we  
251 developed specific siRNAs to perform combined RNA knockdown of PAX6 and KDM5C and  
252 compared this to control (scrambled) siRNA in human iPS-derived neural progenitors (hNPs)  
253 to analyze the effects of PAX6 /KDM5C knockdown (PAX6 siRNA ( $p=0.0005$ ) and KDM5C  
254 siRNA ( $p=0.0002$ )) on the expression levels of *DLL1*, *DLL4*, and *HES1*.

255

256 We did not detect any significant differences in the expression of *HES1* after PAX6/KDM5C  
257 RNA knockdown in hNPs compared to control (**Figure 5**). As noted (**Figure 4c**), the *HES1* gene  
258 region was very rich in H3K4me3, suggesting that PAX6/KDM5C may not be dominant  
259 repressive regulators of the expression of this gene. When analyzing the expression of *DLL1*,  
260 we found that the gene expression decreased ( $p=0,0040$ ) after RNA knockdown of  
261 PAX6/KDM5C in hNPs compared to control.

262

263 Lastly, we analyzed the gene expression levels of *DLL4* after RNA knockdown of  
264 PAX6/KDM5C in the hNPs, and found a robust increase ( $p=0,0020$ ) in the mRNA levels  
265 compared to control (**Figure 5**).

266

267 In summary, our results demonstrate that there is a relatively small subset of  
268 developmentally critical genes, including Notch signaling factors, co-occupied by PAX6 and  
269 KDM5C on H3K4me3-negative regions, and that these two factors exert essential control of  
270 proper expression of some of these genes.

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272

273

274 **DISCUSSION**

275

276 Here we have revealed that PAX6 and KDM5C co-occupy and regulate a subset of  
277 developmentally critical genes associated with Notch signaling. Considering the fundamental  
278 role of Notch signaling in neural development, we suggest that this regulatory mechanism  
279 may play a role in pathologies associated with aberrant PAX6 and/or KDM5C activity.

280

281 This is to our knowledge the first link between KDM5C and regulation of Notch signaling  
282 factors, and it will be of immediate interest to investigate whether effects on Notch signaling  
283 could be involved in the consequences of aberrant KDM5C function<sup>37,43</sup>. In contrast, Pax6  
284 has been linked to Notch signaling in various contexts, but that Pax6 is binding to regions  
285 close to almost all known factors directly involved in Notch signaling has not been previously  
286 understood. In fact, the analysis revealed PAX6 binding close to all ligands and all receptors,  
287 except NOTCH3, as well as more distant members, such as DLK1 (G.G and O.H., unpublished  
288 observations). It is likely that this has not been shown before due to the large number of  
289 genes to which PAX6 is binding and directly regulating<sup>31</sup>, and it is not until analyzing the co-  
290 occupied regions with KDM5C that this subpopulation of genes is revealed.

291

292 PAX6 has previously been linked to chromatin modifying and remodeling factors, such as  
293 CBP/p300 and members of the SWI/SNF and BAF complexes (cf. <sup>44</sup>, reviewed in <sup>31</sup>). Less is  
294 however known about the mechanisms underlying transcriptional repression by Pax6. Our  
295 findings show that Pax6 and KDM5C co-occupy regions with low H3K4me3 but many peaks  
296 of PAX6 found at regions with low H3K4me3 were not occupied by KDM5C and thus  
297 presumably not active (**Figure 3a**). Therefore, alternative mechanisms of repression should  
298 be considered. It was recently shown that Pax6 binds to the histone deacetylase HDAC1 with  
299 functional implications for lens development<sup>36</sup>. HDAC1 is however not ubiquitously  
300 expressed in neural progenitors <sup>11</sup>, and in the hNPs used in the present study, another class I  
301 HDAC, HDAC2, is the most prominently expressed (M. Lam and A. Falk, personal  
302 communications). Class I HDACs such as HDAC1 and HDAC2 can be members of the so called  
303 NURD complex that among other things regulate H3K27 acetylation, and indeed we found in  
304 the transcriptional assays that PAX6 overexpression resulted in a decrease in H3K27  
305 acetylation (**Figure 1**). Future studies will aim at elucidating whether PAX6 can interact with

306 other class I HDACs and possibly the NURD complex in hNPs to regulate the H3K27  
307 acetylation.

308

309 As demonstrated in Figure 5, simultaneous RNA knockdown of PAX6 and KDM5C did not  
310 result in an increased gene expression of all genes assessed. At a first glance, this may seem  
311 like a contradiction, but the variations in the chromatin landscape surrounding the  
312 PAX6/KDM5C binding sites must be taken into account. Thus, in the case of *DLL1*, there are  
313 multiple PAX6 binding sites in the gene but only one overlapping peak with KDM5C (**Figure**  
314 **4a**). It may therefore be hypothesized that the decrease in *DLL1* gene expression was a net  
315 result by knocking down PAX6 not only as a repressor but also as an activator, and that PAX6  
316 may be predominantly an activator of the *DLL1* gene. Further, the chromatin landscape in  
317 the vicinity of the PAX6/KDM5C binding site at the *HES1* gene was very rich in H3K4me3  
318 (**Figure 4c**), pointing to an ancillary role for PAX6/KDM5C in the regulation of *HES1* gene  
319 expression.

320

321 When analyzing the transcriptional outcome, it should be noted that the chromatin is  
322 organized in a 3-dimensional manner, and it is the nanoorganization - or nanoarchitecture -  
323 in coordination with the chemical milieu that will provide the optimal conditions for  
324 transcriptional regulation. Such parameters are extremely dynamic, especially in progenitor  
325 cells. A common model of the outcome of chromatin modifications is the “gas and brake” of  
326 a car, where H3K4me3 is compared to the gas and H3K27me3 to the brake. The model is of  
327 course knowingly a simplification, but staying with the car analogy, a simplified model for  
328 transcriptional regulation by chromatin modifications could rather be compared to a  
329 “clutch”, as previously proposed<sup>11</sup>, where the state of transcription and nanoarchitecture  
330 will influence the effect of changes in transcription and chromatin modifying factor levels. As  
331 the surrounding chemical architecture will determine the overall transcriptional activity, the  
332 effect(s) of simple RNA knockdown or overexpression will depend on the overall  
333 enhancer/promoter activity, and the effects will be different depending on the baseline of  
334 the transcription of the gene assessed, the number of transcription factor binding sites, the  
335 nature of the modification etc. To test this hypothesis, future studies should be utilizing  
336 controlled genetic and epigenetic editing in neural progenitors in a 3D system. Our findings  
337 reveal a relatively small population of genes in neural progenitors with low levels of

338 H3K4me3 that are co-occupied by Pax6 and KDM5C, and a subset of those are genes  
339 encoding factors involved in Notch signaling.

340

341

## 342 **METHODS**

343

### 344 *Bioinformatic analysis*

345 Relevant and publicly available datasets were identified via literature search and  
346 sratools/2.8.0 was used to download raw sequencing reads in fastq format from The  
347 Sequence Read Archive<sup>37,38</sup>. Best practices ChIP-seq data processing and analyses as outlined  
348 by ENCODE consortium were followed to analyse the data. Briefly, TruSeq adapters were  
349 removed from the data as well as poor quality reads using trimmomatic/0.32. Alignment  
350 against reference GRCm38.p5 genome was done with bowtie/1.1.2 suppressing all multiple  
351 alignments. Duplicated reads and/or reads overlapping with the blacklisted genomics  
352 regions with artificially high signals were removed with  
353 picard/2.0.1 (<https://broadinstitute.github.io/picard/>). Peak-independent quality metrics,  
354 including strand cross-correlation, were calculated with phantompeakqualtools/1.1 and  
355 deepTools/1.1.2 to assess quality of ChIP-seq libraries (strength of enrichment). Peaks were  
356 called with MACS/2.1.0<sup>45</sup> using extension size as calculated from strand cross-correlation  
357 (200 and 190 for S1 and S3 respectively). Peaks called were pooled across replicates where  
358 feasible using BEDTools/2.26.0. Annotation to the closest genes and overlapping analyses of  
359 ChIP-seq peaks were done using R/Bioconductor package ChIPpeakAnno<sup>46</sup>. Genes associated  
360 with peaks were analysed for over-represented GO and reactome terms using R/  
361 Bioconductor package goseq<sup>47</sup>.

362

### 363 *GAL4 plasmids and DNA cloning*

364 GAL4–PAX6 was cloned into the pcDNA5/FRT/TO expression vector by standard procedures  
365 and subsequently used for recombination into the genomic DNA of TReX 293 Flp-In cells  
366 (Invitrogen) as described in details previously<sup>41</sup>. The GAL4–PAX6 constructs were sequence-  
367 verified by DNA sequencing. GAL4–EZH2 (control) was a kind gift from Dr. Kristian Helin.

368

### 369 *Cell culture neuroepithelial stem cells*

370 The generation of AF22, a control iPS cell line, has been described elsewhere<sup>48</sup>. Briefly, to  
371 culture long-term self-renewing neuroepithelial-like stem cells or neural progenitors (hNPs),  
372 hiPS cell colonies were induced to differentiate to neural tissue and hNPs cultures were  
373 captured and maintained by passaging them at the ratio of 1:3 every third day essentially as

374 previously described<sup>48</sup>. Briefly, hNPs cultures were maintained in hNPs medium (DMEM/F-  
375 12, GlutaMAX containing N<sub>2</sub> supplement, FGF2 (10 ng ml<sup>-1</sup>), B27 (0.1%), Pen/Strep (all from  
376 Invitrogen), and EGF (10 ng ml<sup>-1</sup>; Peprotech, Rocky Hill, NJ, USA)), and harvested using  
377 TrypLE-Express (Gibco) for passage into 0.1 mg poly-L-ornithine (Sigma-Aldrich) and  
378 1 µg ml<sup>-1</sup> laminin L2020 (Sigma-Aldrich) coated (PLO/L-coated) plates at 1:3 ratio.

379

#### 380 *Chromatin Immunoprecipitation (ChIP-qPCR)*

381 Chromatin immunoprecipitation (ChIP) was performed using HighCell# ChIP kit (Diagenode)  
382 and performed according to the manufacturer's protocol. 6,3 micrograms of the specific  
383 antibody (H3K4me3, H3K9ac and H3K27ac all Rabbit mAb from Cell Signaling Technology)  
384 were used in each IP. Purified, eluted DNA and 1% DNA Input was analyzed by qPCR using  
385 Platinum™ SYBR™ Green qPCR SuperMix-UDG (ThermoFisher Scientific) together with site-  
386 specific primers. The analyzes were performed in triplicates per each biological sample using  
387 the standard curve method to account for potential differences in primer efficiency,  
388 therefore relative occupancy for a specific antibody in different regulatory regions could be  
389 compared. Statistical analyzes were performed using GraphPad Prism (version 7.0, GraphPad  
390 Software, La Jolla, CA, USA).

391

#### 392 *RNA knockdown*

393 For siRNA-mediated knockdown of PAX6 RNA, KDM5C RNA, and control, Amaxa  
394 Nucleofector™ Kits for Human Stem Cells (Lonza) was used according to the manufacturer's  
395 protocol. ON-TARGET plus SMARTpool siRNA for PAX6, KDM5C, and control were obtained  
396 from Dharmacon. Following nucleofection, hNPs were seeded in 6 well plates in hNPs media  
397 under proliferative conditions, as described in the cell culture method section. The results  
398 were analyzed 48h later by RT-qPCR.

399

#### 400 *RT-qPCR*

401 Total RNA was extracted using RNeasy Mini Kit (Qiagen), cDNA was synthesized using High  
402 Capacity cDNA Reverse Transcription Kit (Applied Biosystems, now ThermoFisher Scientific),  
403 and RT-qPCR was performed using Platinum™ SYBR™ Green qPCR SuperMix-UDG  
404 (Invitrogen, now ThermoFisher Scientific) together with site specific primers. mRNA  
405 expression levels were normalized to the housekeeping human TATA-binding protein (TBP)

406 mRNA (RT<sup>2</sup> qPCR Primer Assays, Qiagen) in the GAL4 assay and to the human housekeeping  
407 hypoxanthine guanine phosphoribosyltransferase (HPRT) (RT<sup>2</sup> qPCR Primer Assays, Qiagen)  
408 mRNA when performing siRNA analyses.

409

410

411



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413

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578 **FIGURE LEGENDS**

579

580 **Figure 1: Pax6 occupancy is enriched at genomic regions with low H3K4me3 and Pax6 can**  
581 **exert repression associated with a decrease in H3K4me3 in transcriptional assays.** (a)  
582 Genome wide analysis of ChIP-Seq experiments revealed that the vast majority of Pax6  
583 peaks were found in regions with low H3K4me3. (b) Top Reactome terms for Pax6 peaks. (c)  
584 Top Reactome Terms for high H3K4me3 levels. (d) A Gal4-Pax6 construct exerts repression in  
585 a Dox-sensitive transcriptional assay. 2-way ANOVA shows the effect of DOX treatments (n  
586 (number of biological replicates) = 3 in each group. \*\*\* $p=0.0008$ , the data represent the  
587 mean  $\pm$  SEM). (e) The repressor function of the Gal4-Pax6 fusion construct is associated with  
588 a decrease in H3K4me3 and H3K27ac but not H3K9ac levels.

589

590 **Figure 2: KDM5C is binding to H3K4me3-positive as well as -negative genes.** (a) Analysis of  
591 ChIP-Seq experiments demonstrated KDM5C peaks both at H3K4me3-positive and -negative  
592 regions. (b) Top Reactome terms for KDM5C peaks. The most prevalent term was “neuronal  
593 system”.

594

595 **Figure 3: A subset of peaks where Pax6 and KDM5C overlapped with H3K4me3-negative**  
596 **regions was found in the proximity of genes associated with Notch signaling.** (a) Analysis of  
597 Pax6, KDM5C, and H3K4me3 peaks revealed several interesting subpopulations of genes.  
598 Notably 177 peaks were found where Pax6 and KDM5C overlapped at regions with low  
599 H3K4me3 levels, but it should be noted that the vast majority of Pax6 peaks did not overlap  
600 with KDM5C or H3K4me3-positive regions. (b) The top Reactome terms for  
601 Pax6+/KDM5C+/H3K4me3- regions were found to be associated with Notch signaling.

602

603 **Figure 4: The H3K4me3 landscapes in the vicinity of the Pax6/KDM5C peaks at *Dll1*, *Dll4*,**  
604 **and *Hes1* genes display major differences.** There were major differences in levels of  
605 H3K4me3 in the vicinity of the Pax6+/KDM5C+ peaks close to the genes encoding *Dll1* (a),  
606 *Dll4* (b), and *Hes1* (c). Shaded areas highlight regions with enriched Pax6 and KDM5C peaks,  
607 and low H3K4me3. Horizontal bars mark significant peaks of Pax6 (blue), KDM5C (green),  
608 and H3K4me3 (orange).

609

610 **Figure 5: Effects on gene expression levels of *DLL1*, *DLL4*, and *HES1* after RNA knockdown**  
611 **of *PAX6* and *KDM5C* in human iPS-derived neural progenitors.** (a) The RNA knockdown of  
612 *PAX6* and *KDM5C* (left) after specific siRNA administration was significant (*PAX6* siRNA (n=3,  
613 \*\*\* $p=0.0005$ ; unpaired, two-tailed t-test) and *KDM5C* siRNA (n=3, \*\*\* $p=0.0002$ ; unpaired,  
614 two-tailed t-test). There was a significant decrease in *DLL1* (middle) gene expression (n=3,  
615 \*\* $p=0.0040$ ; unpaired, two-tailed t-test), a significant increase in *DLL4* gene expression (n=3,  
616 \*\* $p=0.0020$ ; unpaired, two-tailed t-test), and no significant difference in *HES1* mRNA levels  
617 (right) after the simultaneous knockdown of *PAX6* and *KDM5C* mRNAs in hNPs. Error bars  
618 represent  $\pm$  SEM.

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## 622 SUPPLEMENTARY FILES

623

624 **Supplementary File 1:** Detailed methods and results of the bioinformatic analysis.

625

626 **Supplementary Figure 1:** (a) Three different *PAX6*-GAL4 clones exerted repression in the  
627 assay in a similar range as the Polycomb-associated repressor *EZH2*. (b) The protein product  
628 of the GAL4-*PAX6* construct was specifically induced by DOX treatment.

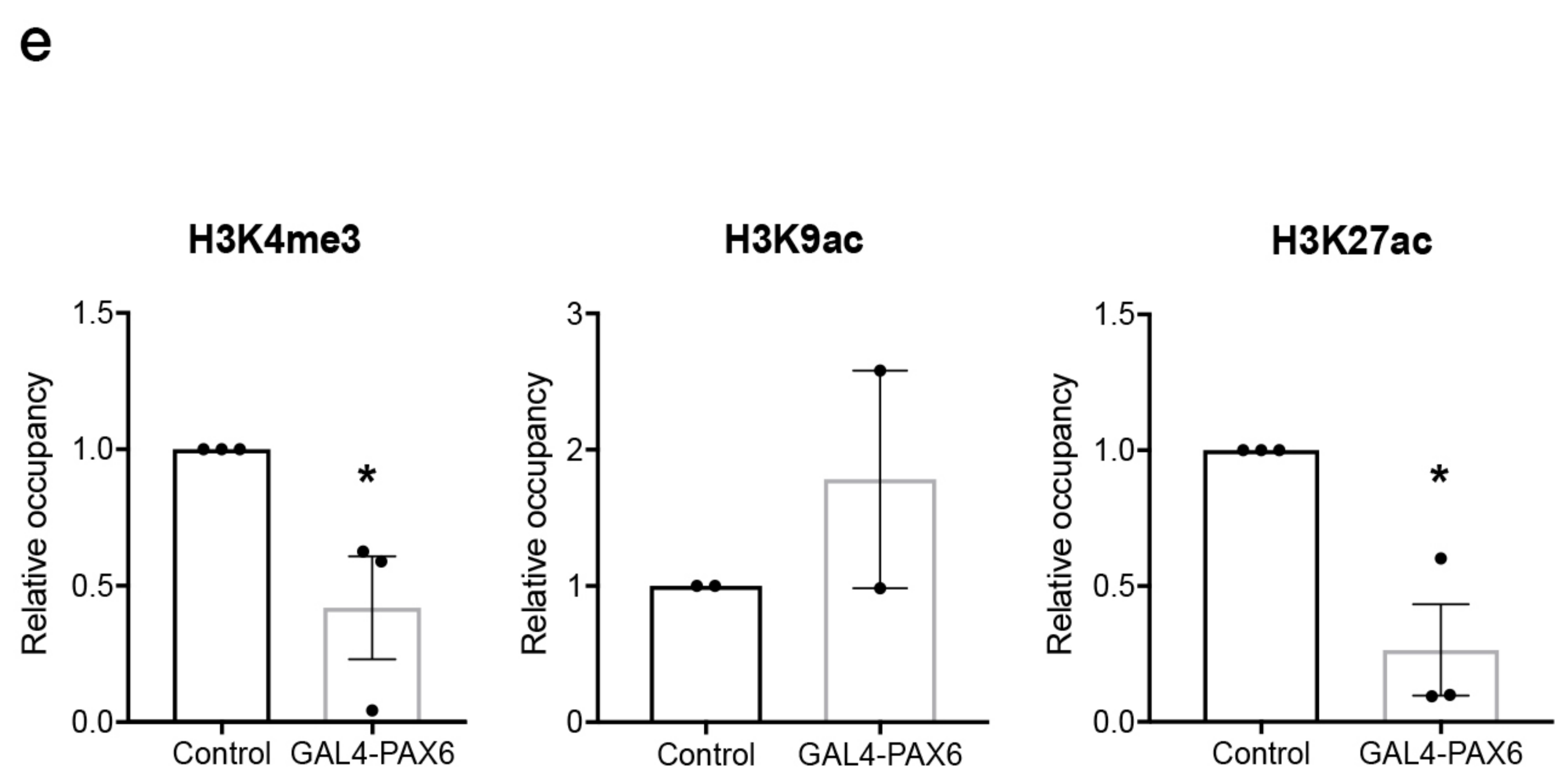
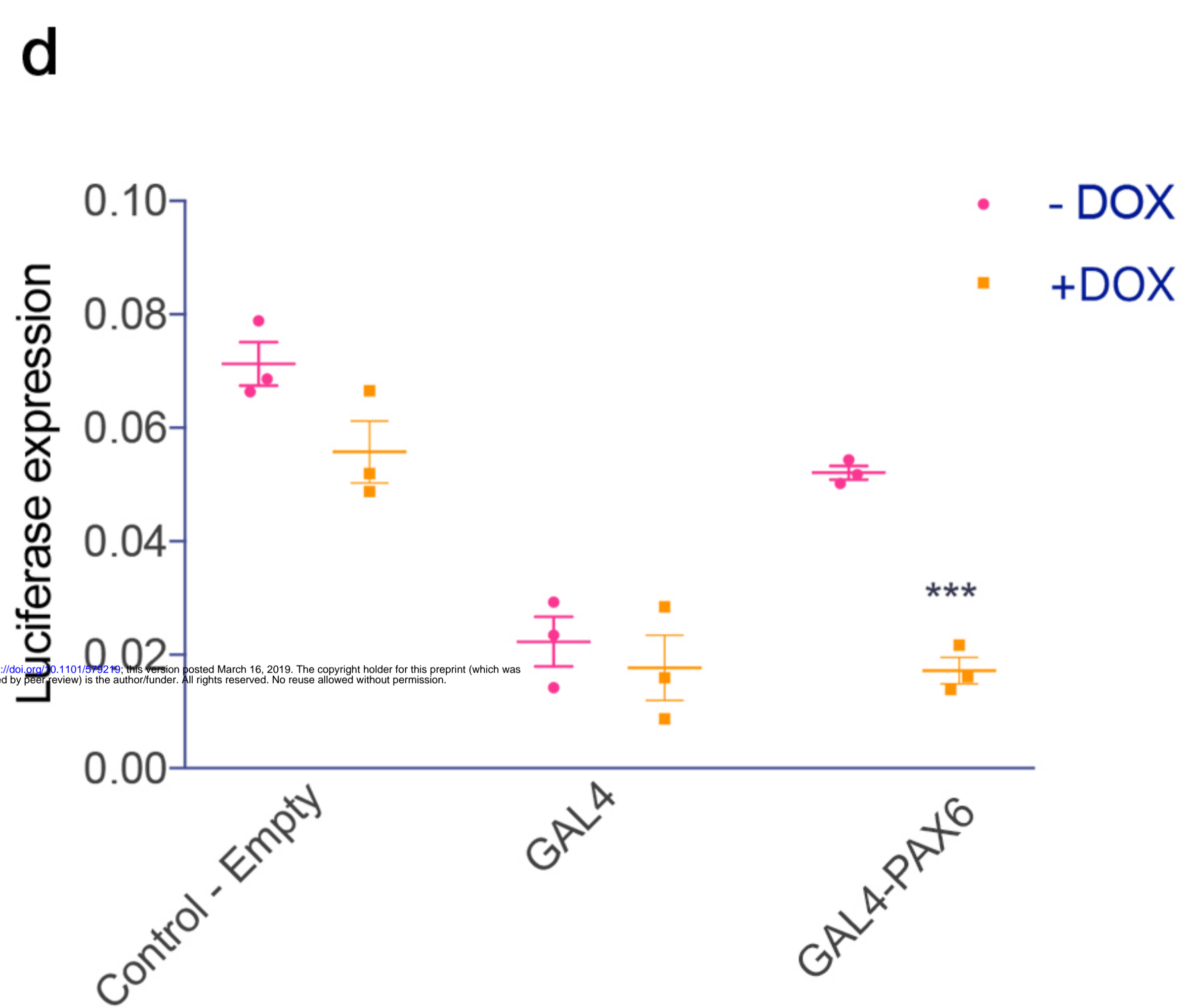
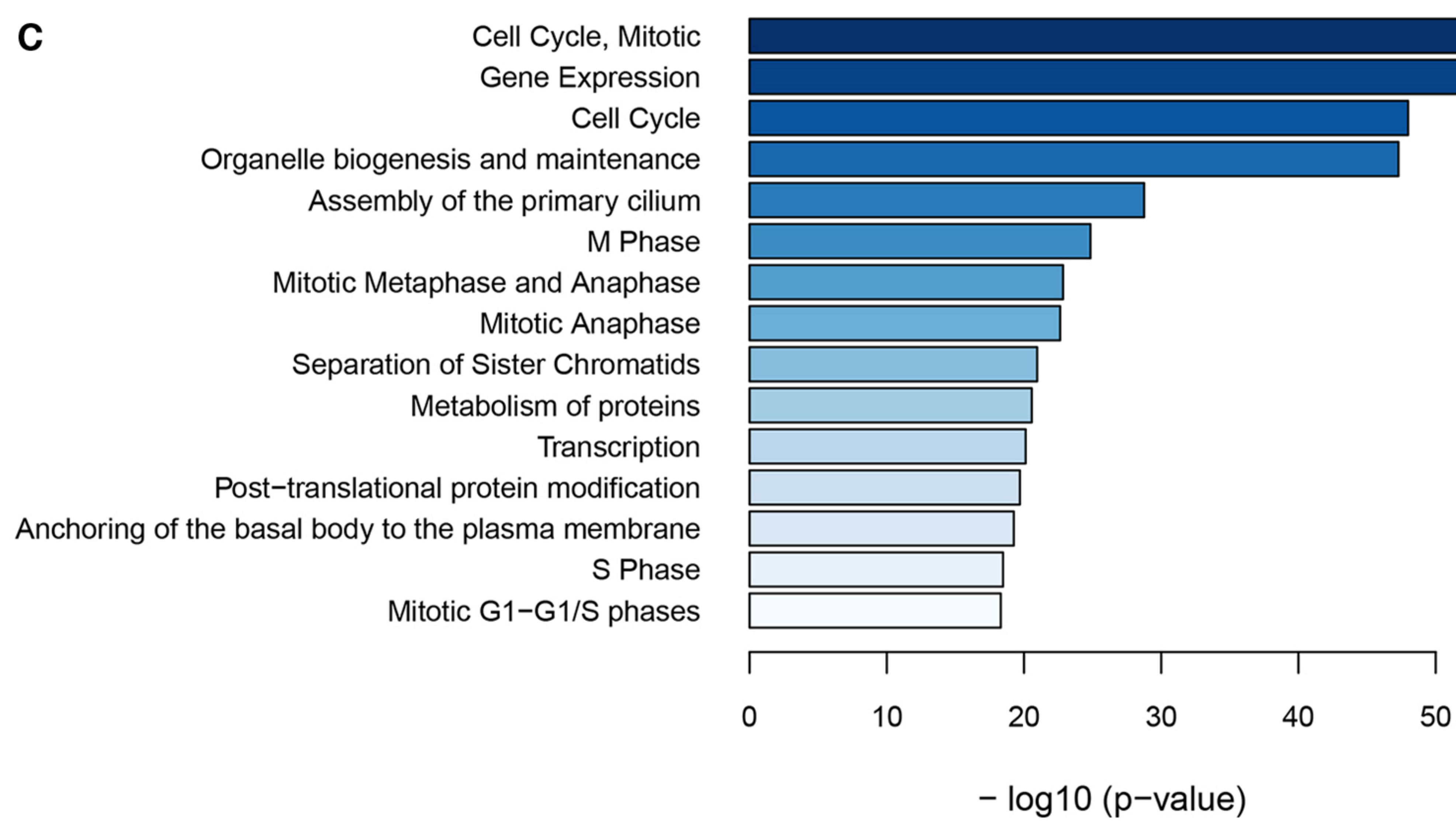
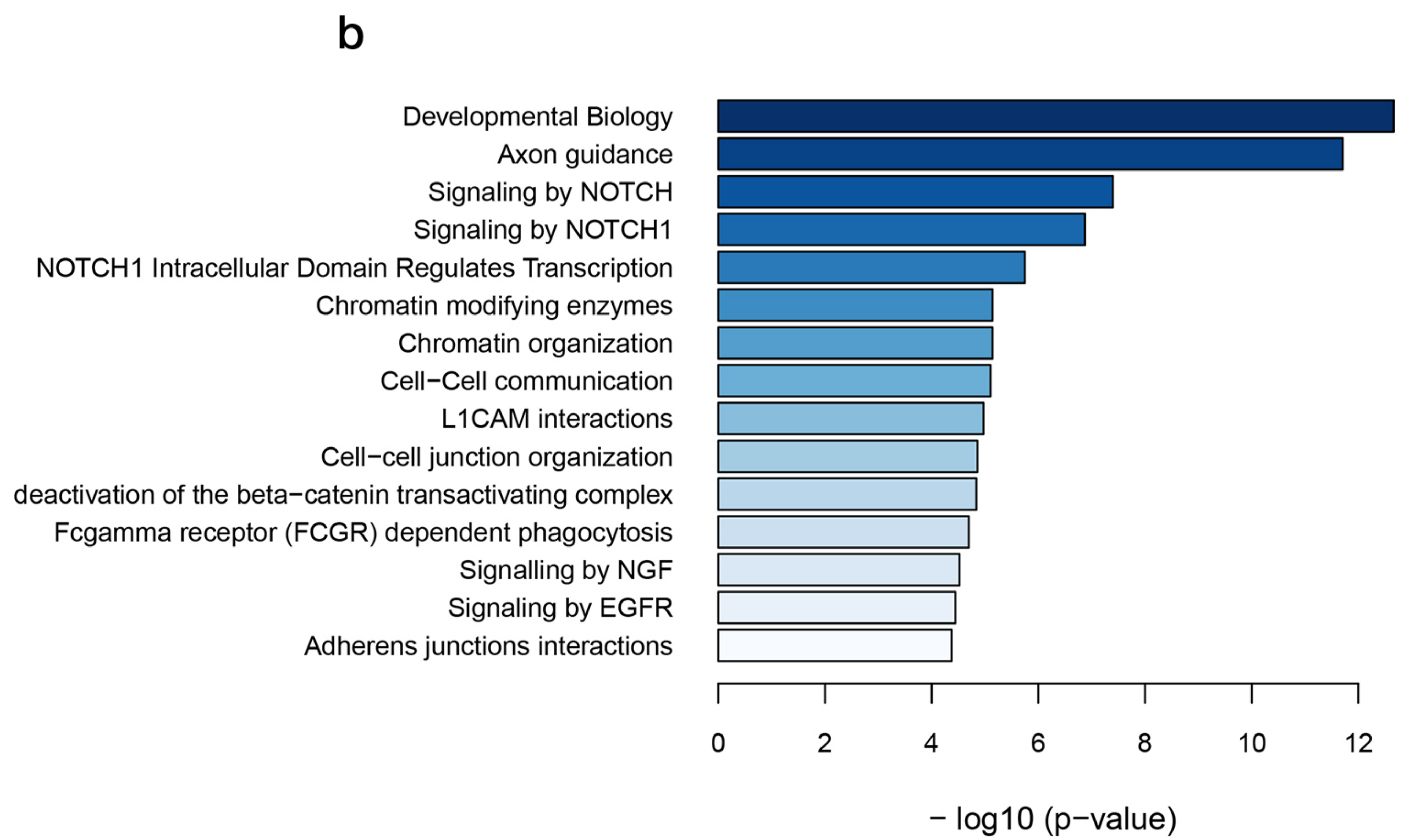
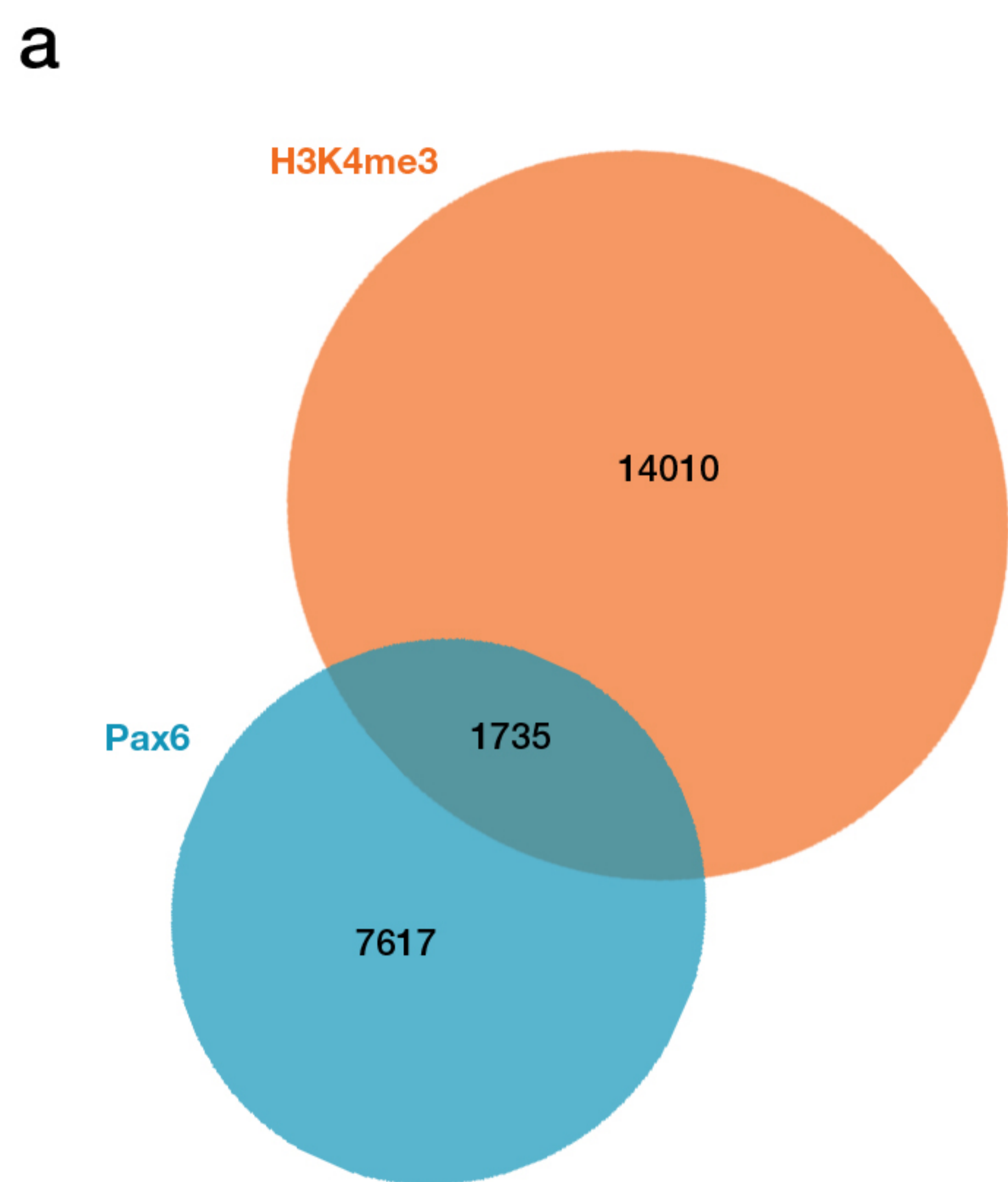
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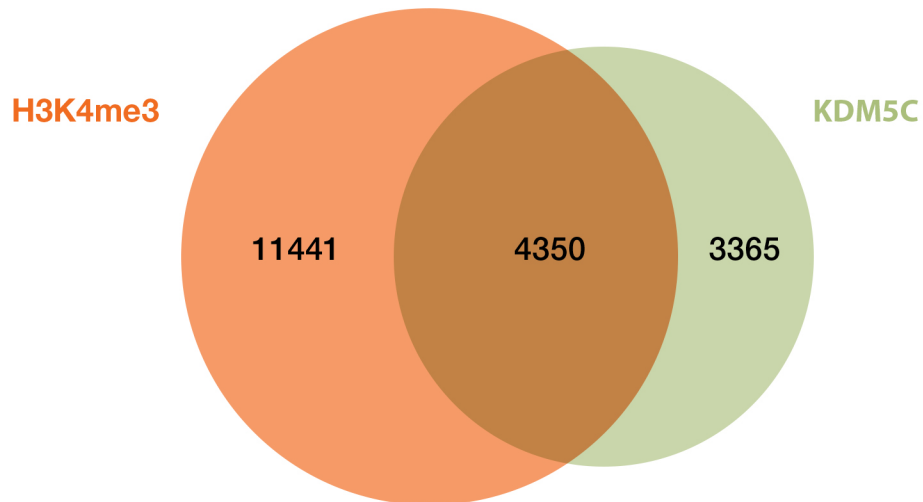
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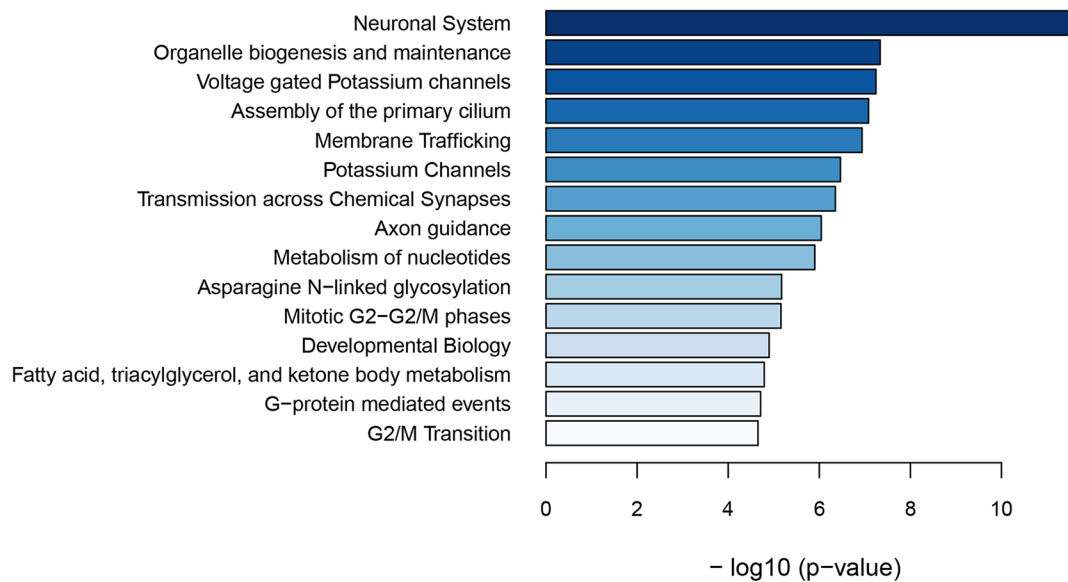




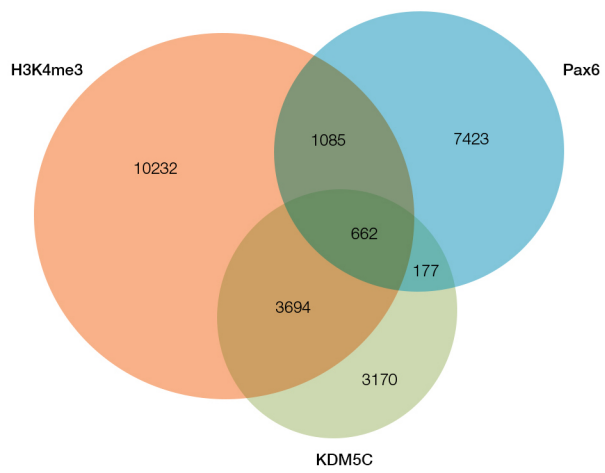
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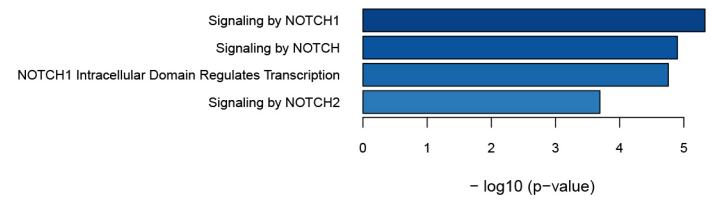
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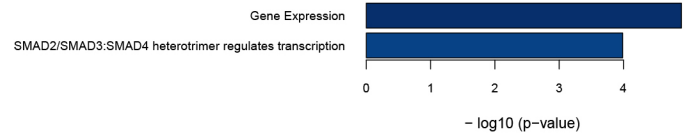
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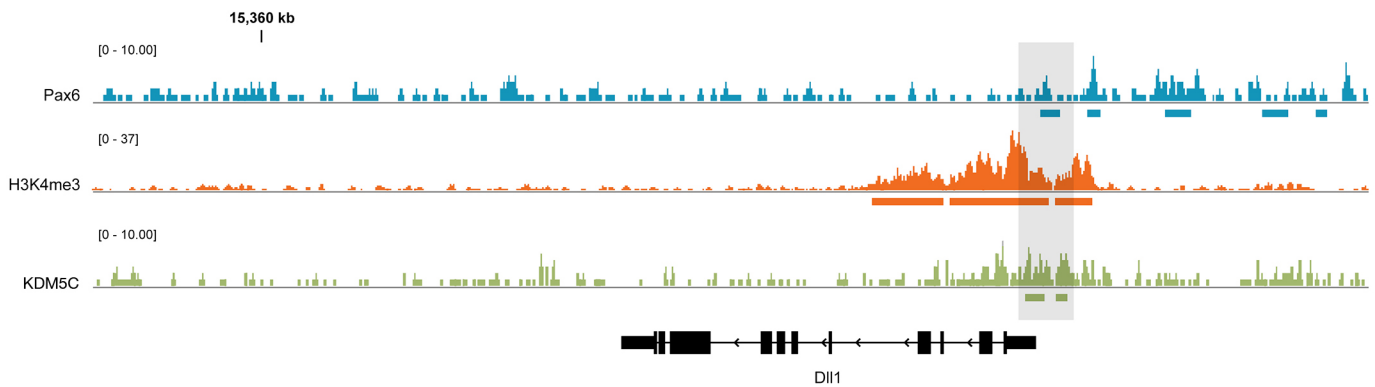
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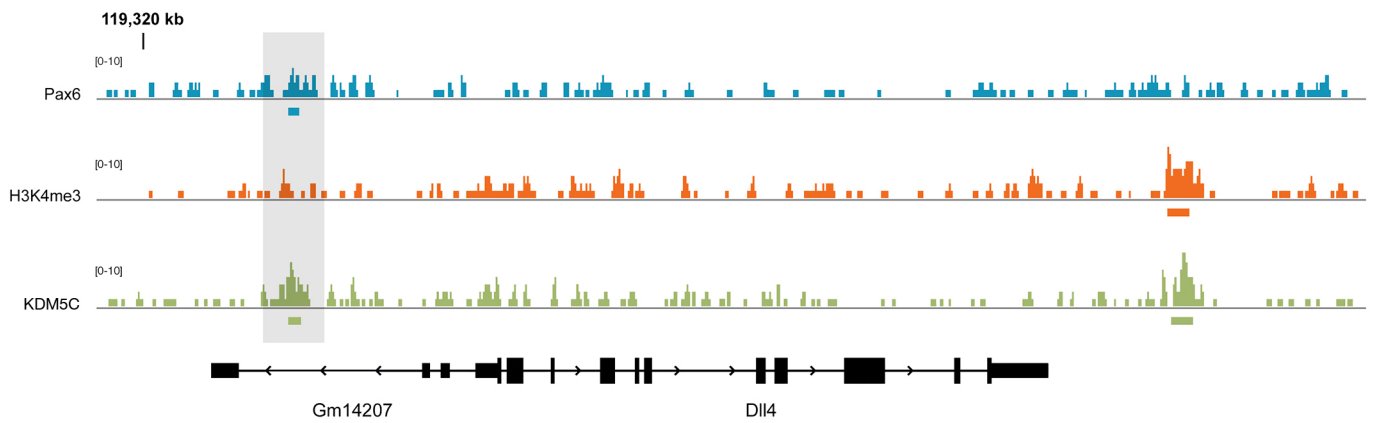
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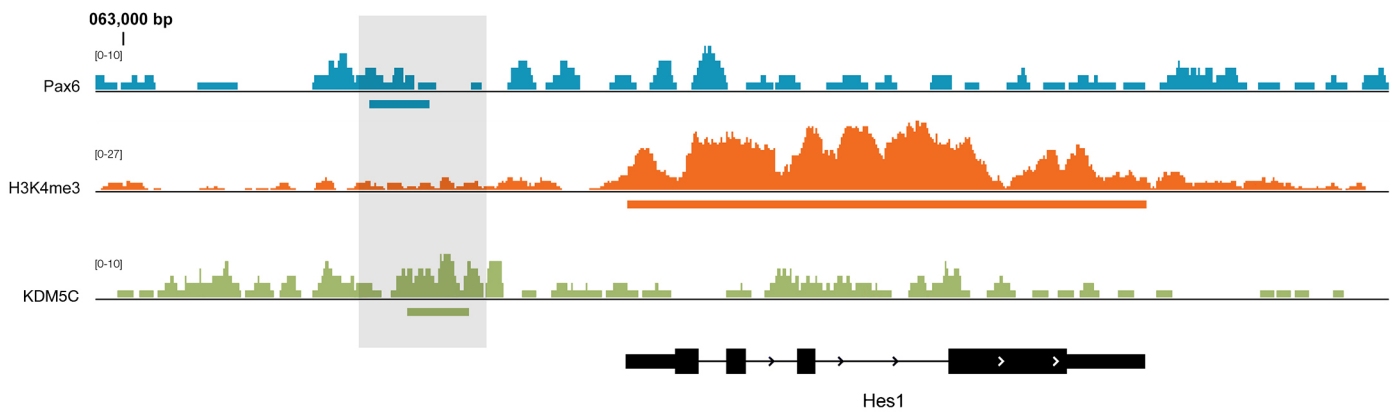
**a**



**b**



**c**



a

