

1 **TITLE**

2

3 Combinatorial transcription factor binding encodes cis-regulatory wiring of  
4 forebrain GABAergic neurogenesis

5

6

7 **AUTHORS**

8 Rinaldo Catta-Preta<sup>1,2,§</sup>, Susan Lindtner<sup>3,§</sup>, Athena Ypsilanti<sup>3</sup>, James Price<sup>3</sup>, Armen  
9 Abnoui<sup>4,5</sup>, Linda Su-Feher<sup>1</sup>, Yurong Wang<sup>1</sup>, Ivan Juric<sup>4</sup>, Ian R. Jones<sup>10,11</sup>, Jennifer A.  
10 Akiyama<sup>6</sup>, Ming Hu<sup>4</sup>, Yin Shen<sup>10,11</sup>, Axel Visel<sup>6,7,9</sup>, Len A. Pennacchio<sup>6,7,8</sup>, Diane Dickel<sup>6</sup>,  
11 John L R Rubenstein<sup>3,\*</sup>, Alex S Nord<sup>1,\*</sup>

12

13

14

15 **AFFILIATIONS**

16 <sup>1</sup> Department of Neurobiology, Physiology and Behavior, and Department of Psychiatry  
17 and Behavioral Sciences, University of California, Davis, Davis, CA 95618, USA

18 <sup>2</sup> Current Address: Department of Genetics, Blavatnik Institute, Harvard Medical  
19 School, Boston, MA 02115, USA

20 <sup>3</sup> Nina Ireland Laboratory of Developmental Neurobiology, Department of Psychiatry  
21 and Behavioral Sciences, UCSF Weill Institute for Neurosciences, University of  
22 California, San Francisco, San Francisco, CA 94143, USA

23 <sup>4</sup> Department of Quantitative Health Sciences, Lerner Research Institute, Cleveland  
24 Clinic Foundation, Cleveland, OH 44106, USA

25 <sup>5</sup> Current Address: NovaSignal, Los Angeles, CA 90064, USA

26 <sup>6</sup> Environmental Genomics and Systems Biology Division, Lawrence Berkeley National  
27 Laboratory, Berkeley, CA 94720, USA

28 <sup>7</sup> U.S. Department of Energy Joint Genome Institute, Walnut Creek, CA 94598, USA

29 <sup>8</sup> Comparative Biochemistry Program, University of California, Berkeley, Berkeley, CA  
30 94720, USA

31 <sup>9</sup> School of Natural Sciences, University of California, Merced, Merced, CA 95343,  
32 USA

33 <sup>10</sup> Institute for Human Genetics, Department of Neurology, University of California, San  
34 Francisco, San Francisco, CA 94143, USA

35 <sup>11</sup> Department of Neurology, University of California, San Francisco, CA 94143, USA

36 <sup>12</sup> Current Address: Octant, Inc, Emeryville, CA 94608, USA

37

38 § These authors contributed equally

39 \* Co-senior authors; corresponding authors

40 **ABSTRACT**

41

42 Transcription factors (TFs) bind combinatorially to genomic cis-regulatory elements  
43 (cREs), orchestrating transcription programs. While studies of chromatin state and  
44 chromosomal interactions have revealed dynamic neurodevelopmental cRE  
45 landscapes, parallel understanding of the underlying TF binding lags. To elucidate the  
46 combinatorial TF-cRE interactions driving mouse basal ganglia development, we  
47 integrated ChIP-seq for twelve TFs, H3K4me3-associated enhancer-promoter  
48 interactions, chromatin and transcriptional state, and transgenic enhancer assays. We  
49 identified TF-cREs modules with distinct chromatin features and enhancer activity that  
50 have complementary roles driving GABAergic neurogenesis and suppressing other  
51 developmental fates. While the majority of distal cREs were bound by one or two TFs, a  
52 small proportion were extensively bound, and these enhancers also exhibited  
53 exceptional evolutionary conservation, motif density, and complex chromosomal  
54 interactions. Our results provide new insights into how modules of combinatorial TF-  
55 cRE interactions activate and repress developmental expression programs and  
56 demonstrate the value of TF binding data in modeling gene regulatory wiring.

57

## 58 INTRODUCTION

59

60 Neurogenesis in the subpallial embryonic basal ganglia (BG) produces the cells that  
61 differentiate into GABAergic and cholinergic neurons that make up mature BG  
62 structures, as well as GABAergic interneuron populations that migrate to areas such as  
63 the cortex and amygdala<sup>1</sup>. Comparative analysis of BG cell types and structures  
64 suggest strong evolutionary conservation of development and anatomy across the >560  
65 million years of vertebrate phylogenetic divergence<sup>2,3</sup>. Many transcription factors (TFs)  
66 have been identified that control patterning and cell type specification in the BG and the  
67 brain overall, with homeobox TF genes playing central and deeply evolutionarily  
68 conserved roles<sup>4-7</sup>. Homeobox TFs are a large family of proteins that include a  
69 homeobox domain capable of recognizing a target DNA motif<sup>8</sup>. Homeobox TFs,  
70 alongside other TF classes, bind in a combinatorial and competitive manner at cis-  
71 regulatory elements (cREs) to direct dynamic expression patterns necessary for brain  
72 development and function<sup>9-11</sup>. Studies in simpler organisms have revealed  
73 combinatorial homeobox TF expression codes determining neuronal identity<sup>12,13</sup>.  
74 Genetic studies in mice have shown a number of homeobox TFs to be critical for all  
75 stages of mammalian Central Nervous System (CNS), including BG development<sup>6,14</sup>.  
76 These studies show that many individual TFs directly activate or repress transcription in  
77 developing brain, presumably via combinatorial and context-dependent TF interactions.

78

79 While single TFs have been studied at various stages of neurodevelopment, it remains  
80 largely unknown how TFs within and across diverse homeobox and other TF families  
81 work together to establish gene regulatory interactions *in vivo* in developing mammalian  
82 brain<sup>15,16</sup>. More specifically, it is unknown in the developing BG and brain overall how  
83 TFs with similar or diverse binding motifs overlap in genomic targets and which  
84 combinations of TFs bind which cREs, and if cREs that are bound by the same set of  
85 TFs have similar regulatory function and evolutionary history. Characterization of TF-  
86 cRE regulatory interactions at scale is also needed to understand how sets of TFs  
87 interact to control chromatin landscapes underlying neurodevelopment. More broadly,  
88 most combinatorial TF binding studies that compare more than a few TFs in a single  
89 system have been done in less complex organisms or *in vitro* cell models, thus there  
90 remain major questions regarding how sets of TFs bind to regulatory DNA targets  
91 during mammalian embryonic development and what the relationships are between cis-  
92 trans interactions between TFs and regulatory targets and chromosomal DNA  
93 interactions. We addressed these questions via integrating ChIP-seq data from 12 TFs,  
94 9 of them representing 6 distinct homeobox classes, with chromatin state and  
95 chromosomal interactions identified using H3K4me3 PLAC-seq, and established the  
96 activity of representative TF-bound cREs in transgenic mouse enhancer assays (Figure  
97 1A).

## 98 RESULTS

99

### 100 A TF-anchored model of regulatory interactions in E13.5 mouse basal ganglia

101

102 We performed epigenetic experiments on micro-dissected embryonic day (E)13.5  
103 mouse BG, when GABAergic and cholinergic neurogenesis is ongoing in the medial,  
104 lateral, and caudal ganglionic eminences (MGE, LGE, and CGE)<sup>17</sup>. Dissections included  
105 the ventricular zone (VZ), where neural precursor stem cells are located and expanding,  
106 the subventricular zone (SVZ), which includes intermediate progenitors and early born  
107 neurons, and the mantle zone (MZ), which is made up of maturing and migrating  
108 immature GABAergic and cholinergic neurons<sup>17</sup>. We performed ChIP-seq targeting five  
109 TFs (ARX, ASCL1, GSX2, NR2F1, and PBX1/2/3) and combined this new data with  
110 seven previously published BG ChIP-seq TF datasets (DLX1, DLX2, DLX5, LHX6,  
111 NKX2.1, OTX2, and SP9)<sup>18-21</sup> (See Figure S1 and Online Methods for computational  
112 and experimental details). The PBX antibody used in ChIP-seq experiments detected  
113 PBX1, PBX2, and PBX3 proteins, but results here are referred to as PBX1 for simplicity.  
114 This representative TF set in this study includes: 1) TFs that establish regional identity  
115 and control proliferation and are expressed most highly in neural progenitors (ASCL1,  
116 GSX2, NR2F1, OTX2); 2) TFs that activate neurogenic transcriptional programs and are  
117 expressed most highly in the VZ-SVZ transition (DLX1, DLX2, NKX2.1), and 3) TFs that  
118 drive maturation of GABAergic neurons (ARX, DLX5, LHX6, PBX1, SP9) expressed  
119 most strongly in the SVZ and MZ, as shown by expression in single cell RNA-seq and *in*  
120 *situ* data (Figure S1b, S1c)<sup>22</sup>. In addition to diverse functions and expression patterns,  
121 these TFs capture a diverse set of TF families, including 9 homeobox TFs from 6  
122 classes: Distal-less/DLX (DLX1, DLX2, DLX5), LIM (LHX6), HOXL (GSX2), PRD (ARX,  
123 OTX2), and NKL (NKX2.1), as well as three non-homeobox TFs, ASCL1 (bHLH family),  
124 NR2F1 (COUP orphan nuclear receptor family), and SP9 (SP family, buttonhead-like  
125 ZF).

126

127 We first identified the genome-wide targets from ChIP-seq data for each TF, and then  
128 generated a merged set totaling 27,398 loci, with each locus targeted by at least one TF  
129 (see Online Methods). The full set of merged loci with TF binding included 22,297 distal  
130 putative cREs (pREs) and 5,101 promoter-proximal sites that overlapped or were less  
131 than 2kb from a TSS. TF-bound loci were enriched near genes associated with  
132 neurodevelopment and with specific functions that spanned proliferation, neurogenesis,  
133 and neuronal maturation. Individual TF ChIP-seq peak sets varied in number of peaks,  
134 percent distal versus proximal targets, and primary binding motifs (Figure 1E). All TFs  
135 except SP9 had strong enrichment of a primary binding motif centered within ChIP-seq  
136 peaks, indicating mostly direct DNA binding. SP9-bound pREs includes a subset that  
137 contain a putative SP9 primary binding motif<sup>23</sup>, suggesting that while direct binding



138 occurs, the majority of SP9 interactions here are indirect. Illustrating the challenges of  
139 inferring TF interactions via motif analysis alone, six of the TFs (ARX, DLX1, DLX2,  
140 DLX5, GSX2, and LHX6) recognize variations of a highly similar “TAATTA” motif  
141 common to many homeobox TFs<sup>24</sup>. Despite recognizing the same motif, genomic  
142 targets of these 6 TFs varied substantially (Figure S1e, S1f). Considering the set of loci  
143 targeted by these 12 representative TFs, the majority featured a peak call from only one  
144 TF (57.5%), though many pREs featured multiple TF peaks and a small subset (2.7%)  
145 included a ChIP-seq peak from 8-12 of the TFs (Figure 1B).

146  
147 To understand the cis-regulatory contexts associated with TF binding, we integrated  
148 biophysical interactions, chromatin state, and gene expression data. We performed  
149 H3K4me3-anchored Proximity Ligation-Assisted Chromatin Immunoprecipitation  
150 followed by sequencing (PLAC-seq) at 10-kb resolution. We identified 113,048  
151 significant interactions that represented 13,128 PLAC-seq contacts (PSCs). These  
152 interactions comprise 1,974 distinct “ensembles” built from chaining together inclusive  
153 sets of interacting regions (see Online Methods). Most of the interaction ensembles  
154 included only one or two PSC interactions, however, there were a substantial number of  
155 complex ensembles with 5 or more chained PSCs. Many of these complex ensembles  
156 featured extensive interactions between distal and proximal contacts, and nearly all had  
157 overlap with at least one TF-bound pRE (Figure S1g, S1h). Across TF-bound pREs,  
158 48% overlapped PSCs and 30% were within loops formed by these contacts (Figure  
159 1C). Via PLAC-seq interactions, we were able to map nearly half of the TF-bound pREs  
160 to putative regulatory target genes and evaluate relationships between TF-binding and  
161 interaction structure. We additionally segmented the genome into 9 chromatin states via  
162 ChromHMM using ChIP-seq data for H3K4me3, H3K4me1, H3K27ac, and H3K27me3  
163 (Figure S1i, S1j). Lastly, we associated published E13.5 BG RNA-seq to complete the  
164 landscape. As expected, PSCs were biased towards chromatin states featuring  
165 H3K4me3 and were associated with increased gene expression, but were also enriched  
166 for states with H3K27ac, H3K4me1, and H3K27me3, indicating chromosomal  
167 interaction data captures transcriptionally active and bivalent enhancer-promoter  
168 complexes. The *Sp9* locus is an example showing the intersection of TF binding,  
169 chromosomal interactions, and chromatin state (Figure 1D). Together, these datasets  
170 synthesize regulatory interactions between cREs and TFs, offering an integrated map of  
171 the regulome in E13.5 mouse BG.

172

### 173 **Combinatorial TF binding defines distinct enhancer and promoter pRE sets**

174

175 We next sought to define combinatorial binding patterns of the 12 TFs. Comparing peak  
176 overlap has limitations for modeling combinatorial binding due to differences in antibody  
177 and ChIP-seq performance, as well as missing differences in strength and spread of

178 signal, and in complex binding patterns. Thus, to better model TF binding patterns, we  
179 leveraged unbiased K-means clustering implemented in DeepTools<sup>25</sup>, using as input  
180 normalized ChIP-seq neighborhood signal coverage across a 1-kb region centered on  
181 TF-bound loci (Figure 2A). This approach yielded 7 clusters of promoter-proximal pREs  
182 and 18 clusters for distal pREs (Figure 2B, 2C). All TFs exhibited strong enrichment in  
183 specific pRE cluster sets, accompanied by weaker or no binding in other clusters.

184

185 There were two general binding patterns for pREs: clusters with one or two dominant  
186 TFs and an average of 1 to 2 ChIP-seq TF peaks versus clusters with multiple TF  
187 binding (“broadly-bound”) and an average of greater than two TF ChIP-seq peaks.  
188 Clusters 1\_D and 2\_D had the highest TF co-occupancy, with an average of 6-8 of the  
189 12 TFs bound per pRE (Figure 2D): DLX2 was the only TF where ChIP-seq signal was  
190 widespread across most clusters, though binding intensity varied and was absent in  
191 some clusters (e.g., 15\_D and 18\_D, which were exclusively bound by PBX1 and  
192 NR2F1, respectively). NR2F1 was the only TF whose proximal and distal interaction  
193 sets (3\_P and 18\_D) did not have a clear overlap with other TFs. Average pRE width  
194 differed by cluster (Figure 2E), which was driven by increased ChIP-seq signal intensity  
195 and/or local spread. Cis-level motif occurrences in DNA across pRE clusters mirrored  
196 TF binding (Figure 2F). For example, there was high specificity between the ChIP-seq  
197 binding for relevant TF and presence of DNA motif for ASCL1, NKX2.1, NR2F1, PBX1,  
198 and OTX2. There was expected high correlation across TAATTA motif variants for the  
199 set of TFs that bind these motifs (ARX, DLX1, DLX2, DLX3, GSX2, and LHX6), with  
200 highest rate of TAATTA motif occurrence in broadly-bound clusters 1\_D and 2\_D.  
201 Cluster 6\_D featured DLX1 binding and was strongly enriched for tandem TTAA simple  
202 repeats, consistent with repeat element binding or the “decoy” model of gene  
203 expression regulation<sup>26</sup>. We additionally tested all HOMER motifs and found numerous  
204 motifs with cluster-specific enrichment, for example Foxo1, Oct4, and Sox2 motifs in  
205 1\_D and 2\_D (Figure S3b)<sup>27</sup>.

206

207 In summary, clustering by local ChIP-seq neighborhood signal separated TF targets into  
208 distinct pRE groups with specific combinatorial TF binding signatures. pRE sequences  
209 were generally enriched for the cognate motifs of bound TFs, with the expected  
210 exception of SP9. While we expected to identify various discrete combinatorial binding  
211 patterns, somewhat surprisingly, 8% of distal pREs that comprised clusters 1\_D and  
212 2\_D were broadly bound across TFs rather than specific to a particular TF subset. Most  
213 strikingly, the small set of distal loci making up 1\_D were bound by nearly all TFs, with  
214 representation across all 9 homeobox TFs, and were further distinguished by  
215 representing the extremes for ChIP-seq signal for TAATTA-binding homeobox TFs.

216

217

218 **Combinatorial TF binding reveals cREs with distinct neurodevelopmental roles,**  
219 **cell-type specificity, and regulatory function**

220

221 We next tested if pREs with different TF binding patterns similarly exhibit differences in  
222 chromatin state, regulatory targets, cell-type specific chromatin accessibility,  
223 chromosomal interactions, and sequence features (Figure 3). We compared chromatin  
224 states across pRE clusters (Figure 3A). Proximal pRE clusters were enriched for active  
225 and bivalent promoter states (H3K4me3 with or without H3K27me3), consistent with  
226 constitutive and developmentally-regulated promoters. Distal pREs sets separated into  
227 groups with differential representation of enhancer-relevant states: no histone marks or  
228 repressed (H3K27me3), active or bivalent (H3K27ac without or with H3K27me3),  
229 inactive or poised (no marks or H3K4me1 without H3K27ac), and a mix of active,  
230 poised, and repressed states. Clusters featuring a single TF were more likely to exhibit  
231 inactive or repressed states, whereas broadly-bound pREs were active or bivalent.  
232 Exceptions were 12\_D, with ASCL1-specific TF binding and active enhancer states, and  
233 4\_D, with binding across several TFs but inactive/poised states.

234

235 We used a neighborhood-based approach (GREAT) for target gene assignment and  
236 functional annotation enrichment analysis of pRE clusters (Figure 3B). PLAC-seq  
237 defined gene target assignment showed overall agreement (Figure S3a). Beyond  
238 shared general enrichment for neurodevelopmental pathways, contrasts emerged in  
239 target genes and pathways across TF-pRE sets. Combining chromatin state-based  
240 activity inference and pathways enrichment revealed signatures of activating, mixed  
241 activating/repressive/inactive, and repressive TF-cRE modules. Broadly-bound pREs,  
242 both distal (1\_D, 2\_D, 3\_D, 7\_D, and 8\_D) and proximal (1\_P and 2\_P) regulated a  
243 specific program of subpallial development and GABAergic neuron differentiation.  
244 These pREs target genes regulating GABAergic neurogenesis and associated  
245 processes such as axon guidance and migration. Contrasting this activation, TF-pRE  
246 modules 9\_D, 3\_D, 8\_D, and 1\_P repress transcriptional programs associated with  
247 earlier embryogenesis, as well as other organ systems and CNS structures. For  
248 example, repression of skeletal and renal systems and endoderm and mesoderm  
249 programs. We identified mixed activation and repression of neural precursor  
250 proliferation directed by specific TF-pRE binding. For example, ASCL1-specific pREs  
251 (12\_D) activated neural precursor expansion, while 9\_D, 3\_D and 8\_D were associated  
252 with bivalent and repressive regulatory states for interneuron differentiation. TF-specific  
253 promoter pRE modules were most strongly enriched for housekeeping functions (e.g.,  
254 RNA splicing and DNA metabolism).

255

256 We intersected pRE clusters with a published set of open chromatin regions (OCRs)  
257 annotated to specific cell types in adult and developing forebrain via single nucleus

258 (sn)ATAC-seq<sup>28</sup> (Figure 3C). 63% of pREs overlap OCRs overall, with 26% of pREs  
259 overlapping a developmental cell-type specific OCR compared to only 3% for adult cell-  
260 type specific OCRs. Neurodevelopmental OCRs separated into cell-type specific sets  
261 for neural progenitors and distinct sub-pallial (SP) and pallial (P) populations of maturing  
262 excitatory and inhibitory neurons. The two TF-pRE modules with strongly active  
263 chromatin and the broadest TF binding, 1\_D and 2\_D were again outliers with the  
264 highest overlap with cell-type specific developmental OCRs (70% and 60%,  
265 respectively), overlapping OCRs from combined SP/P neural progenitors and three  
266 early stages of maturing SP neurons. In contrast, 12\_D, 9\_D, and 3\_D modules had  
267 reduced but still high neurodevelopmental OCR overlap, but with different cell-type OCR  
268 classes. 12\_D was enriched for neural progenitor OCRs, while 3\_D and particularly 9\_D  
269 were enriched in OCRs mapping to pallial early excitatory neurons. TF-pRE clusters  
270 further showed stage specific cis-regulatory programs within the BG, for example 12\_D  
271 and 3\_D had enrichment for progenitor OCRs while 4\_D was enriched for early and  
272 differentiating SP neuron OCRs. Overall, integration of chromatin state, functional  
273 enrichment, and snATAC-seq OCRs reveals combinatorial TF binding in embryonic BG  
274 directs activation of GABAergic neurogenesis while repressing earlier and alternative  
275 developmental programs, including repressing pallial and excitatory fates.

276  
277 Intersection of pREs and PLAC-seq interaction ensembles showed differences across  
278 TF-pRE clusters (Figure 3D). Broadly-bound pRE clusters featured higher than average  
279 rates of inclusion in PSCs and participated in ensembles with increased average  
280 number of PSCs. Among interaction ensembles with more PSCs, complex interactions  
281 between distal pREs as well as distal and proximal pREs, were common, consistent  
282 with a biophysical structure model of TF-cRE biomolecular condensates in the nucleus  
283 that increase recruitment efficiency of co-factors and RNA polymerase complexes<sup>29-31</sup>.  
284 Lastly, we compared evolutionary sequence conservation using the maximum  
285 vertebrate Phastcons element s score for each pRE (Figure 3E). Average proximal pRE  
286 cluster scores ranged from 410, equivalent to a random sample of promoter proximal  
287 intervals, to 580 for 1\_P and 2\_P. Distal clusters spanned from 365, similar to randomly  
288 selected distal regions, to the maximum conservation score of 662 for cluster 1\_D.  
289 There was a strong relationship between number of TFs bound and evolutionary  
290 conservation for distal pREs; again, cluster 1\_D was the extreme case.

291

### 292 **Enhancer activity in developing mouse telencephalon is predicted by** 293 **combinatorial TF binding**

294

295 To assess whether distal elements bound by multiple TFs are *bona fide* enhancers  
296 active in the developing BG, we identified pREs that overlapped with VISTA enhancer  
297 elements<sup>32</sup> and classified as active in SP (n=60), P (n=57), SP and P (n= 70), and non-

298 telencephalic brain (non-tel, n=75) (Figure 4E, Supplementary Table 3). As a  
299 comparison set, we included tested VISTA elements that had no reproducible enhancer  
300 activity (n=121). Distal pREs with high levels of combinatorial binding (8-12 TFs,  
301 clusters 1\_D and 2\_D) overlapped 48% of all VISTA enhancers with SP activity, 33%  
302 with SP and P activity, and 25% with P activity, but only 8% of those with non-  
303 telencephalic brain activity and 3% with no activity (Figure 4A, 4B). Conversely, VISTA  
304 elements without BG TF binding made up 53% of those with non-telencephalic activity  
305 and 64% with no activity (64%). Individual TF ChIP-seq peaks showed similar  
306 enrichment for VISTA enhancers with subpallial activity versus non-telencephalic  
307 enhancers (Figure S4a, S4d). These results indicate that broadly-bound distal pREs  
308 indeed function as enhancers *in vivo* in developing mouse telencephalon, with clear  
309 enrichment for subpallial activity. Strikingly, ChIP-seq of the 12 TFs here identified most  
310 BG-active enhancers in the VISTA database, as well as many enhancers active in other  
311 tissues. This suggests that developmental enhancers feature complex TF binding  
312 driving both activation, here in embryonic BG, as well suppression of activity in other  
313 cells and tissues.

314  
315 Elements in the VISTA database were identified via criteria that may bias this set  
316 towards general enhancer activity, such as ultra-conservation<sup>34</sup> and forebrain p300  
317 ChIP-seq<sup>35</sup>. To directly test our TF-based pRE predictions and towards generating a  
318 resource of subpallial enhancers, we identified 84 novel TF-bound pRE loci and tested  
319 them using the same transgenic enhancer assay as used in VISTA discovery<sup>32</sup>. In  
320 addition to selecting candidates based on TF binding, we also chose enhancers with  
321 PLAC-seq identified target genes that play critical roles in subpallial development,  
322 enriching the value of enhancers screened here. Individual results for these enhancers  
323 are shown in Figure S4. Similar to findings for the VISTA elements, enhancer activities  
324 of newly-tested pREs with broader BG TF binding were more likely to exhibit subpallial  
325 specificity (Figure 4C). Splitting by cluster, pREs from clusters 1\_D and 2\_D were  
326 particularly enriched for subpallial activity, while cluster 3\_D showed similar specificity in  
327 enhancer activity in the SP, SP+P and P (Figure 4D and Figure S4b, S4c). Six tested  
328 pREs representing 5 clusters are shown with ChIP-seq signal and local H3K27ac and  
329 H3K27me3 in Figure 4D. Finally, we assessed if pRE clusters had differential enhancer  
330 activity in subpallial VZ progenitors versus neurons (examples shown in Figure 4E).  
331 Cluster 1\_D had proportionally higher non-VZ versus VZ enhancer activity; 2\_D had a  
332 balance of VZ and non-VZ enhancer activity, and 3\_D had higher VZ activity (Figure  
333 4F), matching gene ontology analyses and snATAC-seq inferences above. In terms of  
334 individual TFs, only OTX2 and PBX1 showed preferential binding to enhancers active in  
335 the VZ (Figure S4e). ASCL1, DLX1, DLX5 and LHX6 preferentially bind to enhancers  
336 with activity in the SVZ/MZ. Thus, different TF-pREs modules exhibit differential activity  
337 in progenitor versus post-mitotic states and between telencephalon regions.



338

339 **Broadly-bound enhancers are characterized by TAATTA homeobox motif**  
340 **composition and deep evolutionary conservation**

341

342 To test how sequence determinants distinguish the combinatorial TF binding clusters,  
343 we compared frequency of motif occurrence, spacing and position of motif pairs (i.e.,  
344 motif “grammar”), and base-level evolutionary conservation (vertebrate PhyloP score) of  
345 motif and flanking DNA (Figure 5). For this analysis, we generated a merged set of  
346 TAATTA motifs. We further separated TAATTA motifs into “symmetric” instances, where  
347 the motif was identified as overlapping occurrences on sense and antisense strands  
348 (i.e., palindromic), “degenerate” instances identified on only one strand, and “complex”  
349 overlapping instances that largely map to simple “TTAA” repeats. The majority of  
350 TAATTA motifs identified in random regions are degenerate, suggesting symmetric  
351 instances are more likely to be functional. Only 6\_D was enriched for complex TAATTA  
352 motifs, consistent with these pREs harboring simple TTAA repeats. The average  
353 number of TAATTA motifs within pRE had significant range across clusters, and both  
354 motif counts and relative symmetric:degenerate motif ratio showed cluster-specific  
355 patterns (Figure 5A). 1\_D averaged nearly four TAATTA instances per pRE and had the  
356 largest shift towards symmetric over degenerate instances. These patterns show that  
357 1\_D represents an extreme in both binding patterns and sequence composition. The  
358 other broadly bound distal clusters of 2\_D, 3\_D, and 4\_D also all averaged multiple  
359 TAATTA instances and increased ratio of symmetric motifs.

360

361 We next examined spacing and orientation of motif pairs within pREs across clusters.  
362 Overall, cluster-relevant motif pairs were likely to be closely spaced (i.e., within 20-200  
363 bp distance) within pREs (Figure 5B). This is most obvious for pRE clusters with more  
364 motif instances. For example, TAATTA-TAATTA pairs were located in spatial proximity  
365 within pRE clusters featuring broad TF binding, epitomized by 1\_D. 1\_D also exhibited  
366 reduced but still clear proximity between TAATTA-NKX2.1 and TAATTA-PBX1 pairs,  
367 though not for TAATTA-ASCL1. In comparison, 12\_D, bound specifically by ASCL1,  
368 showed clustered ASCL1-ASCL1, but not TAATTA-TAATTA, pairs. There were also  
369 differences in TAATTA-TAATTA pairs across clusters. For example, 4\_D and 16\_D both  
370 feature similar average number of TAATTA instances, but TAATTA-TAATTA pairs are  
371 more likely immediately adjacent in 16\_D, with 26% of pREs featuring a pair of TAATTA  
372 motifs within 4 bp compared to 8% in 4\_D and 10% in 1\_D. We did not identify  
373 canonical orientation or spacing rules for motif pairs in any of the TF-pRE clusters, as  
374 would be predicted if TF complex binding was determined by a “syntax” of motifs with  
375 deterministic spacing. At the upper end of the spectrum for motif density, 1\_D featured  
376 clustered, and at times overlapping, sets of motifs including the primary motifs of TFs  
377 studied here as well as other motifs in the HOMER database. Overall, our findings are



378 consistent with a “billboard” model, where relevant DNA binding sequences are  
379 clustered within a core region of the regulatory DNA element, but do not conform to  
380 strict patterns of orientation or syntax<sup>36</sup>.

381

382 Lastly, we examined base-level PhyloP vertebrate conservation of TF motifs within  
383 pREs (Figure 5C). Overall, motifs expected to be relevant for TF binding showed  
384 increased base-level conservation in line with motif position weight matrix and reduced  
385 relative conservation for flanking sequences, indicating purifying selection on bases  
386 critical for TF binding (see Figure S5a for complete motif by cluster analysis). For  
387 example, average base-level conservation for DLX2 primary motif shows strong  
388 conservation for the TAATTA bases compared to flanking sequence for both cluster  
389 1\_D and 16\_D. Base-level motif conservation also showed differences across pRE  
390 clusters bound by the TF. While background conservation is significantly higher in 1\_D  
391 versus 16\_D, the TAATTA core also shows a larger increase relative to background in  
392 1\_D. For DLX2 TAATTA motifs in 1\_D, increased base-level conservation of flanking  
393 DNA gradually decreases out to +/- 200bp, indicating a conserved core region within  
394 these pREs. We next expanded base-level motif conservation analysis to all motifs in  
395 the Homer database that were enriched in each pRE cluster. Considering the evidence  
396 for the central role of TAATTA motifs in 1\_D, we wondered if similarly strong base-level  
397 conservation was present across other motifs. Motifs from several TF families indeed  
398 exhibited increased conservation, for example HOXD11/Hox, FOXA1/Forkhead,  
399 OCT6/OCT, and SOX6/SOX (Figure 5D). Among motifs with high base-level  
400 conservation in 1\_D, TAATTA motifs are at the top of the range, cementing a special  
401 role for TAATTA motifs and TFs that bind these sequences in 1\_D enhancers.

402

403 Our results indicate that at the sequence level, 1\_D pREs are characterized by  
404 increased number of symmetric TAATTA motifs, which are located in proximity to each  
405 other and to other motifs in a highly-conserved and motif-rich core region. To illustrate  
406 these patterns, four representative newly-characterized 1\_D pREs are depicted in  
407 Figure 5E. These 1\_D enhancers capture the overall patterns of this cluster, giving  
408 examples of the motif clustering but variability of spacing and organization as well as  
409 the base-level motif conservation both across the enhancer core as well as of critical  
410 nucleotides within each motif. These results provide evidence that enhancers at the  
411 extreme upper end of evolutionary conservation indeed feature dense hubs of cis-trans  
412 regulatory interactions during embryonic development that are likely to drive strong  
413 selective constraint.

414

415 Our results highlight the complex regulomes of neurodevelopmental TFs targeted by the  
416 TFs profiled here, potentially associating distinct genes via common chromatin  
417 interaction ensembles. Actively regulated chromosomal regions, particularly those

418 harboring TF genes, feature complex physical interaction landscapes anchored by  
419 differential combinations of TFs that act to activate or repress enhancer activity in the  
420 developing BG. As an example, for the interaction ensemble including the *Pbx1* gene,  
421 we identified 31 pREs that serve as PSCs (Figure 6A) and transgenic testing verified  
422 activity for three enhancers predicted to be activated in BG and corresponding pallial or  
423 non-CNS activity for enhancers correctly predicted to be repressed in BG (Figure 6B).

424

## 425 DISCUSSION

426

427 Application of epigenomic profiling resolved to cell type specificity have revolutionized  
428 understanding of cis-regulatory landscapes underlying neurodevelopment, including in  
429 the embryonic BG<sup>37</sup>. Missing from these advances has been parallel comprehensive  
430 understanding of TF components of gene regulatory wiring, and, specifically, how  
431 combinatorial TF binding to cREs directs transcriptional activation and repression.  
432 Studies focused on individual or a small set of TFs have provided valuable insights  
433 about necessity and sufficiency of specific TFs during neurodevelopment and in the BG<sup>1</sup>  
434 and cortex<sup>38</sup>. Yet an integrated perspective of TF binding during brain development has  
435 been lacking due to technical and computational barriers, particularly for ChIP-seq on  
436 *ex vivo* embryonic brain tissues. *In silico* and *in vitro* efforts to use TF co-expression and  
437 motif analyses have provided insights into contributions of TF networks even without  
438 assaying genomic interactions<sup>39–41</sup>. However, modeling roles of TFs without  
439 interrogating genomic binding is problematic due to shared target motifs and  
440 overlapping expression within homeobox and other TF families, and due to presence of  
441 indirect TF interactions with regulatory DNA. Further complicating functional modeling,  
442 our results corroborate previous understanding that not all TF binding events are  
443 equivalent regarding regulatory function<sup>42,43</sup>. Illustrating these complexities, all tested  
444 TFs (except for NR2F1) participated in more than one distinct combinatorial TF binding  
445 pattern that had different functional roles, six of the homeobox TFs bound the same  
446 family of TAATTA motifs, SP9 primarily bound cREs via indirect mechanisms. This  
447 study revealed patterns of TF-cRE interaction and regulatory function that would be  
448 masked if only considering chromatin accessibility or individual TF binding patterns.  
449 This work represents an initial build of an embryonic BG “regulome” made up of TF-cRE  
450 modules that integrates information for TF binding, chromosomal interactions, chromatin  
451 state and transcriptional activity, and cRE regulatory function.

452

453 A motivating factor for this study was to define complex patterns of the combinatorial TF  
454 binding in the developing brain, going beyond previous work on individual TFs to build  
455 models similar to efforts in other organisms and for *in vitro* systems<sup>50–53</sup>. We had  
456 previously found that DLX TFs had a largely overlapping set of binding targets, and  
457 NKX2-1 and LHX6 had overlapping and distinct binding targets<sup>18,20</sup>. Here we combined

458 published and novel TF ChIP-seq across twelve TFs, capturing diversity of TF families  
459 and neurodevelopmental function. Our work illustrates context-dependent combinatorial  
460 TF control of activating and repressive cRE modules and gene regulatory programs  
461 across proliferating and post-mitotic neuronal populations in embryonic mouse BG  
462 (Figure 7A). We show that TF-cRE interactions are required for proper activation and  
463 repression of enhancer activity associated with sub-pallial regional identity, GABAergic  
464 neurogenesis, and cortical interneuron specification (Figure 7B). These different TF-  
465 cRE modules act in parallel to generating complex developmental expression patterns,  
466 for example at the *Pbx1* locus (Figure 7C). These results reveal the molecular and  
467 genomic basis underlying findings from earlier TF knockout mouse studies for *Gsx2*,  
468 *Nkx2-1*, and *Otx2* showing shifts in telencephalic regional identities<sup>21,27,44</sup>, and for *Arx*,  
469 *Ascl1*, *Dlx1/2/5*, *Nr2f1*, *Pbx1*, and *Sp9* showing decreased GABAergic neurogenesis  
470 and differentiation<sup>19,22,45–49</sup>. Our integrated characterization of embryonic BG TF-pRE  
471 modules and the 74 newly-defined enhancers represent a rich resource for future  
472 studies of transcriptional control of enhancer function, enhancer-driven cell labeling, and  
473 neuronal fate mapping.

474

475 Among the most important insights from our analysis was the overlap and strength of  
476 TF binding across 11 of the 12 TFs at a relatively small set of distal pREs. These  
477 findings support previous studies that TF binding overlap is associated with increased  
478 enhancer activity and conservation<sup>4,54,55</sup>. For example, pREs with OCT4, SOX2, and  
479 NANOG combined binding had stronger evolutionary conservation and enhancer  
480 function in ESCs than sites with single TF binding<sup>56</sup>. Indeed, 1\_D included an  
481 enrichment for elements that were identified as among the most conserved non-coding  
482 sequences in the mouse genome<sup>57</sup>. Our findings build context around observations  
483 made regarding what have been deemed “ultraconserved” enhancers, including that  
484 these enhancers are key partners for developmental TFs, are strongly enriched for  
485 activity in the developing brain, that they are enriched for TA-rich motifs and robustly  
486 bound in vivo by homeobox TFs in this study<sup>58–60</sup>, but whose defects are nonetheless  
487 non-lethal<sup>61</sup>. To this picture, our results show that BG-active deeply-conserved  
488 enhancers are broadly bound by TFs, feature rich and often overlapping motif  
489 composition and extreme base-level conservation (Figure 7D). Our results show that  
490 homeobox TFs, and TAATTA-binding TFs in particular, bind this set of ancestral  
491 enhancers, which regulate genes that act at the top of signaling pathways that regulate  
492 the regulators of brain development<sup>62,63</sup>. Our study further supports the model where  
493 these extensive TF-enhancer interactions are involved in establishing and maintaining  
494 complex transcription-associated local biophysical interactions<sup>64</sup>. Our results frame a  
495 model where cREs that are broadly TF bound during neurodevelopment represent the  
496 extreme end of the spectrum for sequence composition, conservation, and regulatory  
497 activity, and as such are exceptional and ancient class of enhancers.

## 498 **AUTHOR CONTRIBUTIONS**

499 Conceptualization, R.C.-P., A.S.N., S.L., and J.L.R.R.; Methodology and Investigation,  
500 S.L. (ChIP-seq, RNA-seq), Y.S. (PLAC-seq experimental), A.A., M.H. (PLAC-seq  
501 computational), J.D.P. and L.S.-F. (RNA-seq), D.E.D., L.A.P., and A.V. (enhancers in  
502 mice), and R.C.-P. and Y.W. (bioinformatics, modeling); Software, R.C.-P., A.S.N., and  
503 A.A.; Formal Analysis, R.C.-P., A.S.N., S.L., and J.L.R.R.; Writing – Original Draft, R.C.-  
504 P., S.L., A.S.N., and J.L.R.R.; Writing – Review & Editing, R.C.-P., S.L., J.D.P., D.E.D.,  
505 A.S.N., and J.L.R.R.; Funding Acquisition, J.L.R.R. and A.S.N.; Supervision, J.L.R.R.  
506 and A.S.N.

507

508

## 509 **STATEMENT OF COMPETING INTERESTS**

510 J.L.R.R. is co-founder, stockholder, and currently on the scientific board of Neurona, a  
511 company studying the potential therapeutic use of interneuron transplantation. The  
512 other authors declare no competing interests.

513

514

## 515 **ACKNOWLEDGEMENTS**

516 University of California, San Francisco (UCSF) and Davis (UCD) cores supported this  
517 work. Sequencing was carried out at the UCSF Center for Advanced Technology and  
518 IHG Genomics Core, UCD DNA Core. R.C.-P. was supported by a Science Without  
519 Borders Fellowship (Conselho Nacional de Desenvolvimento Científico e Tecnológico -  
520 CNPq, Brazil). L.S.-F. was supported by the UCD Schwall Fellowship in Medical  
521 Research and Werner and Jacobsen Fellowship, and National Institute of General  
522 Medical Sciences (NIGMS)-NIH T32-GM008799. This work was supported by the  
523 following research grants: National Institute of Mental Health (NIMH) R01 MH081880  
524 and NIMH R37/R01 MH049428 (to J.L.R.R.); NIH/NIGMS R35 GM119831 (to A.S.N.);  
525 NIH U01DA052713 (to Y.S.). A.V., L.A.P., and D.E.D. were supported by NIH grants  
526 R01MH117106, R01HG003988, and R01NS062859. Research was conducted at the  
527 E.O. Lawrence Berkeley National Laboratory and performed under Department of  
528 Energy Contract DE-AC02-05CH11231, University of California.

529

530

## 531 **SUPPLEMENTARY MATERIAL**

532

533 Supplementary Tables S1-S6

534 Supplementary Figure S1 (Related to Figure 1)

535 Supplementary Figure S2 (Related to Figure 2)

536 Supplementary Figure S3 (Related to Figure 3)

537 Supplementary Figure S4 (Related to Figure 4)

538 Supplementary Figure S5 (Related to Figure 5)

539  
540  
541  
542  
543  
544  
545  
546  
547  
548  
549  
550  
551  
552  
553  
554  
555  
556

## FIGURE LEGENDS

**Figure 1 – TF Binding Profiles and Basic Genomic Features.** (A) Schematics of the scope of the present study, showing 3D structures combinatorially bound by TFs in transcriptionally active chromatin (H3K4me3-marked). The model was validated by enhancer transgenic mouse assays. (B) Distribution of combined bound loci between distal and proximal regions, segmented by the number of TFs sharing locus position. (C) Pie chart showing the distribution of loci in relation to the loops formed by H3K4me3-mediated PLAC-seq contacts (PSC). (D) *Sp9* locus showing TF binding and PLAC-seq interactions with VISTA enhancers *hs242*, *hs243*, *hs244*, *hs245*, *hs574*, *hs860*, and *hs953*. PLAC-seq contacts are displayed as arcs and contact maps (adj.  $p < 0.01$ ). (E) Doughnut plot showing individual TF number of binding loci, split into proximal and distal from gene TSS, with the associated core motifs and their average distribution around peak centers. In parentheses are the enrichments over background and percent of target motifs. See also Figure S1.

## Figure 2 – Organization of Bound Loci into Clusters of Similar Binding

**Neighborhood Profiles.** (A) Schematics showing the several binding patterns captured by investigating local neighborhood around ChIP-seq peak summits, as well the subsequent clustering and following genomic profile characterization. (B) and (C) Heatmaps representing each TF coverage around 1 kb of each called peak in proximal and distal regions, respectively. Row blocks and columns depict TFs and clusters, respectively. Within each row block, each line represents the coverage color-codes for intensity of ChIP-seq signal (intensity grows in the black-to-yellow-to-red direction). Within each cluster, lines with same position across the TFs represent the same genomic locus. Numbers (n = ) indicate the number of peaks called within each cluster. (D) Distribution of mean number of TFs bound to each locus across clusters. Mean distributions were calculated by sampling without replacement (N=1000). Random means was calculated by randomly sampling the genome (N=27398). (E) Distribution of mean number of TFs sharing loci across binding clusters, and compared to a random sample, calculated by sampling without replacement (N=1000). (F) Heatmap showing the relative enrichment of core binding motifs for each of the TFs across clusters. See also Figure S2.

## Figure 3 – Genomic and Functional Features of TF-Bound RE Clusters.

(A) Frequency of occurrence of peaks across chromatin states and binding clusters, split into distal and proximal. As reference, in between the two heatmaps is one derived from assigning chromatin states to a random loci sample. Color codes represent the percentage of peaks by cluster. (B) Gene ontology analysis of genes hitting interaction



580 contact points (PSC) split into clusters, displaying select brain-specific or general terms.  
581 **(C)** Bar plot depicting the intersection of our binding clustering with clusters determined  
582 by single-nucleus ATAC-seq<sup>28</sup>, showing putative neuronal cell differentiation states  
583 across binding clusters. **(D)** Dot plot showing the mean ensemble size across binding  
584 clusters in function of the percent of loci in the cluster colocalizing with PSCs. **(E)**  
585 Distribution of means of PhastCons scores across clusters for 60 vertebrates. Top  
586 panels are distal peaks, and proximal ones are on the bottom panels. References were  
587 random distal and proximal genomic regions of random widths. See also Figure S3.

588

589 **Figure 4 – VISTA and Novel Enhancer Activity across Bound Loci.** **(A)** Heatmap of  
590 combinatorial TF binding (percentage enrichment, 0-12 TFs) on VISTA enhancers that  
591 have subpallial (SP), pallial and subpallial (SP+P), pallial (P), non-telencephalic (non-  
592 tel), and no activity (inactive). **(B)** Stacked bar plot showing the percentage of newly  
593 identified regulatory sequences with high (8-12 TFs), intermediate (5-7 TFs), low (3-4  
594 TFs), and very low (1 TF) binding in BG showing spatial regional activity. **(C)** Stacked  
595 bar plot depicting percentage of novel enhancers across binding clusters showing  
596 restricted spatial enhancer activity in the subpallium, pallium and shared among them.  
597 **(D)** Six pREs representing different clusters that were tested for activity in transgenic  
598 mouse assays<sup>33</sup>. Clusters are shown in the left column, enhancer names are written in  
599 turquoise, and the success ratios are listed next to the name (i.e., 6/6 depicts 6 embryos  
600 with forebrain activity out of 6 embryos tested). Schemas predict the regulated genes by  
601 the tested enhancers (turquoise). The grey arrow depicts the orientation of the TSS.  
602 Green bars show the normalized binding of BG TFs, with color intensity proportional to  
603 ChIP-seq intensity. The specific TFs bound are shown above the top bar. Wholemounds  
604 (WM) and three sections representing the LacZ expression are shown. H3K27ac  
605 (green) and H3K27me3 (red) histone ChIP-seq results from the GE are shown to the  
606 right; the turquoise bars correspond to the tested genomic regions. Cx: Cortex; GE:  
607 Ganglionic Eminences; L: LGE; M: MGE; C: CGE. **(E)** Coronal brain section  
608 schematization showing: 1. the subregions of the primordial BG (LGE and MGE) as well  
609 as the cortex (top left hemisection) ; 2. the subregional laminae of the GEs (VZ, SVZ,  
610 and MZ; top right hemisection). Hemisections from 2 VISTA enhancers with specific sub  
611 regional activity are shown below with *hs1056* showing activity in the VZ and SVZ of the  
612 MGE (bottom left hemisection) and *hs566* showing activity in the mantle zones of the  
613 MGE and LGE (bottom right hemisection). **(F)** Bar plot depicting cluster classification of  
614 enhancers with VZ and non-VZ activity (n=99). See also Figure S4.

615

616 **Figure 5 – Arrayed TAATTA motifs anchor deeply-conserved GABAergic**  
617 **enhancers.** **(A)** Relative number of TAATTA motifs within each RE across clusters  
618 separated by symmetric, degenerate, and complex instances. **(B)** Histogram showing  
619 distribution of distance in base pairs between all motif pair occurrences within REs for



620 selected clusters and motif pairs. **(C)** Average base-level sequence conservation  
621 (vertebrate PhyloP score) for TAATTA motif and flanking DNA for 1\_D (top) and 16\_D  
622 (bottom left) within 10bp of motif, and for 1\_D out to 400bp of motif (bottom right). **(D)**  
623 TAATTA motifs exhibit the strongest base-level conservation across TF motif families  
624 enriched in 1\_D REs. Enriched motifs with significant base-level conservation increase  
625 compared to 10bp flanking sequence labeled, primary motifs from BG TFs in bold. **(E)**  
626 Four representative 1\_D REs with enhancer activity. Target gene, evolutionary  
627 conservation, BG TF binding, and enhancer activity in E12.5 mouse telencephalon. **(F)**  
628 Motif and evolutionary conservation landscape for enhancers in (d) showing motif  
629 clustering and overlap with conserved regions across core 500bp (top) and at single-  
630 base resolution (bottom) for selected intervals. Legend shows colors for BG TF primary  
631 motifs and all Homer motifs. See also Figure S5.

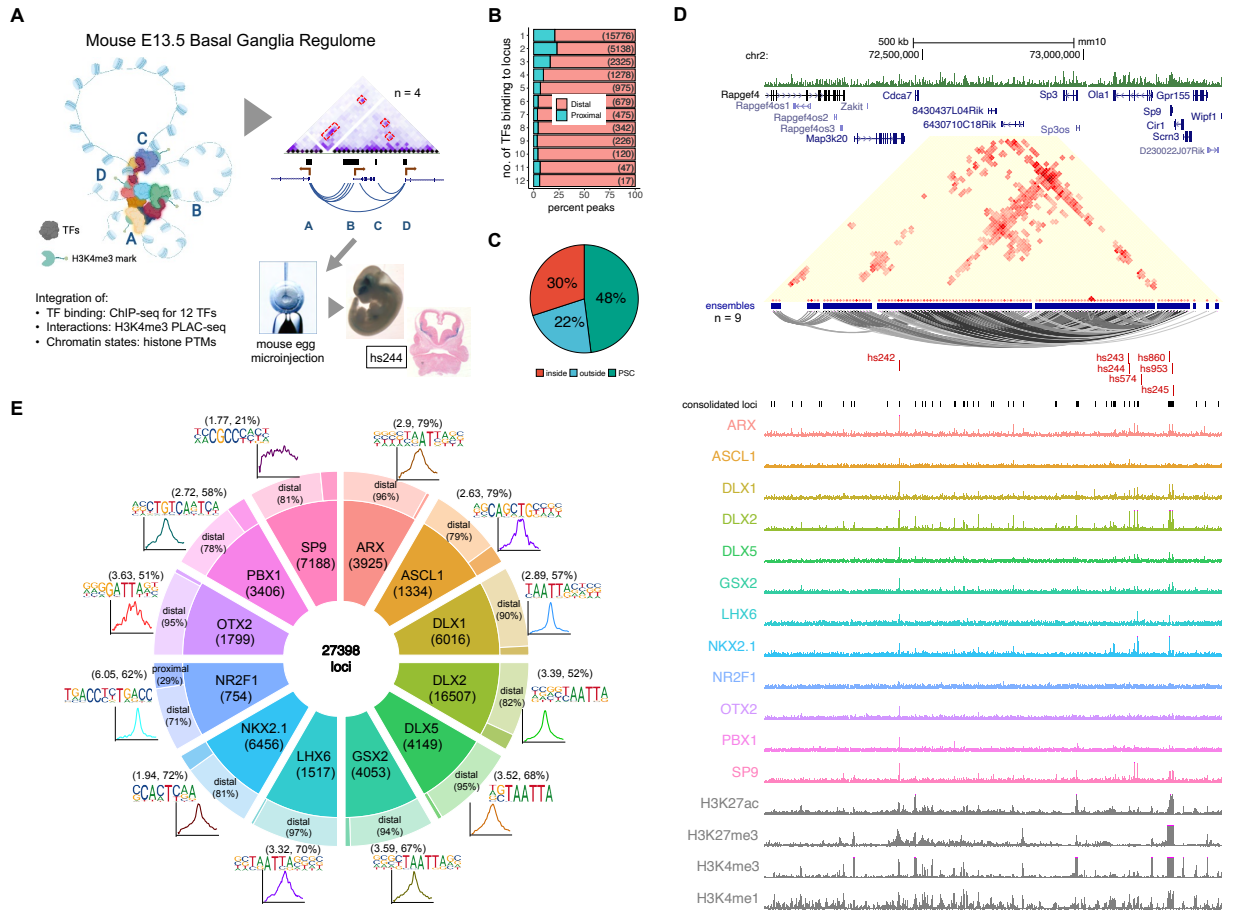
632

633 **Figure 6 – *Pbx1* Genomic Locus and Associated Putative Enhancers. (A)**  
634 Representation of the *Pbx1* locus, showing nearby genes, and the ensemble of PLAC-  
635 seq contacts creating a tridimensional structure made of multiple loops. Bound genomic  
636 loci are noted underneath in black, and enhancers are marked in turquoise and red  
637 (active and inactive, respectively). **(B)** Six enhancers around the *Pbx1* locus exhibited  
638 activity in transgenic mouse assays. Of these, 1\_D and 2\_D enhancers were active in  
639 subpallium, while one 9\_D enhancer was active in in pallium and the other 9\_D and a  
640 4\_D enhancer showed non-telencephalic activity.

641

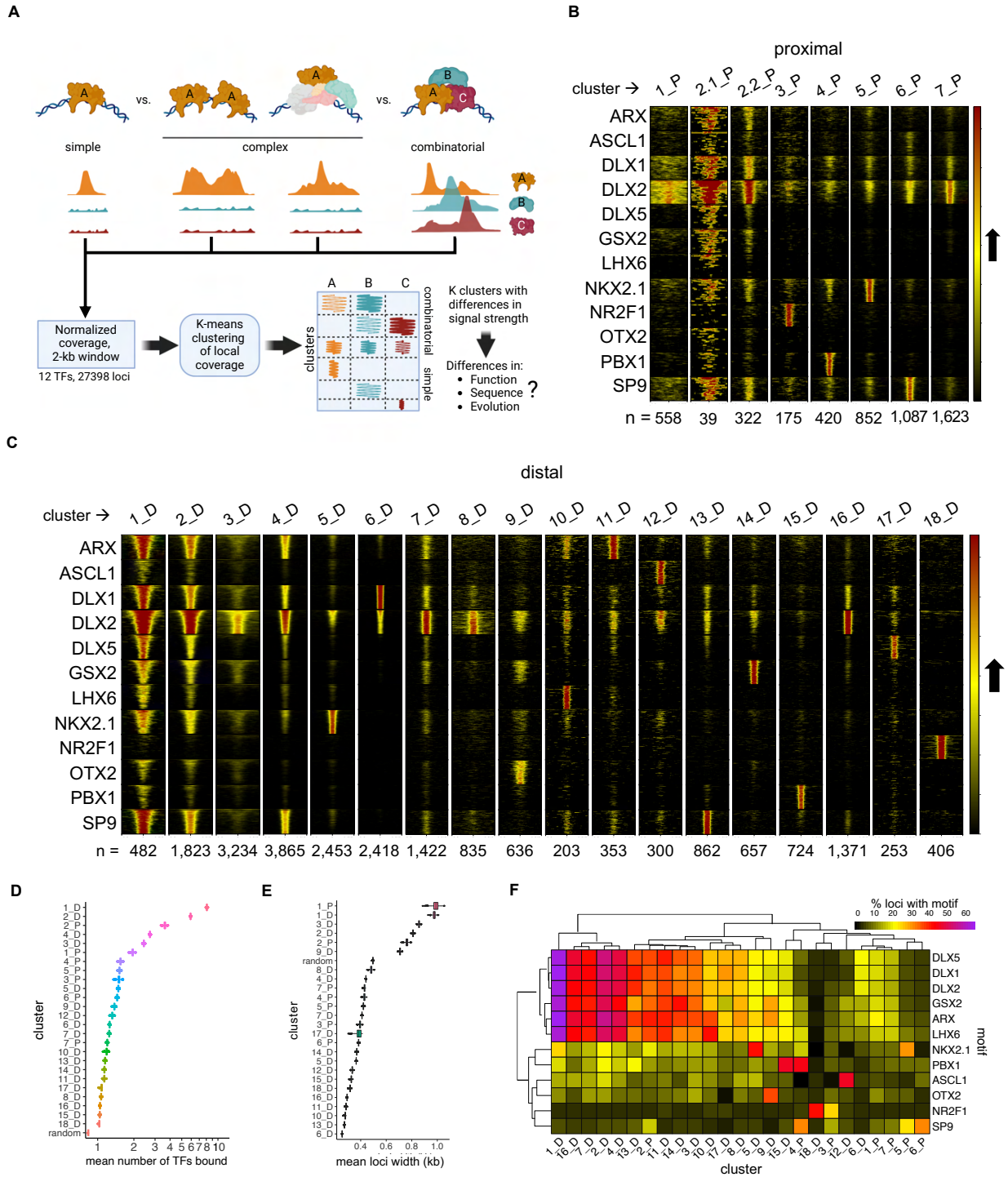
642 **Figure 7 – Cis-trans interactions underlying gene regulation driving GABAergic**  
643 **neurogenesis. (A)** Chromatin accessibility maps identify pREs, but TF binding is  
644 necessary to understand mechanisms and functional relevance of pRE activity. TF  
645 binding can direct either activation or repression of enhancer activity. Here we identify  
646 pRE-TF modules that drive specific regulatory activity in developing mouse BG, with  
647 representative examples depicted. Bold BG modules in (A) are highlighted in (B). **(B)**  
648 Combinatorial TF binding defines context-dependent patterns of enhancer activation  
649 and repression in embryonic BG. Three example cis-trans modules identified here are  
650 shown, with the enhancer activity and schematic of activity across VZ, SVZ, and MZ.  
651 **(C)** Developmental TF genes (i.e. *Pbx1*) relevant to embryonic BG have complex cis-  
652 regulatory landscapes and generally include multiple cis-trans regulatory modules. **(D)**  
653 Comparison of enhancers with simple versus complex TF binding identified in  
654 embryonic BG. Enhancers with exceptional TF binding also feature high density of TF  
655 binding motifs, complex chromosomal contacts, strong evolutionary conservation across  
656 the vertebrate tree (human, chicken, zebrafish conservation represented), and  
657 increased base pair size. Abbreviations: Imm. CIN: immature cortical interneurons, Imm.  
658 PN: immature projection neurons, BG NPC: basal ganglia neural progenitor cell, BG  
659 IPC: basal ganglia intermediate progenitor cell.

660 **Figure 1**  
661



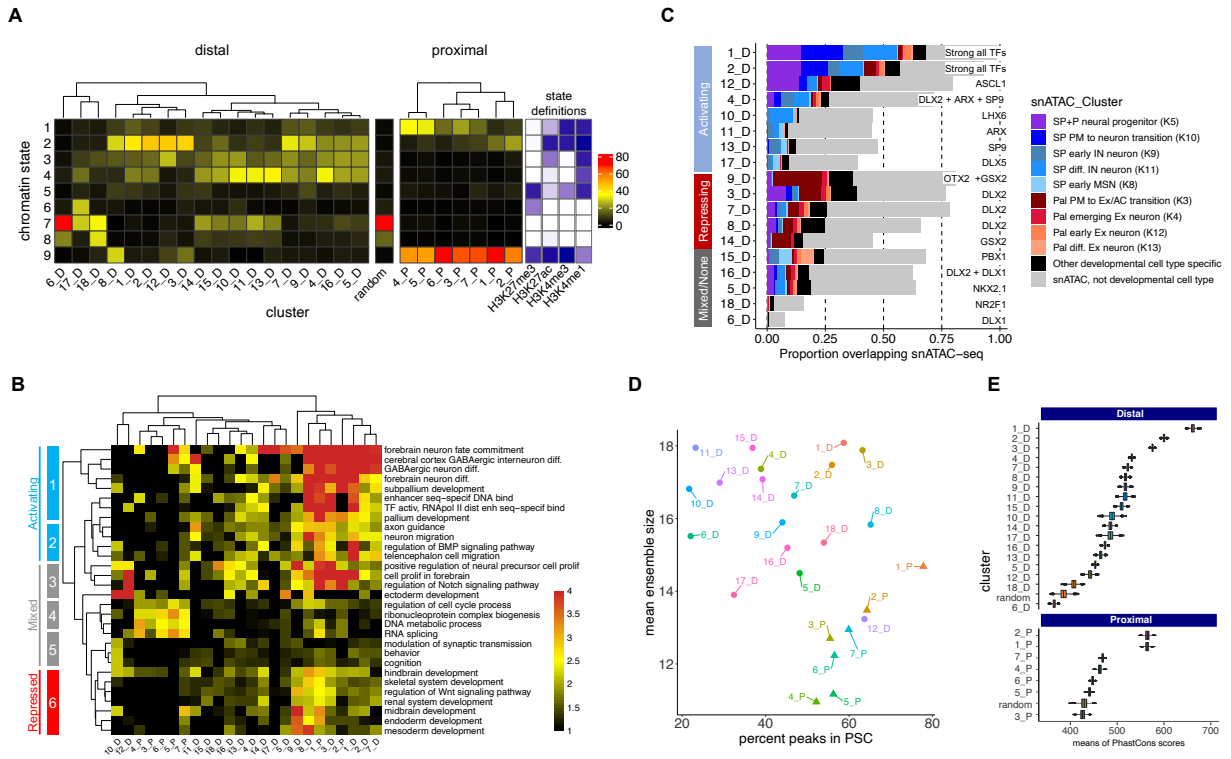
662  
663

664 **Figure 2**  
665



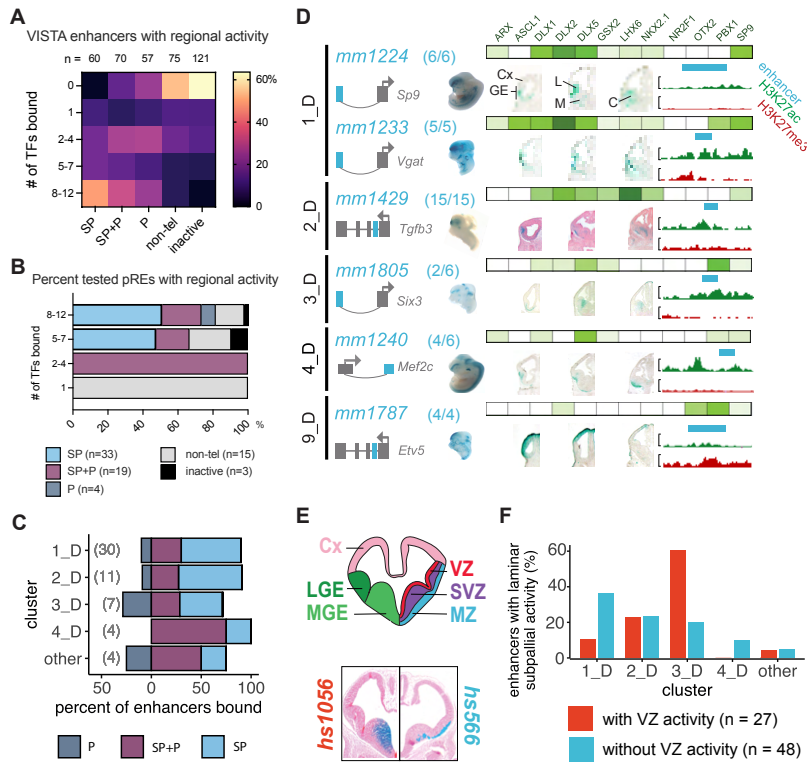
666  
667

668 **Figure 3**  
669



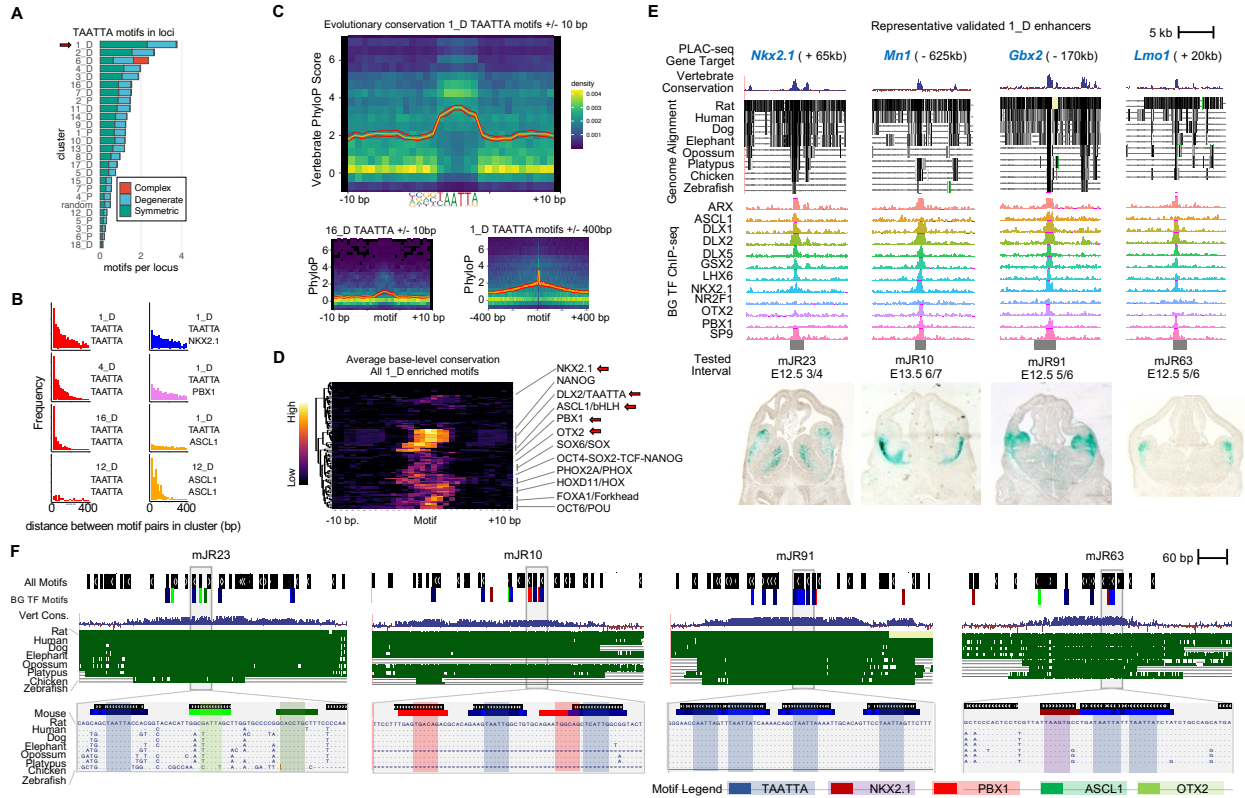
670  
671

672 **Figure 4**  
673



674  
675

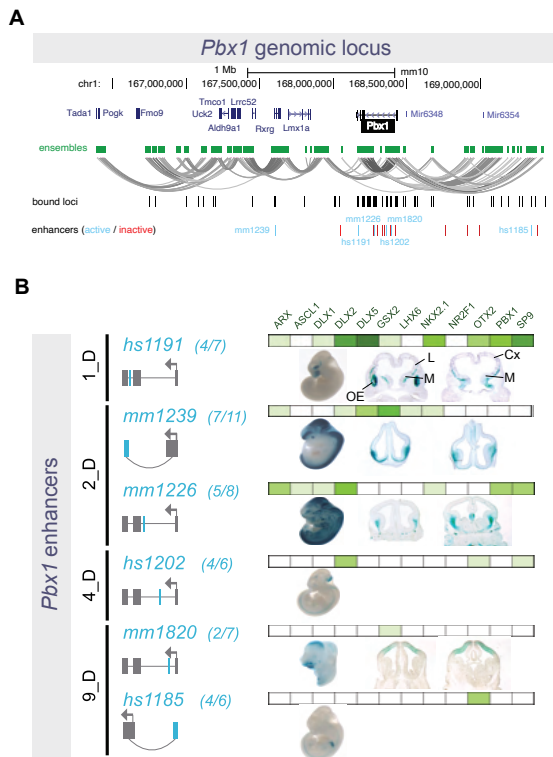
676 **Figure 5**  
677



678  
679



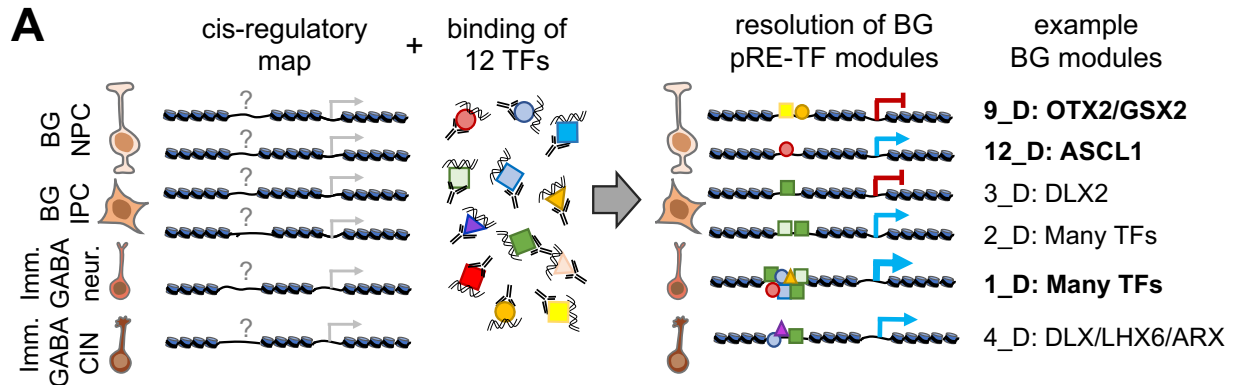
680 **Figure 6**  
681



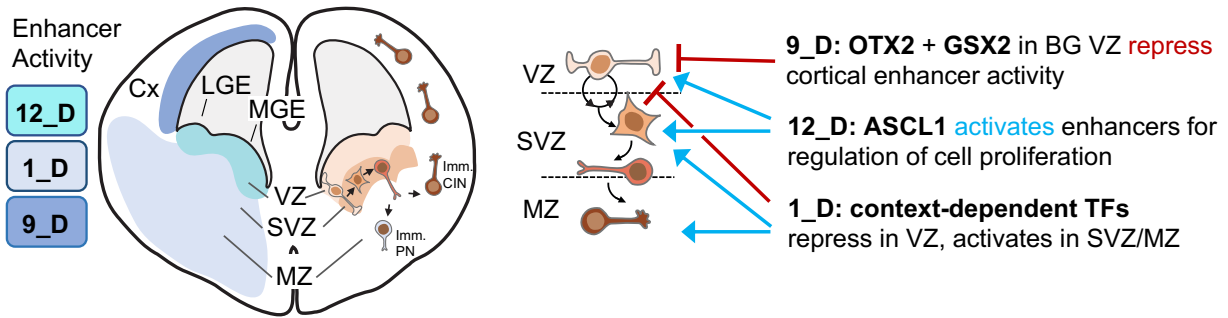
682  
683

684  
685

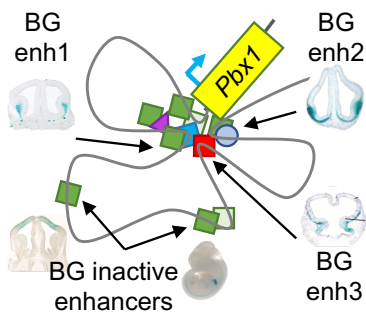
**Figure 7**



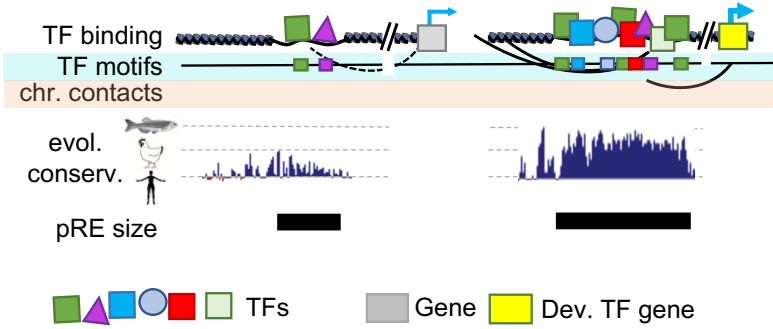
**B** Combinatorial TF binding controls context-dependent enhancer activation/repression in BG



**C** complex cis-trans ensembles (e.g. *Pbx1*)



**D** simple TF Binding vs exceptional TF binding



686  
687

688

## 689 MATERIAL AND METHODS

690

### 691 *Experimental Model and Subject Details*

692

#### 693 Mice

694 All procedures and animal care were approved and performed in accordance with  
695 National Institutes of Health and the University of California San Francisco Laboratory  
696 Animal Research Center (LARC) guidelines. For the RNA-seq experiment, an equal  
697 number of males and females were used. ChIP-seq and native histone ChIP-seq was  
698 performed on *Mus musculus* CD1 strain at developmental stage E13.5. The embryos  
699 were not assessed genotypically for gender since we used a pool of embryos and  
700 therefore expect a roughly equal number of male and females.

701

### 702 *Method Details*

703

#### 704 TF Chromatin Immunoprecipitation (ChIP)

705 ChIP was performed using antibodies against DLX1, DLX2, DLX5<sup>18</sup>, NKX2.1 (Santa  
706 Cruz Biotechnology, Cat# sc-13040), LHX6<sup>20</sup>, OTX2 (Published in Hoch, Lindtner, Price  
707 and Rubenstein - R&D, Cat# AF1979), SP9<sup>65</sup>, ARX (Santa Cruz, Cat# sc-48843),  
708 ASCL1 (BD, Cat# 556604), GSX2<sup>66</sup>, NR2F1 (R&D biosystems, Cat# PP-H8132-10),  
709 and PBX1/2/3 (Santa Cruz, Cat# sc-888). Basal ganglia were dissected in cold PBS  
710 from CD1 embryos (2 L/Ab for DLX2, SP9, NR2F1; 3 L/Ab for DLX1, DLX5, NKX2.1,  
711 LHX6, ARX, ASCL1, and GSX2; 2 L/Ab for OTX2). The basal ganglia consisted of the  
712 LGE, MGE and CGE progenitor and mantle zones except the NKX2.1 and LHX6 ChIPs  
713 for which the medial ganglia were used. The dissected basal ganglia were either fixed in  
714 1% formaldehyde at RT for 10 min (LHX6, NKX2.1, OTX2, PBX, SP9) or fixed in 1.5%  
715 formaldehyde at RT for 20 min (ARX, ASCL1, DLX1, DLX2, DLX5, GSX2, NR2F1,  
716 OTX2), neutralized with glycine, and washed gently in PBS. The fixed cells were lysed  
717 with a hypotonic buffer (50 mM Tris pH 7.5 / 0.5% NP40 / 0.25% sodium deoxycholate /  
718 0.1% SDS / 150 mM NaCl) to obtain the nuclei; these were then lysed in 1% SDS buffer  
719 and the chromatin was sheared into 300-1000 bp fragments by sonicating for 40 cycles  
720 (30 sec on and 45 sec off) using a bioruptor (Diagenode). Immunoprecipitation (IP)  
721 reactions were performed with the sheared chromatin diluted 1/10 times with "dilution  
722 buffer" (0.01% SDS, 1.1% Triton X- 100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1,  
723 167mM NaCl, usually in 6 ml. Antibody was then added to either 5 µg (ARX, DLX1,  
724 DLX2, DLX5, NR2F1, OTX2, PBX, SP9) or 8 µg (ACSL1, GSX2, LHX6, NKX2.1)  
725 specific antibodies. Negative control ChIP reactions used either IgG (5µg) or blocking  
726 peptide (DLX antigen used for immunizing rabbits; 50x molar excess, ARX, LHX6,  
727 NKX2.1, PBX 400x molar excess). Antibody/chromatin complexes were purified using

728 Dynabeads (Invitrogen) and washed extensively in “wash buffer” (low salt, 0.1% SDS,  
729 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl; high salt, 0.1%  
730 SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl; LiCl, 0.25  
731 M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris,  
732 pH 8.1 and TE).

733

734 Complexes were eluted with 1% SDS, 10 mM sodium bicarbonate buffer at 65 °C for  
735 10 min. Eluted chromatin was reverse-crosslinked overnight at 65 °C in the presence of  
736 500 mM NaCl, then subsequently treated with RNase (10 µg/200 µL reaction, 15 min at  
737 37 °C) and Proteinase K (10 µg/200 µL reaction, 60 min at 55 °C) and cleaned using a  
738 ChIP DNA Clean & Concentrator kit (Zymo Research). The chromatin was quality-  
739 controlled (QC) using qPCR to check for enrichment of genomic DNA fragments that  
740 were expected, and not expected, to have the different TF binding.

741

742 Libraries were prepared using an Ovation Ultralow DR Multiplex System (Nugen), size-  
743 selected in the range of 300 bp on a chip from BluePippin (Sage Science) and lastly QC  
744 tested on a Bioanalyzer (Agilent). The libraries were sequenced as single-end 50-bp  
745 reads on a HiSeq 4000 (Illumina) at the Center for Advanced Technology (UCSF).

746

#### 747 Native histone ChIP

748 Each native histone ChIP was performed starting with ~250,000 nuclei from WT E13.5  
749 basal ganglia. The native ChIP was performed as described earlier<sup>18</sup>. Briefly, nuclei  
750 were extracted and digested with micrococcal nuclease (MNase, Sigma). A population  
751 of mono- and di-nucleosomes were used in chromatin immunoprecipitation assays.  
752 Antibodies used were specific to H3 monomethyl lysine-4 (H3K4me1, Abcam, ab8895),  
753 H3 trimethyl lysine-4 (H3K4me3, Abcam, ab8580), H3 trimethyl lysine-27 (H3K27me3,  
754 Active Motif, 39157), and H3 acetylated lysine 27 (H3K27ac, Abcam, ab472).  
755 Immunoprecipitated DNA was washed, isolated, and cleaned as for the TF ChIP-seq  
756 described above.

757

#### 758 PLAC-seq

759 PLAC-seq libraries for E13.5 basal ganglia were prepared similar to a previously  
760 published protocol<sup>67</sup>. 3 to 7 million cells were used for each library. If the cells appeared  
761 aggregated, they were dissociated with gentle MACS dissociator or Dounce  
762 homogenizer. Each PLAC-seq library was prepared using *DpnII* as the restriction  
763 enzyme and Dynabeads M-280 sheep anti-rabbit IgG (Invitrogen #11203D) mixed with  
764 5 µg of H3K4me3 (04-745, Millipore) for the chromatin immunoprecipitation step.  
765 Finally, libraries were prepared with the Illumina TruSeq adaptors and the final libraries  
766 were sent for paired-end sequencing on the HiSeq X Ten (150-bp reads) equipment.

767

768 Transgenic enhancer assays

769 All transgenic enhancer assays were performed at Lawrence Berkeley National  
770 Laboratory (LBNL) under the approval of the Animal Welfare and Research Committee  
771 (AWRC), as previously described<sup>18</sup>. In short, candidate enhancers were PCR-amplified  
772 and cloned into an *hsp68*-promoter- *lacZ* reporter vector<sup>68</sup>. Transgenic assays were  
773 performed according to published methods<sup>68,69</sup>. The enhancer-reporter vector was  
774 linearized and injected into the pronucleus of FVB strain single cell stage mouse  
775 embryos (E0.5). Embryos were implanted into surrogate CD-1 strain *Mus musculus*  
776 mothers and were then collected and stained for reporter gene expression at E11.5,  
777 E12.5, or E13.5. The resulting embryos were not assessed phenotypically for gender,  
778 which is not outwardly obvious at these ages. Therefore, we expect that a roughly equal  
779 number of male and female embryos were assessed. Embryos were excluded from  
780 analysis only if they did not harbor the transgene or if they were not at the correct  
781 developmental stage. No comparisons were made between cohorts of transgenic  
782 embryos, so randomization and experimenter blinding were unnecessary and not  
783 performed. Sample sizes were determined empirically based on our experience  
784 performing >2,000 transgenic enhancer assays. Only *LacZ* activity patterns that were  
785 observed in more than 30% of embryos resulting from independent transgene  
786 integration events of the same construct were considered reproducible.

787

788 Histology and Regional Activity Scoring

789 Brains were fixed, cryopreserved and embedded as described previously<sup>70</sup>. *LacZ*  
790 activity of candidate enhancers were annotated by at least two experts in the field by  
791 annotating activity in the ventricular zone and the subventricular zone/mantle zone of  
792 the ganglionic eminences.

793

794 Computational and Statistical Analysis

795

796 PLAC-seq

797 We detected the statistically significant long-range chromatin interactions from  
798 H3K4me3-associated proximity (PLAC-seq) data, using the MAPS pipeline<sup>71</sup>. Only intra-  
799 chromosomal interactions for autosomal chromosomes were selected, at a 10-kb  
800 resolution in the range 20 kb to 1 Mb. Raw reads from sequencing (FASTQ files) were  
801 mapped to the mm10 genome annotation using BWA mem. After duplicate, chimeric  
802 and low-quality read removal<sup>71</sup>, we split the mapped reads into short- and long-range  
803 reads, for distances between pair ends less than 1 kb and in the 1 kb-1 Mb range,  
804 respectively. We used the short-range and long-range reads to measure protein  
805 immunoprecipitation (IP) efficiency and detect long-range chromatin interactions,  
806 respectively.

807

808 For ascertaining meaningful interactions, we called peaks from ChIP-seq experiments  
809 on GE cells using H3K4me3 antibody using MACS2<sup>72</sup>. Only 10-kb bin pairs for which at  
810 least one end overlapped with called ChIP-seq peaks were used in the analysis. We  
811 fitted a positive Poisson regression model on all selected 10-kb bin pairs with more than  
812 one raw count, taking into consideration bias factors including linear genomic distance  
813 between two interacting bins, restriction enzyme cut site frequency, GC content,  
814 mappability score, and H3K4me3 antibody efficiency measured by the number of short-  
815 range reads in each bin. After model-fitting, we obtained expected contact frequency, p-  
816 value and false discovery rate (FDR) for each 10-kb bin pairs. We filtered only  
817 statistically significant bin pairs, defined as those with (1) raw contact frequency  $\geq 12$ ,  
818 (2) normalized contact frequency (observed/expected contact frequencies)  $\geq 2$ , and (3)  
819 FDR  $< 0.01$ .

820

821 We defined singletons as isolated significant chromatin interactions that passed the very  
822 stringent FDR of  $1 \times 10^{-4}$  (to reduce potential false positives) after merging adjacent  
823 chromatin interactions together. Contiguous contact point intervals from the table  
824 generated as above were merged, and the resulting interaction graphs were produced  
825 and analyzed under the denomination *interaction ensembles*, using a custom R script.

826

### 827 RNA-seq Data Analysis

828 Gene expression in GEs at E13.5 were expressed as  $\log_2(\text{RPKM})$  and calculated from  
829 the mean of read counts assigned to genes using the UCSC annotation. RNA-seq data  
830 was generated as part of a study previously reported<sup>18</sup>, and was used without further  
831 modifications.

832

### 833 ChIP-seq Data Analysis

834 The basic analysis pipeline is depicted in the Figure S1a. Quality-controlled FASTQ  
835 files<sup>73</sup> containing the reads were further cleaned-up from the remaining adapter  
836 sequences using Trim Galore version 0.4.5<sup>74</sup>. The resulting reads were aligned to the  
837 mouse (mm10) genome using BWA version 0.7.9a<sup>75</sup>, and duplicates removed with  
838 Samtools version 1.8<sup>76</sup>. Peaks of enriched binding regions against both negative binding  
839 and input DNA control were called using MACS version 2.1<sup>72</sup> with p-value cutoffs of  $1 \times$   
840  $10^{-4}$ .

841

842 To infer co-occurrence of TF binding at each genomic locus, we combined the peaks  
843 called from 6 published and 6 novel TF ChIP-seq datasets, as described above. Narrow  
844 peaks of TFs were overlapped and merged into one dataset using custom R scripts,  
845 where they were annotated for neighboring gene regulatory regions and filtered against  
846 repeat, blacklisted, and gapped regions. For each TF with more than one replicate, we  
847 selected one that was representative of the whole set; we ran Pearson correlation



848 analysis on genome wide coverage using DeepTools version 2.5.3<sup>25</sup> and determined  
849 that replicates for the same TF were consistent.

850

851 Hypothesizing that genomic context of binding is determined not only by the binding loci  
852 but also by their neighborhood, we used DeepTools to cluster read coverage for each  
853 TF around 1 kb (each side) of each peak in the combined data set at 10-bp resolution  
854 employing the K-means strategy. The data set was split into proximal and distal regions,  
855 defined as those either overlapping or not with putative gene promoters (respectively  
856 2000 and 200 bp upstream and downstream of TSSs), respectively. The number of  
857 clusters was initially set to 3 for each distal/proximal subset and increased by one until  
858 no further patterns were visually captured by the authors, except slight variations in the  
859 clusters bound by multiple TFs. We visually assessed the clusters to determine  
860 patterns. In the distal subset, one of the clusters encompassed more than 8000 peaks;  
861 we further clustered that cluster using the same strategy, and found additional patterns  
862 as outlined above. In the proximal subset, two clusters had similar binding profiles, and  
863 one of them was too small to allow statistical analysis; they were combined, and shown  
864 in Figure 2b as 2.1\_P and 2.2\_P.

865

#### 866 Motif Analysis

867 Core motifs for each of the 12 TFs were determined using HOMER version 4.9<sup>77</sup> in the  
868 called peaks for each individual TF. We performed *de novo* motif discovery with  
869 standard parameters, 300 bp up- and downstream of TF peaks. We compared the  
870 highest significant discovered motifs with HOMER known motif and JASPAR<sup>78</sup>  
871 databases, and inferred the core motif based on similitude. For all TFs except SP9 the  
872 core motif was the most significant one (lowest p-value from the HOMER analysis). The  
873 motif enrichment in the combined data set was determined for each cluster, distal and  
874 proximal loci combined, and overall, for all motifs present in the HOMER known motif  
875 database with p-value < 10<sup>-125</sup> and enrichment > 1.5 (unless otherwise noted). We  
876 removed from the analysis all motifs from proteins encoded by genes not expressed in  
877 E13.5 GEs, as determined by the RNA-seq experiment.

878

879 For each individual TF and bound loci cluster, the average motif coverage enrichment  
880 plot around 300 bp of peaks was established using HOMER annotation output and  
881 custom R scripts. For the coverage analysis on clustered bound loci, we averaged the  
882 signals from the homeobox-containing motifs among our 12 TFs. The heatmap in Figure  
883 2f reflects individual core motif prevalence, whose relative enrichment is displayed in  
884 Figures S2a and S2b, under different scopes. The average core motif coverage plots  
885 across the different binding clusters are shown in Figure S2d.

886

887 Integration of orthogonal data types

888 To make the inferences described in the text, we integrated transcriptomic (RNA-seq),  
889 binding (ChIP-seq), epigenomic (histone ChIP-seq), and interactomic (PLAC-seq) data,  
890 using custom R scripts (Figure S1a). We overlapped binding loci with chromatin  
891 interactions (both ensembles and single interactions), and histone (H3K27ac,  
892 H3K27me, H3K4me3, and H3K4me1) coverage across the mm10 genome. Those  
893 intersection loci were further stratified into the neighborhood binding clusters, as laid out  
894 above. We also overlapped those stratified loci against transcriptional enhancers from  
895 the VISTA database<sup>32</sup>, as well as novel ones generated as part of this study.

896

897 Additionally, gene-ensemble association was annotated, based on the presence of  
898 annotated gene promoters in interaction ensembles. Those interactions were used to  
899 infer a gene expression model from the transcriptional regulation by three-dimensional  
900 chromatin combinatorially-bound by the TFs. In doing so, we also employed  
901 transcriptomic data in published mouse E13.5 GEs<sup>18</sup>.

902

903 Chromatin State Inference

904 We created a 9-state HMM model of chromatin states using E13.5 BG WT histone data  
905 previously reported<sup>18</sup> and ChromHMM<sup>79</sup> and assigned chromatin states based on the  
906 emission probabilities. The number of states was determined by the minimum time for  
907 convergence of the algorithm as well as assessment for biological relevance. Symbols  
908 were assigned to each state parallel to previously published assessment<sup>80</sup>. Our model  
909 chromatin states were assessed as below.

910

911

HMM state	Assigned symbol	Chromatin state
1	EnhLo	active enhancer with transcriptional history
2	EnhTx	highly active enhancer with transcriptional history
3	Enh	active enhancer without transcriptional history
4	EnhPoi	poised/weak enhancer
5	EnhBiv	bivalent enhancer with transcriptional history
6	ReprPC	polycomb-repressed locus
7	Quies	no signal (quiescent)
8	TxWk	weak transcriptional signal
9	TssBiv	bivalent promoter element

912

913

914 Other inferences

915 Non-Gaussian distribution comparisons were performed by computing sample means  
916 (N = 10000) and comparing the means distributions by pairwise t-test; samples were  
917 collected without replacement with a size of 75-80% of the sampling space. For the log  
918 likelihood comparisons between observed and expected, the expected value was  
919 derived from the frequency of the category in each class as per the overall frequency of  
920 that category (Figure S3).

921

922 We compared our results with published data to validate our inferences. We intersected  
923 our neighborhood binding clusters with the clusters derived from the analysis of single-  
924 nucleus chromatin accessibility (scATAC-seq) of developing and post-natal mouse  
925 forebrain<sup>28</sup>, using custom R scripts. Predominant cell fates in those clusters emerged  
926 from that overlap, allowing the inference of TF roles in the regulation of forebrain  
927 development in the GEs.

928

929 For the assessment of overall and base-level evolutionary conservation of stratified  
930 genomic loci as previously described, we used published Vertebrate Phastcons (60  
931 species)<sup>81</sup> and PhyloP (59-way)<sup>82</sup> scores for the mm10 mouse genome, respectively,  
932 downloaded from the UCSC genome browser  
933 (<http://hgdownload.cse.ucsc.edu/goldenPath/mm10/>).

934

935 We further validated our model by comparing the inferences with previously published  
936 findings of regulatory roles of DLX2<sup>18</sup> and NKX2.1<sup>20</sup> in activating (a.RE) and repressing  
937 (r.RE) gene expression.

938

939

940 Comparison with Cortex TF binding

941 To make the comparison against known TF binding in developing cortex at a similar  
942 developmental stage, we reanalyzed published cortex ChIP-seq datasets<sup>38</sup> under the  
943 same parameters herein, and overlapped those binding loci with those obtained in our  
944 study, using custom R scripts.

945

946

947 Data and Code Availability

948 This article made use of published and unpublished genomic and epigenomic data that  
949 are deposited in the NCBI database, as outlined below. The data can be downloaded  
950 from ([https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GEO accession](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GEO%20accession)), as below.

951

952

Data type	Source	GEO accession
E13.5 GE ChIP-seq		
ARX	this study	GSE222183
ASCL1	this study	GSE222183
DLX1	Lindtner et al, 2019	GSE124936
DLX2	Lindtner et al, 2019	GSE124936
DLX5	Lindtner et al, 2019	GSE124936
GSX2	this study	GSE222183
LHX6	Sandberg et al, 2016	GSE85705
NKX2.1	Sandberg et al, 2016	GSE85705
NR2F1	this study	GSE222183
OTX2	Hoch et al, 2015	GSE69724
PBX1	this study	GSE222183
SP9	this study	GSE222183
E13.5 GE PLAC-seq	this study	GSE222183
E13.5 GE RNA-seq		
DLX1/2	Lindtner et al, 2019	GSE124936
NKX2.1	Sandberg et al, 2016	GSE85705

953

954

955 Data can be visualized in UCSC track hub format whose information is provided on  
956 Nord Lab GitHub page

957 (<https://github.com/NordNeurogenomicsLab/Publications/tree/master/Catta->

958 [Preta\\_XXX\\_2023](https://github.com/NordNeurogenomicsLab/Publications/tree/master/Catta-Preta_XXX_2023)). All R scripts were created in-house and can be provided upon

959 request.

960

961

## 962 REFERENCES

963 1. Rubenstein, J. L. R. & Campbell, K. Chapter 18 - Neurogenesis in the basal  
964 ganglia. in (eds. Rubenstein, J., Rakic, P., Chen, B. & Kwan, K. Y. B. T.-P. and C.  
965 T. S. in the D. C. N. S. and P. N. S. (Second E.) 399–426 (Academic Press,  
966 2020). doi:<https://doi.org/10.1016/B978-0-12-814405-3.00018-7>.

967 2. Grillner, S. & Robertson, B. The Basal Ganglia Over 500 Million Years. *Curr. Biol.*  
968 **26**, R1088–R1100 (2016).

969 3. Stephenson-Jones, M., Samuelsson, E., Ericsson, J., Robertson, B. & Grillner, S.  
970 Evolutionary Conservation of the Basal Ganglia as a Common Vertebrate  
971 Mechanism for Action Selection. *Curr. Biol.* **21**, 1081–1091 (2011).

972 4. Nitta, K. R. *et al.* Conservation of transcription factor binding specificities across  
973 600 million years of bilateria evolution. *Elife* **4**, e04837 (2015).

974 5. Holland, P. W. H. & Takahashi, T. The evolution of homeobox genes: Implications

975 for the study of brain development. *Brain Res. Bull.* **66**, 484–490 (2005).

976 6. Rubenstein, J. L. R. & Puelles, L. Homeobox Gene Expression during  
977 Development of the Vertebrate Brain. in (ed. Pedersen, R. A. B. T.-C. T. in D. B.)  
978 vol. 29 1–63 (Academic Press, 1994).

979 7. Sánchez-Higuera, C. *et al.* In vivo Hox binding specificity revealed by systematic  
980 changes to a single cis regulatory module. *Nat. Commun.* **10**, 3597 (2019).

981 8. Bürglin, T. R. & Affolter, M. Homeodomain proteins: an update. *Chromosoma* **125**,  
982 497–521 (2016).

983 9. Darieva, Z. *et al.* A competitive transcription factor binding mechanism determines  
984 the timing of late cell cycle-dependent gene expression. *Mol. Cell* **38**, 29–40  
985 (2010).

986 10. Göke, J. *et al.* Combinatorial Binding in Human and Mouse Embryonic Stem Cells  
987 Identifies Conserved Enhancers Active in Early Embryonic Development. *PLOS*  
988 *Comput. Biol.* **7**, e1002304 (2011).

989 11. Charest, J. *et al.* Combinatorial Action of Temporally Segregated Transcription  
990 Factors. *Dev. Cell* **55**, 483–499.e7 (2020).

991 12. Salomone, J. *et al.* Conserved Gsx2/Ind homeodomain monomer versus  
992 homodimer DNA binding defines regulatory outcomes in flies and mice. *Genes*  
993 *Dev.* **35**, 157–174 (2021).

994 13. Feng, S. *et al.* Transcription factor paralogs orchestrate alternative gene  
995 regulatory networks by context-dependent cooperation with multiple cofactors.  
996 *Nat. Commun.* **13**, 3808 (2022).

997 14. Vollmer, J.-Y. & Clerc, R. G. Homeobox Genes in the Developing Mouse Brain. *J.*  
998 *Neurochem.* **71**, 1–19 (1998).

999 15. Kribelbauer, J. F. *et al.* Context-Dependent Gene Regulation by Homeodomain  
1000 Transcription Factor Complexes Revealed by Shape-Readout Deficient Proteins.  
1001 *Mol. Cell* **78**, 152–167.e11 (2020).

1002 16. Cho, G., Nasrallah, M. P., Lim, Y. & Golden, J. A. Distinct DNA binding and  
1003 transcriptional repression characteristics related to different ARX mutations.  
1004 *Neurogenetics* **13**, 23–29 (2012).

1005 17. Batista-Brito, R. & Fishell, G. B. T.-C. T. in D. B. Chapter 3 The Developmental  
1006 Integration of Cortical Interneurons into a Functional Network. in *Development of*  
1007 *Neural Circuitry* vol. 87 81–118 (Academic Press, 2009).

1008 18. Lindtner, S. *et al.* Genomic Resolution of DLX-Orchestrated Transcriptional  
1009 Circuits Driving Development of Forebrain GABAergic Neurons. *Cell Rep.* **28**,  
1010 2048–2063.e8 (2019).

1011 19. Liu, Z. *et al.* Sp9 Regulates Medial Ganglionic Eminence-Derived Cortical  
1012 Interneuron Development. *Cereb. Cortex* **29**, 2653–2667 (2018).

1013 20. Sandberg, M. *et al.* Transcriptional Networks Controlled by NKX2-1 in the  
1014 Development of Forebrain GABAergic Neurons. *Neuron* **91**, 1260–1275 (2016).

- 1015 21. Hoch, R. V., Lindtner, S., Price, J. D. & Rubenstein, J. L. R. OTX2 Transcription  
1016 Factor Controls Regional Patterning within the Medial Ganglionic Eminence and  
1017 Regional Identity of the Septum. *Cell Rep.* **12**, 482–494 (2015).
- 1018 22. Long, J. E. *et al.* Dlx1&2 and Mash1 transcription factors control striatal patterning  
1019 and differentiation through parallel and overlapping pathways. *J. Comp. Neurol.*  
1020 **512**, 556–572 (2009).
- 1021 23. Pei, J. & Grishin, N. V. C2H2 zinc finger proteins of the SP/KLF, Wilms tumor,  
1022 EGR, Hucbein, and Klumpfuss families in metazoans and beyond. *Gene* **573**,  
1023 91–99 (2015).
- 1024 24. Berger, M. F. *et al.* Variation in Homeodomain DNA Binding Revealed by High-  
1025 Resolution Analysis of Sequence Preferences. *Cell* **133**, 1266–1276 (2008).
- 1026 25. Ramírez, F. *et al.* deepTools2: a next generation web server for deep-sequencing  
1027 data analysis. *Nucleic Acids Res.* **44**, W160–W165 (2016).
- 1028 26. Lee, T.-H. & Maheshri, N. A regulatory role for repeated decoy transcription factor  
1029 binding sites in target gene expression. *Mol. Syst. Biol.* **8**, 576 (2012).
- 1030 27. Sandberg, M. *et al.* Genomic analysis of transcriptional networks directing  
1031 progression of cell states during MGE development. *Neural Dev.* **13**, 21 (2018).
- 1032 28. Preissl, S. *et al.* Single-nucleus analysis of accessible chromatin in developing  
1033 mouse forebrain reveals cell-type-specific transcriptional regulation. *Nat.*  
1034 *Neurosci.* **21**, 432–439 (2018).
- 1035 29. Banani, S. F., Lee, H. O., Hyman, A. A. & Rosen, M. K. Biomolecular  
1036 condensates: organizers of cellular biochemistry. *Nat. Rev. Mol. Cell Biol.* **18**,  
1037 285–298 (2017).
- 1038 30. Boija, A. *et al.* Transcription Factors Activate Genes through the Phase-  
1039 Separation Capacity of Their Activation Domains. *Cell* **175**, 1842-1855.e16  
1040 (2018).
- 1041 31. Sabari, B. R. R. *et al.* Coactivator condensation at super-enhancers links phase  
1042 separation and gene control. *Science (80-. ).* **361**, eaar3958 (2018).
- 1043 32. Visel, A., Minovitsky, S., Dubchak, I. & Pennacchio, L. A. VISTA Enhancer  
1044 Browser—a database of tissue-specific human enhancers. *Nucleic Acids Res.* **35**,  
1045 D88–D92 (2007).
- 1046 33. Visel, A. *et al.* A High-Resolution Enhancer Atlas of the Developing  
1047 Telencephalon. *Cell* **152**, 895–908 (2013).
- 1048 34. Visel, A. *et al.* Ultraconservation identifies a small subset of extremely constrained  
1049 developmental enhancers. *Nat Genet* **40**, 158–160 (2008).
- 1050 35. Visel, A. *et al.* ChIP-seq accurately predicts tissue-specific activity of enhancers.  
1051 *Nature* **457**, 854–858 (2009).
- 1052 36. Kircher, M. *et al.* Saturation mutagenesis of twenty disease-associated regulatory  
1053 elements at single base-pair resolution. *Nat. Commun.* **10**, 3583 (2019).
- 1054 37. Khouri-Farah, N., Guo, Q., Morgan, K., Shin, J. & Li, J. Y. H. Integrated single-cell



- 1055 transcriptomic and epigenetic study of cell state transition and lineage  
1056 commitment in embryonic mouse cerebellum. *Sci. Adv.* **8**, eabl9156 (2022).
- 1057 38. Ypsilanti, A. R. *et al.* Transcriptional network orchestrating regional patterning of  
1058 cortical progenitors. *Proc. Natl. Acad. Sci.* **118**, e2024795118 (2021).
- 1059 39. Riley, T. R. *et al.* SELEX-seq: A Method for Characterizing the Complete  
1060 Repertoire of Binding Site Preferences for Transcription Factor Complexes BT -  
1061 Hox Genes: Methods and Protocols. in (eds. Graba, Y. & Rezsöházy, R.) 255–  
1062 278 (Springer New York, 2014). doi:10.1007/978-1-4939-1242-1\_16.
- 1063 40. Ravasi, T. *et al.* An Atlas of Combinatorial Transcriptional Regulation in Mouse  
1064 and Man. *Cell* **140**, 744–752 (2010).
- 1065 41. Fu, L. *et al.* Predicting transcription factor binding in single cells through deep  
1066 learning. *Sci. Adv.* **6**, eaba9031 (2022).
- 1067 42. Banks, C. J., Joshi, A. & Michoel, T. Functional transcription factor target  
1068 discovery via compendia of binding and expression profiles. *Sci. Rep.* **6**, 20649  
1069 (2016).
- 1070 43. Whitfield, T. W. *et al.* Functional analysis of transcription factor binding sites in  
1071 human promoters. *Genome Biol.* **13**, R50 (2012).
- 1072 44. Yun, K., Potter, S. & Rubenstein, J. L. Gsh2 and Pax6 play complementary roles  
1073 in dorsoventral patterning of the mammalian telencephalon. *Development* **128**,  
1074 193–205 (2001).
- 1075 45. Dickel, D. E. *et al.* Ultraconserved Enhancers Are Required for Normal  
1076 Development. *Cell* **172**, 491-499.e15 (2018).
- 1077 46. Tuttle, R., Nakagawa, Y., Johnson, J. E. & O’Leary, D. D. Defects in  
1078 thalamocortical axon pathfinding correlate with altered cell domains in Mash-1-  
1079 deficient mice. *Development* **126**, 1903–1916 (1999).
- 1080 47. Cobos, I. *et al.* Mice lacking Dlx1 show subtype-specific loss of interneurons,  
1081 reduced inhibition and epilepsy. *Nat. Neurosci.* **8**, 1059–1068 (2005).
- 1082 48. de Melo, J. *et al.* Dlx1 and Dlx2 function is necessary for terminal differentiation  
1083 and survival of late-born retinal ganglion cells in the developing mouse retina.  
1084 *Development* **132**, 311–322 (2005).
- 1085 49. Villaescusa, J. C. *et al.* A PBX1 transcriptional network controls dopaminergic  
1086 neuron development and is impaired in Parkinson’s disease. *EMBO J.* **35**, 1963–  
1087 1978 (2016).
- 1088 50. Ferraris, L. *et al.* Combinatorial binding of transcription factors in the pluripotency  
1089 control regions of the genome. *Genome Res.* **21**, 1055–1064 (2011).
- 1090 51. Vandel, J., Cassan, O., Lèbre, S., Lecellier, C.-H. & Bréhélin, L. Probing  
1091 transcription factor combinatorics in different promoter classes and in enhancers.  
1092 *BMC Genomics* **20**, 103 (2019).
- 1093 52. Ibarra, I. L. *et al.* Mechanistic insights into transcription factor cooperativity and its  
1094 impact on protein-phenotype interactions. *Nat. Commun.* **11**, 124 (2020).

- 1095 53. Stefflova, K. *et al.* Cooperativity and Rapid Evolution of Cobound Transcription  
1096 Factors in Closely Related Mammals. *Cell* **154**, 530–540 (2013).
- 1097 54. Palstra, R.-J. Transcription factor binding at enhancers: shaping a genomic  
1098 regulatory landscape in flux. *Frontiers in Genetics* vol. 3 (2012).
- 1099 55. Dogan, N. *et al.* Occupancy by key transcription factors is a more accurate  
1100 predictor of enhancer activity than histone modifications or chromatin  
1101 accessibility. *Epigenetics Chromatin* **8**, 16 (2015).
- 1102 56. Chronis, C. *et al.* Cooperative Binding of Transcription Factors Orchestrates  
1103 Reprogramming. *Cell* **168**, 442–459.e20 (2017).
- 1104 57. Polychronopoulos, D., King, J. W. D., Nash, A. J., Tan, G. & Lenhard, B.  
1105 Conserved non-coding elements: developmental gene regulation meets genome  
1106 organization. *Nucleic Acids Res.* **45**, 12611–12624 (2017).
- 1107 58. Khoueiry, P. *et al.* Uncoupling evolutionary changes in DNA sequence,  
1108 transcription factor occupancy and enhancer activity. *Elife* **6**, e28440 (2017).
- 1109 59. Sahu, B. *et al.* Sequence determinants of human gene regulatory elements. *Nat.*  
1110 *Genet.* **54**, 283–294 (2022).
- 1111 60. Snetkova, V. *et al.* Ultraconserved enhancer function does not require perfect  
1112 sequence conservation. *Nat. Genet.* **53**, 521–528 (2021).
- 1113 61. Snetkova, V., Pennacchio, L. A., Visel, A. & Dickel, D. E. Perfect and imperfect  
1114 views of ultraconserved sequences. *Nat. Rev. Genet.* **23**, 182–194 (2022).
- 1115 62. Viturawong, T., Meissner, F., Butter, F. & Mann, M. A DNA-Centric Protein  
1116 Interaction Map of Ultraconserved Elements Reveals Contribution of Transcription  
1117 Factor Binding Hubs to Conservation. *Cell Rep.* **5**, 531–545 (2013).
- 1118 63. McCole, R. B., Erceg, J., Saylor, W. & Wu, C. Ultraconserved Elements Occupy  
1119 Specific Arenas of Three-Dimensional Mammalian Genome Organization. *Cell*  
1120 *Rep.* **24**, 479–488 (2018).
- 1121 64. Morgunova, E. & Taipale, J. Structural perspective of cooperative transcription  
1122 factor binding. *Curr. Opin. Struct. Biol.* **47**, 1–8 (2017).
- 1123 65. Zhang, Q. *et al.* The Zinc Finger Transcription Factor Sp9 Is Required for the  
1124 Development of Striatopallidal Projection Neurons. *Cell Rep.* **16**, 1431–1444  
1125 (2016).
- 1126 66. Toresson, H., Potter, S. S. & Campbell, K. Genetic control of dorsal-ventral  
1127 identity in the telencephalon: Opposing roles for Pax6 and Gsh2. *Development*  
1128 **127**, 4361–4371 (2000).
- 1129 67. Fang, R. *et al.* Mapping of long-range chromatin interactions by proximity ligation-  
1130 assisted ChIP-seq. *Cell Res.* **26**, 1345–1348 (2016).
- 1131 68. Pennacchio, L. A. *et al.* In vivo enhancer analysis of human conserved non-  
1132 coding sequences. *Nature* **444**, 499–502 (2006).
- 1133 69. Kothary, R. *et al.* Inducible expression of an hsp68-lacZ hybrid gene in transgenic  
1134 mice. *Development* **105**, 707 LP – 714 (1989).

- 1135 70. Pattabiraman, K. *et al.* Transcriptional regulation of enhancers active in  
1136 protodomains of the developing cerebral cortex. *Neuron* **82**, 989–1003 (2014).
- 1137 71. Juric, I. *et al.* MAPS: Model-based analysis of long-range chromatin interactions  
1138 from PLAC-seq and HiChIP experiments. *PLOS Comput. Biol.* **15**, e1006982  
1139 (2019).
- 1140 72. Zhang, Y. *et al.* Model-based Analysis of ChIP-Seq (MACS). *Genome Biol* **9**,  
1141 R137 (2008).
- 1142 73. Andrews, S. FASTQC. A quality control tool for high throughput sequence data.  
1143 2010. [Http://Www.Bioinformatics.Babraham.Ac.Uk/Projects/Fastqc/](http://www.Bioinformatics.Babraham.Ac.Uk/Projects/Fastqc/) (2010).
- 1144 74. Krueger, F. Trim Galore!: A wrapper tool around Cutadapt and FastQC to  
1145 consistently apply quality and adapter trimming to FastQ files. *Babraham Institute*  
1146 (2015) doi:10.1002/maco.200603986.
- 1147 75. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows–Wheeler  
1148 transform. *Bioinformatics* **25**, 1754–1760 (2009).
- 1149 76. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics*  
1150 **25**, 2078–2079 (2009).
- 1151 77. Heinz, S. *et al.* Simple Combinations of Lineage-Determining Transcription  
1152 Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell  
1153 Identities. *Mol. Cell* **38**, 576–589 (2010).
- 1154 78. Fornes, O. *et al.* JASPAR 2020: update of the open-access database of  
1155 transcription factor binding profiles. *Nucleic Acids Res.* **48**, D87–D92 (2019).
- 1156 79. Ernst, J. & Kellis, M. ChromHMM: automating chromatin-state discovery and  
1157 characterization. *Nat Meth* **9**, 215–216 (2012).
- 1158 80. van der Velde, A. *et al.* Annotation of chromatin states in 66 complete mouse  
1159 epigenomes during development. *Commun. Biol.* **4**, 239 (2021).
- 1160 81. Siepel, A. *et al.* Evolutionarily conserved elements in vertebrate, insect, worm,  
1161 and yeast genomes. *Genome Res.* **15**, 1034–1050 (2005).
- 1162 82. Pollard, K. S., Hubisz, M. J., Rosenbloom, K. R. & Siepel, A. Detection of  
1163 nonneutral substitution rates on mammalian phylogenies. *Genome Res.* **20**, 110–  
1164 121 (2010).
- 1165