

1 **Spatiotemporal transcriptomic atlas of mouse organogenesis**
2 **using DNA nanoball patterned arrays**

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68 **SUMMARY**

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70 Spatially resolved transcriptomic technologies are promising tools to study cell
71 fate decisions in a physical microenvironment, which is fundamental for
72 enhancing our knowledge of mammalian development. However, the
73 imbalance between resolution, transcript capture and field of view of current
74 methodologies precludes their systematic application to analyze relatively
75 large and three-dimensional mid- and late-gestation mammalian embryos.
76 Here, we combined DNA nanoball (DNB) patterned arrays and tissue RNA
77 capture to create SpaTial Enhanced REsolution Omics-sequencing (Stereo-
78 seq). This approach allows transcriptomic profiling of large histological
79 sections with high resolution and sensitivity. We have applied Stereo-seq to
80 study the kinetics and directionality of transcriptional variation in a time course
81 of mouse organogenesis. We used this information to gain insight into the
82 molecular basis of regional specification, neuronal migration and
83 differentiation in the developing brain. Furthermore, we mapped the
84 expression of a panel of developmental disease-related loci on our global
85 transcriptomic maps to define the spatiotemporal windows of tissue
86 vulnerability. Our panoramic atlas constitutes an essential resource to
87 investigate longstanding questions concerning normal and abnormal
88 mammalian development.

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102 INTRODUCTION

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104 Understanding how a single totipotent cell, the zygote, develops into a
105 complex organism such as a mammal in a precisely controlled manner, over
106 time and space, is one of the most fascinating questions in science ever since
107 Aristotle. Mouse is an excellent model mammal to study developmental
108 processes and establish parallels with larger species thanks to the inbred
109 genetic background, susceptibility to genetic manipulation and relatively short
110 gestation period (~21 days). Recent intense efforts on cell atlas using high-
111 throughput single-cell RNA-sequencing (scRNA-seq) technologies have
112 provided an increasingly detailed view of mouse developmental gene
113 expression dynamics (Cao et al., 2019; He et al., 2020). However, except for
114 the early embryonic time points where fewer cells are present (Peng et al.,
115 2019; Xue et al., 2013), topographical transcriptomic information is still mostly
116 lacking. This deficiency complicates the interpretations, in particular the
117 correlation between signaling cues and cell fate decisions. In this regard,
118 embryonic decisions are often regulated by gene expression thresholds rather
119 than binary mechanisms. The outstanding need for precise coordination of
120 complex signals and the myriad embryonic cell types in development is
121 exemplified by genetic disorders like situs inversus, where alterations in the
122 response to nodal flow cause inversion of the internal anatomy (Okada et al.,
123 2005). Systematic spatially resolved analysis of gene expression is thus
124 strictly necessary to dissect the intricacies of mammalian development.

125 Several remarkable methodologies have been recently developed that
126 allow spatially resolved transcriptomic profiling of tissue sections. They can be
127 divided in three main classes based on their detection method: physical
128 segmentation using microdissection coupled to sequencing (Nichterwitz et al.,
129 2016; Peng et al., 2019), pre-designed probes for either single-molecule
130 hybridization (Eng et al., 2019; Zhuang, 2021) or amplification and *in situ*
131 decoding (Alon et al., 2021; Lee et al., 2014; Wang et al., 2018), and
132 barcoded probes (Cho et al., 2021a; Rodrigues et al., 2019; Srivatsan et al.,
133 2021; Stahl et al., 2016; Vickovic et al., 2019; Yao et al., 2020). These
134 techniques all have specific advantages and disadvantages in resolution and
135 applicability but share a major caveat in the limited field of view. Although

136 small parts of developing mouse tissues (Di Bella et al., 2021; Stickels et al.,
137 2021) or even whole mouse embryos (Peng et al., 2019; Srivatsan et al., 2021;
138 Yao et al., 2020) have been analyzed using these approaches, no available
139 technology can profile samples of the size of whole mouse embryos in mid- or
140 late-stage gestation with high definition. This poses a tremendous challenge
141 for systematically studying the differentiation continuum that happens across
142 different areas of the same tissue and for comparing different tissues
143 simultaneously. Here, we report the development of a new DNB-based
144 technology, Stereo-seq, that combines high resolution and sensitivity with
145 large field of view to generate the first panoramic transcriptomic atlas of
146 mouse organogenesis.

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149 RESULTS

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151 **DNB patterned arrays enable large field-of-view spatially resolved** 152 **transcriptomics with high definition**

153 DNB sequencing is based on rolling circle amplification of DNA fragments
154 (Drmanac et al., 2010), whose products are effectively docked into defined
155 positions of a lithographically etched chip for *in situ* sequencing through
156 automated high-throughput microscopic imaging. We envisaged that these
157 features could lay the foundation for a spatially resolved transcriptomic
158 technology with high-resolution and large field of view, which we developed as
159 following. Standard DNB chips have spots with approximately 220 nm
160 diameter and a center-to-center distance of 500 or 715 nm (**Figure 1A, step**
161 **1**). This provides up to 400 spots for tissue RNA capture per 100 μm^2 (average
162 size of a mammalian cell). DNB templates containing random barcodes are
163 deposited on the patterned array, incubated with primers, and sequenced to
164 obtain the data matrix containing the coordinate identity (CID) of every DNB
165 (**Figure 1A, step 2**). The use of random barcode-labeled DNB allows a large
166 spatial barcode pool size (4^{25}). Next, unique molecular identifiers (UMI) and
167 polyT sequence-containing oligonucleotides are ligated onto each spot
168 through hybridization with an oligonucleotide sequence containing the CID
169 (**Figure 1A, step 3**). Frozen tissue sections (10 μm thickness) are loaded onto

170 the chip surface, followed by fixation, permeabilization to capture the tissue
171 polyA-tailed RNA and finally reverse transcription plus amplification (**Figure**
172 **1A, step 4**). Amplified barcoded cDNA is collected, used as template for
173 library preparation and sequenced together with the CID (**Figure 1A, step 5**).
174 Computational analysis of sequencing data generated this way allows high-
175 resolution spatially resolved transcriptomics (**Figure 1A, step 6**). We named
176 this approach Stereo-seq (SpaTial Enhanced REsolution Omics-sequencing).
177 Importantly, Stereo-seq has a larger number of spots per 100 μm^2 , with
178 smaller spot size and center-to-center distance, than other available methods
179 (**Figure 1B**). This makes it particularly suitable for detecting transcriptomic
180 gradients with high resolution and sensitivity. So far, we have used Stereo-seq
181 chips of up to an effective area of 13.2 cm x 13.2 cm for different tissue sizes,
182 subdivided into 50 mm^2 , 100 mm^2 and 200 mm^2 smaller chips for profiling
183 sections from the mouse olfactory bulb ($\sim 10.5 \text{ mm}^2$), a whole mouse brain
184 ($\sim 52.8 \text{ mm}^2$) and whole mouse embryos (~ 7.1 to $\sim 97.6 \text{ mm}^2$), respectively
185 (**Figure S1A**). These capture areas are substantially larger than those
186 achieved by other available technologies (**Figure 1B**), and we anticipate the
187 application of unsliced chips to much larger tissues such as whole human
188 brain sections (**Figure S1A**).

189 To benchmark our technology, we first profiled the mouse olfactory bulb
190 because it is a widely used model tissue for spatially resolved transcriptomic
191 approaches (Lebrigand et al., 2020b; Rodriques et al., 2019; Vickovic et al.,
192 2019). Stereo-seq captured numbers ranging on average 69 UMI per 2 μm
193 (diameter) bin (bin 3, 3 \times 3 DNB), 1,450 UMI per 10 μm (diameter) bin (bin 14,
194 14 \times 14 DNB, equivalent to ~ 1 medium size cell) and 133,776 UMI per 100 μm
195 (diameter) bin (bin 140, 140 \times 140 DNB) (**Figure 1C and S1B**). This is
196 superior to other available technologies including Seq-Scope (848 UMI on
197 average per 10 μm diameter bin) (Cho et al., 2021a) when compared at the
198 same bin resolution.

199 Conventional cell taxonomy analysis with single-cell RNA-seq (scRNA-
200 seq) uses algorithms such as Leiden clustering to group cells based on
201 transcriptome similarity (Traag et al., 2019). Applying this method to spatially
202 resolved data, however, is problematic due to the lack of consideration of
203 spatial coordinates. Thus, we developed a spatially-constrained clustering

204 algorithm especially suited for high-resolution spatially resolved transcriptomic
205 data (**see Methods**), which clustered bins over a continuous area from which
206 tissue domains are annotated. This approach showed high level of
207 consistency between biological replicates corresponding to two adjacent
208 sections of the mouse olfactory bulb ($R^2 = 0.963$) (**Figure 1D, S1C, and S1D**).
209 The distribution of specific markers (*Cdhr1*, *Pcp4*, *Slc17a7*, *Sox11* and *Th*)
210 captured by Stereo-seq exhibited remarkable similarity to *in situ* hybridization
211 (ISH) data (Lein et al., 2007), and showed clearer patterns than HDST or
212 Slide-seqV2 (**Figure 1E, S1C, and S1E**).

213 To further demonstrate the robustness of Stereo-seq and prove its
214 scalability, we profiled an entire adult mouse coronal brain section.
215 Unsupervised spatially-constrained clustering of the Stereo-seq data identified
216 different anatomical structures including the cortex and subcortical regions
217 such as hippocampus, thalamus and striatum (**Figure 1F**). This is so far the
218 largest brain spatial atlas profiled by a genome-wide sequencing based
219 spatial technology. Differentially expressed gene (DEG) and gene ontology
220 (GO) enrichment analyses confirmed the unique identity of the observed
221 structures (**Figure S2A and S2B**). When focusing on specific elements such
222 as the hippocampus (*Ncdn*⁺ and *Neurod6*⁺), thalamus (*Prkcd*⁺), hypothalamus
223 (*Sparc*⁺) and striatum (*Penk*⁺), among others, we observed specific spatial
224 distribution of known markers (**Figure S2C**). Notably, we used a specific dye
225 for nucleic acids before *in situ* permeabilization to assess the resolution of the
226 data after sequencing. When zooming into a specific region corresponding to
227 the cortex, we observed substantial colocalization of the dye and the
228 aggregated transcripts detected by Stereo-seq (**Figure 1G**). This confirmed
229 that Stereo-seq captures transcripts at cellular resolution.

230 We concluded that Stereo-seq can be used to spatially characterize the
231 transcriptomic pattern of complex tissues in an unbiased multiplexed manner
232 with high definition and unprecedented field of view.

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234 **Spatially resolved transcriptomic atlas of mouse organogenesis using** 235 **Stereo-seq**

236 We postulated that the unique features of Stereo-seq would allow us to create
237 panoramic transcriptomic maps of middle- and late-gestation mouse embryos

238 for which full body sections cannot be profiled using other available
239 technologies. This would also showcase the strength of Stereo-seq in
240 measuring spatiotemporal transcriptional dynamics at full scale. To this aim,
241 we created the beginning of Mouse Organogenesis Spatiotemporal
242 Transcriptomic Atlas (MOSTA), an expandable and interactive database for
243 spatial transcriptomic studies of mouse organogenesis. As proof of principle,
244 we profiled 14 sagittal sections (two sections per embryo) from C57BL/6
245 mouse embryos at E9.5 (~7.1 mm²), E10.5 (~10.9 mm²), E11.5 (~19.7 mm²),
246 E12.5 (~30.2 mm²), E13.5 (~55.2 mm²), E15.5 (~93.2 mm²) and E16.5 (~97.6
247 mm²). These stages cover most of the key events in mouse organogenesis
248 (Cao et al., 2019).

249 To gain an unbiased global view of MOSTA, we segmented our individual
250 embryo datasets into 50 × 50 DNB bins (bin 50 resolution, 36 μm diameter). In
251 total, we retrieved transcriptomic information for 616,125 bins. The average
252 number of captured genes per bin in each embryo ranged from 1,088 at E12.5
253 to 3,153 at E11.5, and of UMI from 3,473 at E13.5 to 10,939 at E11.5 (**Figure**
254 **S3A**). Unsupervised spatially-constrained clustering showed the expected
255 major organs and systems (e.g., nervous system, heart, liver, lung, kidney,
256 adrenal gland, gastrointestinal tract, genitourinary tract, skin, bone and
257 muscle) at each respective time point, whose external boundaries closely
258 resembled the anatomical structures (**Figure 2A, S3B, and S3C**). DEG
259 analysis showed the expected correspondence with emerging tissue-specific
260 cell identities at individual stages (**Figure S4**). This was further confirmed by
261 plotting specific marker genes on the spatial maps across all embryonic time
262 points (**Figure 2B and S5**). Since Stereo-seq captures very high density of
263 signals, we also performed spatial re-clustering of bins for selected tissues,
264 which produced detailed structures containing different subregions (**Figure**
265 **2C**). For example, re-clustering of the E10.5 heart identified the atrium (*Myh6*⁺)
266 and ventricle (*Myh7*⁺/*Myh2*⁺); of the E15.5 limb the cartilage primordium
267 (*Col2a1*⁺), dermis (*Dcn*⁺) and epidermis (*Krt15*⁺); of the E16.5 spinal cord the
268 marginal (*Scng*⁺), mantle (*Snrpn*⁺/*Plxna2*) and ventricular zones
269 (*Snrpn*⁺/*Plxna2*⁺); and of the E16.5 adrenal gland the medulla (*Dbh*⁺), cortex
270 (*Fdx1*⁺) and capsule (*Col1a2*⁺). Notably, reanalysis of E10 mouse embryo
271 DBiT-seq data (Liu et al., 2020) showed significantly fewer 50 μm-diameter

272 spots (902) than 36 μm diameter (bin 50)-bins (7,386) were calculated using
273 Stereo-seq in an individual E10.5 section. Consequently, unsupervised
274 clustering or mapping of specific genes failed to identify discernable
275 anatomical structures with DBiT-seq (**Figure S6A and S6B**). This discrepancy
276 is related to the large inter-channel space (50 μm) of DBiT-seq.

277 To study cell function enrichments within specific areas contained in the
278 individual tissue clusters generated with Stereo-seq, we applied Hotspot
279 (DeTomaso and Yosef, 2021), an algorithm that measures non-random
280 variation to recognize informative gene programs. The latter is important
281 because amalgamation of signals from different cell types and states in
282 complex tissues such as the embryo can otherwise complicate the
283 interpretations. We identified 28 gene modules for E9.5, 28 for E10.5, 33 for
284 E11.5, 30 for E12.5, 24 for E13.5, 23 for E15.5 and 28 for E16.5 (**Figure 2D,**
285 **2E, and S7-S13; Table S1**). GO enrichment analysis demonstrated that most
286 of these spatial gene modules correspond to features related to region-
287 specific biological processes such as muscle development (module G1),
288 neurogenesis (module G2), skin development (module G4), digestive system
289 (module G13) and ossification (module G14) at E16.5 (**Figure 2D, 2E, and**
290 **S13A; Table S1**). Then, we used a set of manually annotated cell types from
291 a recent scRNA-seq atlas of E9.5-E13.5 mouse embryos (Cao et al., 2019) to
292 assign putative cell identities to our spatial modules, observing good
293 correlation. For example, genes specific to hepatocytes and erythroid lineage,
294 megakaryocytes or neutrophils showed strong co-enrichment in the same
295 module starting from E11.5 but were not earlier, consistent with the fact that
296 the definitive hematopoiesis only occurs in the liver from E11.5 onwards
297 (Lewis et al., 2021) (**Figure S9C**). Likewise, radial glial cells are the source of
298 neuroblasts (Merkle et al., 2004) and both cell types co-localized in brain
299 regions since E11.5. In addition, jaw and tooth progenitor cells co-localized
300 with osteoblasts and connective tissue progenitor cells also after E11.5. The
301 complete set of cell assignments for all tested time points can be found in
302 **Figure S7-S13**.

303 Therefore, MOSTA generated by Stereo-seq constitutes a unique
304 resource to investigate the molecular basis of tissue patterning and the
305 emergence of cell identities during mouse organogenesis. Our publicly

306 available interactive data portal can be accessed at
307 <https://db.cngb.org/stomics/mosta/>.

308

309 **Spatiotemporal dynamics of gene regulation in mouse organogenesis**

310 Cell identity maintenance and cell state dynamics are governed by intrinsic
311 and inducible gene regulatory networks (Aibar et al., 2017). These networks
312 are often studied in the form of modules of co-expressed genes that are co-
313 regulated by common sets of transcription factors, or regulons (Aibar et al.,
314 2017). Although this data can be extracted from scRNA-seq datasets applying
315 algorithms such as SCENIC (Aibar et al., 2017), the lack of spatial information
316 muddles the establishment of reliable associations. We used SCENIC to
317 calculate the frequencies of predicted transcription factor binding events in
318 each of the individual time points of MOSTA. This identified many regulons
319 presenting regional specificity at each stage. To achieve a global overview of
320 these transcriptional programs and how they are interconnected, we applied
321 again Hotspot, which categorized them into a series of regulon modules
322 **(Table S2)**. Many of these modules contained sets of transcription factors
323 known to be involved in the formation of specific tissues and/or specific
324 developmental processes **(Figure 3A and S14-S19)**. For example, at E9.5 we
325 identified 23 modules containing a total of 410 regulons corresponding to
326 different tissues **(Figure 3A)**. Among these, GATA4 was enriched in the heart
327 (module R1), GATA1 in the blood (module R2), SOX2 in the brain (module
328 R4), DLX6 in the branchial arch (module R10) FOXA2 in the notochord
329 (module R11) and HNF4A in the liver (module R18) **(Figure 3B)**.

330 We focused on the brain for more detailed investigation because the
331 relatively large dimensions, unparalleled complexity and prolonged
332 developmental time makes particularly challenging the study of regulons with
333 single-cell sequencing approaches. First, we performed unsupervised
334 clustering of E9.5 brain regulons. This showed four major neuroepithelial
335 clusters (*Pax6*⁺ and/or *Sox2*⁺) corresponding to the telencephalon,
336 diencephalon, midbrain, and hindbrain, with a smaller neuroblast-like cluster
337 (*Nhlh1/2*⁺) distributed in the diencephalon and more obviously in the hindbrain
338 **(Figure 3C and S20A)**. Several transcription factors with known functions in
339 brain development showed differential activity among these regions **(Figure**

340 **3D-3F and S20B-S20D**). For example, the telencephalon was enriched in
341 EMX1/2, which regulate proliferation and specification of radial glial cells
342 (Bishop et al., 2003). The diencephalon was enriched in transcription factors
343 involved in thalamus specification (e.g., TCF7L2, BARHL2 and DBX1) (Lipiec
344 et al., 2020; Vue et al., 2007). EN1/2 were enriched in the anterior and
345 posterior midbrain and are known to regulate dopaminergic neuron
346 specification (Alves dos Santos and Smidt, 2011). Mid- and hindbrain shared
347 some regulons including BARHL1, which is known to be specifically enriched
348 in the adult cerebellum (part of the hindbrain) (Li et al., 2004), and LMX3.
349 Likewise, the early neuroblast regulon was enriched in transcription factors
350 characteristic of this cell type (e.g., SOX4, NEUROD1 and LHX1) (Liu et al.,
351 2000) (**Figure 3D and S20B**).

352 We next systematically examined the regulons that direct regional brain
353 specification across different time points. We focused on E9.5 and E12.5-
354 E16.5 because of the enhanced capture of related structures. We identified
355 regulon clusters controlling the formation and expansion of the diencephalon,
356 pallium, subpallium, midbrain, and hindbrain (**Figure 3G**). In the diencephalon,
357 the neuroepithelial regulons (e.g., DBX1 and BARHL2) identified at E9.5
358 became progressively inactivated after E12.5, whereas TCF7L2, BARHL2 and
359 DBX1 increase until E16.5 (**Figure 3H**). Another group of regulons comprising
360 factors associated with thalamus maturation (e.g., RORA and TFAP2B)
361 (Vitalis et al., 2018) appeared at E15.5 and remained high at E16.5. Several
362 regulons were shared between all stages (e.g., LEF1 and SHOX2) but their
363 activity increased towards E16.5, suggesting that fine-tuning their levels
364 orchestrates the transition from specification to maturation. Regulons in the
365 pallium (originated from the telencephalon) at later stages were divided into
366 the ventricular zone (VZ) and mantle zone (MZ) (**Figure 3G**). E9.5
367 telencephalon regulons progressively declined after this time point (**Figure**
368 **3H**). Many of the VZ-specific regulons showed transcription factors related to
369 early neurogenesis (e.g., EOMES, LHX2 and E2F family members) (Greig et
370 al., 2013) and remained high from E12.5 onwards (**Figure 3H**). In contrast,
371 those involved in MZ formation were related to neuronal maturation (e.g.,
372 CUX2 and BCL6), appeared at E13.5 and increased until E15.5 or E16.5.
373 Subpallium regulons showed strong enrichment of DLX homeobox

374 transcription factors (DLX1/2/5/6) from E13.5 to E16.5 (**Figure S20E and**
375 **S20F**), consistent with their role in GABAergic interneuron production (Sultan
376 et al., 2013). Midbrain and hindbrain largely showed similar sets of regulons
377 associated with neuron progenitor at early stage (e.g., E2F1, E2F5 and SP3),
378 but presented different set of transcription factors related to neuronal
379 maturation at later stages in each region (e.g., LEF1 and DMBX1 in the
380 midbrain, and HOXA5 and HOXB5 in the hindbrain) (**Figure S20E and S20F**).

381 We next investigated the spatial expression of diffusible extracellular cues
382 (morphogens) at the so-called secondary organizer centers of the developing
383 brain. These morphogens are responsible for inducing changes in gene
384 regulatory programs that cause regionalization in the brain (Tannahill et al.,
385 2005). We studied the earlier stages (E9.5 and E12.5) because the biggest
386 morphogenetic events of the brain happen during that period. Analysis of
387 gene modules identified with Hotspot showed good correspondence with the
388 known location of key secondary organizer centers including the floor plate
389 (FP), roof plate (RP), anterior neural ridge (ANR), zona limitans intrathalamica
390 (ZLI) and isthmus organizer (ISO) (**Figure S21A and S21B**). Spatial
391 correlation analysis of ligands in these gene modules revealed distinct
392 clusters in each stage (**Figure S21C and S21D**). These morphogen clusters
393 included ligands from the Sonic hedgehog (SHH), Wnt, bone morphogenetic
394 proteins (BMP), fibroblast growth factors (FGF) and R-spondin (RSPO)
395 families (Tannahill et al., 2005). We used this information to generate a spatial
396 morphogen map of the developing brain (**Figure 3I and S21E**). Examples of
397 morphogens involved in dorsoventral patterning included *Wnt1*, *Wnt3a*, *Wnt9a*
398 and *Rspo1/2/3* at the RP, whereas *Shh* was mainly enriched at the FP and to
399 a less extent at the ZLI. Morphogens involved in anteroposterior patterning
400 included FGF family members such as *Fgf8* and *Fgf15* at the ANR, ZLI and
401 ISO. We noticed that regulons at these secondary organizers were quite
402 different and variable during development. For example, EN1/2 were enriched
403 at the ISO in both E9.5 and E12.5 embryos, while the ZLI was enriched in
404 DMBX1 and PAX6 at E9.5 or PRDM16 and MAFF at E12.5. In addition,
405 FOXA1/2/3 were enriched at the FP and OTX2 in the RP (**Figure S22**). These
406 results highlight the utility of Stereo-seq to study coordinated transcriptional
407 responses to signaling cues at large scale.

408 Regulons for other tissues also showed a gradual spatiotemporal
409 transition. For example, in the skeleton we identified regulons consisting of
410 the early condensation regulators SOX5 and SOX9 (Kozhemyakina et al.,
411 2015), which promote the transition from mesenchymal progenitors in the
412 sclerotome (E9.5-E11.5) to the early (cartilage primordium, E12.5 and E13.5)
413 and late (chondrocyte, E15.5 and E16.5) chondrogenic lineage (**Figure S23A-**
414 **S23C**). Likewise, we detected key regulators of chondrocyte to osteoblast
415 maturation such as SP7 and XBP1 (Kozhemyakina et al., 2015), which
416 correlated with a switch in the pattern of extracellular matrix production from
417 *Col9a3* to *Col22a1*.

418 In summary, MOSTA can be used to infer the gene regulatory networks
419 behind patterning events driving organogenesis. Systematic analysis of other
420 regulons, morphogens and tissues can be performed using our interactive
421 portal.

422

423 **Regulation of cell diversification in the telencephalon**

424 The precise regulation of neuronal cell fate is critical for the development of
425 brain architecture, of which the neocortex is particularly interesting because of
426 the diversity of neuronal subtypes and the sophisticated migration trajectories
427 (Greig et al., 2013). We first focused on the late stage telencephalon (E15.5),
428 when the pallium and subpallium become apparent. Unsupervised spatially-
429 constrained clustering at bin 20 resolution (20 × 20 DNB, 14 μm diameter)
430 identified clusters corresponding to known anatomical regions (**Figure 4A**).
431 These clusters displayed specific markers (**Figure S24A and S24B**). Briefly,
432 we could clearly distinguish distinct pallium clusters including the dorsal
433 pallium (DPall), medial pallium (MPall, also known as hippocampal allocortex)
434 and ventral pallium (VPall). The DPall included clusters corresponding to the
435 VZ, subventricular zone (SVZ), intermediate zone (IZ), cortical plate (CP) and
436 marginal zone (MarZ). All subpallium regions expressed markers related to
437 the generation and maturation of cortical interneurons (e.g., *Dlx5* and *Gad2*;
438 **Figure S24A and S24B**) (Greig et al., 2013), and also markers corresponding
439 to known anatomical areas including the central subpallium (CSPall) and
440 paraseptal subpallium (PaSe) (<https://developingmouse.brain->

441 [map.org/static/atlas](https://www.brain-map.org/static/atlas)). Through enrichment of known genes, we further located
442 the striatum and lateral ganglionic eminence (LGE) (*Bcl11b*, *Foxp1/2*, *Isl1* and
443 *Ebf1*), medial ganglionic eminence (MGE) and preoptic area (POA) (*Nkx2.1*,
444 *Lhx6* and *Lhx8*) (**Figure 4A and S24C**). Of note, the spatial clustering showed
445 two clusters along the rostral to caudal direction at the CP (**Figure 4A**). DEG
446 analysis revealed graded distribution of genes related to axonogenesis and
447 neuron projection development along this axis (**Figure S25A and S25B**),
448 reflecting different kinetics of projection neuron specification from partially
449 fate-restricted postmitotic progenitors (Greig et al., 2013). Interestingly, we
450 also observed a gradient of expression for morphogens such as *Nrg3* along
451 the rostral to caudal direction (Muller et al., 2018; Nowakowski et al., 2017)
452 (**Figure S25C and S25D; Table S3A**).

453 We then applied SPOTlight (Elosua-Bayes et al., 2021) to map the cell
454 types into the spatial maps using a recently published neocortex single-cell
455 dataset (Di Bella et al., 2021). This detected a variety of neuronal and non-
456 neuronal cell types (**Figure 4B and S26A**). The VZ and SVZ largely showed
457 radial glial cells and intermediate progenitor cells, which decline at the IZ
458 being replaced by immature neurons. For cortical projection neurons, we
459 observed corticothalamic projection neuron (CThPN) in the inner CP in the
460 vicinity of immature neurons, and subcerebral projection neurons (SCPN)
461 mainly in the outer CP and more sparsely in the IZ. Consistent with our
462 findings above, we observed higher level of upper layer cortical projection
463 neurons (UL.CPN) in the rostral cluster of the CP, while deeper layer cortical
464 projection neurons (DL.CPN) had similar distribution.

465 To detect the directionality of gene expression changes in the developing
466 telencephalon, we applied Dynamo (**see Methods**), an analytical framework
467 that measures transcriptional dynamics to predict the emergence of cell fates
468 via vector field reconstruction (Qiu et al., 2021). In this regard, over 10% of
469 the Stereo-seq captured transcripts contained intronic reads (**Figure S26B**),
470 comparable to the proportion captured by scRNA-seq (La Manno et al., 2018).
471 Spatial RNA velocity analysis revealed two strong directional flows of
472 transcriptional changes, one from VZ and SVZ towards the CP, and the other
473 from the subpallium to the cortical layers above it. The former direction is
474 consistent with the path of neuronal specification from progenitor cells

475 migrating radially towards the MZ, while the latter is consistent with the
476 tangential migration of interneurons from the MGE into the neocortex at
477 specific layers including the SVZ and IZ (Lodato and Arlotta, 2015) (**Figure**
478 **4C**). This agrees with the observed spatial probability score of cortical
479 interneurons, projection neurons and neuron progenitors (**Figure 4B**). To
480 study the regulators that guide interneuron and cortical neuron migration, we
481 performed pseudotime analysis along the migration paths (**Figure 4D and 4E**;
482 **see Methods**). Interestingly, this showed both known regulators and unknown
483 potential regulators of neuronal migration. For example, the ephrin receptor
484 tyrosine kinases *Epha3*, *Epha4* and *Epha5*, were identified in the tangential
485 path of interneurons (Bevins et al., 2011) (**Figure S26C**). Like wise, for radial
486 migration, we identified *Sox2* and *Pax6* associated with early neurogenesis,
487 and *Satb2* with projection neuron maturation (Alcamo et al., 2008; Gomez-
488 Lopez et al., 2011). Other identified regulators are listed in **Table S3** and
489 could be important for further study.

490 We next investigated the dynamic changes in projection neurons and
491 interneurons across developmental stages in neocortex regions from E11.5 to
492 E16.5 embryos. Unsupervised spatially-constrained clustering revealed
493 clusters corresponding to specific layers in each group (**Figure S27A**). Single-
494 cell projection into these spatial maps revealed striking cell type diversification
495 during these stages. We observed that interneurons appear in the CP at
496 E13.5 and expand at E15.5 and E16.5. In contrast, CPN appeared early
497 (E12.5) and expanded and concentrated later at the IZ at E15.5 and E16.5.
498 SCPN appeared at E12.5 and resided at the upper CP layer at later stages,
499 while CThPN appeared later and resided at the inner CP layer (**Figure 4F**,
500 **S27B, and S27C**). Cell cycle analysis further confirmed the dynamics from
501 early progenitor to later postmitotic transition at the CP (**Figure S27D**).

502 Our results thus support the existence of spatiotemporal hierarchies
503 governing cell-type diversification in the developing telencephalon
504 (Nowakowski et al., 2017). Further scrutiny of this dataset using MOSTA
505 database will be invaluable for defining associations with potential functional
506 significance in the brain or other tissues.

507

508 **Deciphering developmental disease susceptibility using MOSTA**

509 Developmental diseases have been classically regarded as those producing
510 gross neonatal manifestations. Yet, many genetic conditions only manifested
511 after birth have a developmental, often unnoticed, component because their
512 target genes are also expressed during development (Boycott and Ardigo,
513 2018). For example, although Huntington's disease is a late-manifesting
514 neurological disorder affecting mostly the striatum, it is also associated with
515 abnormalities in cortex development (Barnat et al., 2020). Nowadays, scRNA-
516 seq datasets are becoming widely used to predict the origin and mechanisms
517 of genetic diseases including developmental diseases (Watanabe et al., 2019).
518 Yet, the cell capture bias and lack of contextual microenvironment affects the
519 conclusions.

520 Given its unique characteristics, MOSTA could be particularly useful to
521 dissect the developmental origins of mammalian genetic diseases, especially
522 for understanding what tissue regions are originally affected and when,
523 potentially shedding light into both big and subtle phenotypes. A major
524 advantage compared to data generated with other spatially resolved
525 technologies is the ability to look at multiple areas of the same tissue and
526 multiple tissues simultaneously across different embryonic stages. To
527 showcase this, we first took the 1,987 disease list (including 1,940 genes)
528 from the Deciphering Developmental Disorders (DDD) database (Firth et al.,
529 2011), filtered them to select the top 729 genes based on the expression
530 threshold in our own dataset (**Table S4**), and projected both the filtered and
531 unfiltered genes onto our spatial transcriptomic maps for all developmental
532 time points. This showed enrichment in different tissues including the brain,
533 heart, liver, blood, bone, muscle and others (**Figure 5A and S28**). Some of
534 these disease-related genes were expressed in most or all embryonic stages,
535 whereas others had a restricted window. Similarly, although many genes were
536 mainly enriched in one tissue (in some cases confined to a specific region) or
537 system, others were shared between several. For example, *Wnt5a*, which is
538 related to Robinow syndrome, located in the craniofacial region, genital ridge,
539 and brain (FP and neocortex) since early stages, consistent with the disease
540 phenotypes (Menezes et al., 2010) (**Figure 5B**). *NKX2-1*, mutated in brain-
541 lung-thyroid syndrome (Shetty et al., 2014), was mainly enriched in brain
542 (hippocampus and subpallium) and lung. *Lmod3*, which is associated with

543 nemaline myopathy (Berkenstadt et al., 2018), was enriched in the skeletal
544 muscle.

545 Autism encompasses a complex and multifactorial (genetic and
546 environmental) group of neuropsychiatric diseases with recognized
547 developmental roots but poorly understood mechanisms (Lord et al., 2020).
548 Clarifying the diversity of tissues affected by autism besides the brain is
549 important for designing patient-specific management therapies, as it is often
550 associated with non-behavioral manifestations. We projected a battery of 161
551 autism-related genes (Liberzon et al., 2015) onto our spatial embryo maps,
552 observing grouping in different tissues (**Figure 5C; Table S4**). Approximately
553 1/3 of the genes were enriched in the central nervous system (CNS), mostly
554 the brain, with predominance or not for certain regions. Some autism-related
555 genes were expressed along the whole developmental timeline, whereas
556 others were more restricted (**Figure 5C**). For example, *Otx2* was enriched in
557 the midbrain, subthalamus and pallium at different stages, whereas *Tbr1* was
558 located mainly in the MZ of the pallium. Interestingly, a large group of autism
559 related genes were enriched in non-neural tissues (e.g., heart, liver, muscle,
560 bone and lung). For example, *Dmpk* was widely expressed in muscle but not
561 in the brain, suggesting that abnormalities of the former tissue indirectly cause
562 the autism phenotype (**Figure 5D**).

563 These results demonstrate the utility of MOSTA for defining the
564 spatiotemporal windows of genetic disease vulnerability during development,
565 and for establishing expected and unexpected causal relationships. Manual
566 inclusion of other disease datasets can be used for case-specific explorations.

567

568

569 **DISCUSSION**

570

571 Topographic transcriptomic information is necessary to dissect the molecular
572 events driving mammalian development. Stereo-seq is a spatially resolved
573 transcriptomic technology with high resolution, sensitivity and large field of
574 view. These methodological parameters are all fundamental for reliably
575 profiling the transcriptomic heterogeneity of complex tissues and organisms
576 such as mammalian embryos. Whole mouse embryo-scale spatially resolved

577 transcriptomes have been reported recently (Liu et al., 2020; Srivatsan et al.,
578 2021) but they contained low density of detected signals and were restricted
579 to only one stage of organogenesis. The sparsity of gene detection in those
580 studies makes it difficult to analyze gene expression gradients across the
581 developing tissues and, thus, it is hard to recapitulate fine anatomical
582 structures. We have used Stereo-seq to create the first version of MOSTA
583 (<https://db.cngb.org/stomics/mosta/>), an expandable panoramic high-definition
584 transcriptomic resource of mouse organogenesis. MOSTA provides detailed
585 topographic information about the stepwise formation of tissues and the
586 emergence of tissue-specific cell identities across different stages of mouse
587 organogenesis. Examples of the latter include the identification of incipient
588 areas of neuroblast specification in E9.5 brain subregions and of gradients of
589 neuronal specification in the neocortex. One caveat of MOSTA is that some
590 tissues such as the thyroid are underrepresented due to the sectioning
591 procedure, whereas others will benefit from additional sections to fully capture
592 the intricacy of developmental processes. It will also be important to include
593 whole neonatal and adult mice for additional comparisons. In the future, we
594 will add these samples and expand the number of sections with the goal of
595 ultimately achieving 3D-transcriptomic reconstructions. Facilitating this goal,
596 the precise spatial coordinates retrieved by Stereo-seq allow optimal
597 integration of different slides, which might not be achieved with accuracy by
598 techniques using randomly distributed beads. Moreover, being based on
599 standard DNB technology it is relatively easy to enhance the sample
600 throughput using Stereo-seq and systematically profile hundreds of tissue
601 slices in a relatively short time.

602 To demonstrate the utility of MOSTA for inferring the molecular logic
603 behind developmental processes, we have for the first time systematically
604 dissected the spatially resolved gene regulatory networks driving
605 organogenesis. This has identified tissue- and area-specific regulons across
606 different embryonic stages and focusing on the brain we have also shown the
607 correspondence with signaling cues responsible for inducing their
608 diversification. In addition to understanding development, knowledge of these
609 regulons and morphogens might be useful to devise or improve ways to
610 reprogram somatic cells into other lineages *in vivo* or *in vitro* and for fine-

611 tuning differentiation protocols using pluripotent stem cells (Wang et al., 2021).
612 We have also projected the expression of developmental disease loci
613 (including among others monogenic diseases involving transcription factors or
614 morphogens and complex traits like autism) onto our spatial maps,
615 highlighting unexpected associations such as the localization of autism-
616 related genes in the muscle or the liver. Although mice and humans differ in
617 multiple aspects of normal physiology or disease and caution should be taken
618 with the interpretations, this approach has a tremendous potential to uncover
619 novel disease mechanisms. Systematic independent explorations using our
620 interactive portal will provide users worldwide with a unique chance to unlock
621 the black boxes of mouse embryogenesis and developmental diseases. In this
622 regard, expansion with datasets from genetically engineered mice that mimic
623 developmental diseases or bear alteration of developmental pathways will be
624 useful to test or refine any generated models of normal and abnormal
625 development generated with MOSTA.

626 Importantly, larger Stereo-seq chips could enable the profiling of embryos
627 from other mammalian species including primates. Forthcoming optimizations
628 of Stereo-seq will also increase gene and transcript capture, facilitating the
629 assignment of individual cell identities. Similarly, oligonucleotide-labeled
630 antibodies (Stoeckius et al., 2017) could be incorporated into the DNB for
631 simultaneous protein detection of surface markers, which would be useful for
632 identifying specific cell types (e.g., infiltrating immune cells). Likewise, we
633 anticipate that Stereo-seq will have other applications beyond RNA-
634 sequencing such as spatially resolved epigenomics (e.g., chromatin
635 accessibility and DNA methylation profiling). Accordingly, besides
636 development, Stereo-seq and its future technical variations will be highly
637 transformative for multiple research fields across kingdoms of life. Given its
638 characteristics and applicability Stereo-seq also has the potential to move into
639 the routine clinical practice as an extraordinary diagnostic tool complementary
640 to medical imaging and histopathology data.

641

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832

833

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846

847 **AUTHOR CONTRIBUTIONS**

848 J.W., X.X., A.C., L.L., and Y.Li conceived the idea; J.W., X.X., A.C., L.L., Y. Li
849 and M.A.E. supervised the work; A.C., S. Liao., L.L. and M.C. designed the
850 experiment; S. Liao, J.Y. and W.L. generated the Stereo-seq chip with the
851 help from X.C., X.H.^{1,3} and D.F.; M.C., J.X., H.Z., H.X., L.H., P.G. and F.L.
852 performed the majority of the experiments; K.M., L.W., X.L., X.T., H.L. and M.
853 Li performed data preprocessing and quality evaluation; L.W., Y.Lai, X.Q., X.
854 W. and S.H. analyzed the data; Z.L., Y.J., J.P., S.Liu, C.L., Zhi.Wang, Y.Yuan.,
855 Y.H., Q.L., Zhao.Wang, X.H.¹, F.Z., H.K., O.W., B.W. and H.L. provided
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857 J.M., J.P.T, M.Lisby, R.J.C and M.U. gave the relevant advice; M.A.E., J.W.,
858 X.X., L.L., Y.Lai, W.L. and A.C. wrote the manuscript.

859

860 **DECLARATION OF INTERESTS**

861 The chip, procedure and applications of Stereo-seq are covered in pending
862 patents. Employees of BGI have stock holdings in BGI.

863

864

865 **RESOURCE AVAILABILITY**

866

867 **Lead contact**

868 Further information and requests for the resources and reagents may be
869 directed to the corresponding author Xun Xu (xuxun@genomics.cn)

870

871 **Material availability**

872 All materials used for Stereo-seq are commercially available.

873

874 **Data and code availability**

875 All raw data generated by Stereo-seq have been deposited to CNGB
876 Nucleotide Sequence Archive (accession code: CNP0001543
877 (<https://db.cngb.org/search/project/CNP0001543>). All data were analyzed with
878 standard programs and packages. Custom code using open-source software
879 supporting the current study is available from the corresponding authors on
880 request and will be uploaded at <https://github.com/BGIResearch/handleBam>.

881

882

883 **METHODS**

884

885 **Tissue collection**

886 All relevant procedures involving animal experiments presented in this paper
887 are compliant with ethical regulations regarding animal research and were
888 conducted under the approval of the Animal Care and Use committee of the
889 Guangzhou Institutes of Biomedicine and Health, Chinese Academy of
890 Sciences (license number IACUC2021002). Mouse olfactory bulb and brain
891 were dissected from 12-week-old C57BL/6J female mice. E9.5, E10.5, E11.5,
892 E12.5, E13.5, E15.5 and E16.5 embryos were collected from pregnant
893 C57BL/6J female mice. After collection, tissues were snap-frozen in liquid
894 nitrogen prechilled isopentane in Tissue-Tek OCT (Sakura, 4583) and
895 transferred to a -80°C freezer for storage before the experiment. Cryosections
896 were cut at a thickness of approximately 10 µm in a Leica CM1950 cryostat;
897 mouse olfactory bulb and mouse brain were cut coronally, mouse embryos
898 were cut sagittally.

899

900 **Stereo-seq chip preparation**

901 **Generation of Stereo-seq chips.** To generate the patterned array, we first
902 synthesized two oligo sequences: one containing 25 random
903 deoxynucleotides (nt)
904 (TGTGAGCCAAGGAGTTGNAACTGCTGACGTAAGGAGGAGGATGGCGAC
905 CTTATCAGNNNNNNNNNNNNNNNNNNNNNNNNNNNTTGTCTTCCTAAGACC
906 G, Sangon) and the other a fixed sequence with phosphorylation
907 (/5phos/CTTGGCCTCCGACTTAAGTCGGATCGTAGCCATGTCGTTTC,

908 Sangon). These two oligos were ligated by incubation with T4 ligase (NEB;
909 1U/ μ l T4 DNA ligase and 1 \times T4 DNA ligation buffer) and splint oligo
910 (TCGGAGGCCAAGCGGTCTTAGGAA, Sangon, 1 μ M) at 37 $^{\circ}$ C for 2 hours.
911 The products were purified using the Ampure XP Beads (Vazyme, N411-03)
912 and then PCR amplified with the following steps: 95 $^{\circ}$ C for 5 minutes, 12 cycles
913 at 98 $^{\circ}$ C for 20 seconds, 58 $^{\circ}$ C for 20 seconds, 72 $^{\circ}$ C for 20 seconds and a final
914 incubation at 72 $^{\circ}$ C for 5 minutes. PCR products were purified using the
915 Ampure XP Beads (Vazyme, N411-03). DNBs were then generated by rolling
916 circle amplification and loaded onto the patterned chips according to the MGI
917 DNBSEQ-Tx sequencer manual. Next, to determine the distinct DNB-CID
918 sequences at each spatial location, single-end sequencing was performed
919 using a sequencing primer
920 (CTGCTGACGTACTGAGAGGCATGGCGACCTTATCAG, Sangon, 1 μ M) in
921 a MGI DNBSEQ-Tx sequencer with SE25 sequencing strategy. After
922 sequencing, the capture oligo
923 (/5phos/TTGTCTTCCTAAGACNNNNNNNNNTTTTTTTTTTTTTTTTTTTTTTV,
924 Sangon, 1 μ M) including 22 nt poly-T and 10 nt UMI was hybridized with the
925 DNB in 5 \times SSC buffer at 37 $^{\circ}$ C for 30 minutes, and then incubated with T4
926 ligase (NEB, 1 U/ μ l T4 DNA ligase, 1 \times T4 DNA ligation buffer and 0.5%
927 PEG2000) at 37 $^{\circ}$ C for 1 hour. This procedure produces capture probes
928 containing a 25 nt CID barcode, a 10 nt UMI and a 22 nt poly-T ready for poly-
929 A RNA capture. A schematic describing this procedure is included in the
930 MOSTA website (<https://db.cngb.org/stomics/mosta/>).

931 **Calling of CID.** CID sequences together with their corresponding coordinates
932 for each DNB were determined using a base calling method according to
933 manufacturer's instruction of MGI DNBSEQ-Tx sequencer. After sequencing,
934 the capture chip was split into smaller size chips (5 mm \times 10 mm, 10 mm \times 10
935 mm, 10 mm \times 20 mm) ready for use. At this stage, we filtered out all
936 duplicated CID that corresponded to non-adjacent spots.

937

938 **Stereo-seq library preparation and sequencing**

939 **Tissue processing.** Tissue sections were adhered to the Stereo-seq chip
940 surface and incubated at 37 $^{\circ}$ C for 3 minutes. Then, they were fixed in

941 methanol and incubated for 30 minutes at -20°C, after which they were ready
942 to be used for Stereo-seq. Where indicated, the same sections were stained
943 with nucleic acid dye (Thermo fisher, Q10212), or adjacent sections were
944 subjected to tissue histology examination using hematoxylin and eosin staining
945 according to standard procedure. Imaging for both procedures was performed
946 with a Ti-7 Nikon Eclipse microscope.

947 ***In situ reverse transcription.*** Tissue sections placed on the chip were
948 permeabilized using 0.1% pepsin (Sigma, P7000) in 0.01 M HCl buffer,
949 incubated at 37°C for 12 minutes and then washed with 0.1× SSC buffer
950 (Thermo, AM9770) supplemented with 0.05 U/μl RNase inhibitor (NEB,
951 M0314L). RNA released from the permeabilized tissue and captured by the
952 DNB was reverse transcribed overnight at 42°C using SuperScript II
953 (Invitrogen, 18064-014, 10 U/μl reverse transcriptase, 1 mM dNTPs, 1 M
954 betaine solution PCR reagent, 7.5 mM MgCl₂, 5 mM DTT, 2 U/ml RNase
955 inhibitor, 2.5 μM Stereo-TSO and 1× First-Strand buffer). After reverse
956 transcription, tissue sections were washed twice with 0.1× SSC buffer and
957 digested with Tissue Removal buffer (10 mM Tris-HCl, 25 mM EDTA, 100 mM
958 NaCl and 0.5% SDS) at 37°C for 30 minutes. cDNA-containing chips were
959 then subjected to Exonuclease I (NEB, M0293L) treatment for 1 hour at 37°C
960 and were finally washed once with 0.1x SSC buffer.

961 ***Amplification.*** The resulting cDNA were amplified with KAPA HiFi Hotstart
962 Ready Mix (Roche, KK2602) with 0.8 μM cDNA-PCR primer. PCR reactions
963 were conducted as follows: first incubation at 95°C for 5 minutes, 15 cycles at
964 98°C for 20 seconds, 58°C for 20 seconds, 72°C for 3 minutes and a final
965 incubation at 72°C for 5 minutes.

966 ***Library construction and sequencing.*** The concentrations of the resulting
967 PCR products were quantified by Qubit™ dsDNA Assay Kit (Thermo,
968 Q32854). A total of 20 ng of DNA were then fragmented with *in-house* Tn5
969 transposase at 55°C for 10 minutes, after which the reactions were stopped by
970 the addition of 0.02% SDS and gently mixing at 37 °C for 5 minutes after
971 fragmentation. Fragmentation products were amplified as described below: 25
972 ml of fragmentation product, 1× KAPA HiFi Hotstart Ready Mix and 0.3 μM

973 Stereo-Library-F primer, 0.3 μ M Stereo-Library-R primer in a total volume of
974 100 ml with the addition of nuclease-free H₂O. The reaction was then run as:
975 1 cycle of 95 °C 5 minutes, 13 cycles of (98 °C 20 seconds, 58 °C 20 seconds
976 and 72 °C 30 seconds) and 1 cycle of 72 °C 5 minutes. PCR products were
977 purified using the Ampure XP Beads (Vazyme, N411-03) (0.6 \times and 0.15 \times),
978 used for DNB generation and finally sequenced (35 bp for read 1, 100 bp for
979 read 2) on a MGI DNBSEQ-Tx sequencer.

980

981 **Stereo-seq raw data processing**

982 Fastq files were generated using a MGI DNBSEQ-Tx sequencer. CID and
983 UMI are contained in the read 1 (CID: 1-25bp, UMI: 26-35bp) while the read 2
984 consist of the cDNA sequences. CID sequences on the first reads were first
985 mapped to the designed coordinates of the *in situ* captured chip achieved
986 from the first round of sequencing, allowing 1 base mismatch to correct for
987 sequencing and PCR errors. Reads with UMI containing either N bases or
988 more than 2 bases with quality score lower than 10 were filtered out. CID and
989 UMI associated with each read were appended to each read header.
990 Retained reads were then aligned to the reference genome (mm10) using
991 STAR (Dobin et al., 2013) and mapped reads with MAPQ \geq 10 were counted
992 and annotated to their corresponding genes using *handleBam* (available at
993 <https://github.com/BGIResearch/handleBam>). UMI with the same CID and the
994 same gene locus were collapsed, allowing 1 mismatch to correct for
995 sequencing and PCR errors. Finally, this information was used to generate a
996 CID-containing expression profile matrix.

997

998 **Unsupervised spatially-constrained clustering of Stereo-seq data**

999 Expression profile matrix was divided into non-overlapping bins covering an
1000 area of $X \times X$ DNB, with $X \in (1, 3, 14, 50, 140)$ and the transcripts of the
1001 same gene aggregated within each bin. After this step, data were log-
1002 normalized in Scanpy and spatially-constrained unsupervised clustering was
1003 performed. In brief, to include spatial information during clustering, we first
1004 built a spatial k-nearest neighbor graph $G_{spatial}^{k_1}$ (k_1 is by default set to be 8
1005 as each bin has 8 nearest spatial neighbors) using Squidpy and then took the

1006 union with the k-nearest neighbor graph $G_{expression}^{k_2}$ based on transcriptomic
1007 data (k_2 is by default set to be 30). The combined graph
1008 ($G_{combined} = G_{spatial}^{k_1} \cup G_{expression}^{k_2}$) was then used as input for leiden
1009 clustering. Further, each cluster was annotated based on the cluster specific
1010 markers identified by the rank_genes_groups function of scanpy using default
1011 parameters as well as the anatomical annotation based on eHistology
1012 Kaufman Annotations (<http://www.emouseatlas.org/emap/home.html>) or Allen
1013 Brain Atlas (<http://mouse.brain-map.org/>).

1014

1015 **Comparison of Stereo-seq with other published methods**

1016 HDST data (Vickovic et al., 2019) were taken from GSE130682, SLIDE-seqV2
1017 data (Stickels et al., 2021) from the Single Cell Portal of the Broad Institute,
1018 DBiT-seq data (Yao et al., 2020) from GSE137986, and Visium data
1019 (Lebrigand et al., 2020b) from GSE153859, Seq-Scope data were taken from
1020 GSE169706 (Cho et al., 2021a). For **Fig. 1c**, to ensure that proper
1021 comparisons were made, the data of Stereo-seq were binned into bin 3 (3 × 3
1022 resolution, ~2 μm diameter), bin 14 (14 × 14 resolution, ~10 μm diameter) or
1023 bin 140 (140 × 140 resolution, ~100 μm diameter). For **Extended Data Fig.**
1024 **1e**, data from HDST were binned into bin 5 (5 × 5, ~10 μm diameter).

1025

1026 **Spatially-resolved gene regulatory networks**

1027 The analysis of regulon activity was performed by following the standard
1028 SCENIC pipeline (Aibar et al., 2017). The input to SCENIC was the bin 50 (50
1029 x 50 resolution) expression matrix. The expression matrix was loaded into
1030 GENIE3 and the co-expressed gene network for each transcription factor was
1031 constructed. Transcription factor co-expression modules were then analyzed
1032 by RcisTarget and their potential targets further filtered by default parameters.
1033 Filtered potential targets were used to build the regulons. Regulon activity
1034 (Area Under the Curve) was analyzed by AUCCell and the active regulons were
1035 determined by AUCCell default threshold. The activity of regulons for each bin
1036 was then mapped to the physical space. The gene network for **Figure 3F** was
1037 constructed by selecting the target genes of corresponding transcription
1038 factors related to neuronal development, and further visualized by Cytoscape.

1039 **Identification of spatially auto-correlated gene or regulon modules**

1040 Spatially auto-correlated gene or regulon modules were identified using
1041 Hotspot (DeTomaso and Yosef, 2021). The expression matrix for the top
1042 5,000 variable genes and all regulon activity matrix of each embryo were used
1043 as input. For gene module, the data were first normalization by the total UMI
1044 number of each bin, then create knn graph of genes using the
1045 `create_knn_graph` function with the parameters: `n_neighbors = 30` (for regulon,
1046 `n_neighbors = 10`), then genes or regulons with significant spatial
1047 autocorrelation ($FDR < 0.05$) were kept for further analysis. And the modules
1048 were identified using the `create_modules` with the parameters:
1049 `min_gene_threshold = 20` and `fdr_threshold = 0.05` (for the regulon:
1050 `min_gene_threshold = 5` and `fdr_threshold = 0.05`).

1051

1052 **Deconvolution of cell types**

1053 We applied SPOTlight (Elosua-Bayes et al., 2021) to deconvolute and map all
1054 cell types in the murine telencephalon. Mouse embryonic cortex scRNA-seq
1055 data were retrieved from GSE153164 (Di Bella et al., 2021). We chose cell
1056 type with the highest probability out of all cell types provided by the
1057 `spotlight_deconvolution` function with commendatory parameters as the final
1058 cell type for each bin. The cell type specific proportions were projected to the
1059 physical space.

1060

1061 **Rostral-caudal brain gene expression dynamics**

1062 The CP of E15.5 telencephalon was manually dissected into 16 blocks along
1063 the rostral-caudal direction based on spatial coordinates. Variable genes
1064 along the rostral to caudal axis were identified using the `differentialGeneTest`
1065 function of `monocle` (Qiu et al., 2017) based on the coordinates of the blocks.
1066 Then DEG along the rostral-caudal axis were identified with $p < 0.05$.

1067

1068 **RNA velocity analysis**

1069 Analysis was performed using `Dynamo` (Qiu et al., 2021) following the tutorial
1070 found at <https://dynamo-release.readthedocs.io/>. Unspliced and spliced RNA
1071 for each bin (20×20 resolution) were extracted from E15.5 telencephalon
1072 region with `handleBam`. The data matrix was then processed by `Dynamo` to

1073 normalize the expression, select feature genes and perform dimension
1074 reduction via UMAP, followed by default parameters to estimate the kinetic
1075 parameters and gene-wise RNA velocity vectors that were then projected to
1076 the physical space. Specifically, the new “Fokker-Planck” kernel implemented
1077 in Dynamo instead of the conventional correlation or cosine kernel was used
1078 for the projection of high-dimensional RNA velocity vectors to physical space.
1079 Streamlines were used to visualize the velocity vector flows on telencephalon
1080 in which only velocity flows for the relevant domains are visualized. To
1081 facilitate the understanding of gene expression dynamics over space, we first
1082 reconstructed the continuous vector field in the UMAP space, and then
1083 utilized a new method, hodge decomposition, implemented in Dynamo that
1084 takes the simplicial complexes (a sparse directional graph) constructed based
1085 on the learned vector field function to infer the vector field based pseudotime
1086 (vf pseudotime). At last, we also illustrated the kinetics of all genes with
1087 highest PCA loading as well as the DEG identified by spatially-constrained
1088 clustering along the vf pseudotime.

1089

1090 **Spatial gene enrichment analysis of human developmental disease** 1091 **associated genes**

1092 A list of 1,940 genes corresponding to monogenic diseases was retrieved
1093 from DDG2P (version 2.29). The gene set related to autism were taken from
1094 MSigDB (Liberzon et al., 2015). For monogenic disease analysis, genes with
1095 depth-normalized expression value greater than 1(729 genes) were kept for
1096 further analysis. The expression level of indicated organs at selected time
1097 point were aggregated, Z score normalized, and further visualized by heatmap.

1098

1099

1100 **FIGURE LEGENDS**

1101

1102 **Figure 1. Advanced RNA capture from complex tissues by Stereo-seq.**

1103 **A.** Stereo-seq pipeline. *Step 1*, design of the DNB patterned array chip. *Step*
1104 *2*, *in situ* sequencing to determine the spatial coordinates of uniquely
1105 barcoded oligonucleotides placed on each spot of the chip. *Step 3*,
1106 preparation of capture probes by ligating the UMI-polyT containing

1107 oligonucleotides to each spot. *Step 4*, subsequent *in situ* RNA capture
1108 from tissue placed on the chip. *Step 5*, cDNA amplification, library
1109 construction and sequencing. *Step 6*, data analysis to generate the
1110 spatially resolved transcriptome of the profiled tissue.

1111 **B.** Stereo-seq achieves a smaller spot size (upper left), higher resolution
1112 (upper right), higher number of spots per 100 μm^2 (bottom left) and larger
1113 capture area (bottom right) than other reported methods. Samples used
1114 for the comparison included mouse olfactory bulb (Stereo-seq, Visium,
1115 Slide-seqV2 and HDST), E10 mouse embryo (DBiT-seq) and mouse liver
1116 (Seq-Scope) (Cho et al., 2021b; Lebrigand et al., 2020a; Liu et al., 2020;
1117 Stickels et al., 2021; Vickovic et al., 2019). Note that since Seq-Scope
1118 uses a random array, which contains no patterned spots, the size of each
1119 pixel was estimated according to the published dataset.

1120 **C.** Boxplots showing the number of transcripts captured by Stereo-seq at the
1121 indicated resolution in comparison with reported HDST, Slide-seqV2,
1122 Visium, DBiT-seq and Seq-Scope datasets. Samples in those datasets
1123 used for comparison are as in **panel B**.

1124 **D.** Unsupervised spatially-constrained clustering of the mouse olfactory bulb
1125 section analyzed by Stereo-seq data at bin 14 resolution, bins were
1126 colored by their annotation. ONL, olfactory nerve layer. OPL, outer
1127 plexiform layer. GL, glomerular layer. GCL-D, granular cell zone deep.
1128 GCL-E, granular cell layer externa. GCL-I, granular cell layer internal. IPL,
1129 internal plexiform layer. ML, mitral layer. SEZ, subependymal zone. Scale
1130 bar, 500 μm .

1131 **E.** Left: spatial visualization of *Cdhr1* in a mouse olfactory bulb section
1132 analyzed by Stereo-seq. Right: *Cdhr1* ISH data taken from Allen Brain
1133 Atlas. Scale bars, 500 μm .

1134 **F.** Unsupervised spatially-constrained clustering of the mouse brain section
1135 analyzed by Stereo-seq at bin 50 resolution, bins were colored by their
1136 annotation. Scale bar, 500 μm .

1137 **G.** Left: projection of captured DNB signals of the same region squared in the
1138 **panel F**. Scale bar, 50 μm . Right: superimposed nucleic acid staining and
1139 captured DNB signals from the same region squared in the middle panel.
1140 Scale bar, 10 μm .

- 1141 **Figure 2. Spatiotemporal transcriptomic atlas of mouse organogenesis.**
- 1142 **A.** Unsupervised spatially-constrained clustering of mouse embryo sections
1143 across E9.5, E10.5, E11.5, E12.5, E13.5, E15.5, and E16.5, bins were
1144 colored by their annotation. The squares indicate the regions for analysis
1145 in **panel C**. Scale bar, 1 mm.
- 1146 **B.** Spatial visualization of expression of indicated genes for muscle,
1147 epidermis, cartilage primordium, and nervous system. Scale bar, 1 mm.
- 1148 **C.** Spatial visualization of indicated areas in **panel A** identified detailed
1149 anatomical structure for the heart (E10.5), limb (E15.5), spinal cord
1150 (E16.5), and adrenal gland (E16.5). The expression of marker genes for
1151 each anatomical structure is shown on the right side of each area. Scale
1152 bars, 500 μm .
- 1153 **D.** Heatmap showing the genes with significant spatial autocorrelation (3,240
1154 genes, FDR < 0.05) grouped into 28 gene modules on the basis of
1155 pairwise spatial correlations of E16.5 embryo. Selected genes and GO
1156 terms related to representative gene modules are highlighted on the right
1157 side.
- 1158 **E.** Spatial visualization of indicated gene module related to muscle, cartilage
1159 primordium, connective tissue, pancreas, epidermis, GI tract, and nervous
1160 system. Scale bar, 1 mm.

- 1161
- 1162 **Figure 3. Spatially resolved gene regulatory networks of organogenesis.**
- 1163 **A.** Heatmap showing the regulons with significant spatial autocorrelation
1164 (481 regulons, FDR < 0.05) grouped into 23 modules on the basis of
1165 pairwise spatial correlations of E9.5 embryo section, selected regulons
1166 and their corresponding GO terms related to representative regulon
1167 modules are highlighted on the right side.
- 1168 **B.** Spatial visualization of indicated modules and their representative regulon
1169 for heart, blood, brain, branchial arch, notochord, and liver. Scale bar, 500
1170 μm .
- 1171 **C.** Unsupervised spatially-constrained clustering of E9.5 mouse embryonic
1172 brain based on regulon activity. Tel: telencephalon; Die: diencephalon; Mb:
1173 midbrain; Hb: hindbrain. Scale bar, 500 μm .
- 1174 **D.** Heatmap showing the normalized regulon activity score of top

1175 transcription factors corresponding to different anatomical structures of
1176 E9.5 brain.

1177 **E.** Spatial visualization of the indicated regulon activity representing different
1178 anatomical structures of E9.5 brain. Scale bar, 500 μm .

1179 **F.** Gene regulatory network of transcription factors (EMX1/2) representing
1180 telencephalon (EMX1/2), diencephalon (TCF7L2 and DBX1), midbrain
1181 (EN1/2), and mid-hindbrain (BARHL1 and MSX3) of E9.5 brain as
1182 visualized by Cytoscape. Selected target genes related to neuronal
1183 development was shown.

1184 **G.** Unsupervised spatially-constrained clustering of mouse embryonic brain
1185 from E9.5, E12.5, E13.5, E15.5 and E16.5 based on regulon activity
1186 identified anatomical structures of the developing brain. MZ: mantle zone;
1187 VZ: ventricular zone; Pall, pallium; Spall, subpallium; Th, thalamus, Pth,
1188 prethalamus; Hy, hypothalamus; Mb-Hb, mammillary body - hindbrain.
1189 Scale bars, 500 μm .

1190 **H.** Heatmaps showing the normalized regulon activity score of top
1191 transcription factors corresponding to mouse embryonic diencephalon
1192 (top) or pallium (bottom) development.

1193 **I.** Upper left: schematic representation of the secondary organizer centers in
1194 the early embryo brain. Bottom: Spatial scatter plots showing single DNB
1195 that captured the transcripts for the indicated morphogens at the ISO,
1196 ANR, ZLI, RP, and FP at E9.5 (bottom left) and E12.5 (bottom right)
1197 embryos. Plots were superimposed with E9.5 or E12.5 brain structures.
1198 ISO, isthmus organizer; ANR, anterior neural ridge; ZLI, zona limitans
1199 intrathalamica; RP, roof plate; FP, floor plate. Scale bars, 500 μm .

1200

1201 **Figure 4. Cell type diversification during neocortex development.**

1202 **A.** Unsupervised spatially-constrained clustering of the forebrain from E15.5
1203 embryo identified anatomical structures (bin 20 resolution). Bins are
1204 colored by their annotated structures. CP-C, cortical plate - caudal; CP-R,
1205 cortical plate - rostral; MarZ, marginal zone; MPall, medial pallium; VPall,
1206 ventral pallium; CSPall, central subpallium; PaSe, paraseptal subpallium;
1207 StrV, ventricular zone of subpallium; ChP, choroid plexus. Scale bar, 500
1208 μm .

- 1209 **B.** Left: spatial visualization of the indicated cell type proportion score for the
1210 E15.5 forebrain. Right: dotplot showing the cell type composition at
1211 different anatomical structures of the E15.5 forebrain. Ap. Prog, apical
1212 progenitor; Int. prog., intermediate progenitor; Imm. neu., immature
1213 neurons; Mig. Neu. migrating neuron; SCPN, subcerebral projection
1214 neurons; DL. CPN, deeper layer callosal projection neuron; UL. CPN,
1215 upper layer callosal projection neuron; CThPN, corticothalamic projection
1216 neuron; IN, Interneuron. CR, Cajal-Retzius cell. EC, endothelial cell.
1217 VLMC, vascular and leptomeningeal cell. Scale bar, 500 μ m.
- 1218 **C.** RNA velocity streamline plots visualize the developmental trajectory of the
1219 forebrain. Bins are colored by their annotated structures, as in **panel A**.
1220 Scale bar, 500 μ m.
- 1221 **D.** Vector field pseudotime analysis of tangential migration of interneuron
1222 neurons (upper) and radial migration of cortical neuron (bottom). Scale
1223 bar, 500 μ m.
- 1224 **E.** Gene expression heatmap of 680 top PCA genes in a pseudo-temporal
1225 order of tangential migration (upper) and radial migration path (bottom),
1226 genes with highest PCA loading as well as the DEG identified by
1227 unsupervised spatially-constrained clustering were shown in the figure.
- 1228 **F.** Dotplot showing the cell type composition dynamic for the mouse
1229 embryonic cortex at E11.5, E12.5, E13.5, 15.5, and E16.5.

1230

1231 **Figure 5. Association of MOSTA profiles with human developmental**
1232 **disorders.**

- 1233 **A.** Heatmap showing the normalized expression level of 729 genes selected
1234 from the Deciphering Developmental Disorders database in six
1235 representative anatomical structures (brain, bone, muscle, heart, liver and
1236 lung) in embryos from E9.5 to E16.5.
- 1237 **B.** Spatial visualization of expression of indicated genes related to the
1238 human developmental disorders (Robinow syndrome, brain-lung-thyroid
1239 syndrome and nemaline myopathy) in mouse embryos from E9.5 to E16.5.
1240 Disease- related structures are annotated in the figure.
- 1241 **C.** Heatmap showing the normalized expression level of 161 autism-related
1242 genes in six representative anatomical structures (brain, bone, muscle,

1243 heart, liver and lung) in mouse embryos from E9.5 to E16.5.

1244 **D.** Spatial visualization of specific genes associated with autism (*Dmpk*, *Tbr1*,
1245 and *Otx2*) in the embryo brain from E9.5 to E16.5. Disease-related
1246 structures are annotated in the figures.

1247

1248 **Figure S1. Size of stereo-seq chips and assessment of performance,**
1249 **related to Figure 1.**

1250 **A.** Stereo-seq chips of different sizes ranging from 50 mm² to 174.24 cm².

1251 **B.** Spatial visualization of the number of UMI captured by Stereo-seq from
1252 two adjacent mouse olfactory bulb coronal sections at bin 14 resolution.

1253 **C.** Unsupervised spatially-constrained clustering of the mouse olfactory bulb
1254 section (section 2) at bin 14 resolution.

1255 **D.** Pearson correlation coefficient ($R^2 = 0.9666$) of pseudo-bulk profiles from
1256 the two Stereo-seq experiments of mouse olfactory bulb.

1257 **E.** Spatial visualization of the indicated genes in the two independent Stereo-
1258 seq experiments for mouse olfactory bulb and reported HDST, Slide-
1259 seqV2 and ISH datasets (Lein et al., 2007; Stickels et al., 2021; Vickovic
1260 et al., 2019). Scale bars, 500 μ m.

1261

1262 **Figure S2. Stereo-seq detects different anatomical structures in the**
1263 **adult mouse brain, related to Figure 1.**

1264 **A.** Heatmap showing the mean expression level of DEG between the
1265 indicated anatomical structures identified in **Figure 1F**.

1266 **B.** GO analysis of DEG between the indicated anatomical structures.
1267 Selected GO terms (Benjamini-Hochberg corrected *P* value) are shown.

1268 **C.** Spatial visualization of the expression of indicated genes representing
1269 different anatomical structures in a whole adult mouse brain coronal
1270 section analyzed by Stereo-seq at bin 50 resolution. Gene expression
1271 levels were quantified as $\log_2(\text{transcripts per } 10\text{K UMI} + 1)$, which applies
1272 to all similar plots.

1273

1274 **Figure S3. Spatially resolved transcriptomic atlas of mouse**
1275 **organogenesis, related to Figure 2.**

1276 **A.** Violin plot showing the number of transcripts (left) and genes (right)

1277 captured by Stereo-seq at different stages of embryonic development,
1278 related to **Figure 2A**.

1279 **B.** Top: overview of the sampled embryonic time points and sections. Middle
1280 and bottom: spatially-constrained unsupervised clustering of additional
1281 embryo sections across E9.5, E10.5, E11.5, E12.5, E13.5, E15.5, and
1282 E16.5 also identifies anatomical structures (annotation is shown below).
1283 Bins are colored according to different anatomical structures. Scale bar, 1
1284 mm.

1285 **C.** Hematoxylin and eosin staining of embryo sections adjacent to the
1286 corresponding Stereo-seq sections. Scale bars, 1 mm.

1287

1288 **Figure S4. DEG of mouse embryo anatomical structures, related to**
1289 **Figure 2.**

1290 Heatmap showing the normalized expression of DEGs for the indicated
1291 anatomical structures of the mouse embryo sections profiled by Stereo-seq in
1292 **Figure 2A**.

1293

1294 **Figure S5. Expression of selected markers for specific mouse embryo**
1295 **tissues, related to Figure 2.**

1296 Spatial visualization of the expression of DEGs enriched in the indicated
1297 tissues of the mouse embryo sections profiled by Stereo-seq in **Figure 2A**.

1298

1299 **Figure S6. Reanalysis of a mouse E10 embryo dataset generated by**
1300 **DBiT-seq, related to Figure 2.**

1301 **A.** Spatial heatmap indicating the number of UMI captured by Stereo-seq
1302 from an E10.5 embryo section (same as in **Figure 2A**) at bin 50 (left)
1303 compared to an E10 DBiT-seq dataset at the same resolution (right).
1304 Scale bar, 500 μ m.

1305 **B.** Spatial visualization of the expression of the indicated genes in Stereo-
1306 seq (upper panels) and DBiT-seq (lower panels) mouse embryo datasets.
1307 Scale bars, 500 μ m.

1308

1309 **Figure S7-S12. | Hotspot identified spatial gene expression modules in**
1310 **E9.5-E15.5 mouse embryo sections, related to Figure 2.**

- 1311 **A.** Heatmap showing the genes with significant spatial autocorrelation
1312 (2,082, 1,916, 3,062, 2,831, 2,074, 2,480 genes, FDR < 0.05) grouped
1313 into 28, 28, 33, 30, 24 and 23 gene modules based on pairwise spatial
1314 correlations of gene expression in the same embryo sections of **Figure**
1315 **2A** from E9.5 to E15.5, respectively. Selected genes and GO terms
1316 related to representative modules are highlighted on the right side.
- 1317 **B.** Heatmap showing the Pearson correlation of module score for each
1318 spatial autocorrelation module and the expression of sets of specific
1319 genes for each cell type from a reported dataset (Cao et al., 2019).
- 1320 **C.** Spatial visualization of gene modules related to the indicated anatomical
1321 structures. Scale bar, 1 mm.

1322

1323 **Figure S13. Hotspot identified spatial gene expression modules in an**
1324 **E16.5 mouse embryo sections, related to Figure 2.**

- 1325 **A.** Heatmap showing the Pearson correlation of module score for each
1326 spatial autocorrelation module and the expression of sets of specific
1327 genes for each cell type from a reported dataset (Cao et al., 2019).
- 1328 **B.** Spatial visualization of gene modules related to the indicated anatomical
1329 structures. Scale bar, 1 mm.

1330

1331 **Figure S14-S19. | Hotspot identified spatial regulatory modules in E10.5-**
1332 **E16.5 mouse embryo sections, related to Figure 3.**

- 1333 **A.** Heatmap showing the regulons with significant spatial autocorrelation
1334 (553, 554, 500, 380, 467 and 480 regulons, FDR < 0.05) grouped into 26,
1335 31, 25, 20, 28 and 27 regulon modules based on pairwise spatial
1336 correlations of regulon activity in the same embryo sections of **Figure 2A**
1337 from E10.5 to E16.5, respectively. Selected regulons related to
1338 representative modules and their corresponding GO terms are shown.
- 1339 **B.** Spatial visualization of the indicated regulon modules and their
1340 representative regulon for different anatomical structures. Scale bar, 1
1341 mm.

1342

1343 **Figure S20. Stereo-seq reconstructs the spatiotemporal gene regulatory**
1344 **network of the developing mouse brain, related to Figure 3.**

- 1345 **A.** Spatial visualization of the expression of the indicated genes representing
1346 neuroepithelium (left) and early neuroblasts in the E9.5 (right) brain.
- 1347 **B.** Spatial visualization of the indicated regulon activity in early neuroblasts
1348 of the E9.5 brain.
- 1349 **C.** Spatial visualization of the indicated regulon activity representing different
1350 anatomical structure of the E9.5 brain.
- 1351 **D.** GO analysis of target genes for transcription factors corresponding to
1352 different anatomical structures of the E9.5 brain as in **Figure 3F**.
- 1353 **E.** Heatmap showing the normalized regulon activity of top transcription
1354 factors corresponding to the subpallium, midbrain, and hindbrain
1355 development of mouse brain.
- 1356 **F.** Spatial visualization of the indicated regulon activity for the subpallium
1357 (DLX5), midbrain (DMBX1) and hindbrain (HOXA2). Scale bars, 500 μm .

1358

1359 **Figure S21. Characterization of morphogens expressed by secondary**
1360 **organizer centers in the developing mouse brain, related to Figure 3.**

- 1361 **A** and **B.** Spatial visualization of the indicated modules representing
1362 secondary organizer centers of the E9.5 and E12.5 brain.
- 1363 **C** and **D.** Heatmap showing the Spearman's correlation of ligands enriched in
1364 the secondary organizes centers of the E9.5 and E12.5 brain.
- 1365 **E.** The spatial scatter plots showing single DNB that captured the transcripts
1366 of the indicated morphogens at the ISO, ANR, ZLI, RP, and FP organizer
1367 centers at E9.5 (bottom left) and E12.5 (bottom right) embryos. Plots
1368 were superimposed with E9.5 or E12.5 embryo structures. Scale bar, 500
1369 μm .

1370

1371 **Figure S22. Schematic representation of morphogens and regulons at**
1372 **the indicated organizer centers, related to Figure 3.**

1373

1374 **Figure S23. Stereo-seq reconstructs the spatiotemporal gene regulatory**
1375 **networks of the developing mouse skeleton, related to Figure 3.**

- 1376 **A.** Unsupervised spatially-constrained clustering of the developing mouse
1377 skeleton based on regulon activity.
- 1378 **B.** Heatmap showing the normalized regulon activity score of the top

1379 transcription factors enriched in the indicated anatomical structures
1380 during mouse embryonic skeleton development.

1381 **C.** Spatial visualization of the activity of the indicated regulons for
1382 chondrocytes (SOX9) and osteoblasts (SP7).

1383 **D.** Spatial visualization of the expression of the indicated genes representing
1384 the early (*Col9a3*) and late (*Col22a1*) stages of ossification. Scale bar,
1385 500 μm .

1386

1387 **Figure S24. Stereo-seq reconstructs the developing mouse forebrain,**
1388 **related to Figure 4.**

1389 **A.** Heatmap showing the normalized expression of DEGs for the indicated
1390 anatomical structures of the E15.5 forebrain in **Figure 4A**.

1391 **B.** Spatial visualization of indicated genes representing different anatomical
1392 structures of the E15.5 forebrain. Scale bar, 500 μm .

1393 **C.** Spatial visualization of gene enrichment score of MGE, POA, LGE and
1394 Str. Genes related to each structure are labeled on the right side. MGE,
1395 medial ganglionic eminence; POA, preoptic area; LGE, lateral ganglionic
1396 eminence; Str, striatum. Scale bar, 500 μm .

1397

1398 **Figure S25. Rostral-caudal patterning of the CP, related to Figure 4.**

1399 **A.** Manual dissection of the CP based on spatial coordinates. Scale bar, 500
1400 μm .

1401 **B.** Heatmap showing the normalized expression of genes with rostral-caudal
1402 gradient.

1403 **C.** Spatial visualization of module score (left) representing the aggregated
1404 expression level of CP (rostral) specific gene set and representative
1405 genes (right). Scale bar, 500 μm .

1406 **D.** Spatial visualization of module score (left) representing the aggregated
1407 expression level of CP (caudal) specific gene set and representative
1408 genes (right). Scale bar, 500 μm .

1409

1410 **Figure S26. Mapping cell-type localization across forebrain, related to**
1411 **Figure 4.**

1412 **A.** Spatial visualization of cell proportion score representing different cell

1413 types. Scale bar, 500 μ m.

1414 **B.** Violin plot showing the number of spliced and unspliced transcripts.

1415 **C.** Spatial visualization of the expression of indicated genes related to
1416 tangential migration of interneurons. Scale bar, 500 μ m.

1417 **Figure S27. Mapping cell-type localization across neocortex**
1418 **development, related to Figure 4.**

1419 **A.** Unsupervised spatially-constrained clustering of the cortex from E11.5,
1420 E12.5, E13.5, 15.5, and E16.5 of mouse embryo.

1421 **B.** Spatial visualization of the cell proportion score across different stages of
1422 the mouse neocortex.

1423 **C.** Spatial visualization of representative genes for VZ, SVZ, IZ, CP, MarZ
1424 and meninges across different stages of the mouse neocortex.

1425 **D.** Spatial visualization of the cell cycle score across different stages of the
1426 mouse neocortex.

1427

1428 **Figure S28. Spatial mapping of genes associated with human**
1429 **developmental disorders in different mouse embryo stages, related to**
1430 **Figure 5.**

1431 Heatmap showing the normalized expression level of 1,940 genes selected
1432 from the Deciphering Developmental Disorders database in the indicated
1433 organs of E9.5 to E16.5 embryos.

1434

1435 **Table S1. Gene modules for each embryonic stage, related to Figure 2.**

1436

1437 **Table S2. Regulon modules for each embryonic stage, related to Figure**
1438 **3.**

1439

1440 **Table S3. Expression of genes related to the rostral-caudal gradient in**
1441 **the cortical plate, and genes related to the pseudotime of neuronal**
1442 **migration, related to Figure 4.**

1443

1444 **Table S4. Expression of genes related to monogenic diseases and**
1445 **autism, related to Figure 5.**

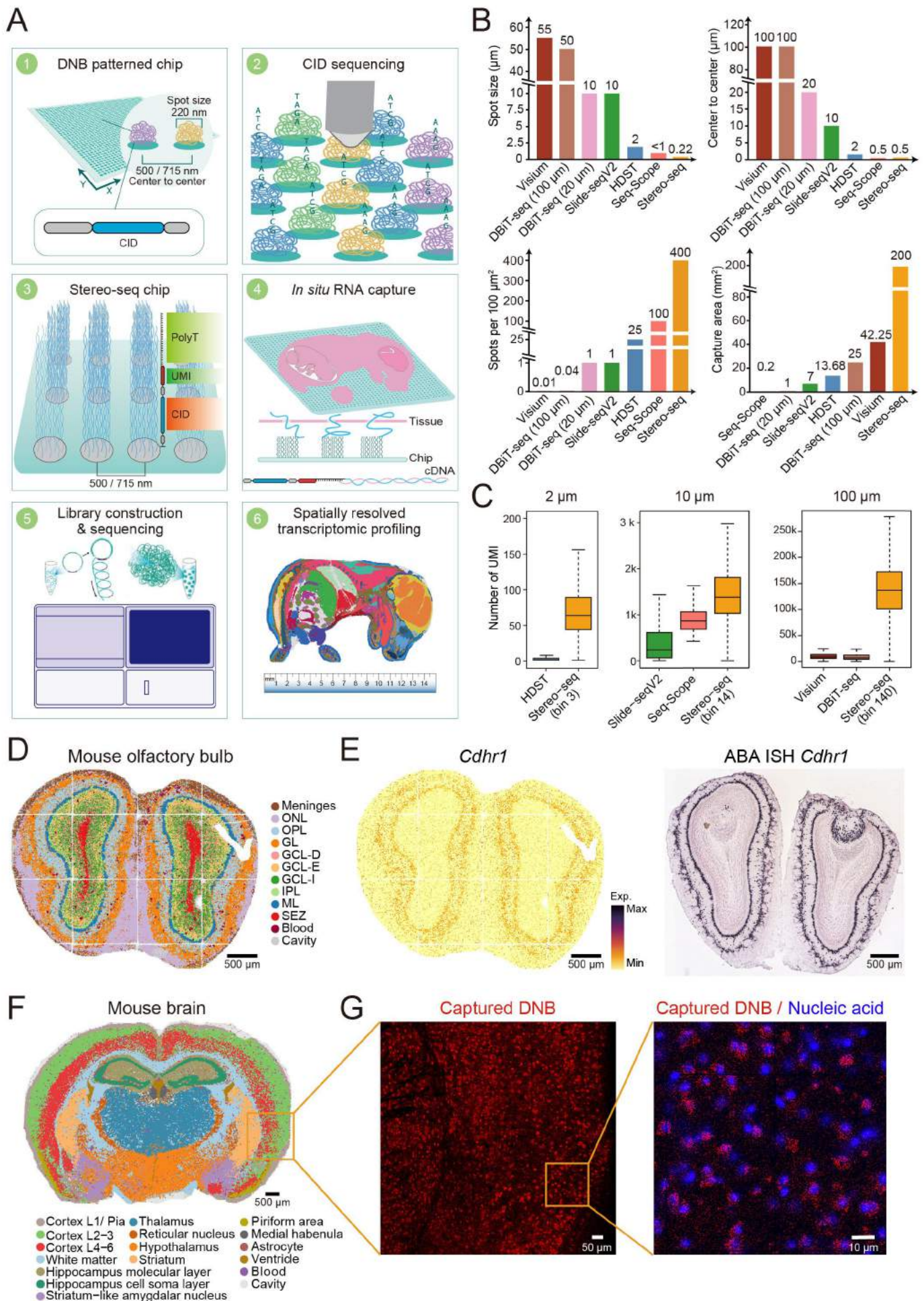
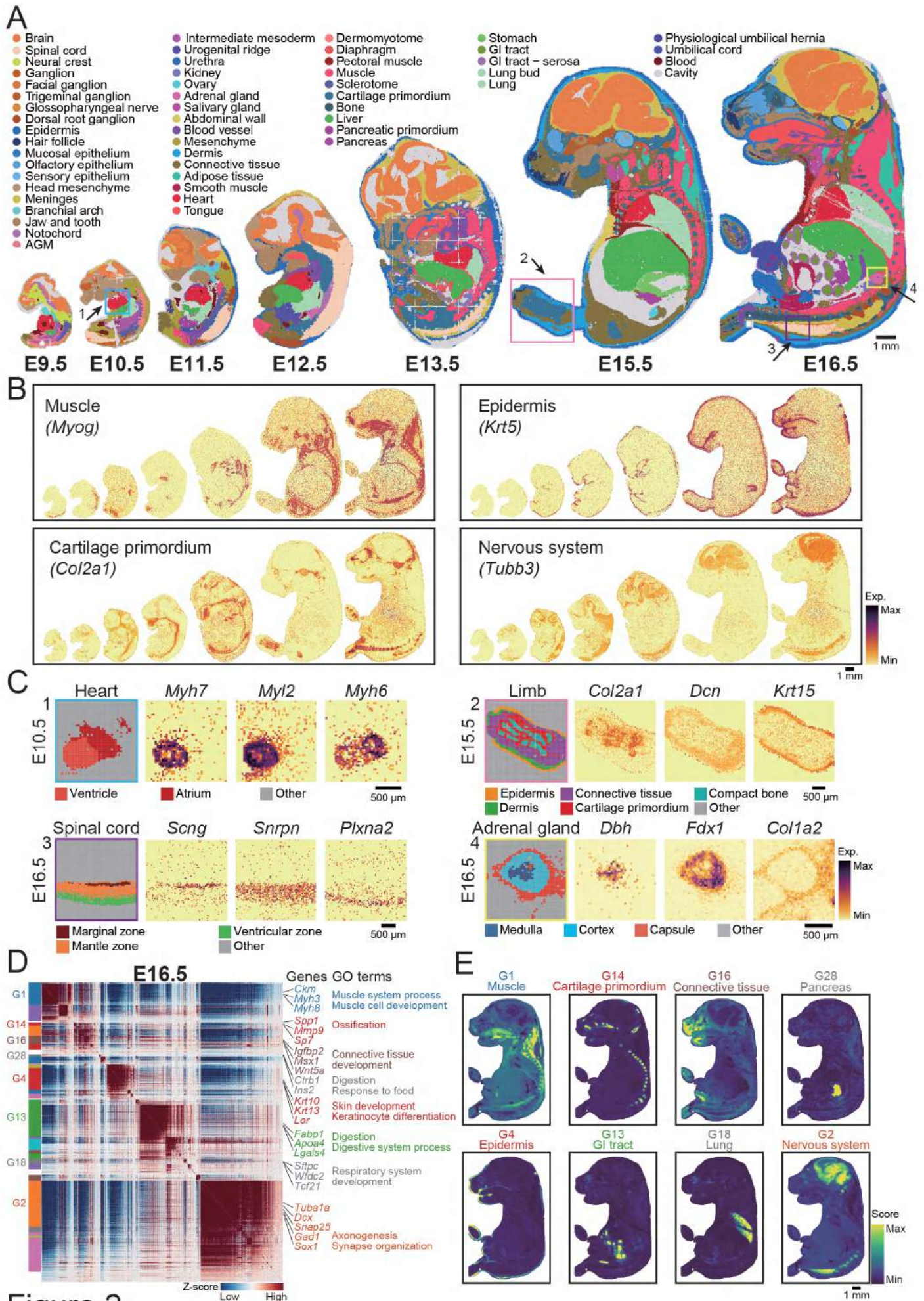


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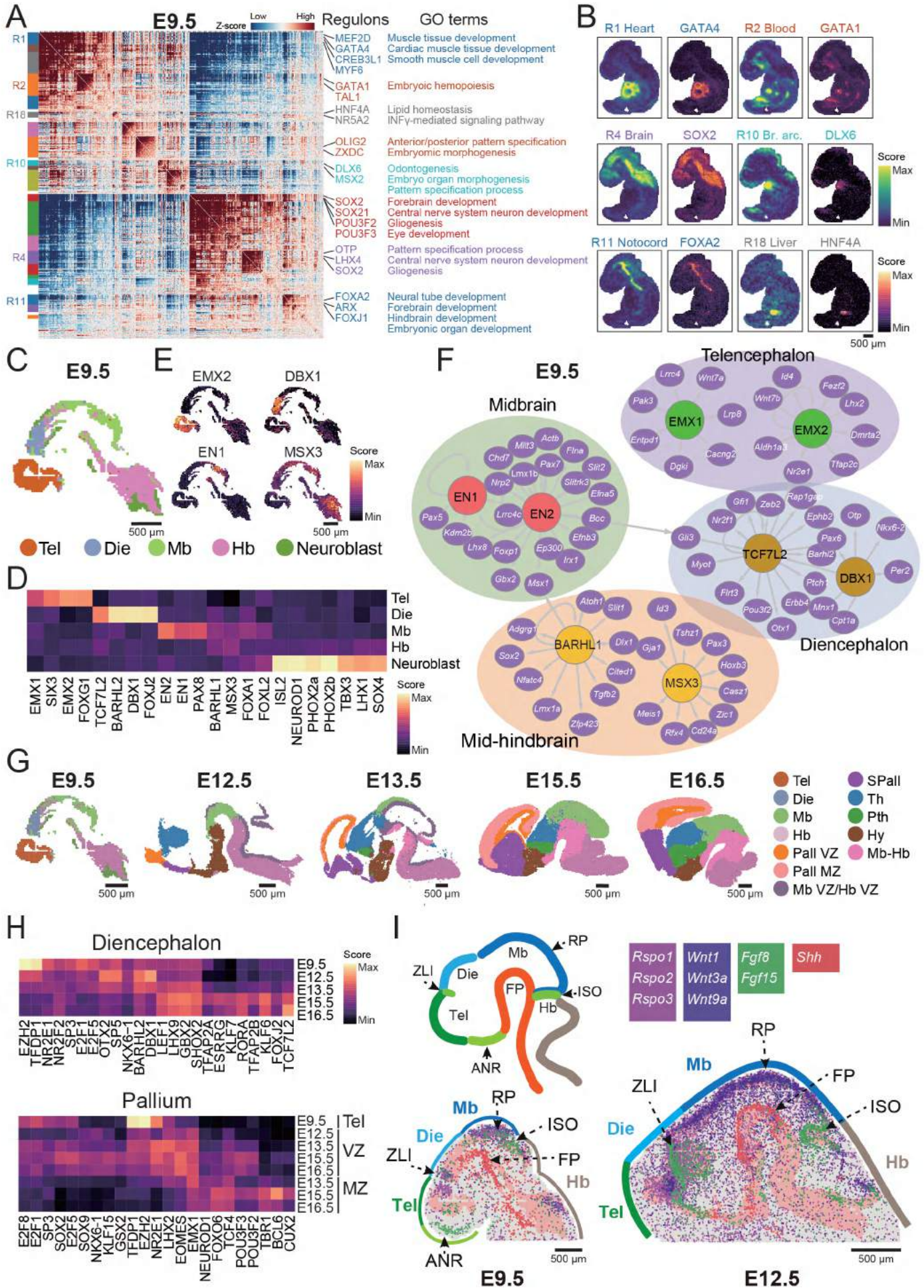


Figure 3

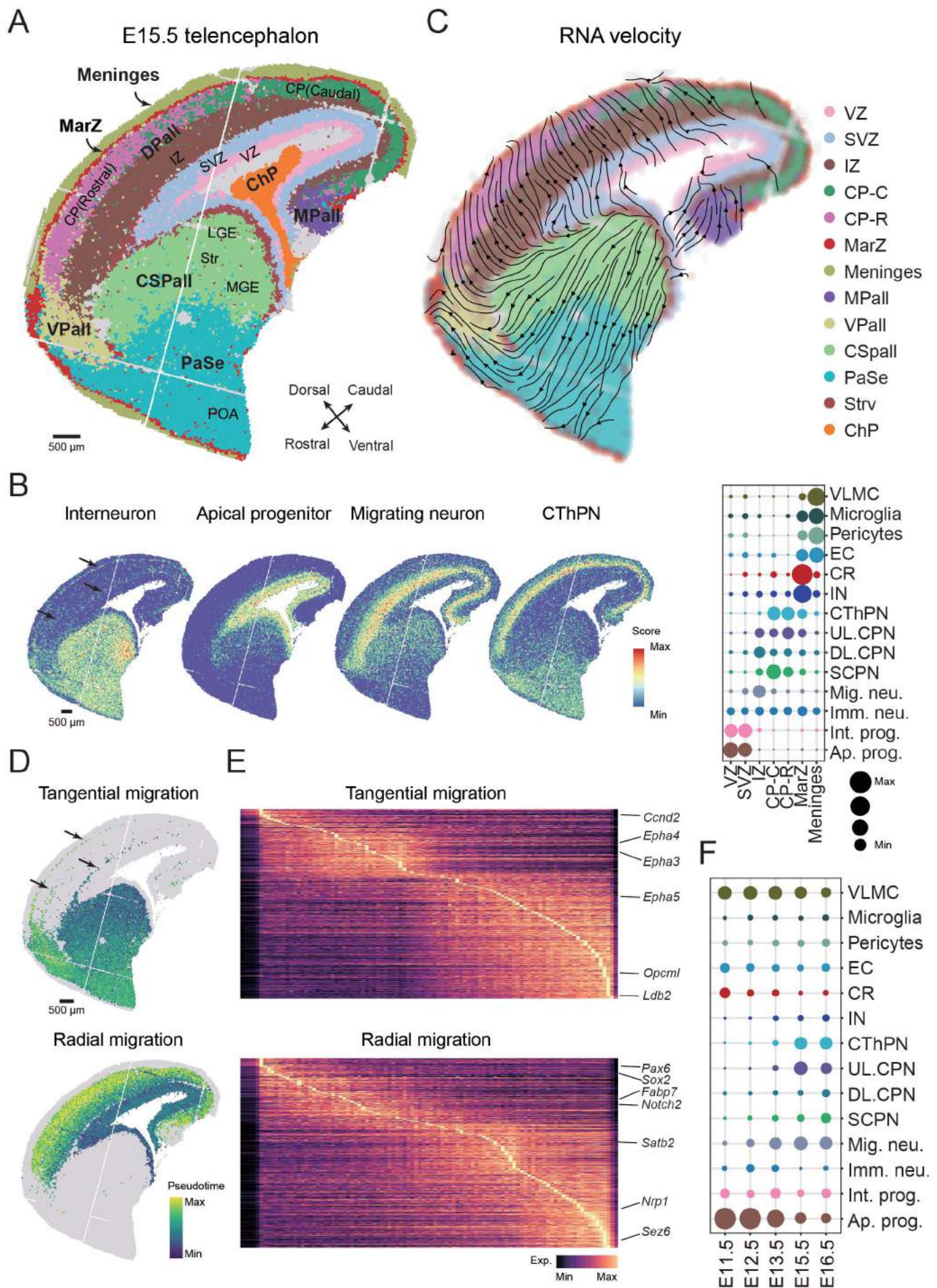


Figure 4

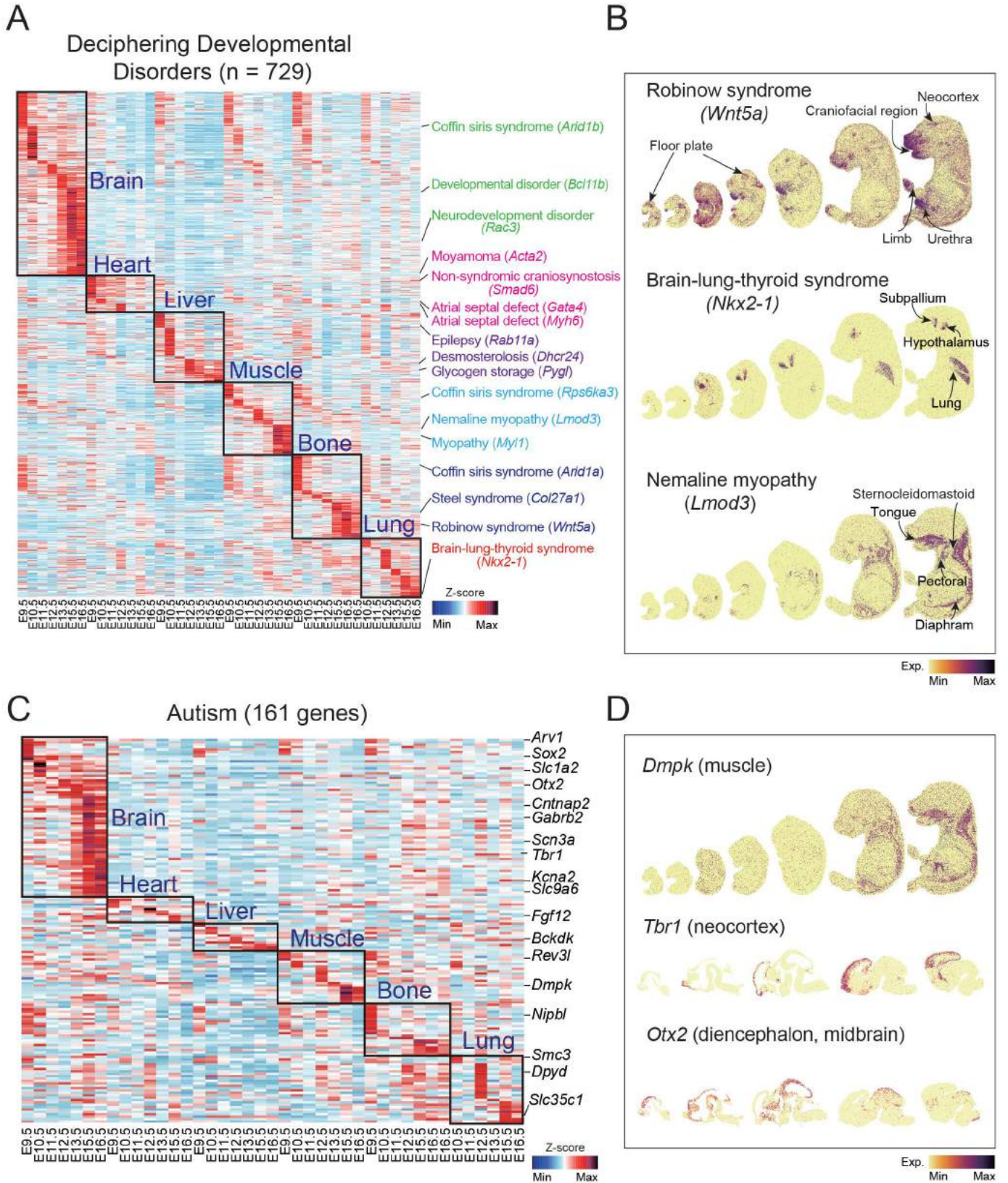


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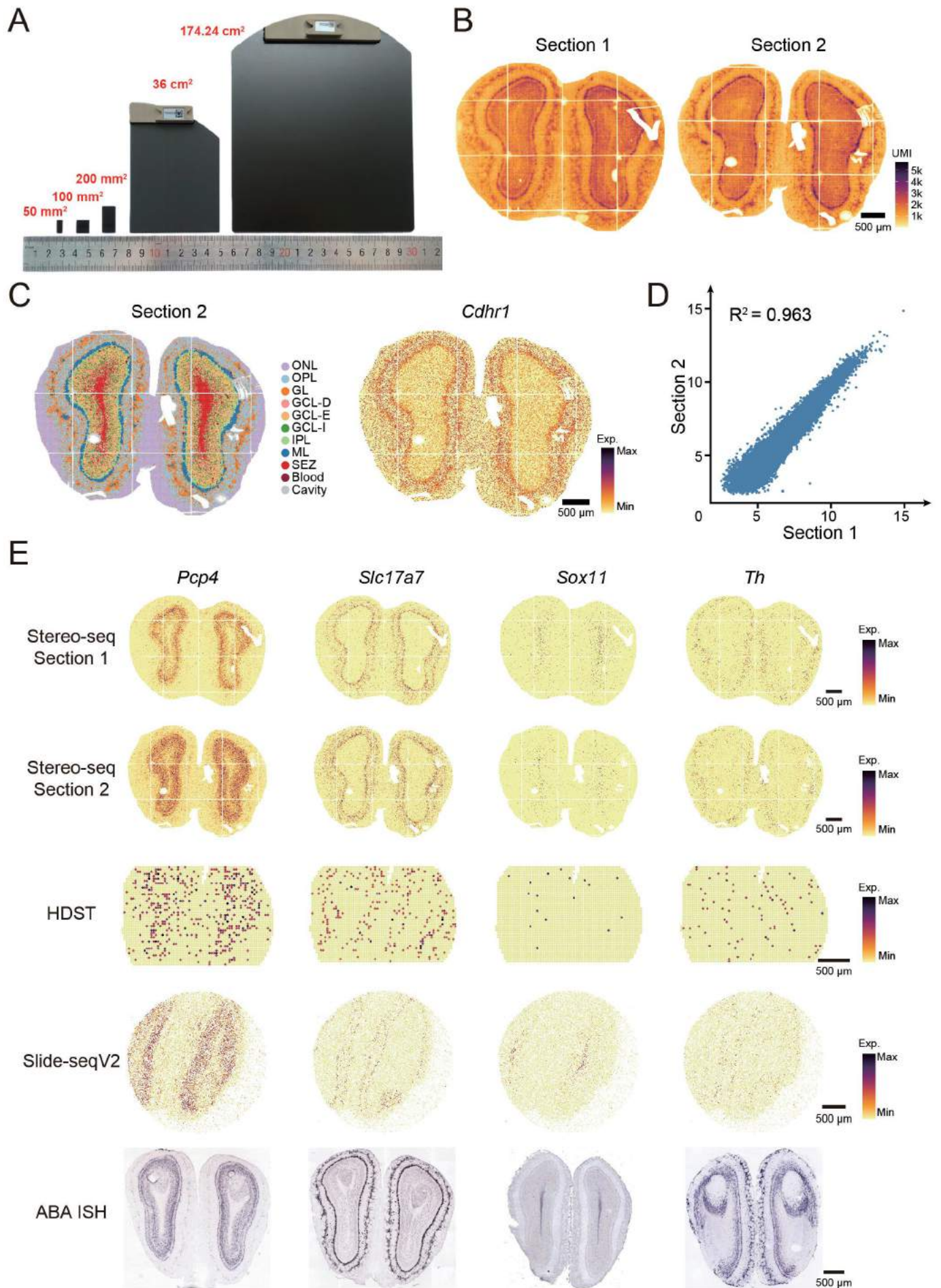


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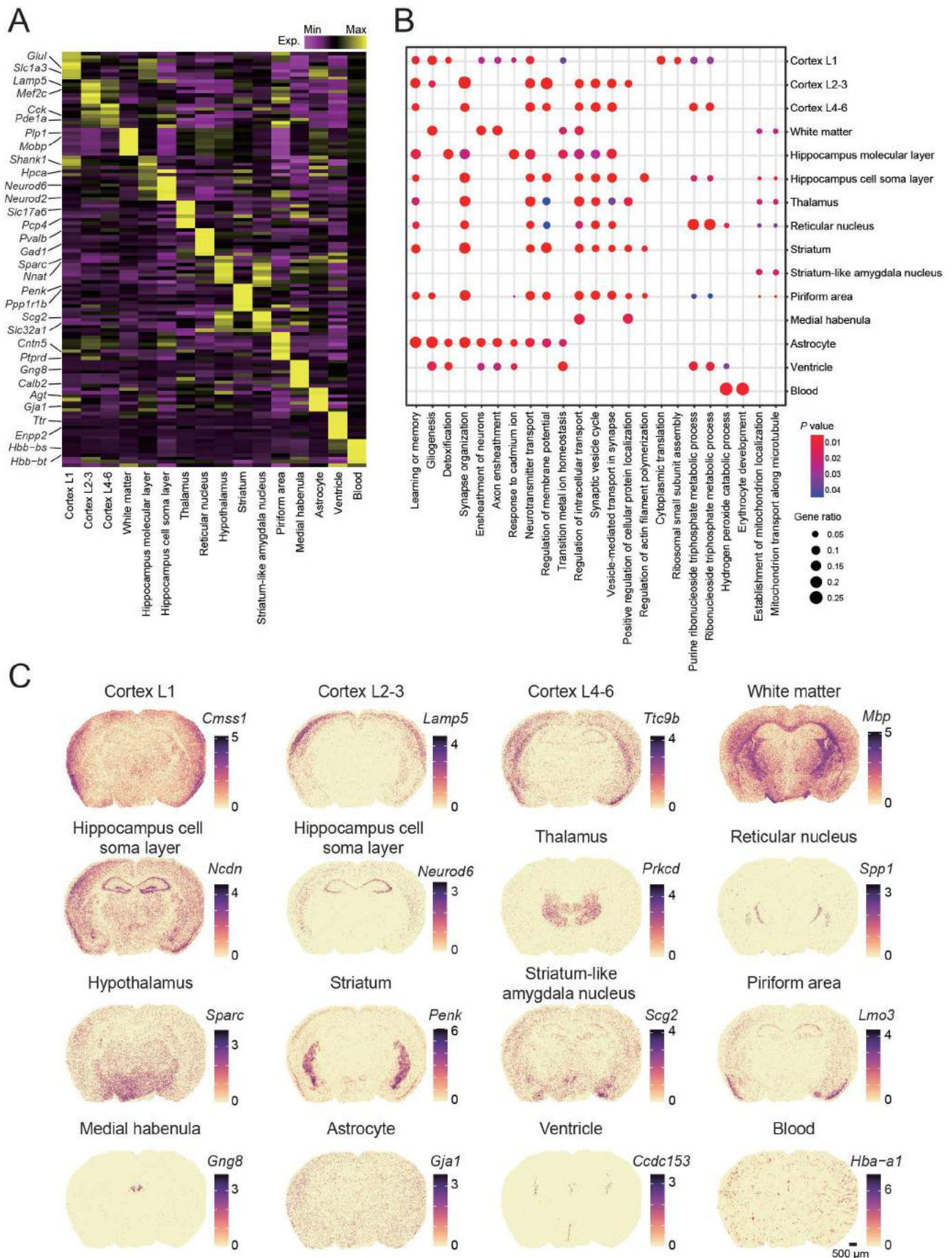


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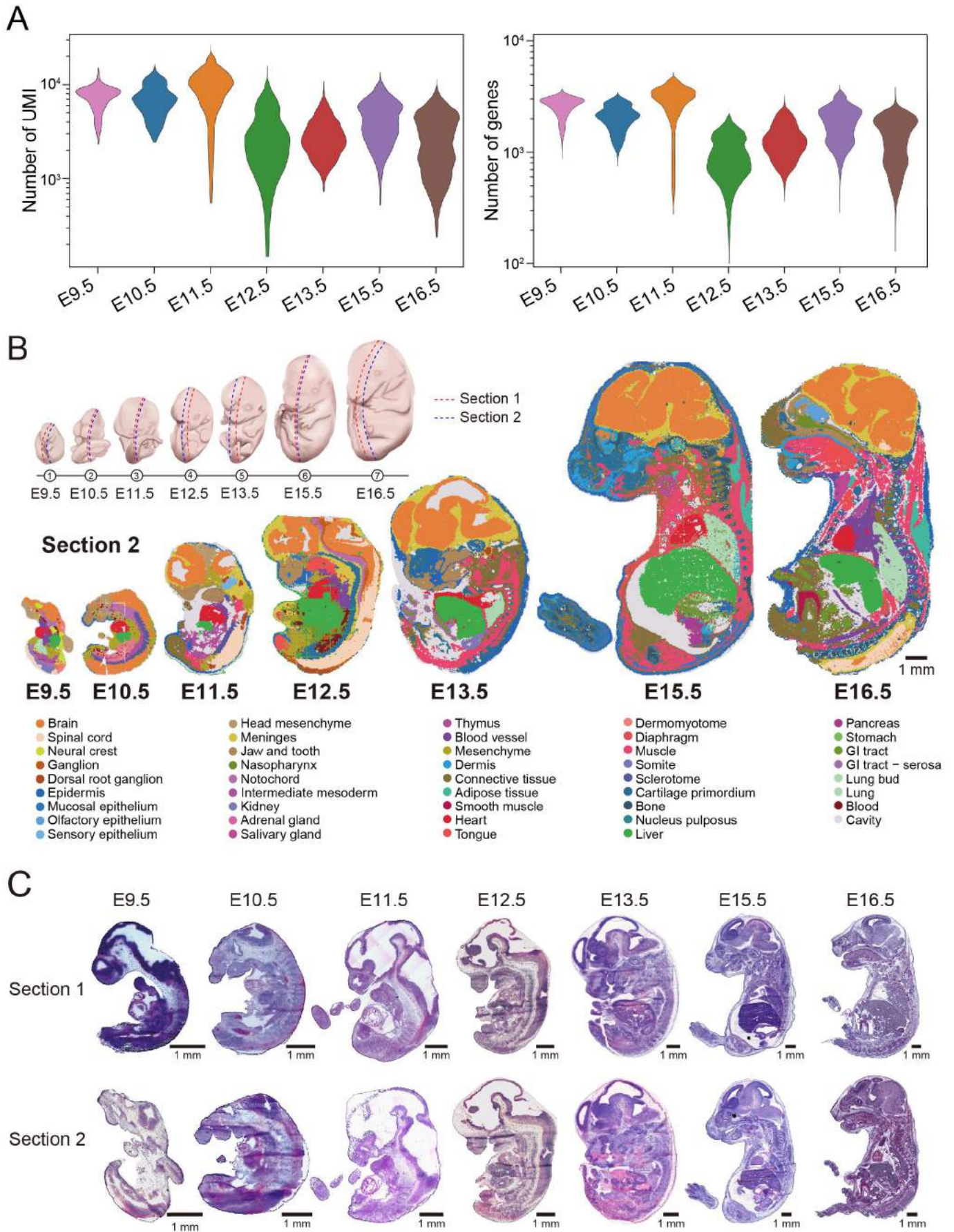


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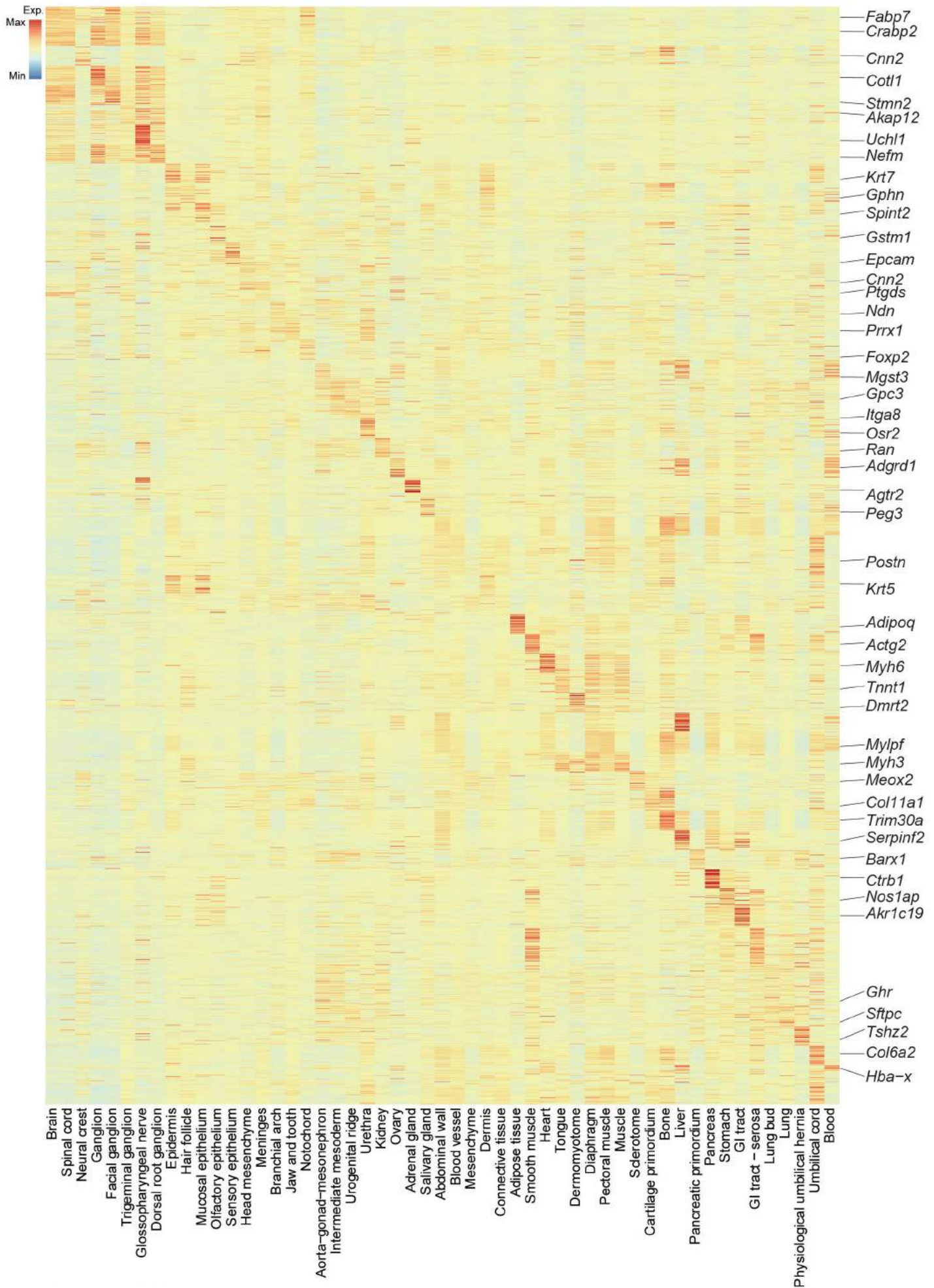


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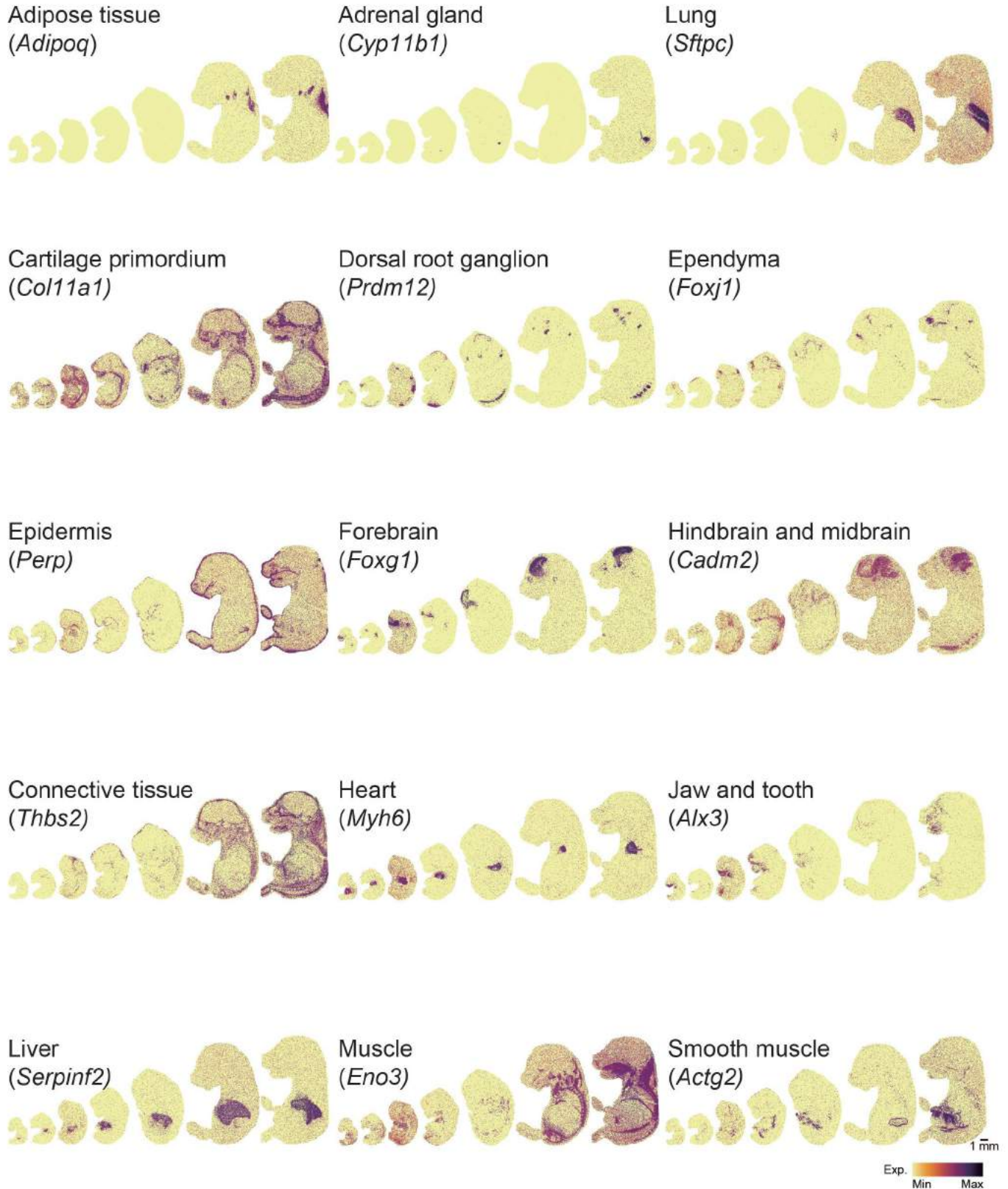


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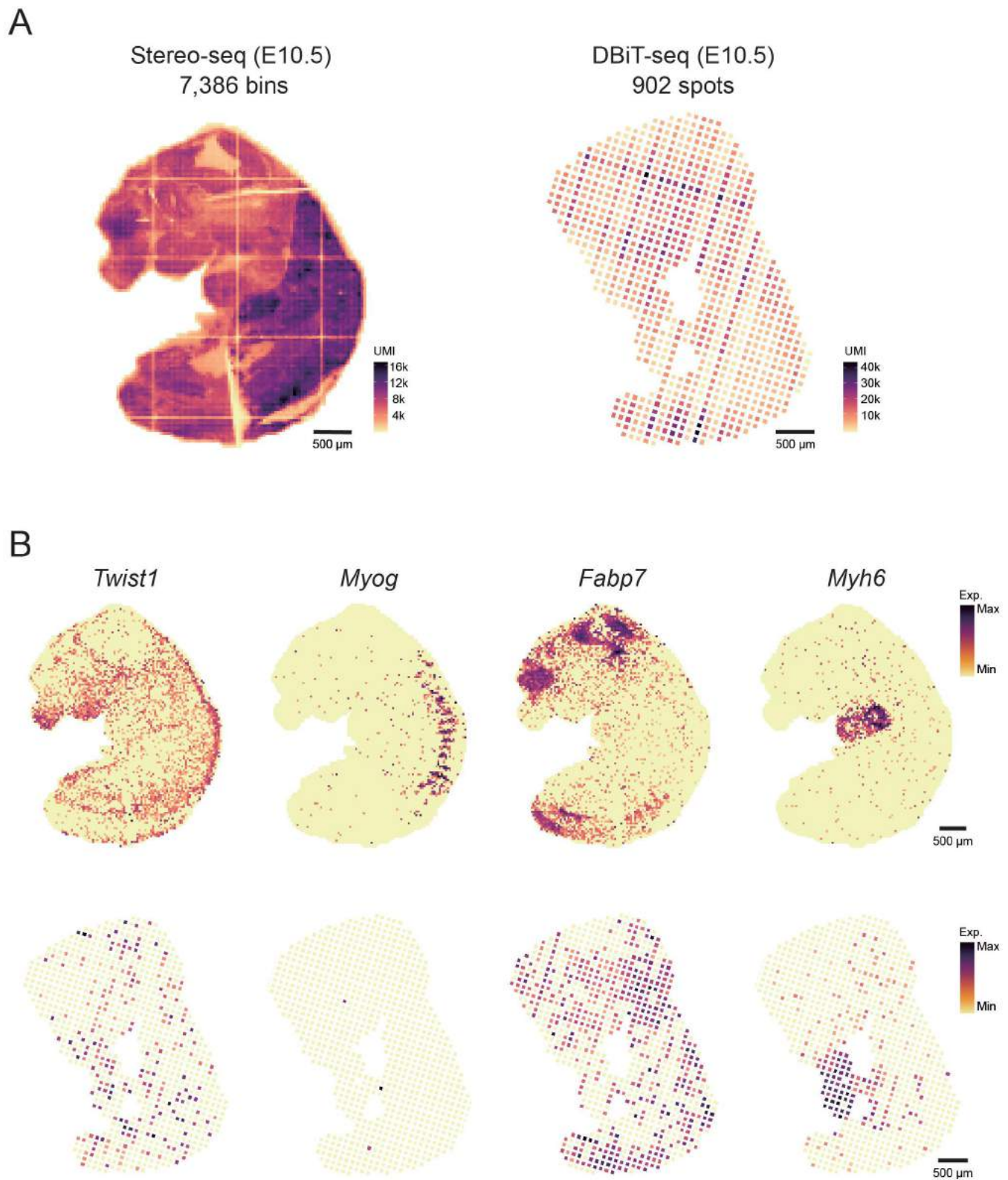


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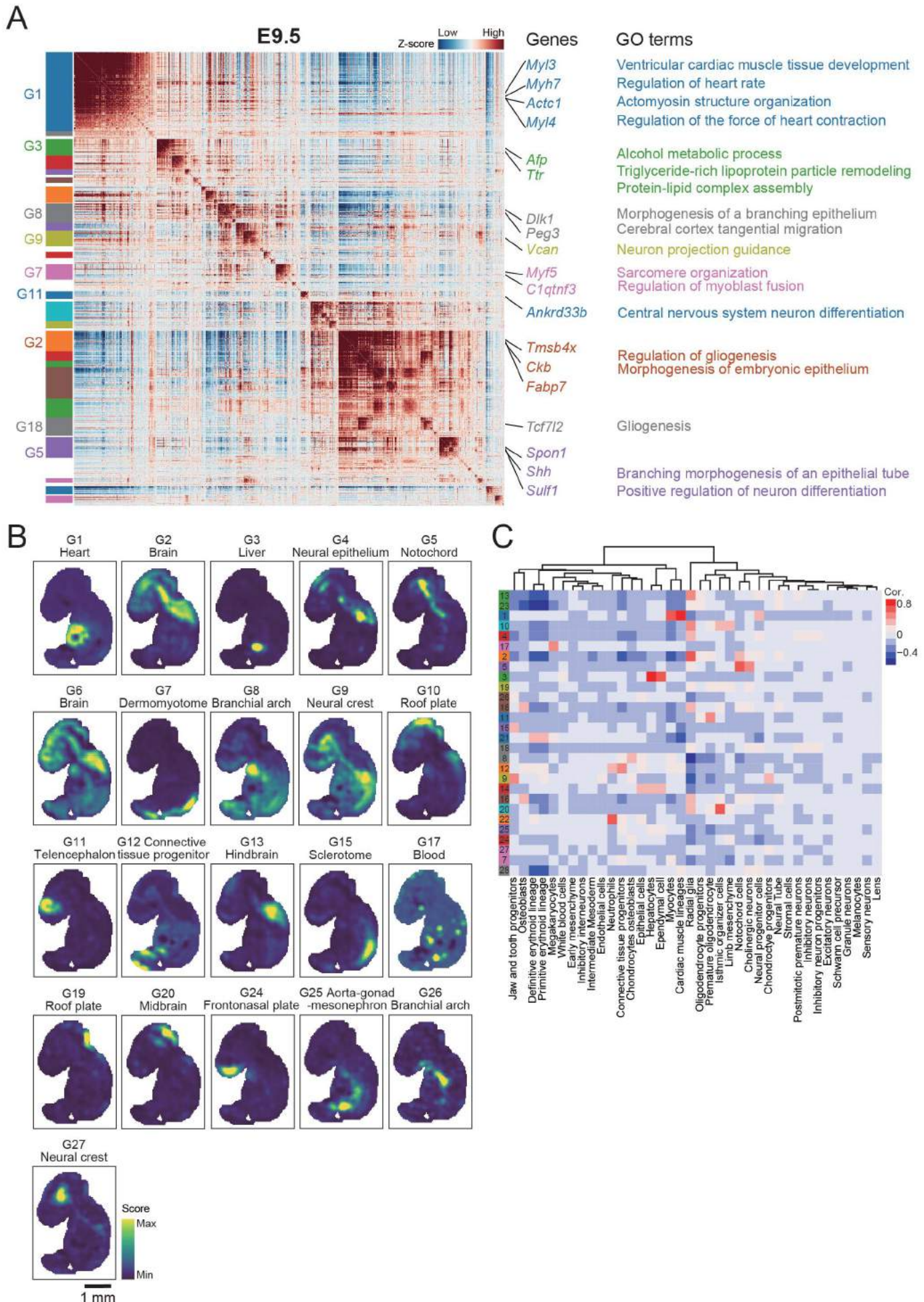


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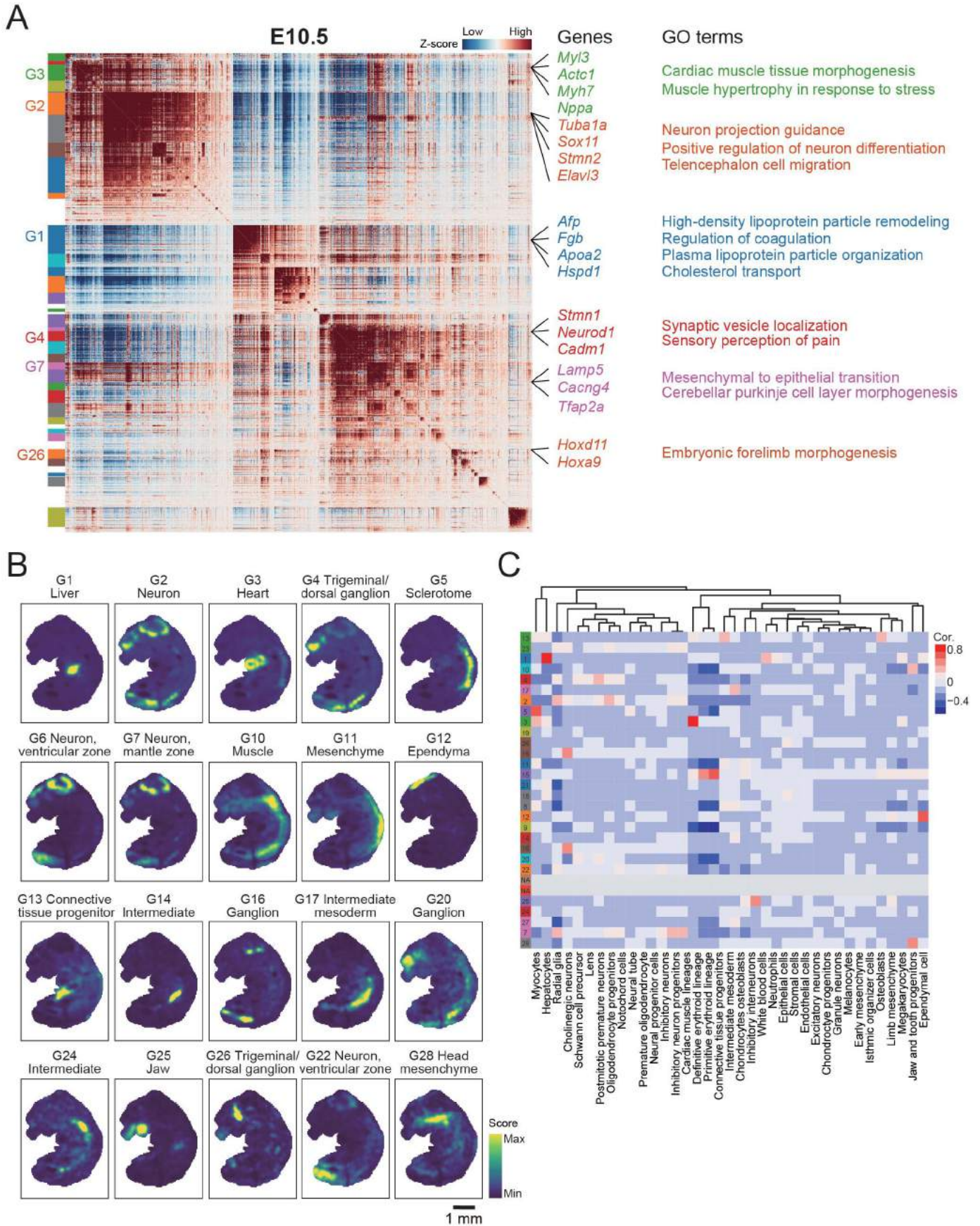


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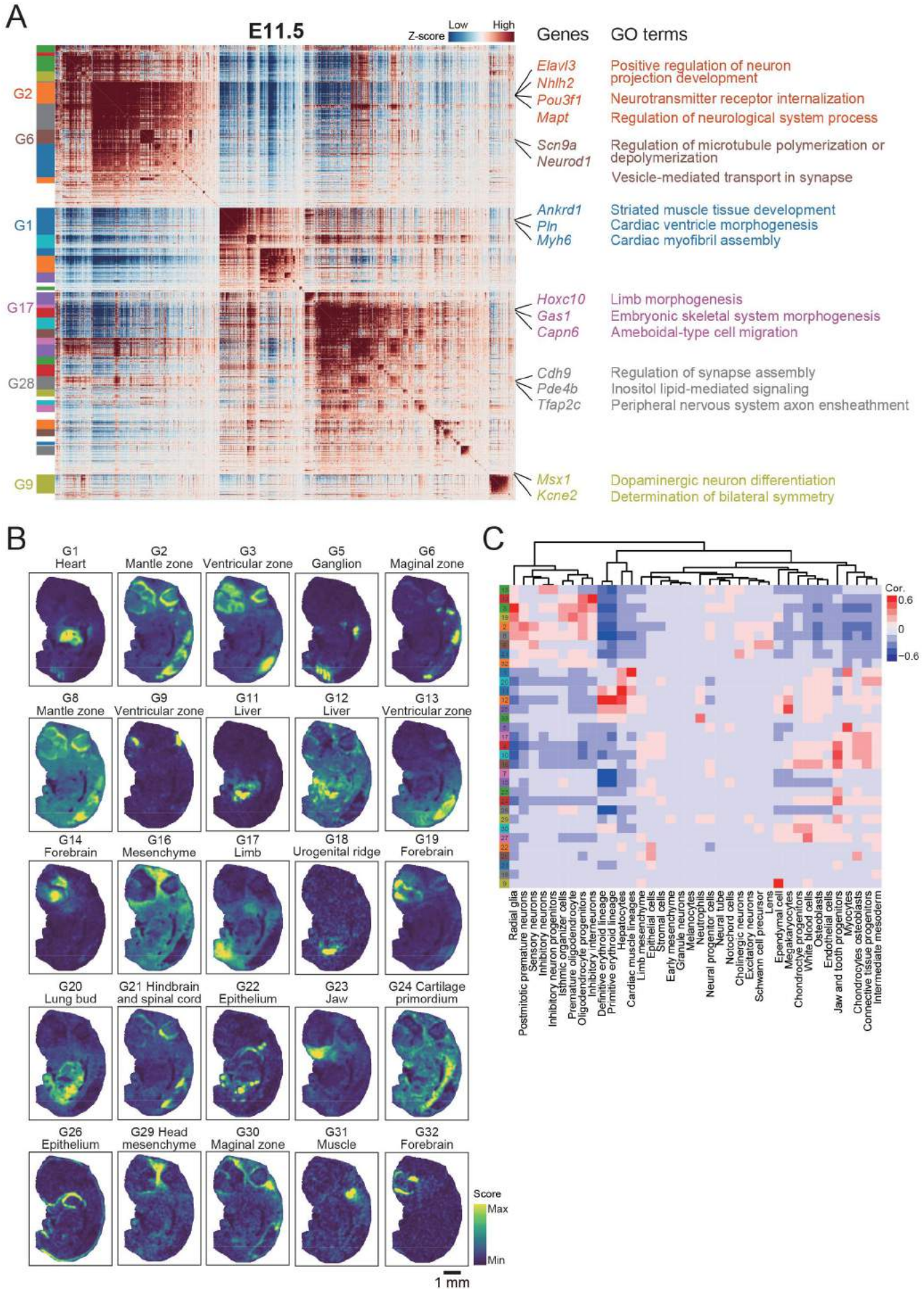


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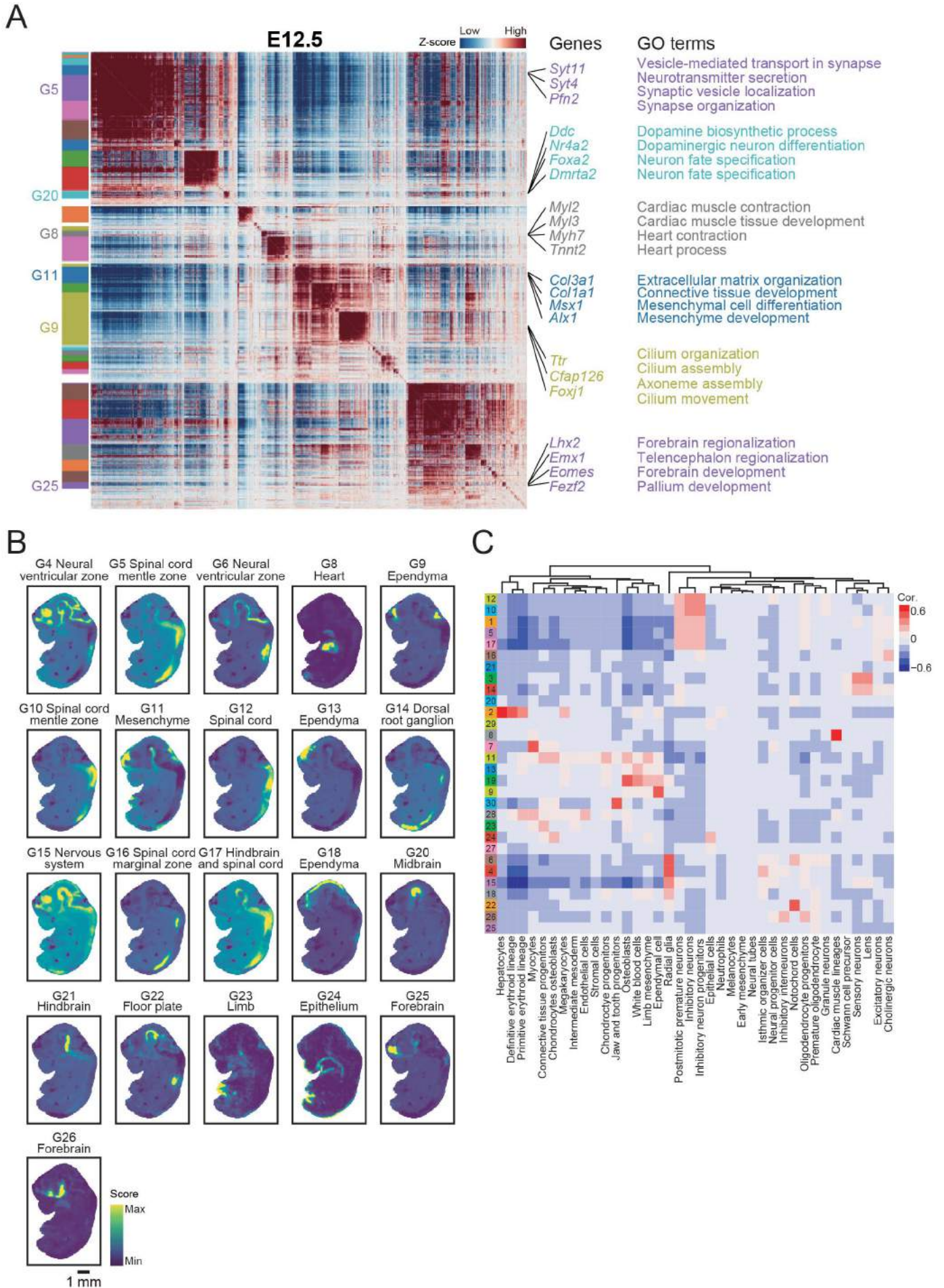


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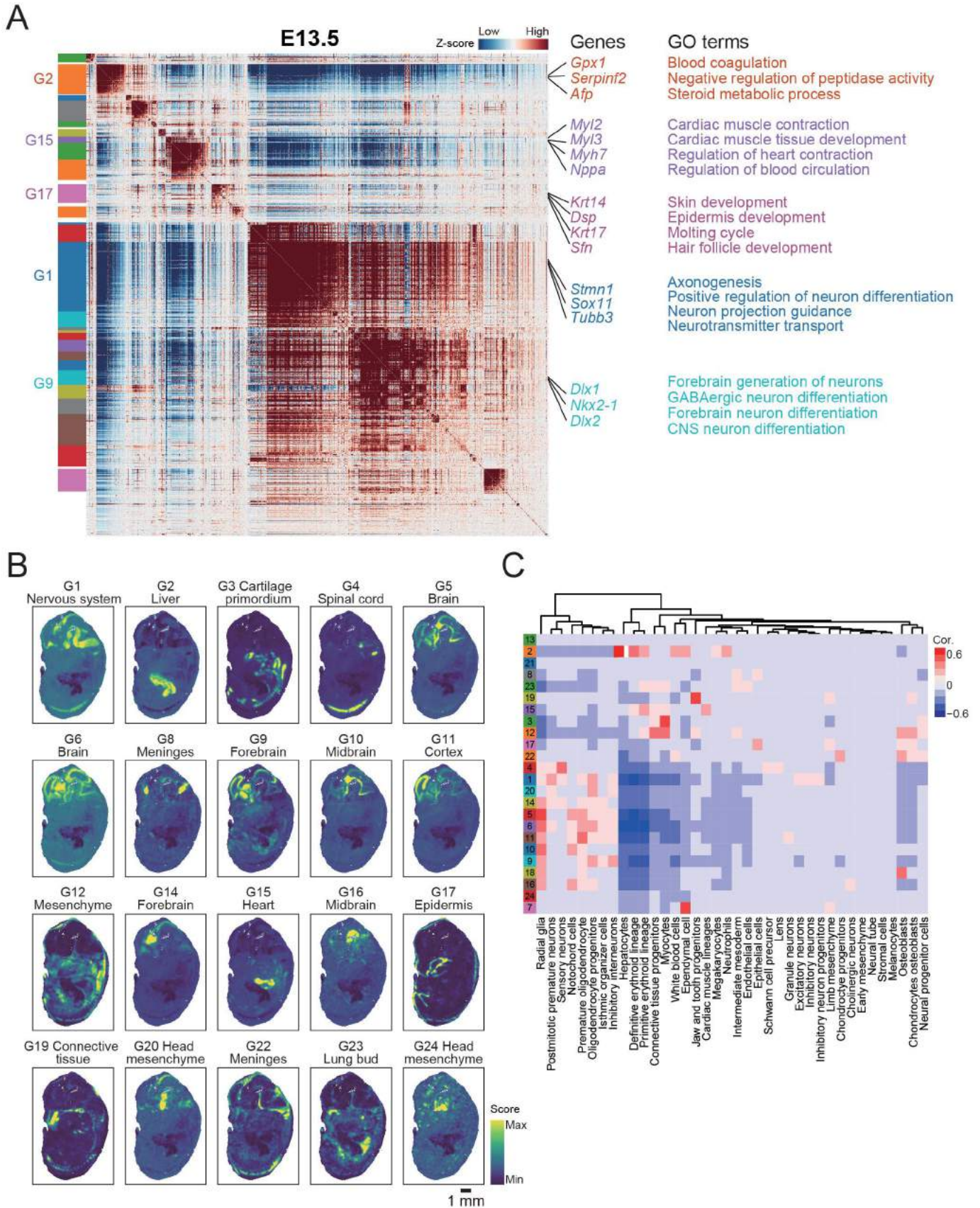


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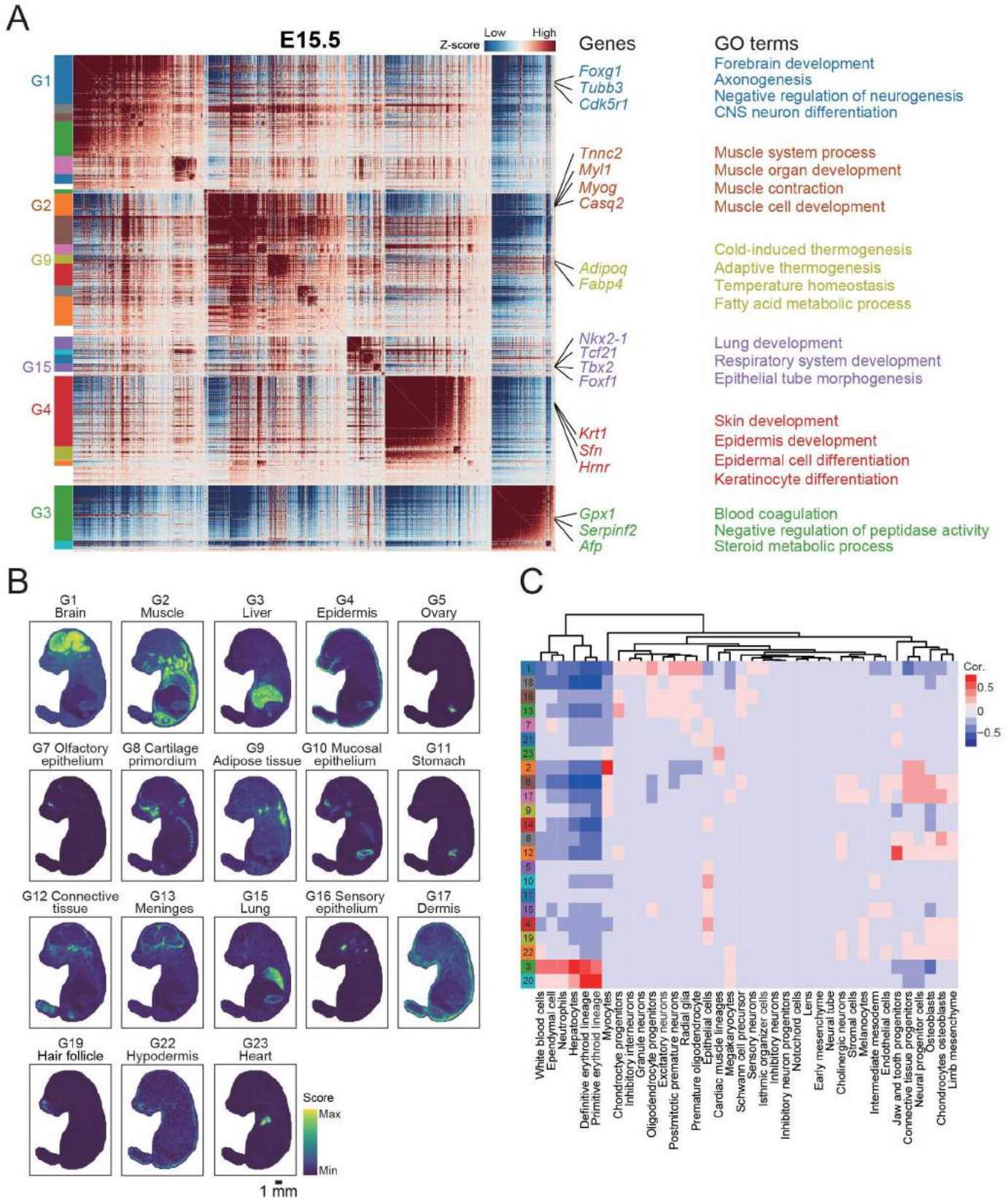


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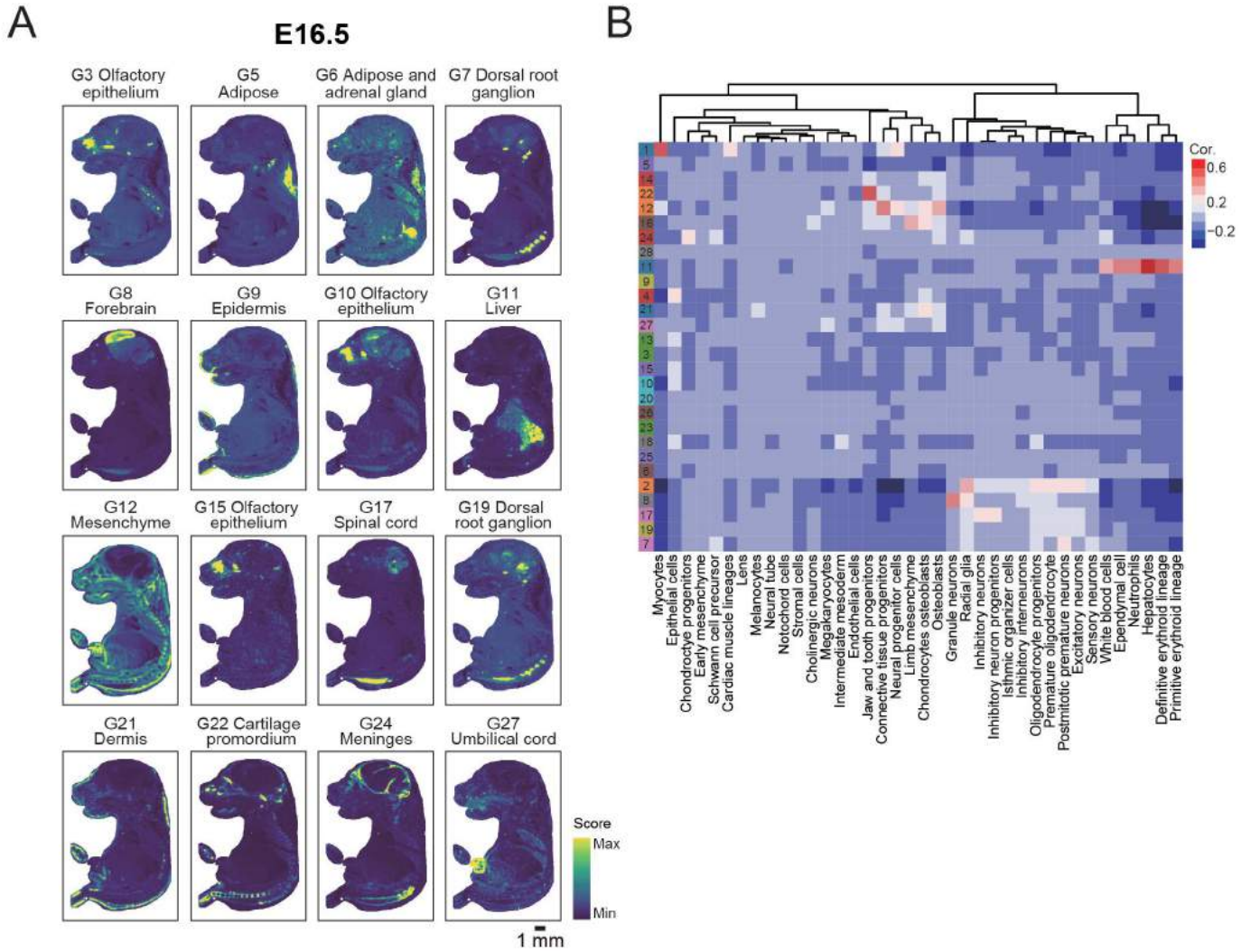


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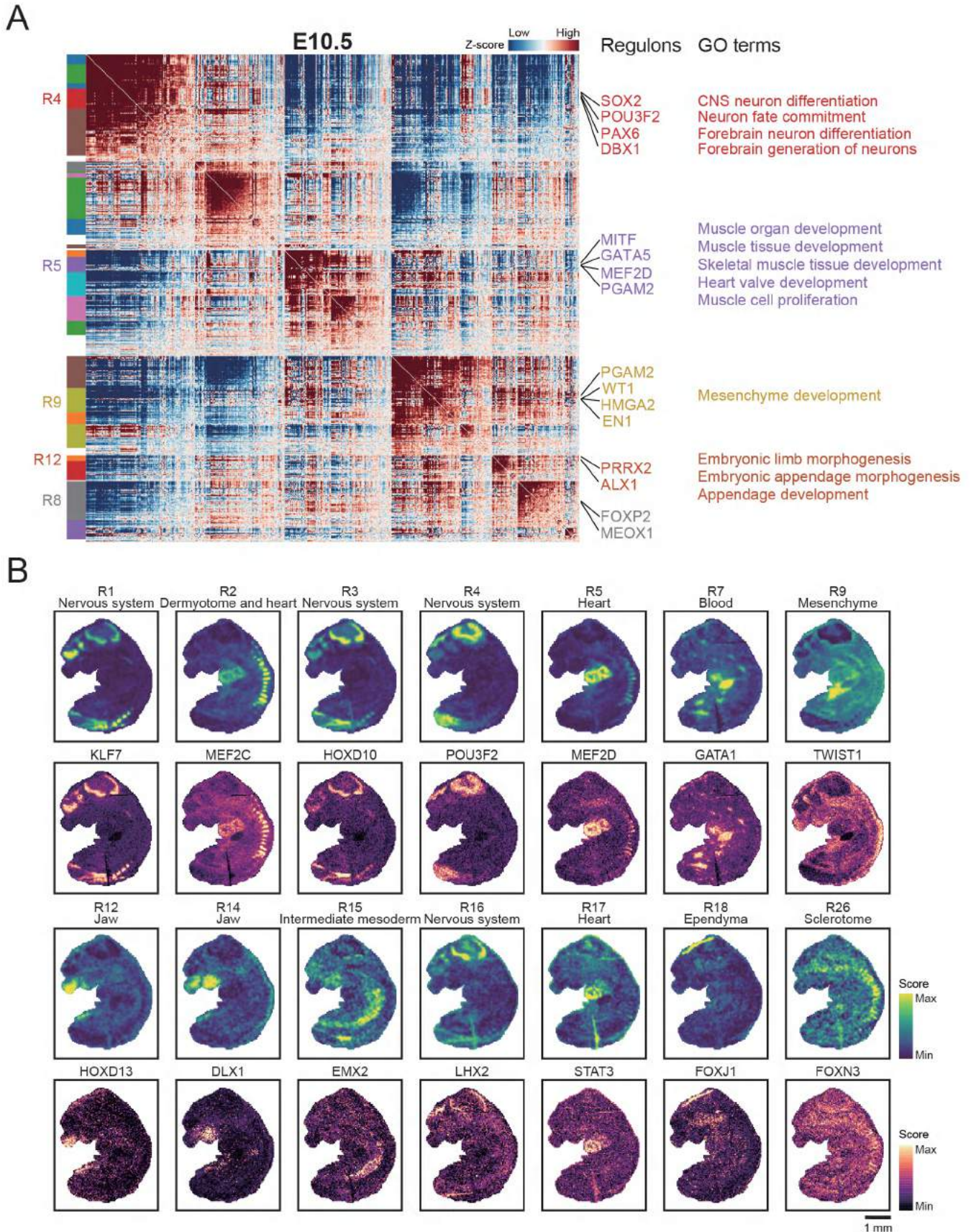


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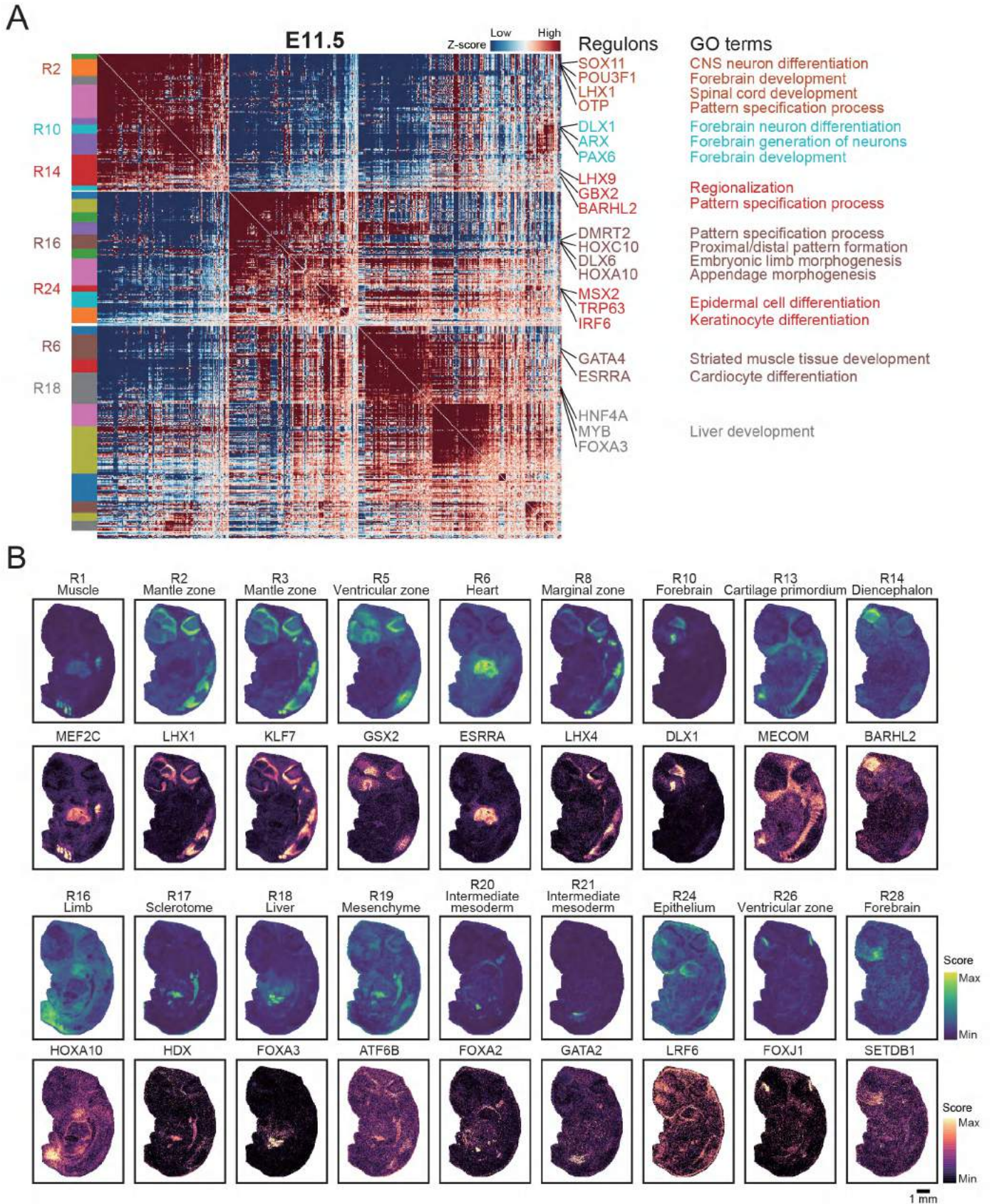


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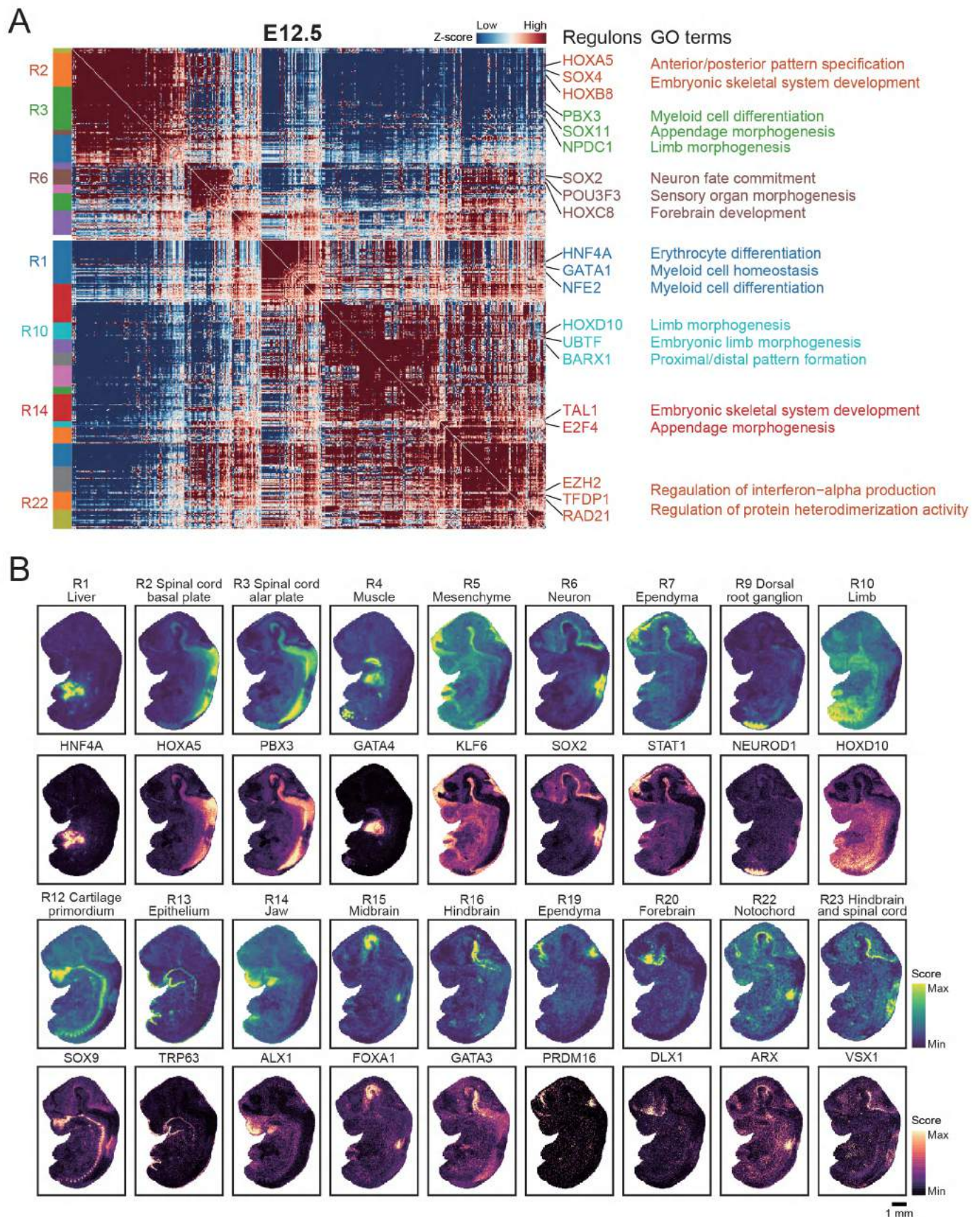


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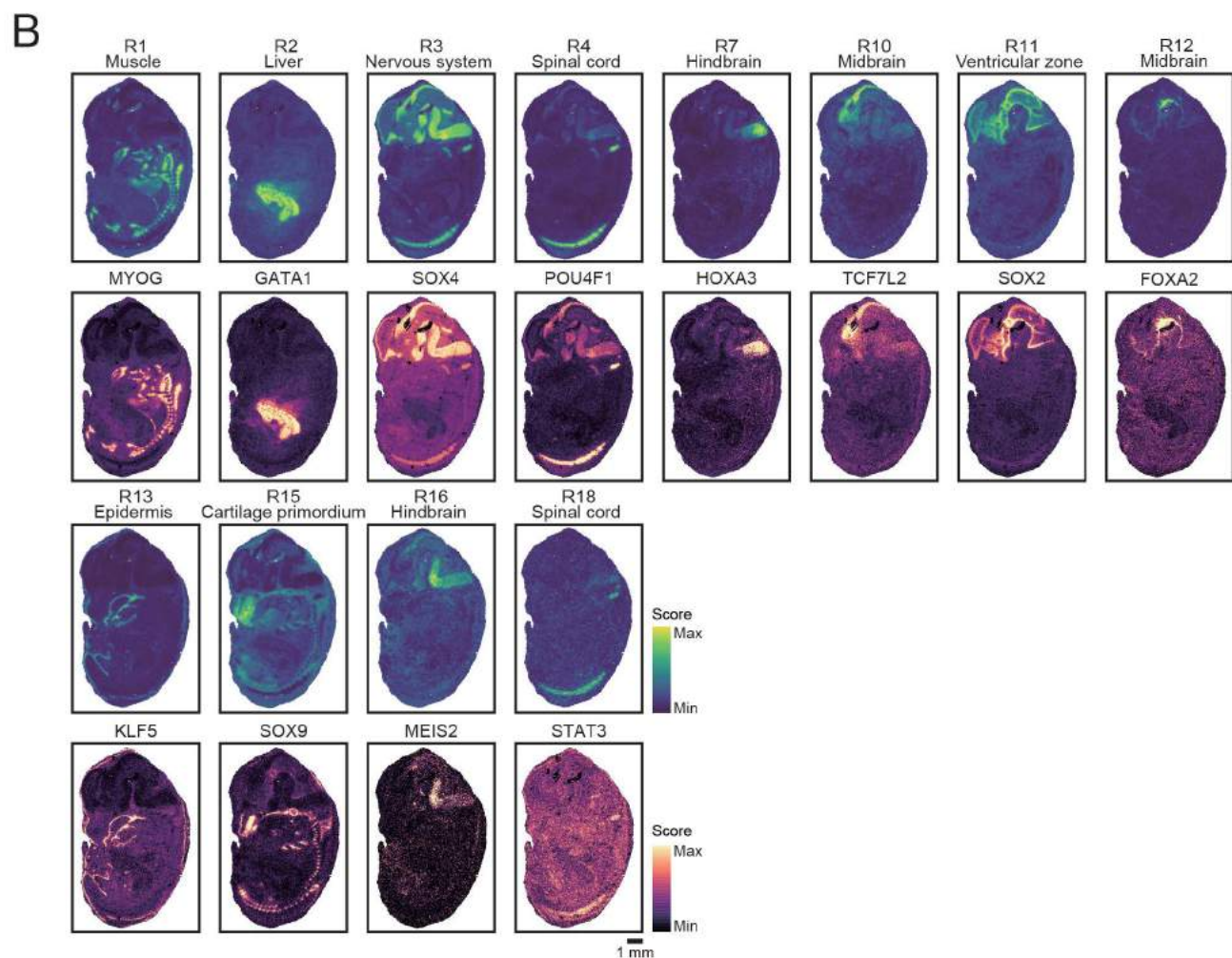
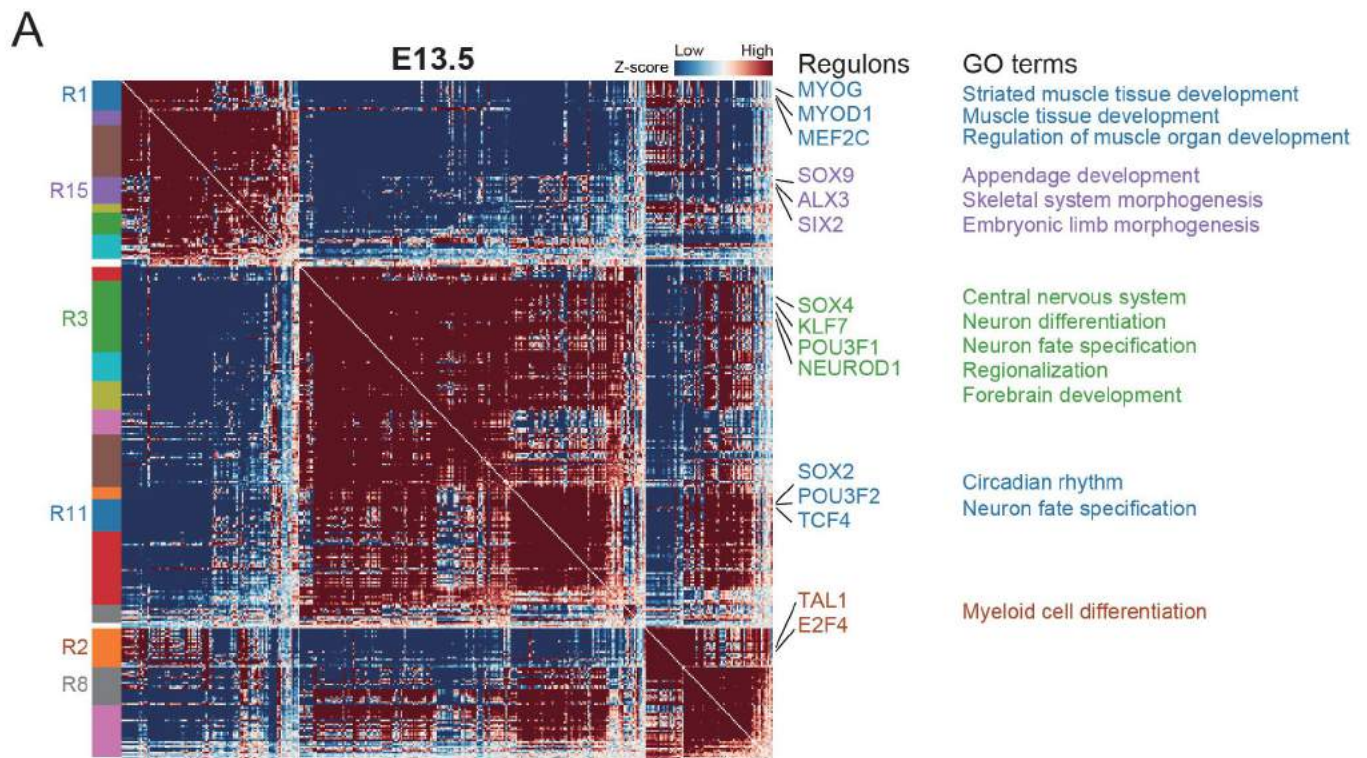


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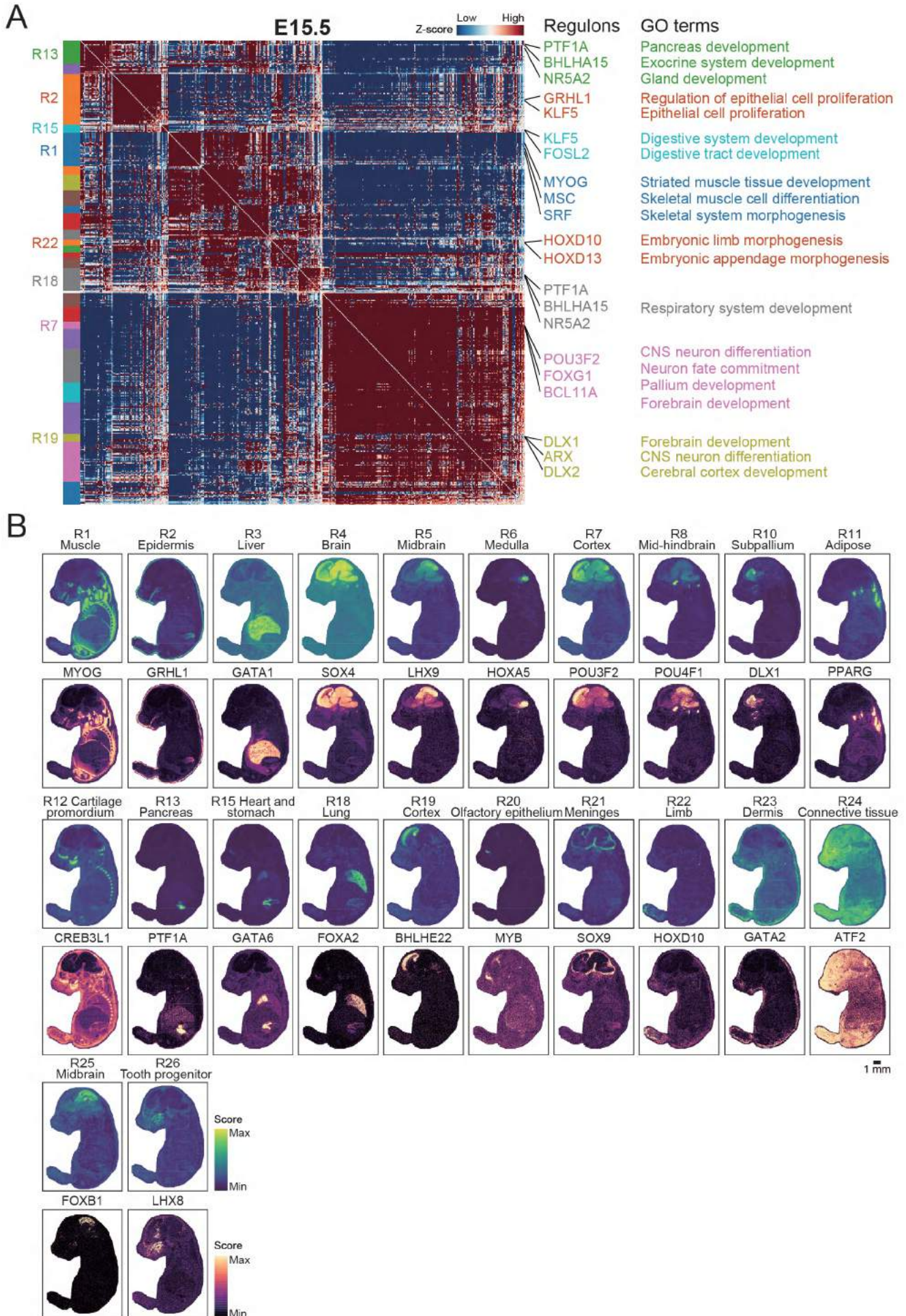


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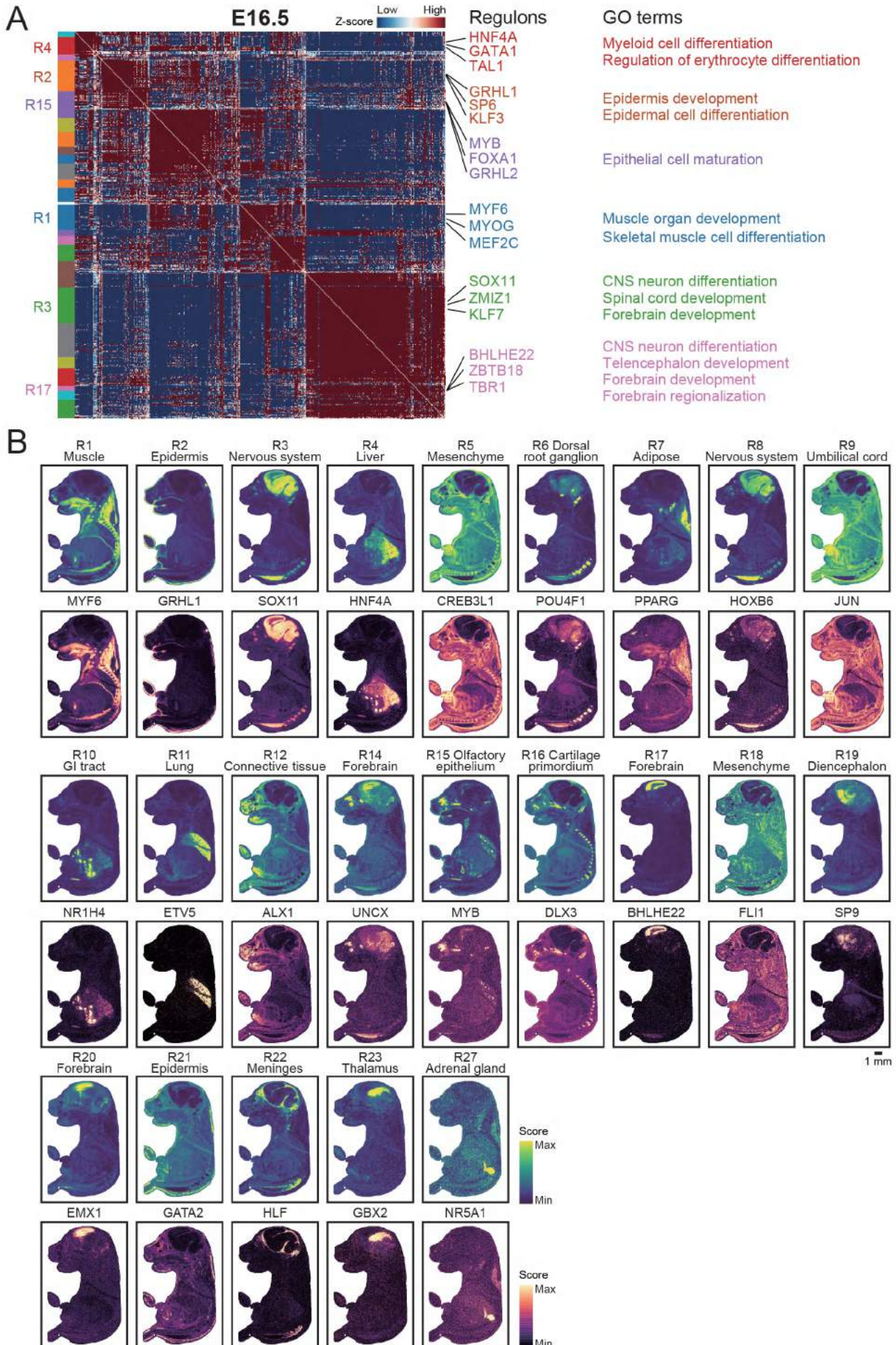


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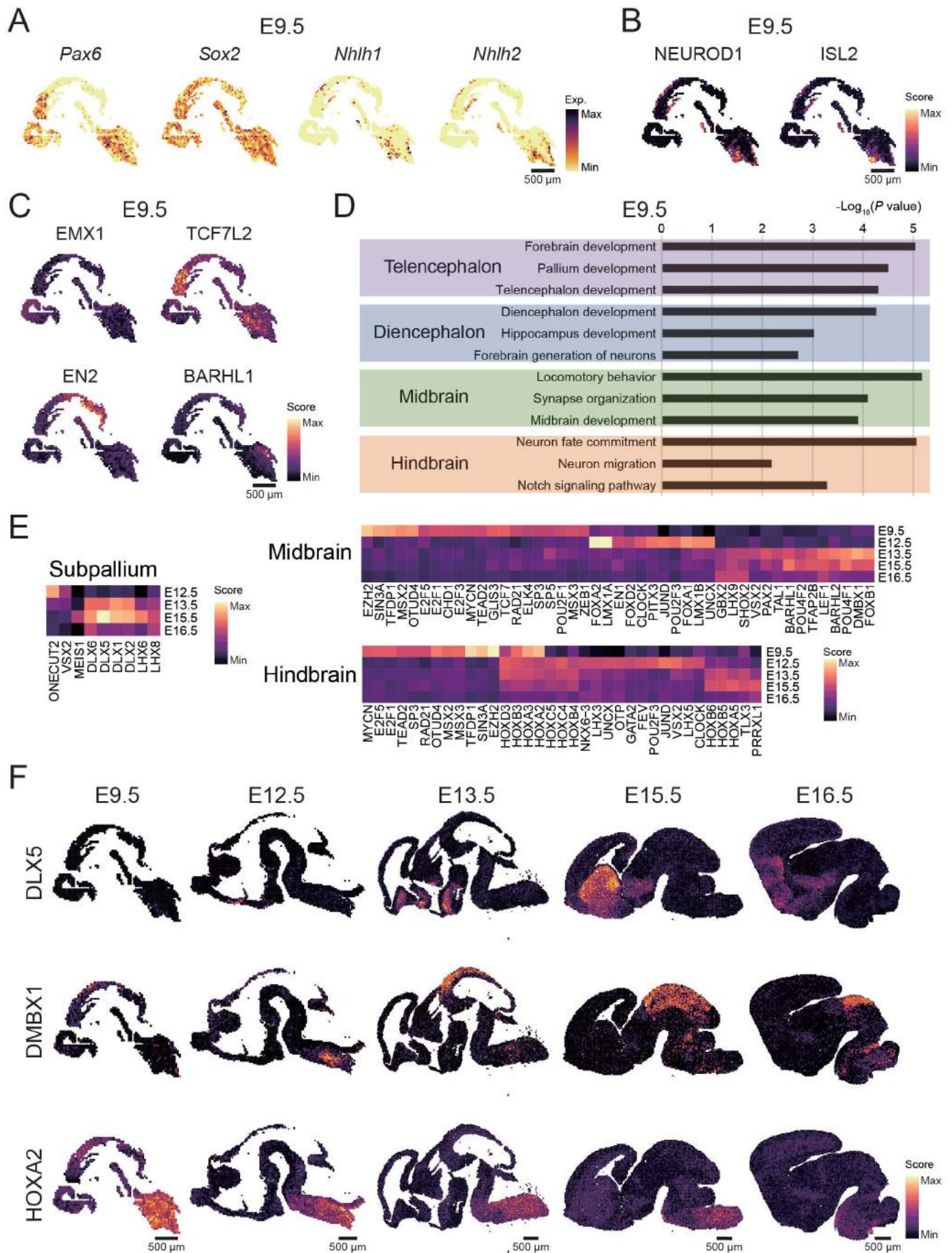


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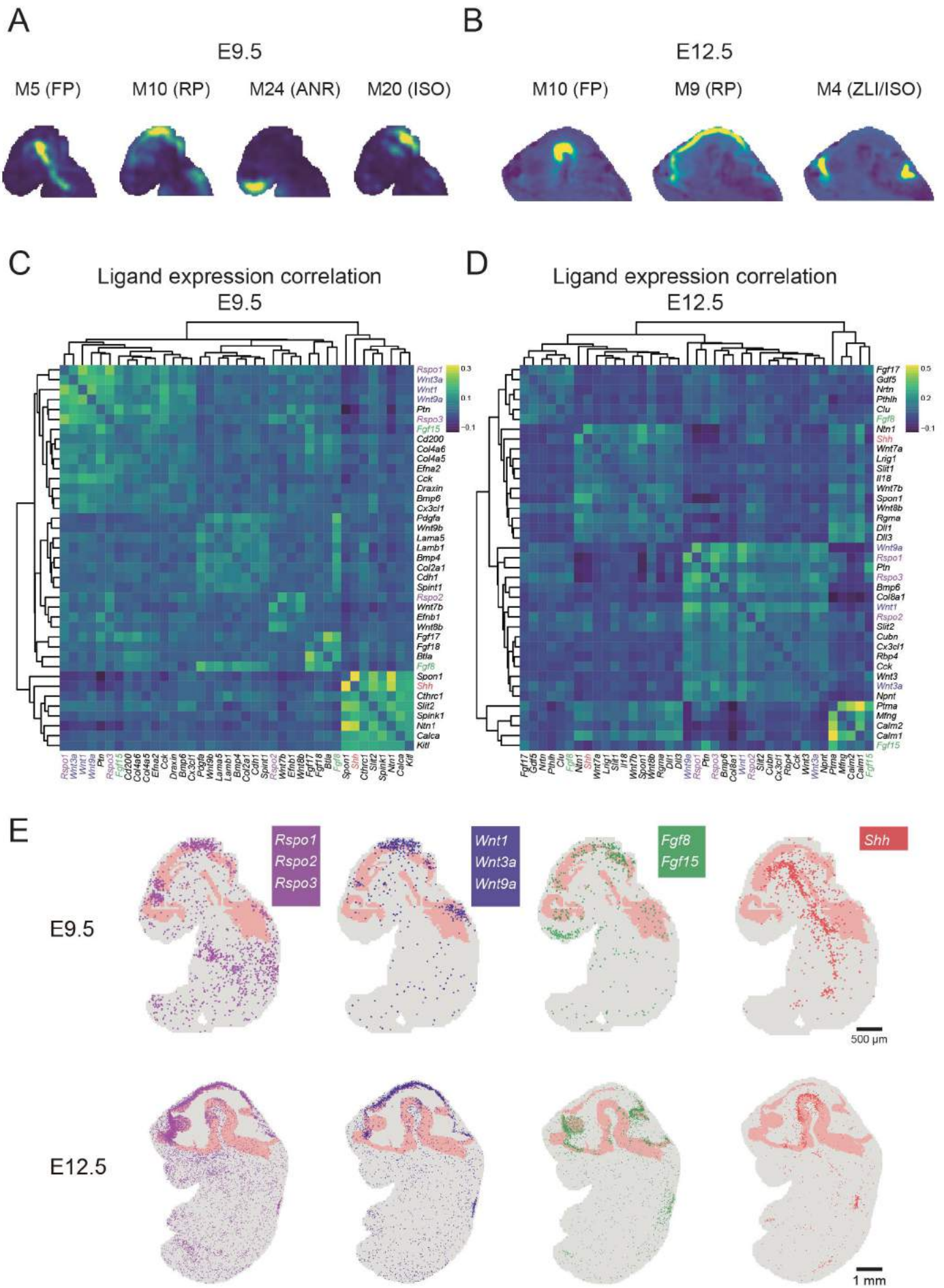
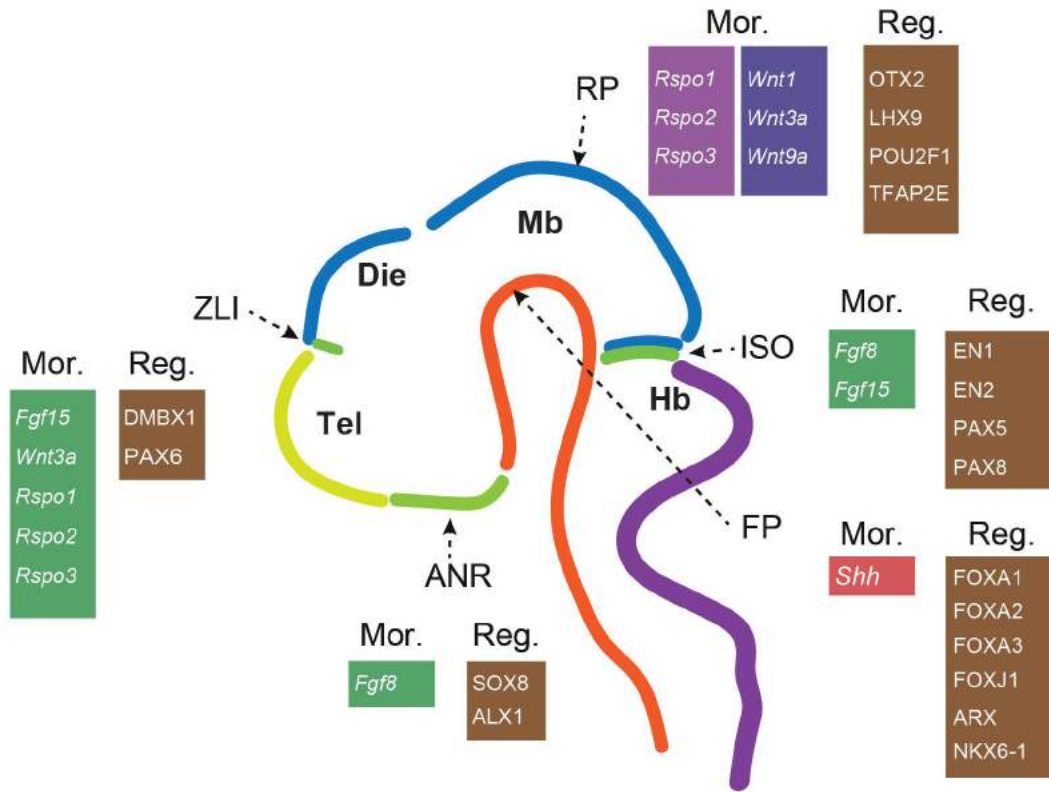


Figure S21

E9.5



E12.5

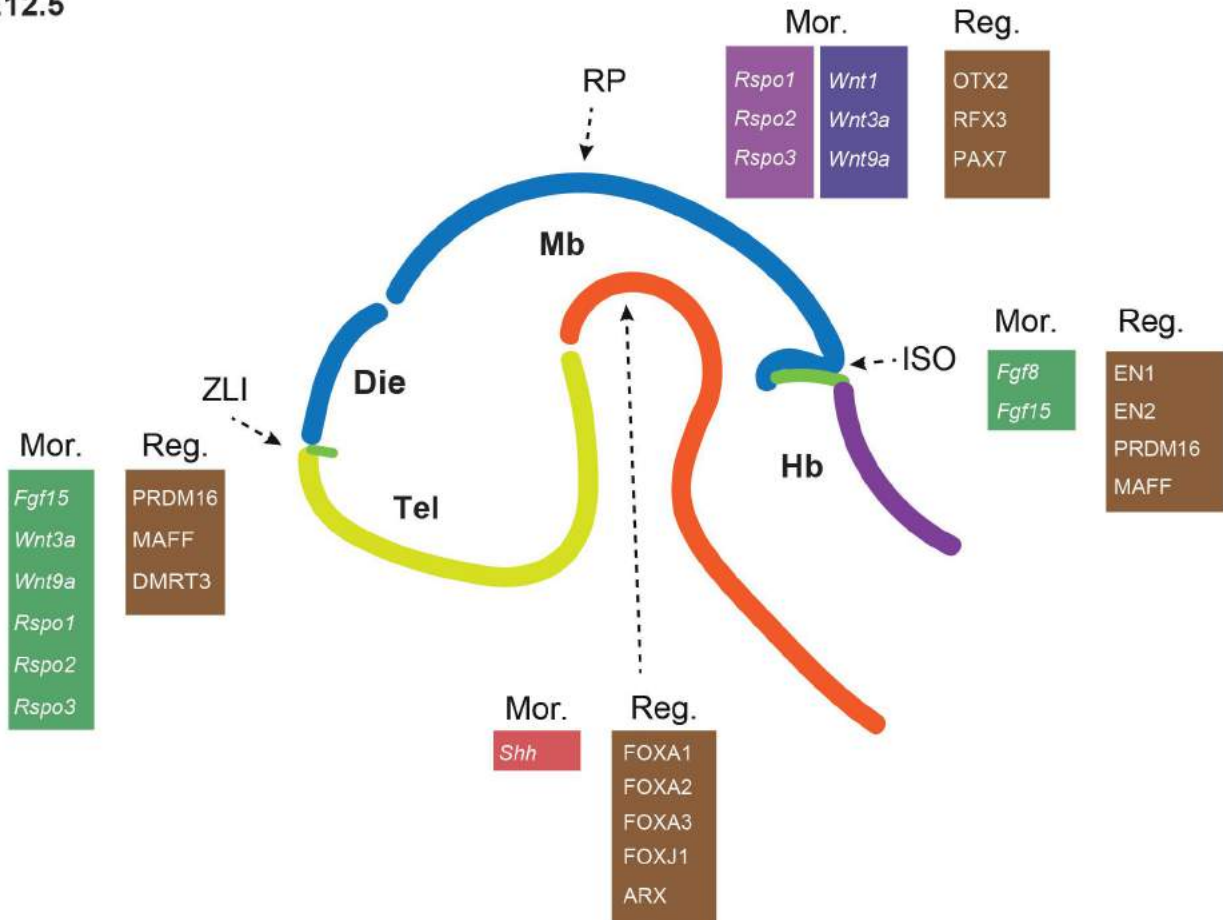


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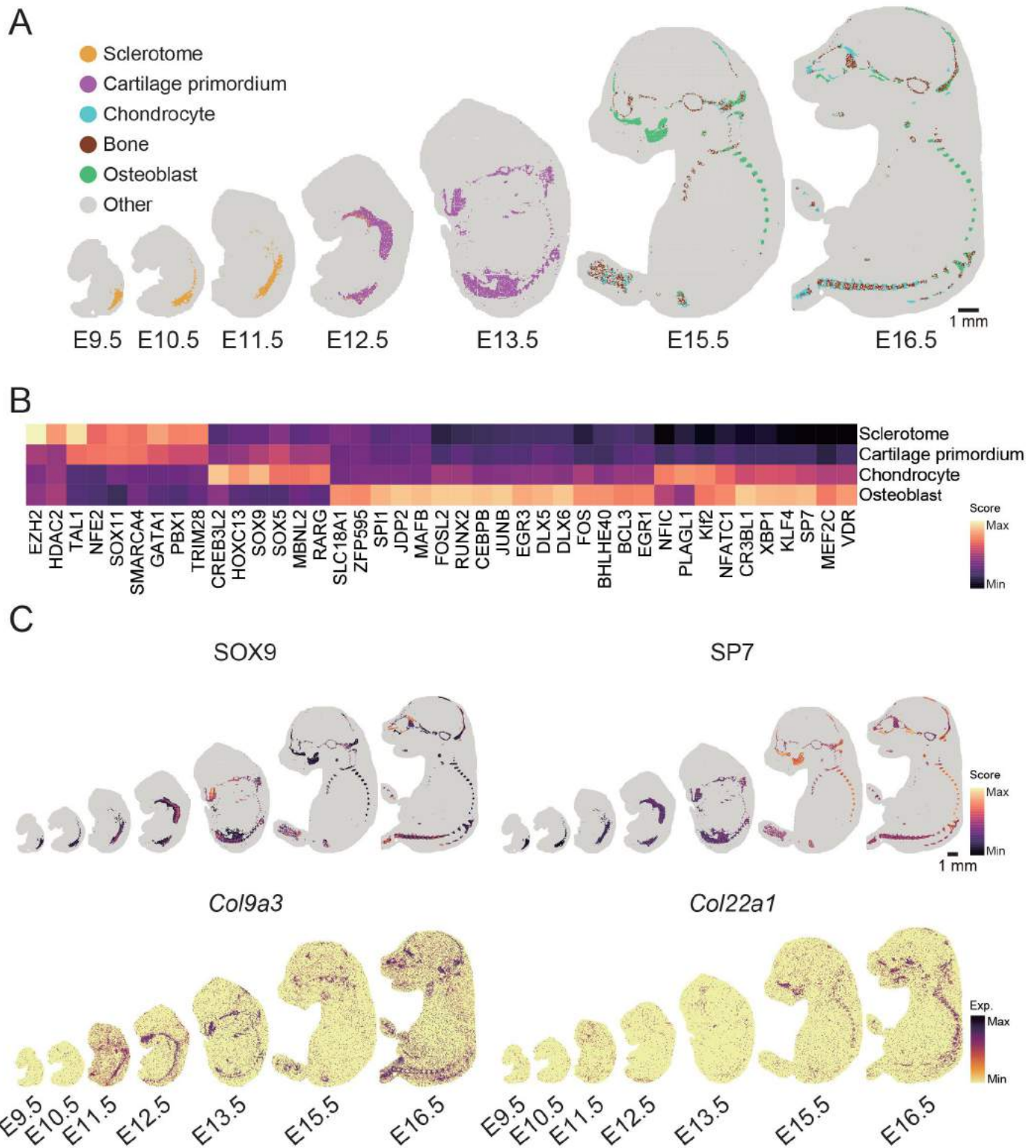


Figure S23

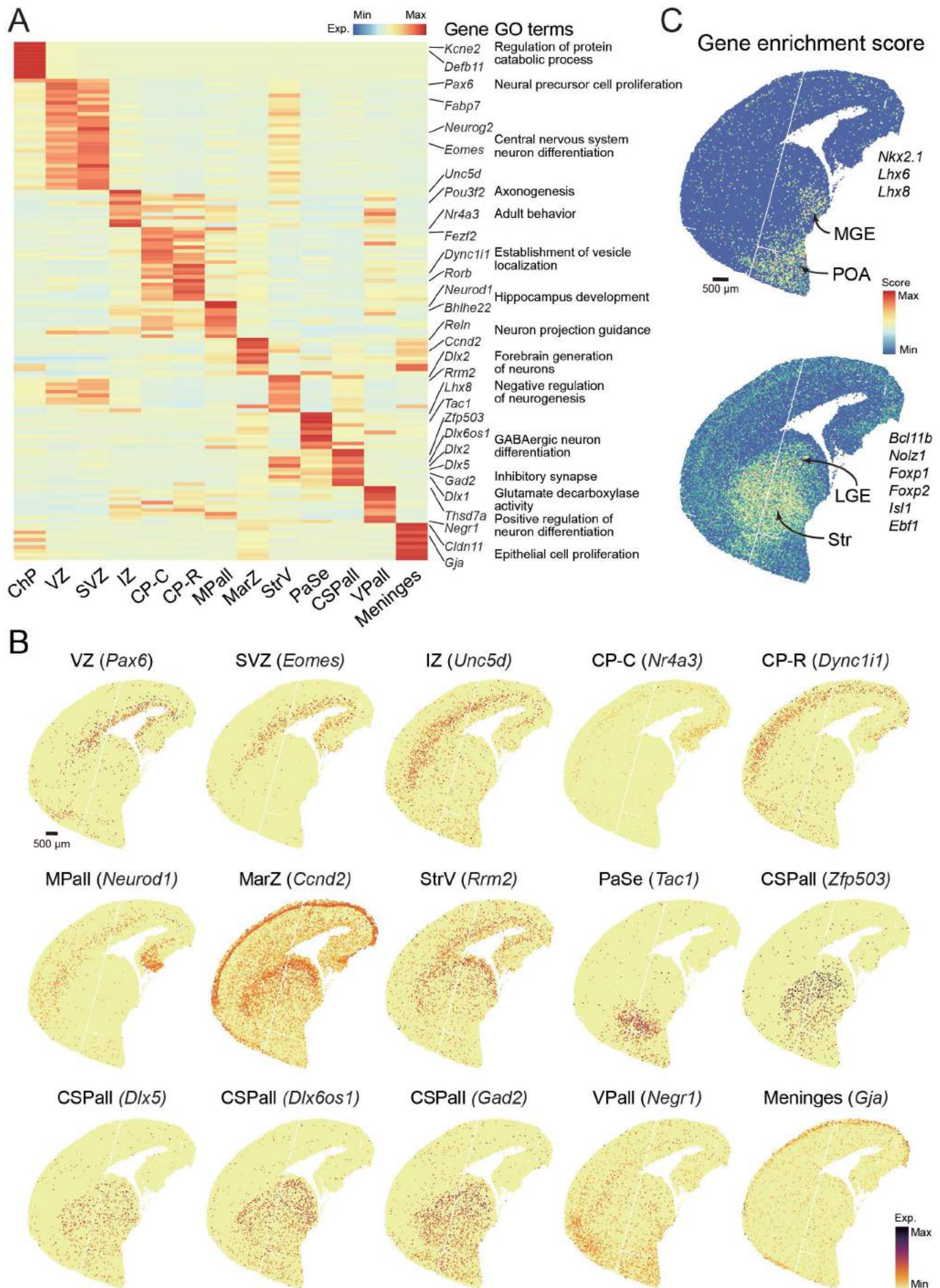


Figure S24

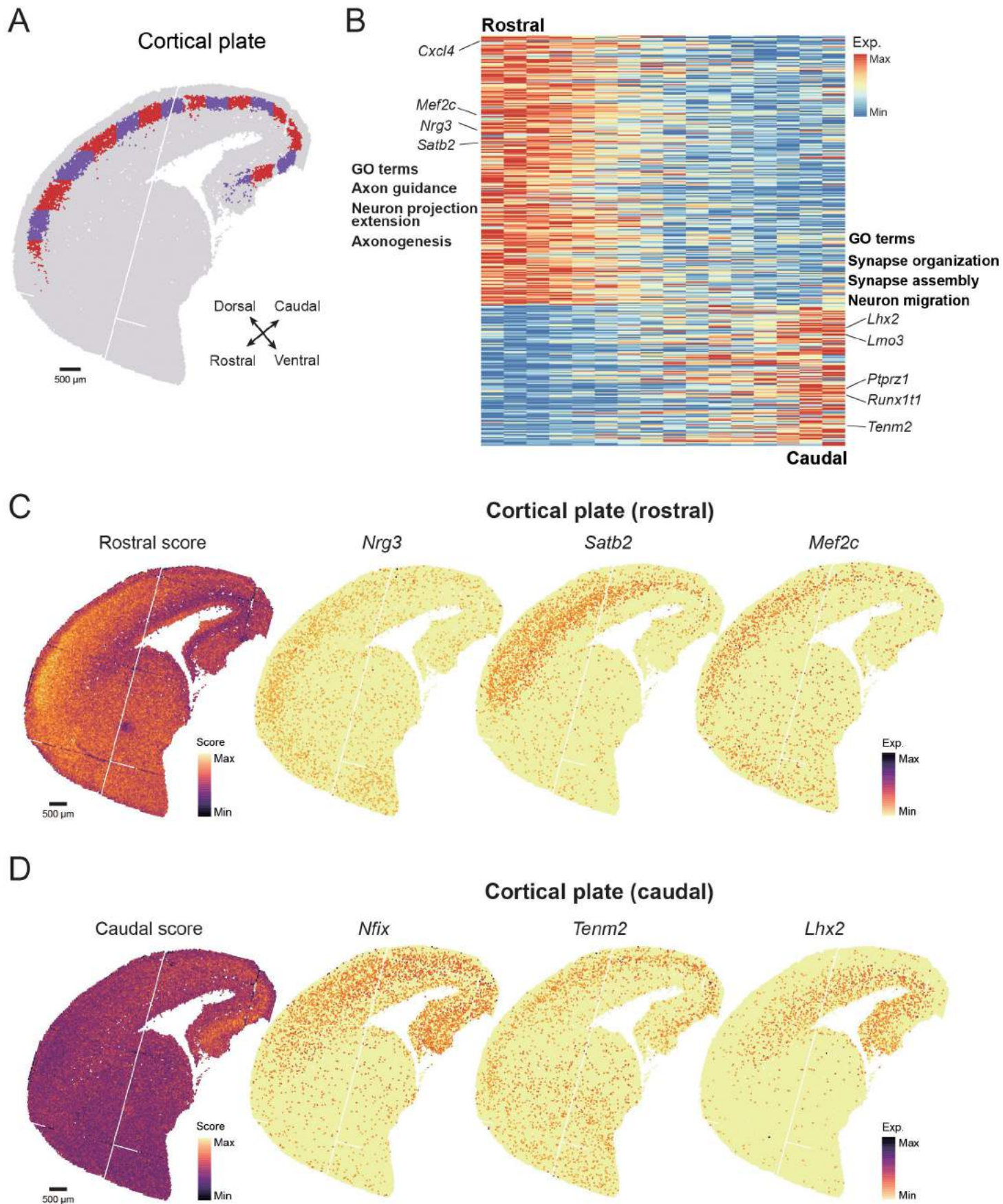


Figure S25

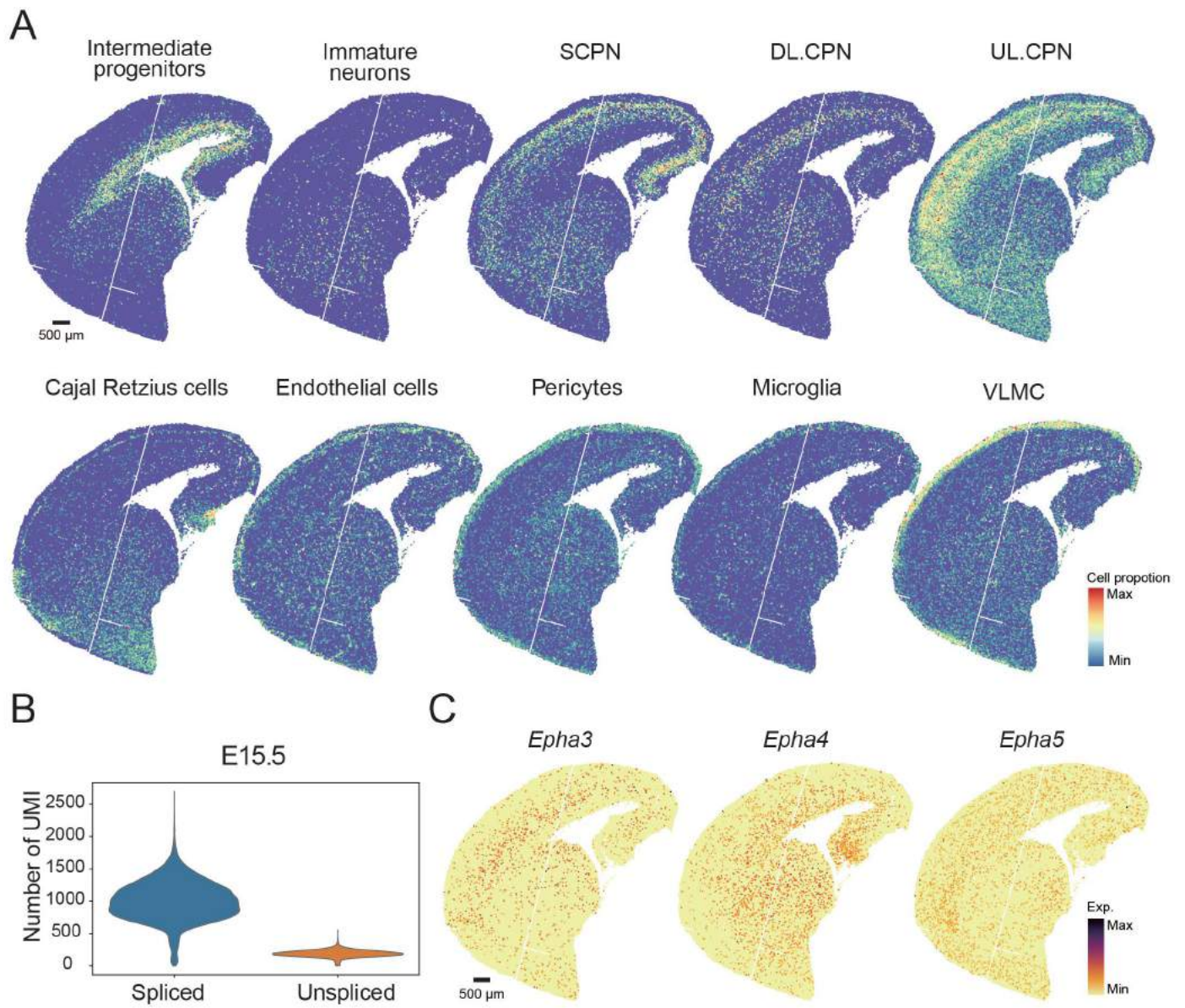


Figure S26

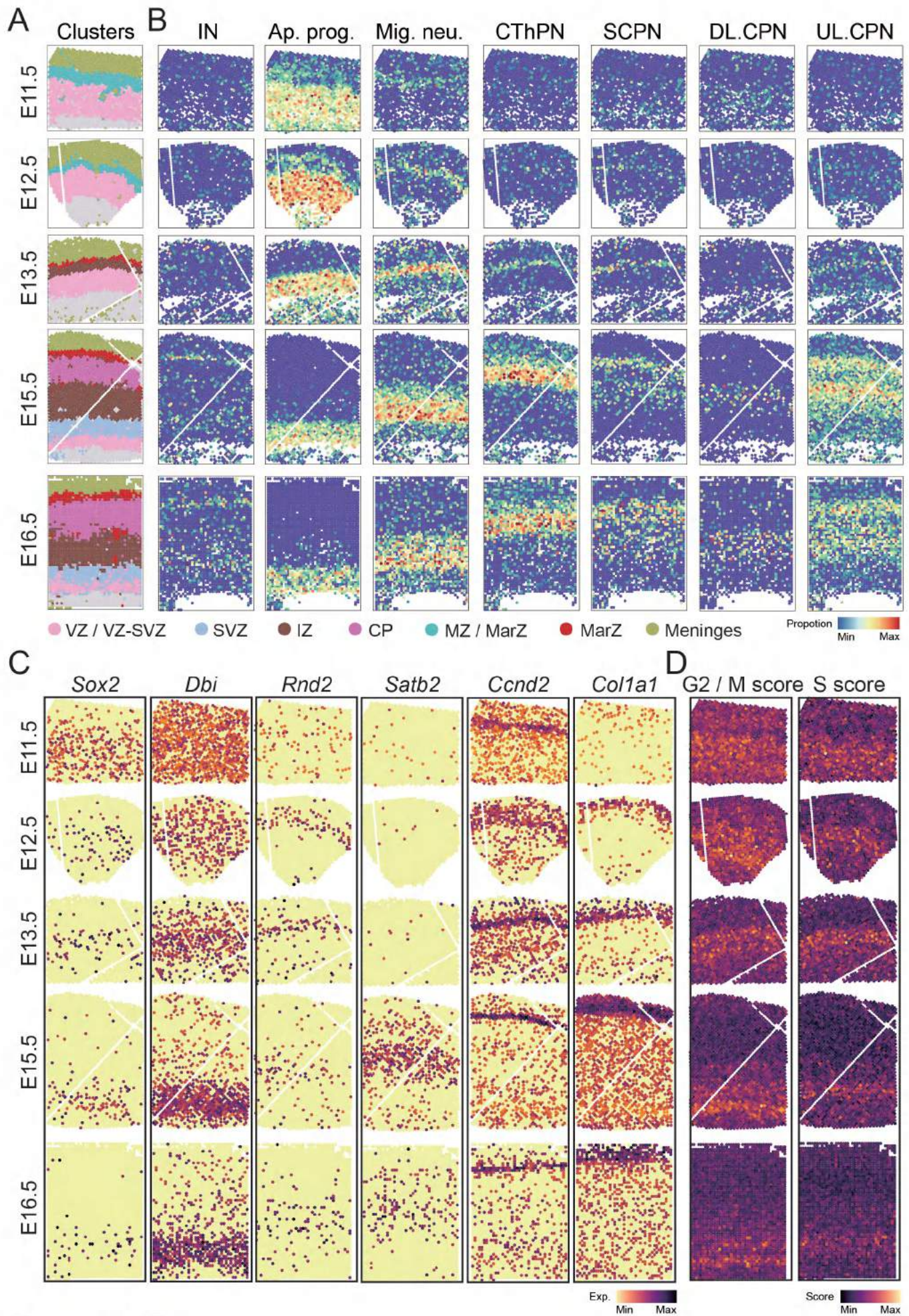


Figure S27

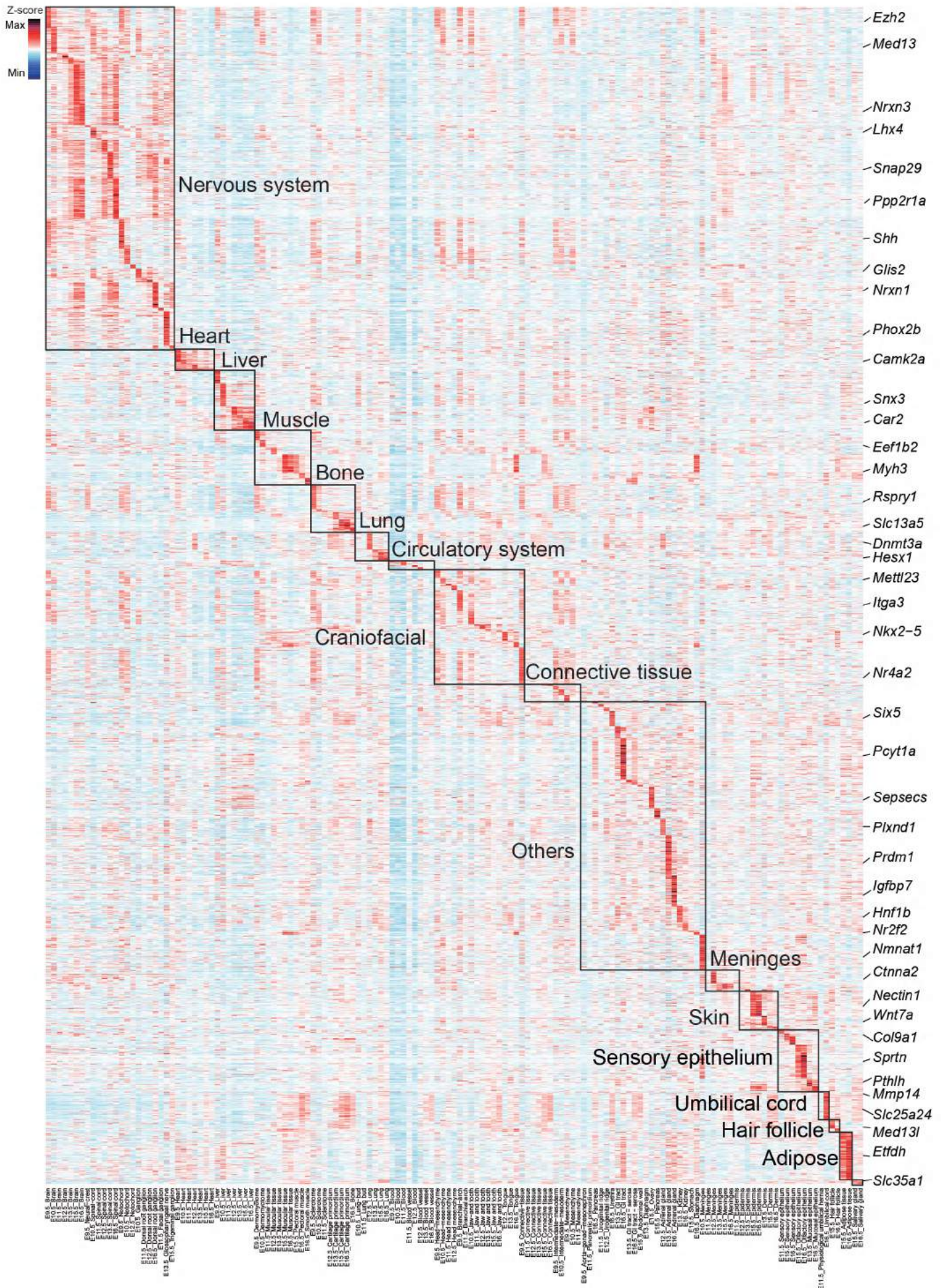


Figure S28