

SUPPLEMENTARY INFORMATION

An in vivo and in vitro spatiotemporal profile of human midbrain development

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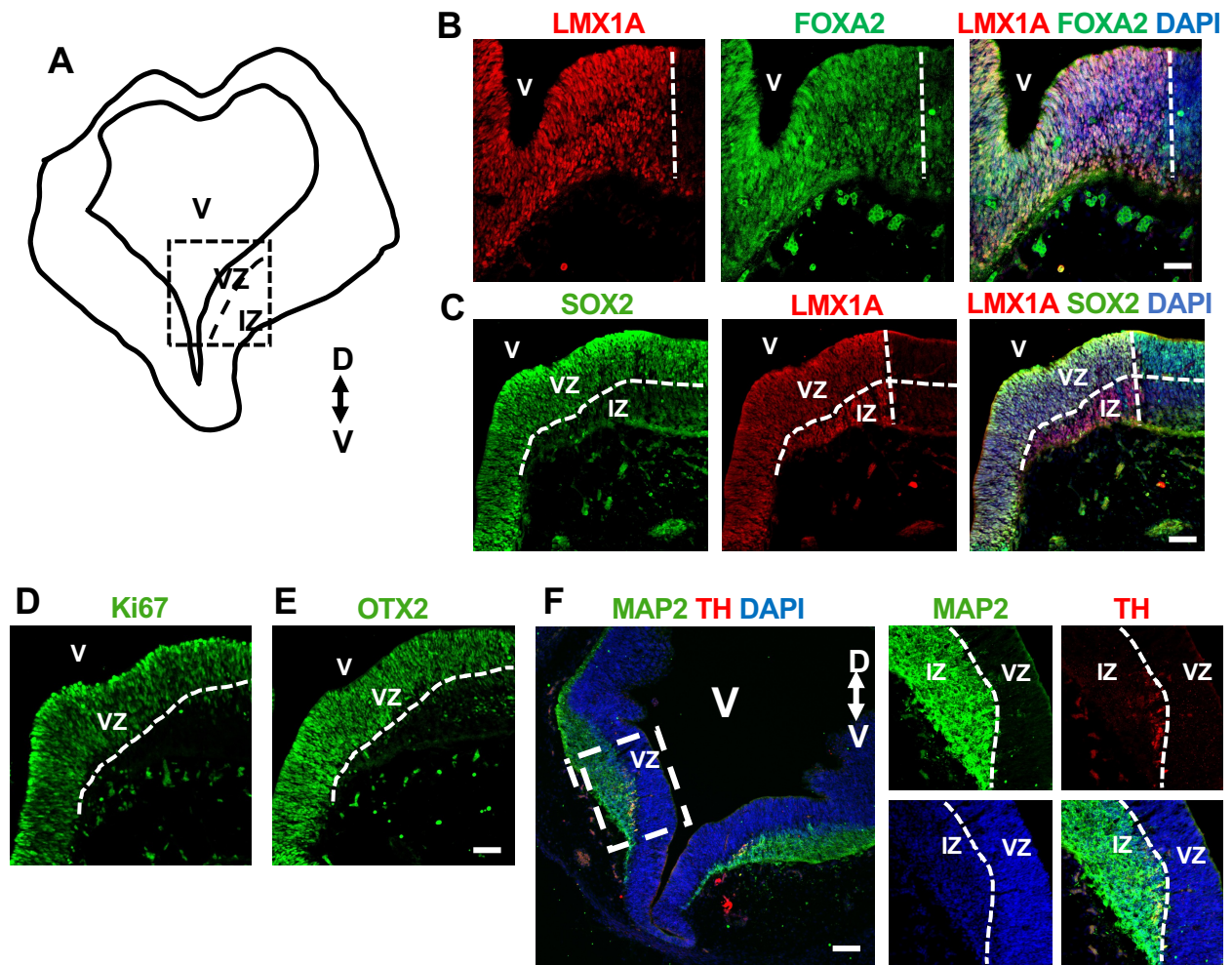
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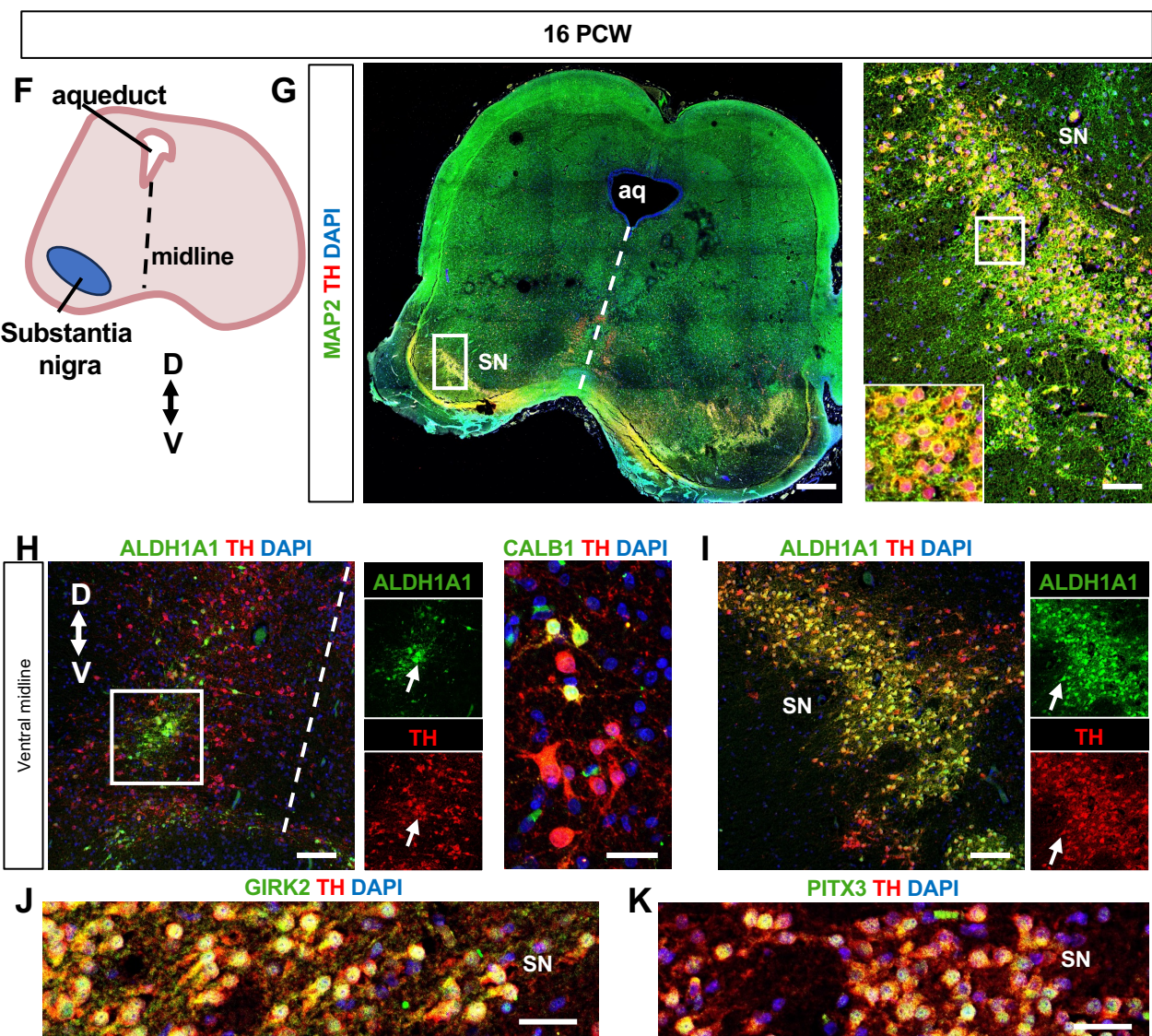
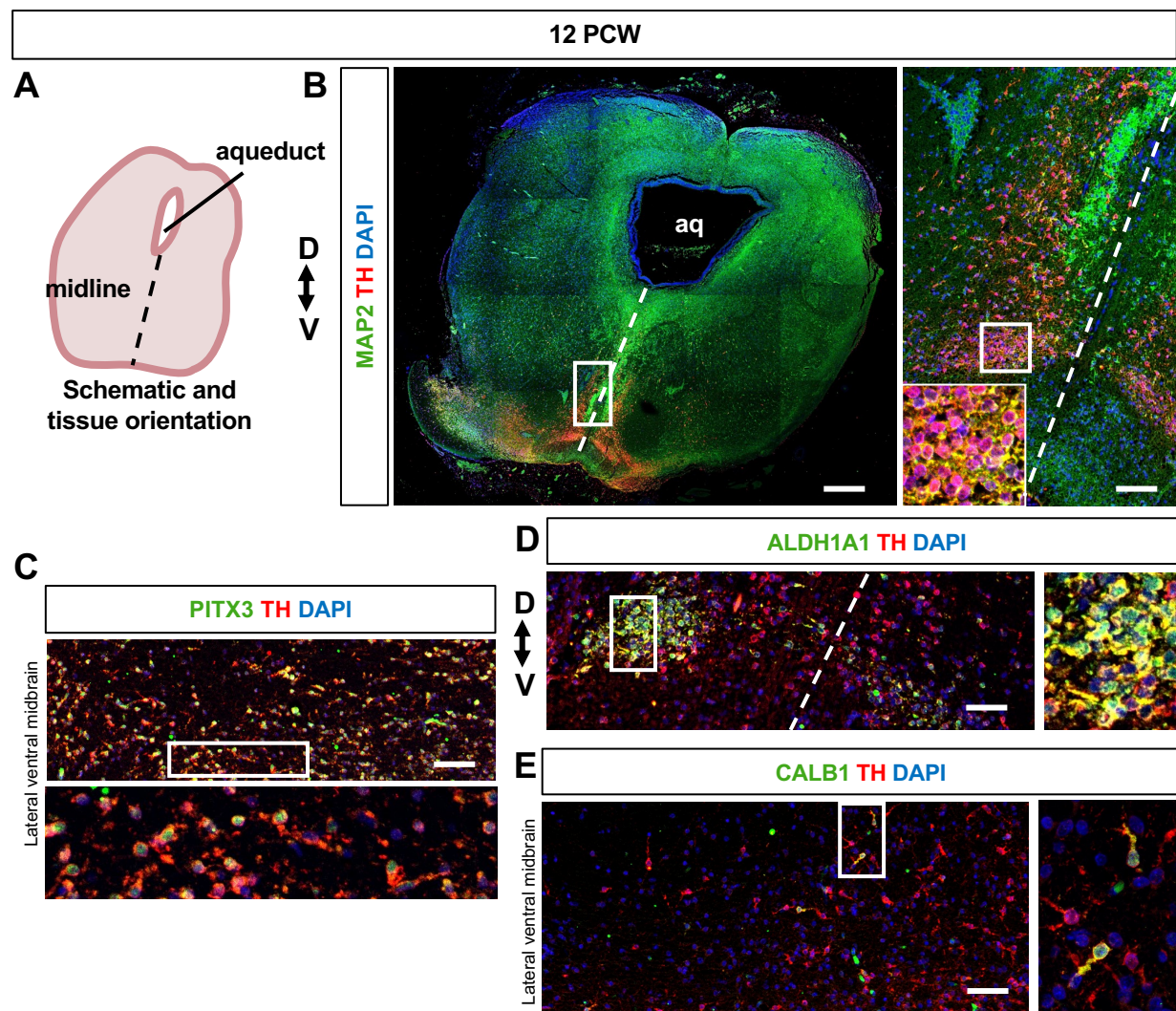
+ These authors equally supervised the project

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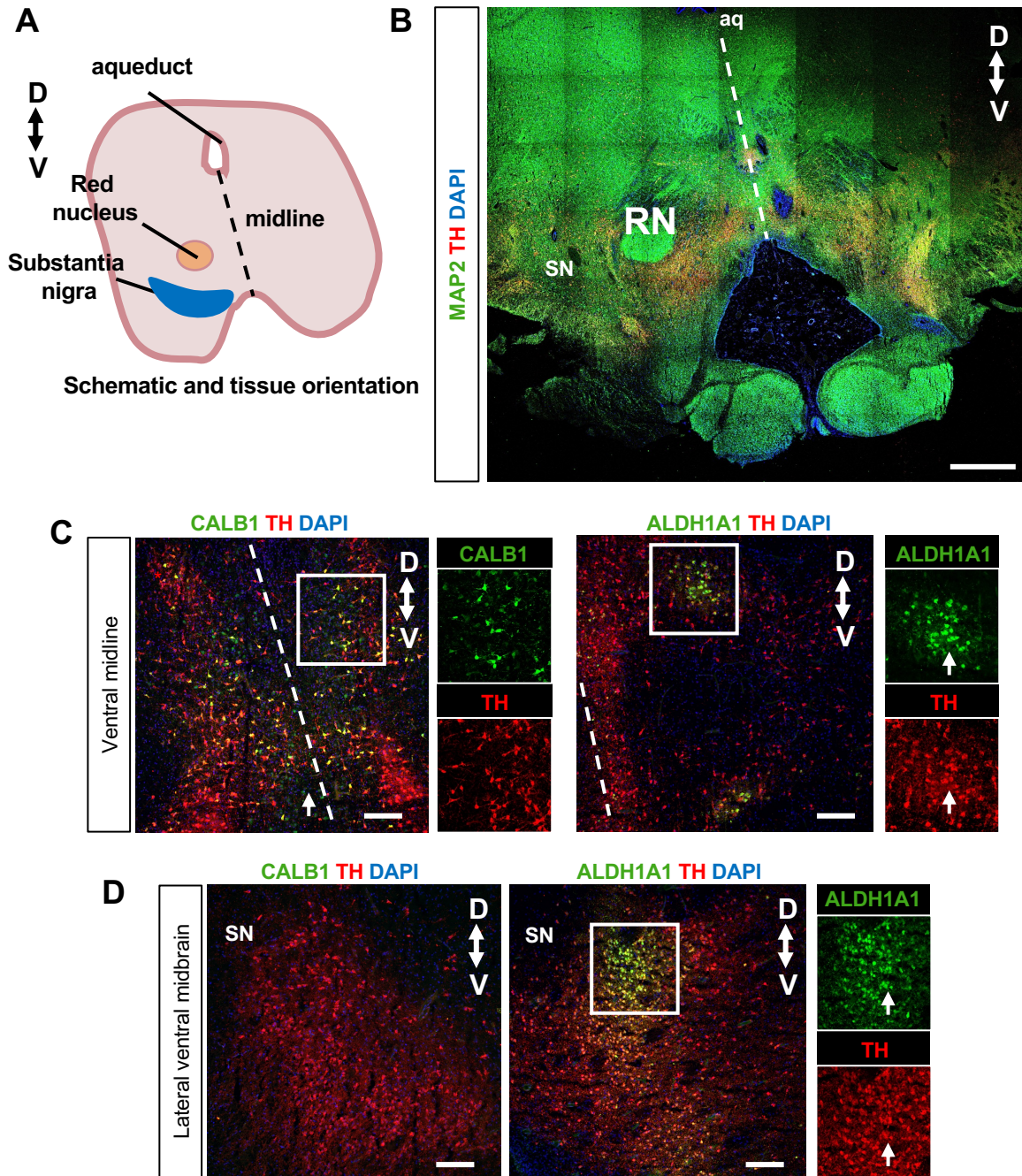
Human fetal midbrain 6 PCW



Supplementary Fig 1 Human ventral midbrain at early stage of development. **A**, Graphical representation of a coronal section of the human midbrain at 6 PCW. Created in BioRender. BUDINGER, D. (2025) <https://BioRender.com/wn4tv4q>. **B**, Immunofluorescence for LMX1A and FOXA2 in the midbrain ventral ventricular zone (VZ). **C**, Immunofluorescence staining for SOX2 and LMX1A in the ventral midbrain. **D-E**, Staining for Ki67 and OTX2 of the human midbrain at 6 PCW. **F**, Immunofluorescence analysis of a coronal section of the human ventral midbrain at 6 PCW for TH and MAP2. Nuclei are stained for DAPI. Lines demarcate the ventral midbrain region and the VZ to IZ boundary Scale bars = 100 μm (B, C, D and E); 200 μm (F). Ventricular zone (VZ), intermediate zone (IZ), ventricle (V).



Supplementary Fig 2 Human midbrain maturation during the early second trimester of fetal development. **A**, Graphical representation of a coronal section of the human midbrain at 12 PCW. Created in BioRender. BUDINGER, D. (2025) <https://BioRender.com/ynycjl1>. **B**, Immunofluorescence analysis of human fetal sample at 12 PCW for MAP2 and TH. Scale bars = 300 μm (*left panel*) and 100 μm (*right panel*). **C**, Immunofluorescence representative image of the lateral ventral human midbrain at 12 PCW for PITX3 and TH. Scale bar = 100 μm . **D**, Representative images of immunofluorescence analysis for ALDH1A1 and TH at the human ventral midbrain ventral midline. Scale bar = 100 μm . **E**, Immunofluorescence for CALB1 and TH of the lateral ventral midbrain. Scale bar = 100 μm . **F**, Graphical representation of a coronal section of the human midbrain at 16 PCW. Created in BioRender. BUDINGER, D. (2025) <https://BioRender.com/rqcf05m>. **G**, Immunofluorescence analysis of human fetal sample at 16 PCW for MAP2 and TH. Scale bars = 400 μm (*left panel*) and 100 μm (*right panel*). **H**, Immunofluorescence analysis of the 16 PCW human midbrain in the ventral midbrain areas for ALDH1A1, CALB1 and TH. Scale bars = 100 μm (*left panel*) and 20 μm (*right panel*). **I**, Immunofluorescence analysis for ALDH1A1 and TH across the lateral ventral midbrain. Scale bar = 100 μm . **J-K**, Immunofluorescence analysis for GIRK2, PITX3 and TH in the lateral ventral midbrain. Scale bar = 30 μm . Nuclei are stained for DAPI. Lines demarcate ventral midline. Aqueduct (aq), dorsal (D), ventral (V), substantia nigra (SN).



Supplementary Fig 3 Ventral midbrain dopaminergic neurons subtypes

distribution at 19 PCW. A, Graphical representation of a coronal section of the human midbrain at 19 PCW. Created in BioRender. BUDINGER, D. (2025)

<https://BioRender.com/yuijdei>. **B**, Immunofluorescence analysis of human fetal

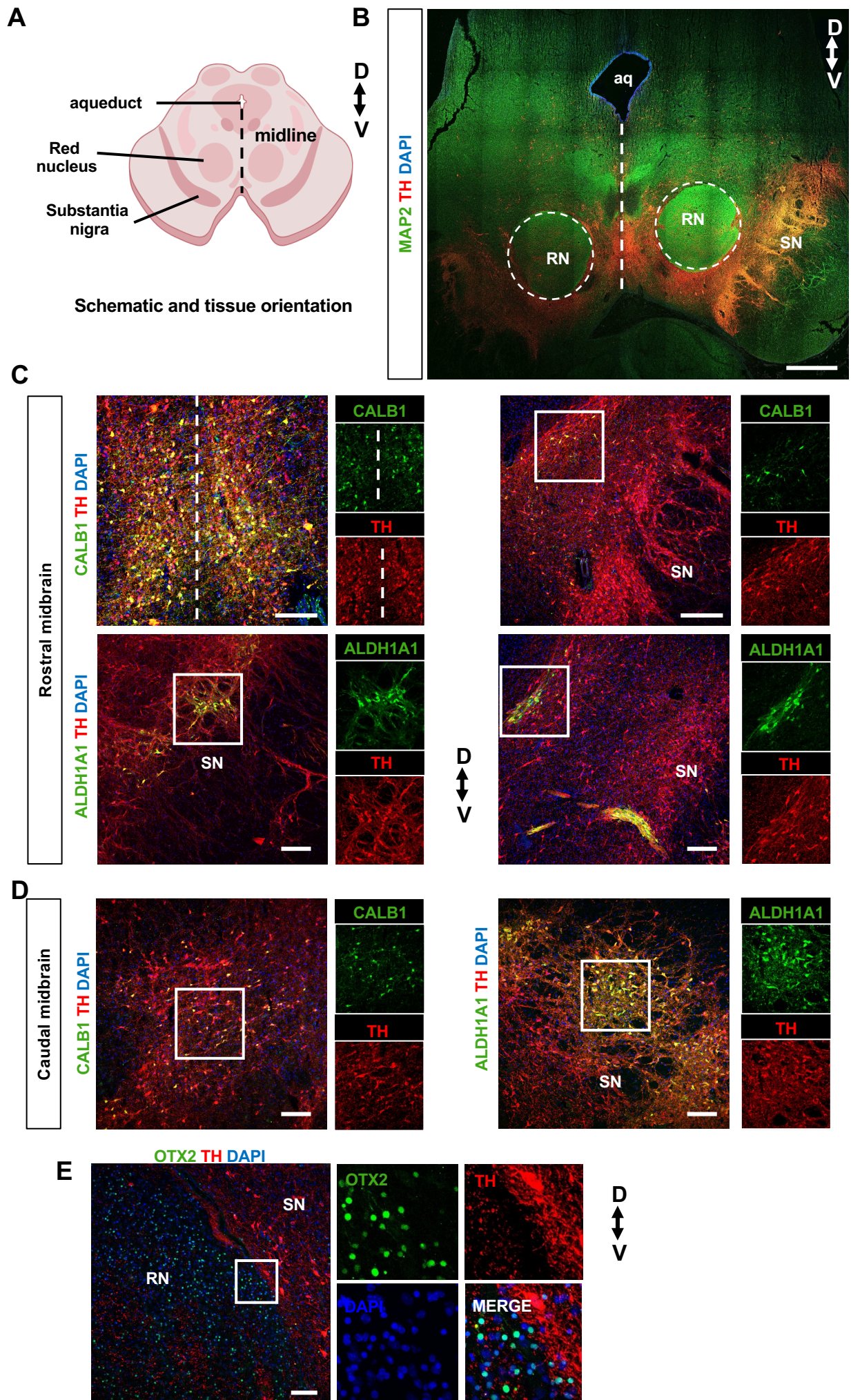
sample at 19 PCW for MAP2 and TH. Scale bar = 300 μ m. **C-D**, Representative

images of immunostaining for CALB1, ALDH1A1 and TH along the ventral midline and

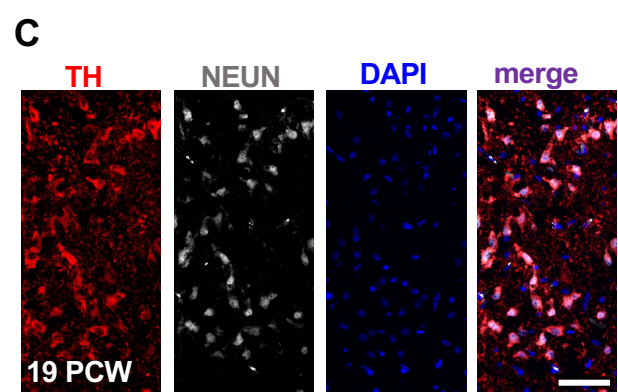
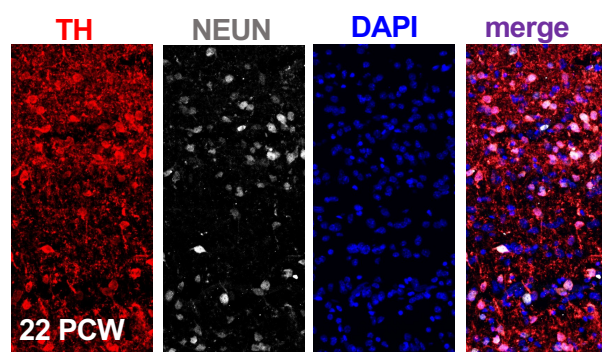
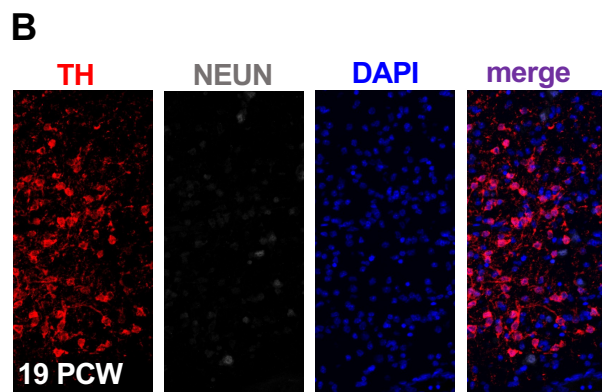
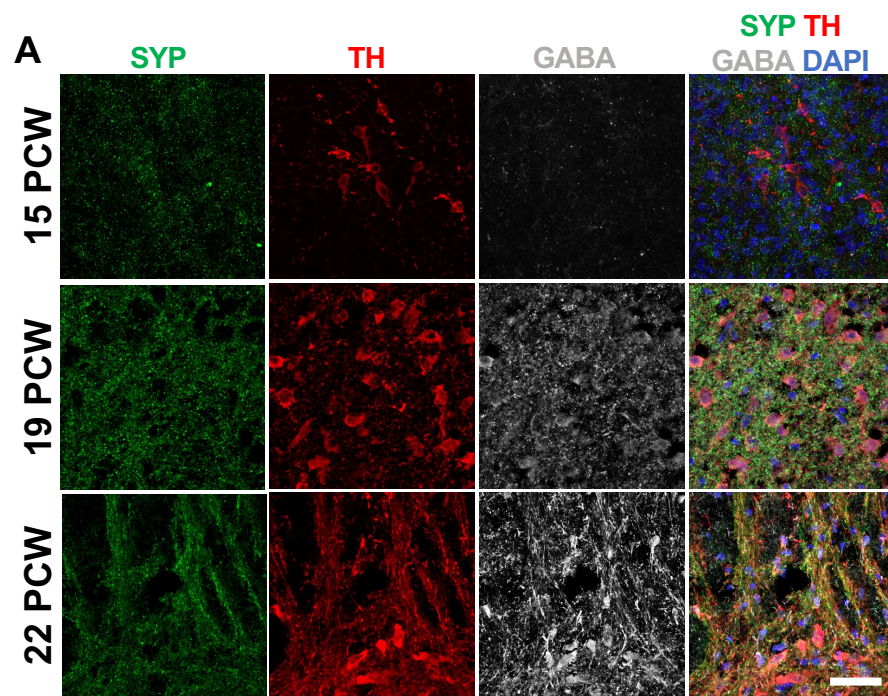
the lateral ventral midbrain of the human midbrain at 19 PCW, respectively. Scale bars

= 100 μ m. Nuclei are stained for DAPI. Lines demarcate ventral midline. Aqueduct

(aq), dorsal (D), ventral (V), substantia nigra (SN), red nucleus (RN).



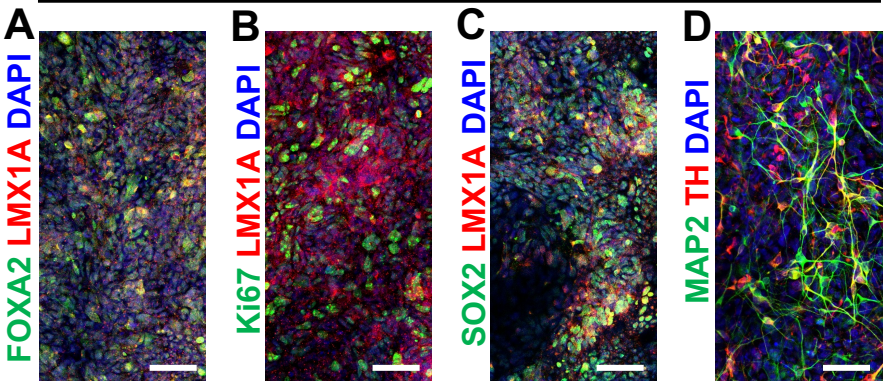
Supplementary Fig 4 Ventral midbrain dopaminergic neurons subtypes distribution at 22 PCW. **A**, Graphical representation of a coronal section of the human midbrain at 22 PCW. Created in BioRender. BUDINGER, D. (2025) <https://BioRender.com/5cmho7m>. **B**, Immunofluorescence analysis of human fetal sample at 19 PCW for MAP2 and TH. Scale bar = 300 μ m. **C-D**, Representative images of immunostaining for CALB1, ALDH1A1 and TH in the rostral and caudal ventral midbrain at 22 PCW, respectively. Scale bars = 100 μ m. **E**, Immunofluorescence analysis of OTX2 and TH expression. Scale bar = 100 μ m. Nuclei are stained for DAPI. Lines demarcate ventral midline. Aqueduct (aq), dorsal (D), ventral (V), substantia nigra (SN), red nucleus (RN).



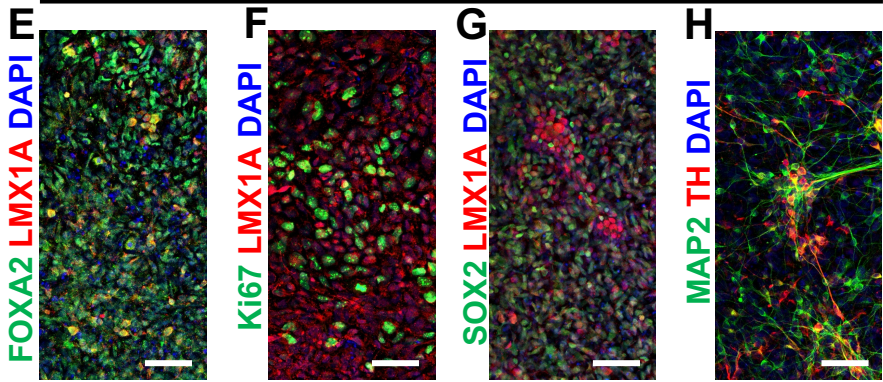
Supplementary Fig 5 Human midbrain dopaminergic neurons subtypes and maturation stage. **A**, Immunofluorescence analysis for Synaptophysin (SYP), TH and GABA of fetal samples at 15, 19 and 22 PCW. **B-C**, Representative immunofluorescence images of radial and tangential migrating dopaminergic neurons (TH+) and maturation stage as indicated by NEUN positivity. Nuclei are stained for DAPI. Scale bars = 50 μ m.

d20 2D mDA

Control 1

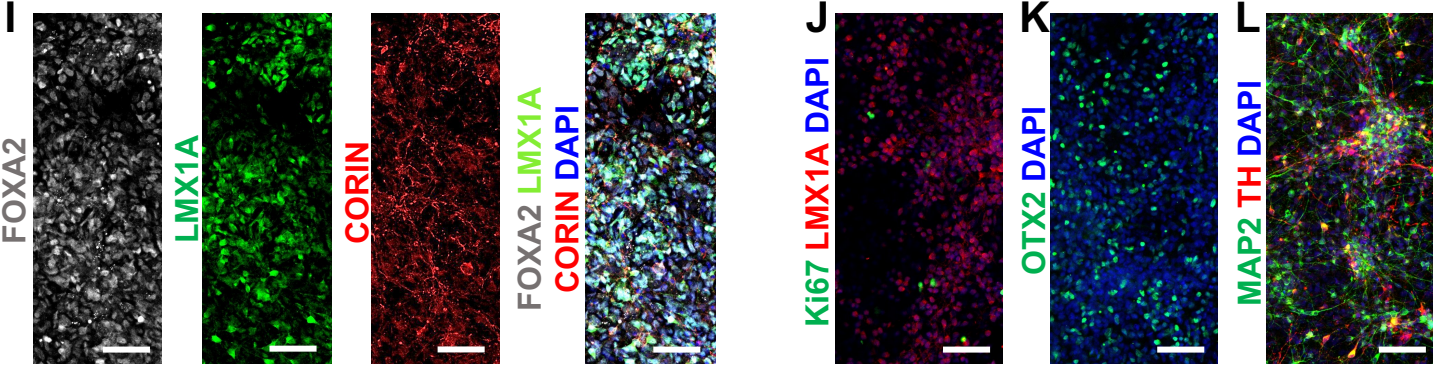


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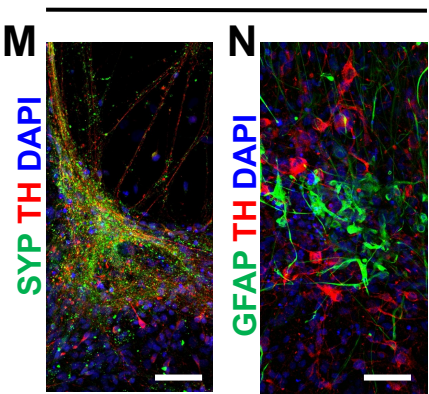
d40 2D mDA

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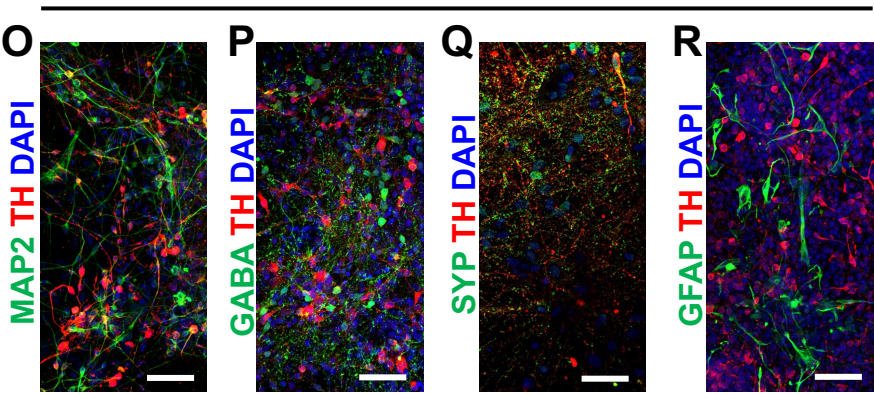


d70 2D mDA

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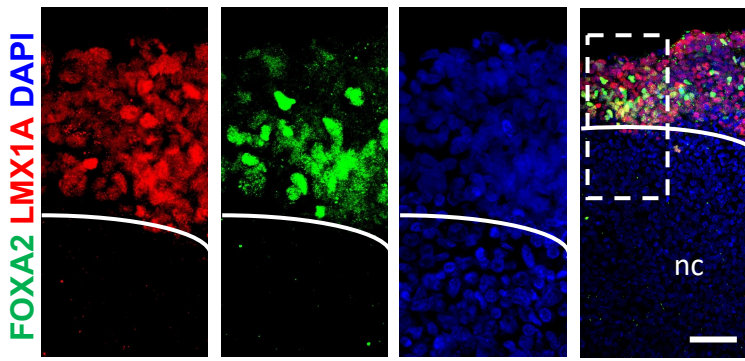
Control 2



Supplementary Fig 6 Characterization of iPSC-derived 2D mDA neuronal cultures. **A-R**, Representative images of immunofluorescence analysis for midbrain and neuronal-related proteins FOXA2, LMX1A, CORIN, SOX2, MAP2, TH, OTX2, GABA, SYP, the glial cell marker GFAP and the cell cycle protein Ki67 in Control 1 and Control 2-derived 2D mDA neural cultures at 20, 40 and 70 days of differentiation. Nuclei are staining with DAPI. Scale bars = 50 μ m.

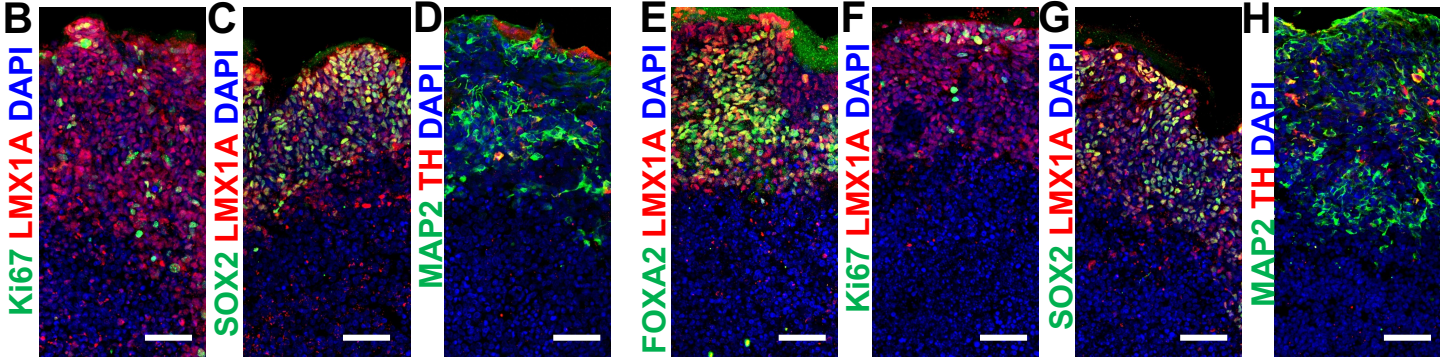
d20 3D MLO

A

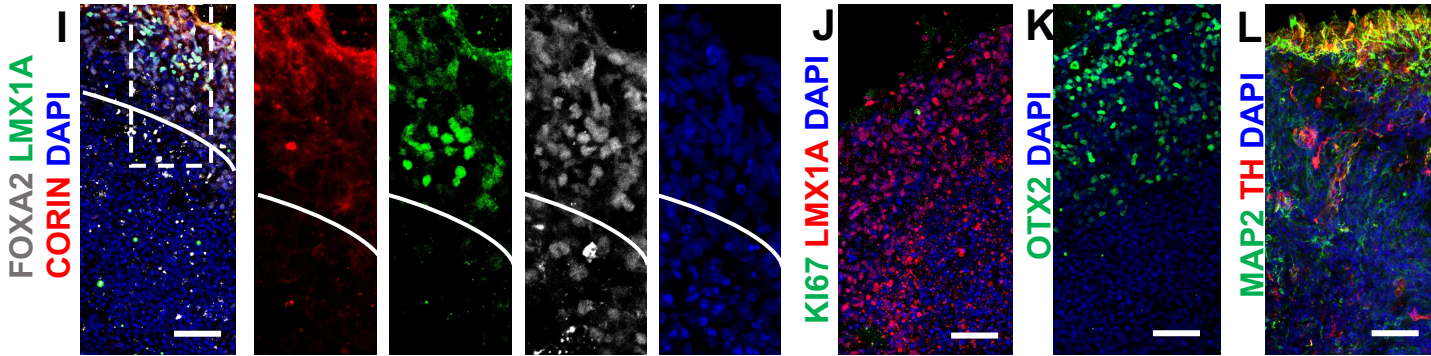


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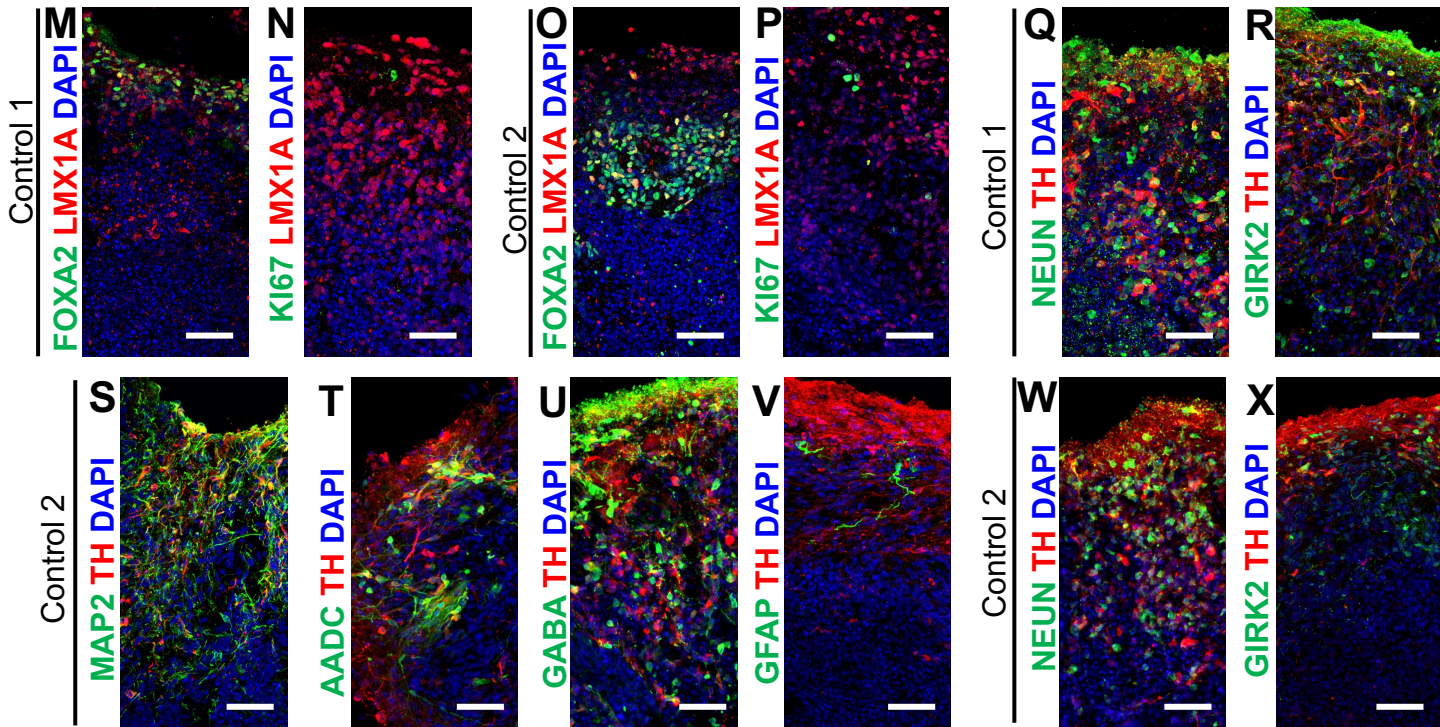
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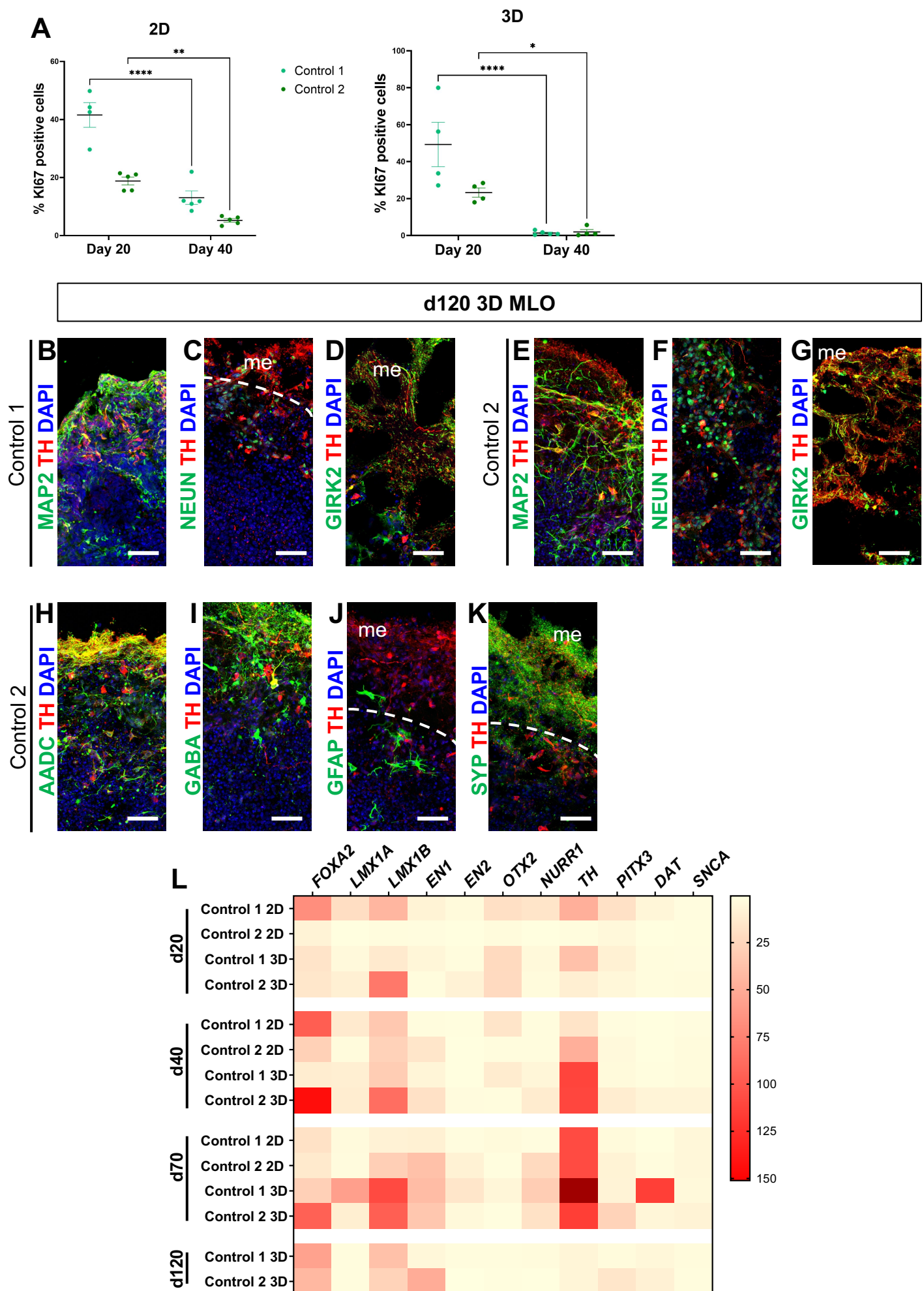
d40 3D MLO



D70 3D MLO

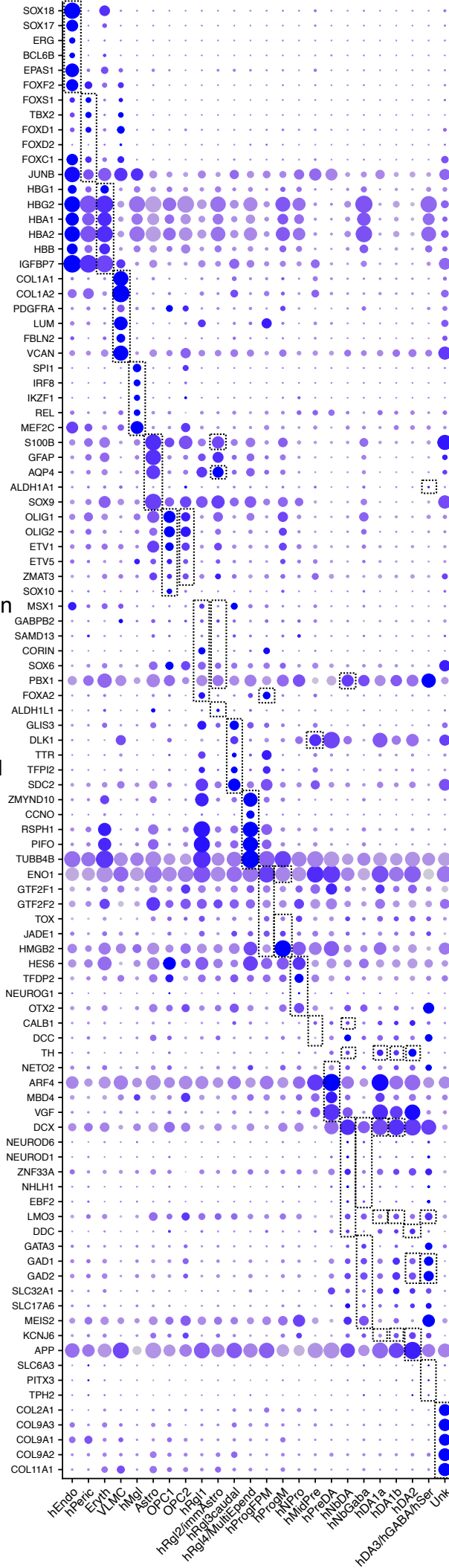


Supplementary Fig 7 Characterization of iPSC-derived MLOs. A-X, Representative images of immunofluorescence analysis for midbrain and neuronal-related proteins FOXA2, LMX1A, CORIN, SOX2, MAP2, TH, AADC, OTX2, GABA, NEUN, GIRK2, the glial cell marker GFAP and the cell cycle protein Ki67 in Control 1 and Control 2-derived MLO at 20, 40 and 70 days of differentiation. Nuclei are staining with DAPI. Scale bars = 50 μ m.

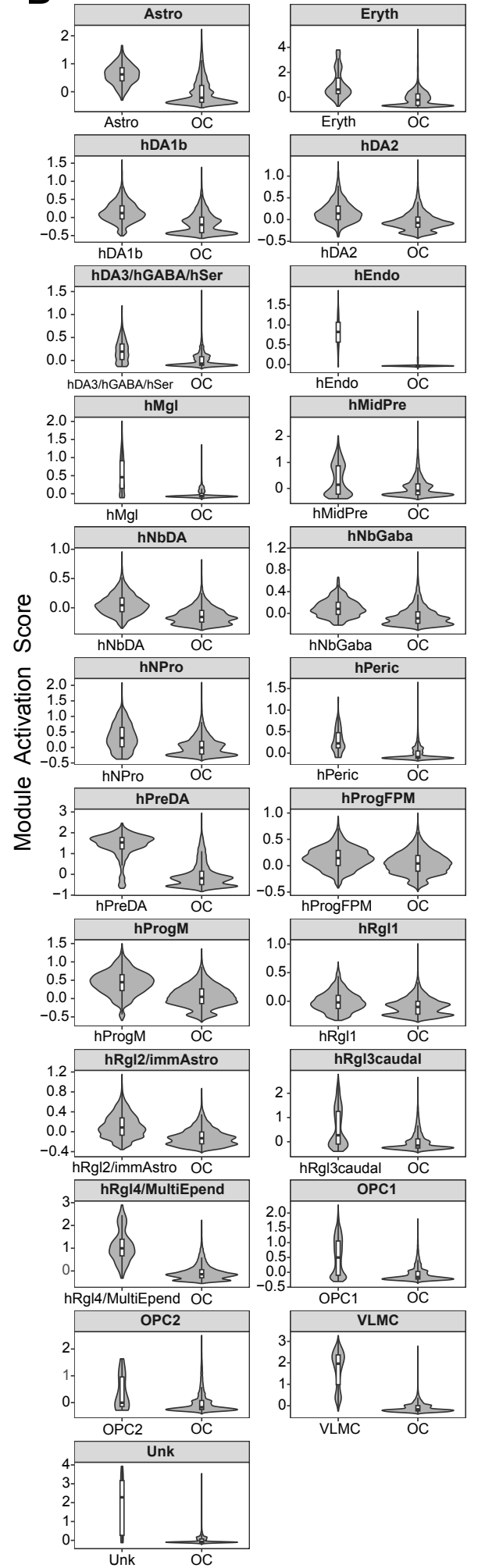


Supplementary Fig 8 Characterization of iPSC-derived MLO at late stage of maturation. **A**, Quantification for KI67+ cells in controls in both 2D cultures and 3D organoids, between d20 and d40 of differentiation. **B-K**, Immunofluorescence images of Control 1 and 2-derived MLO at 120 days of differentiation showing positive cells for TH, MAP2, NEUN, GIRK2, GFAP and SYP. Nuclei are staining with DAPI. Scale bars = 50 μ m. **L**, Quantitative real time PCR (q-RT-PCR) for midbrain related genes in Control 1 and 2-derived 2D mDA (2D) cultures and MLOs at 20, 40, 70 and 120 days of differentiation. Genes expression for *FOXA2*, *LMX1A*, *LMX1B*, *EN1*, *EN2*, *OTX2*, *NURR1*, *PITX3*, *DAT*, *SNCA* is relative to housekeeping gene (*GAPDH*) and normalized to their respective iPSCs (n=1 for each line). Error bars indicate SEM. 2D and 3D cultures at day 20 and day 40 were independently compared using two-tailed Student's *t*-test for all analyses. Matrigel embedding (me). Source data are provided as a Source Data file.

A

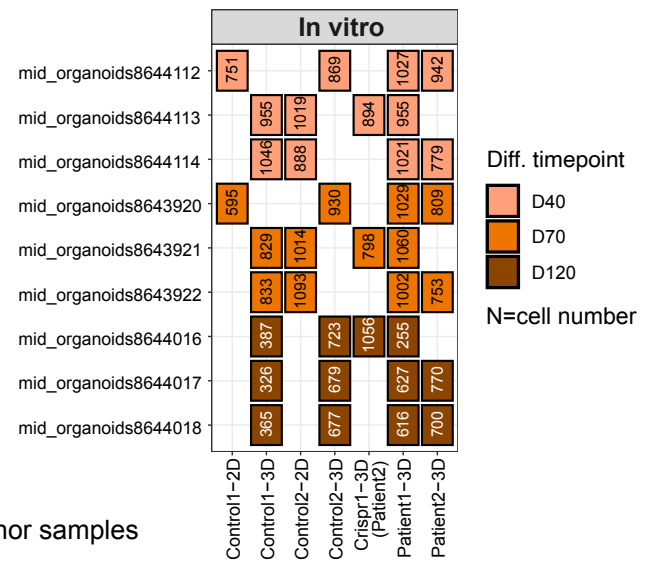
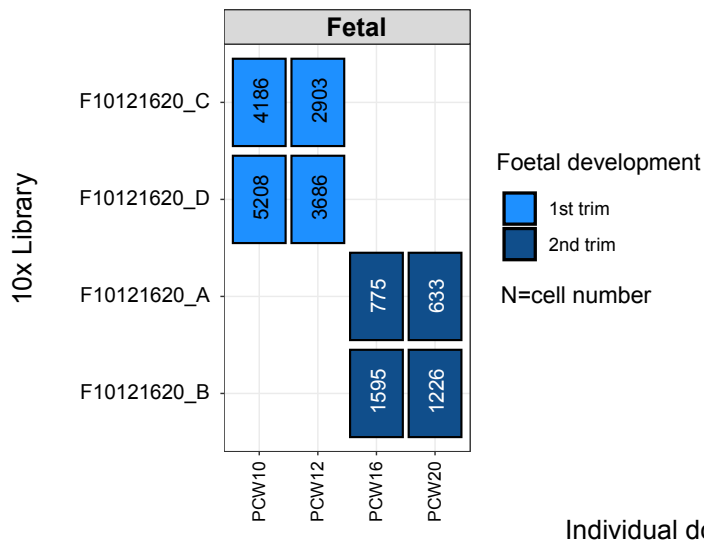


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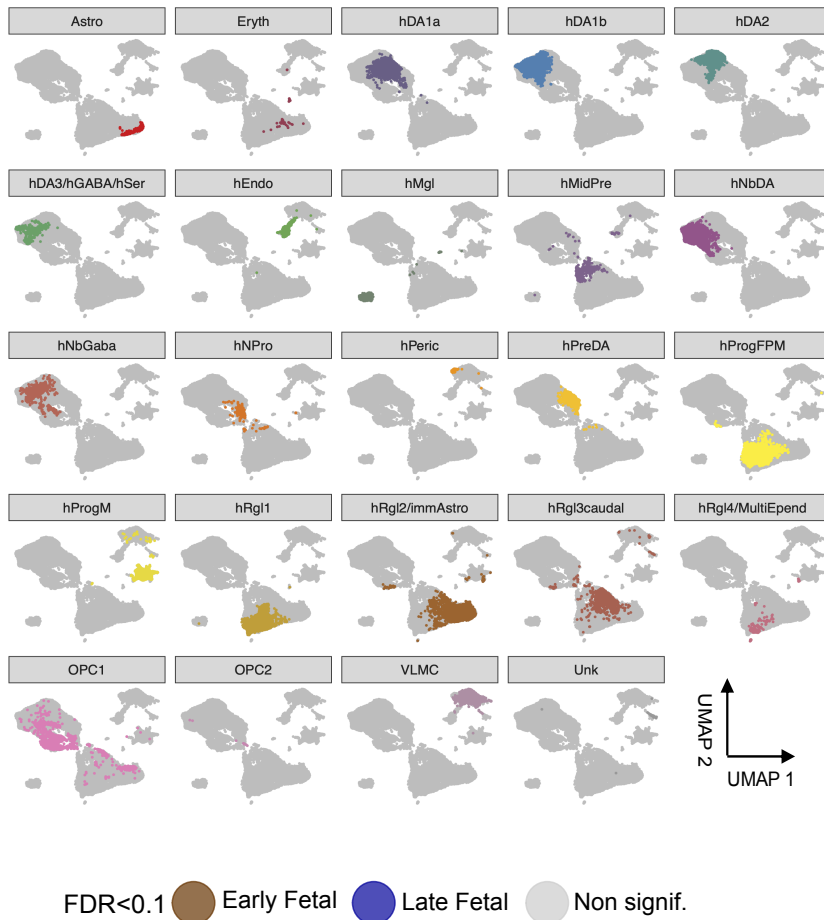


Supplementary Fig 9 High-resolution cell type annotation based on marker genes and gene modules. A, Marker genes used for high-resolution cell type annotation. The blue gradient indicates the scaled average expression per cell type, while the dot size indicates the percentage of cells expressing each gene within the corresponding cell type. For further details on marker genes and abbreviations, see **Supp Data S3. B,** Violin plots showing the distribution of activation score for cell-type specific gene modules (Supp Data S3), comparing the cell type of interest to other cell types (OC).

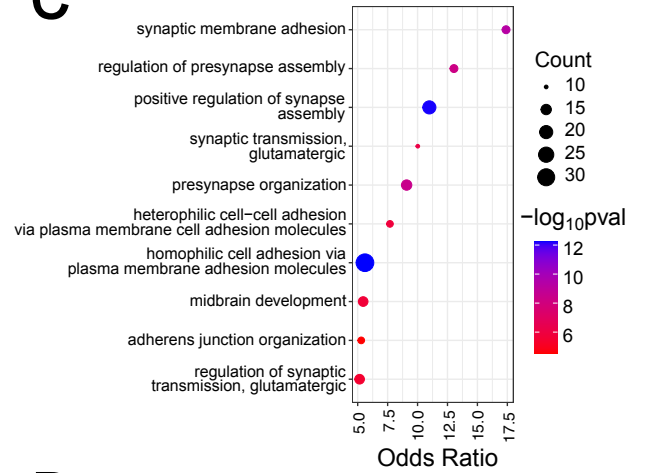
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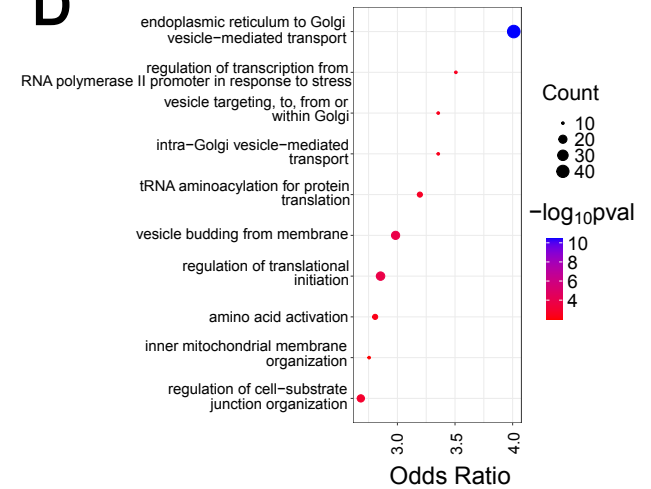
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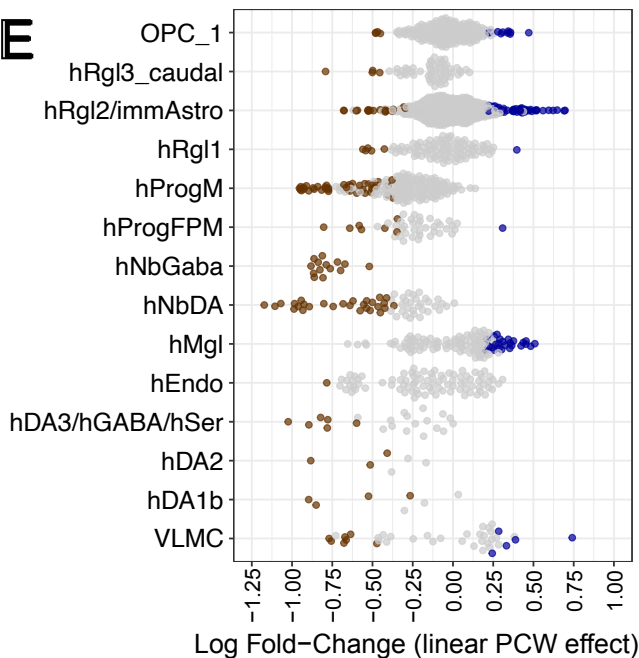
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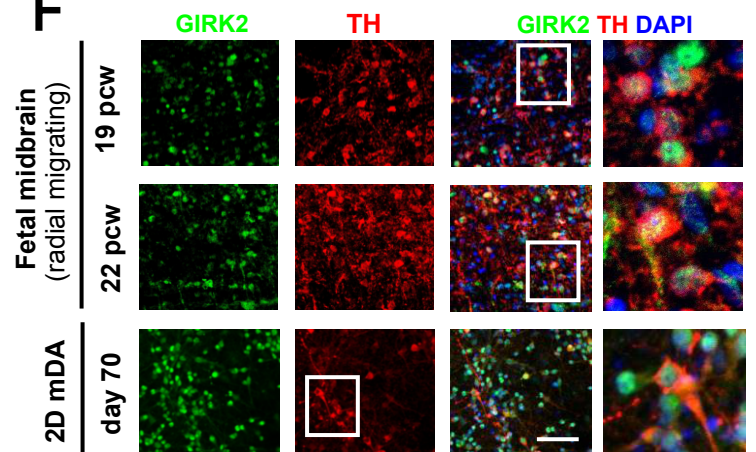
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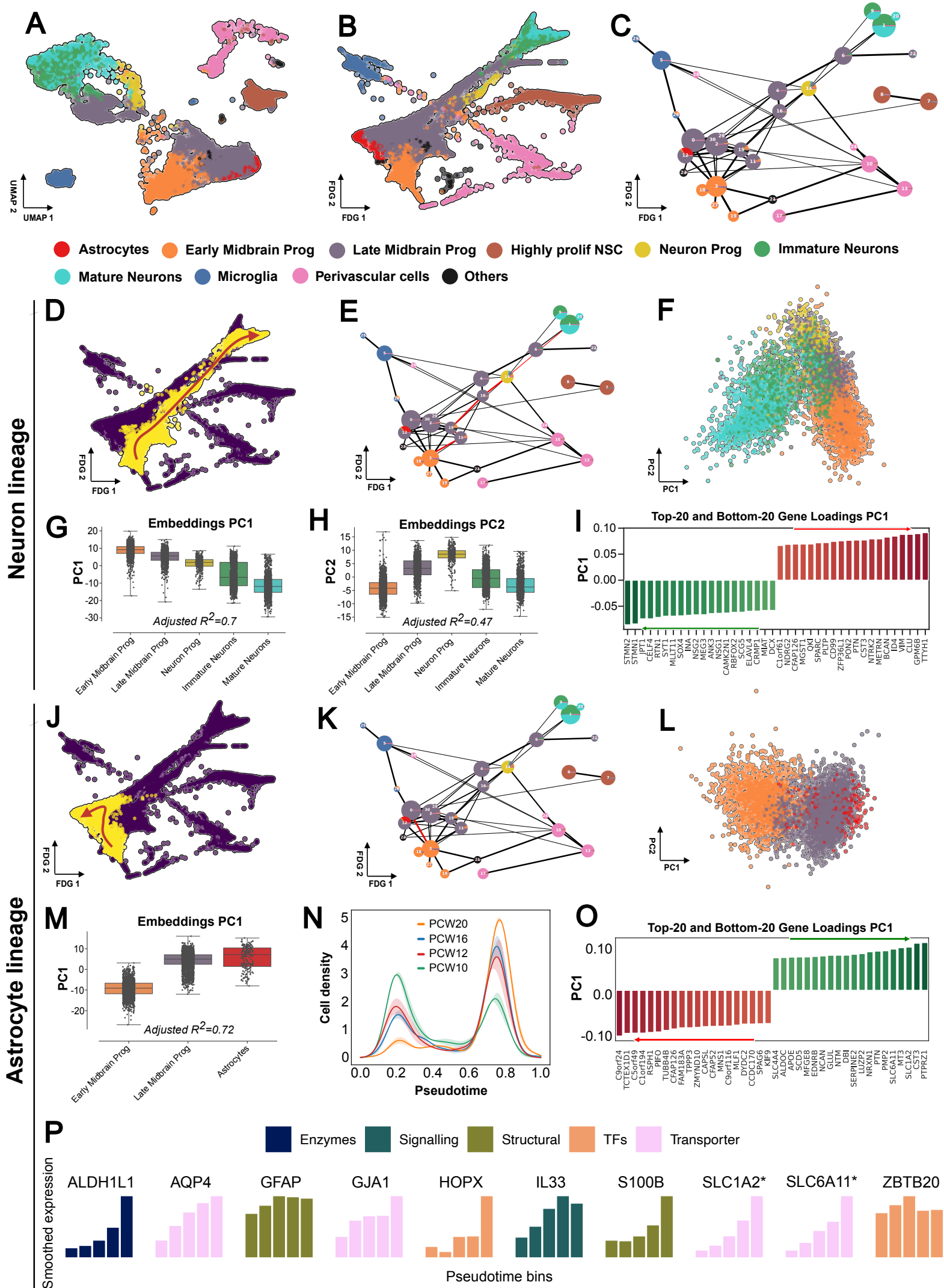
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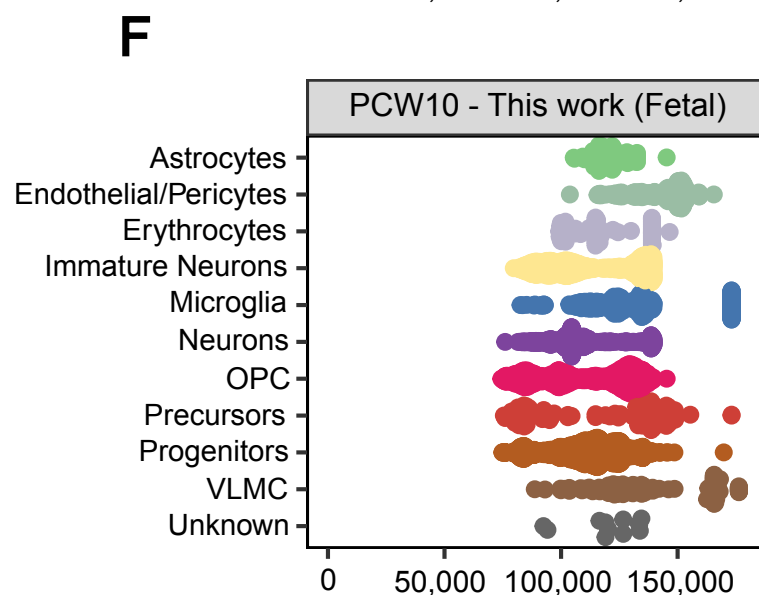
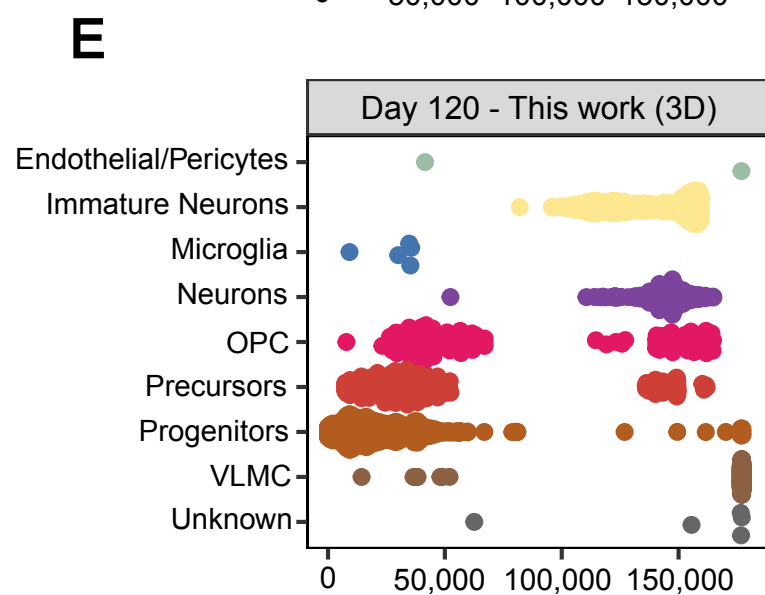
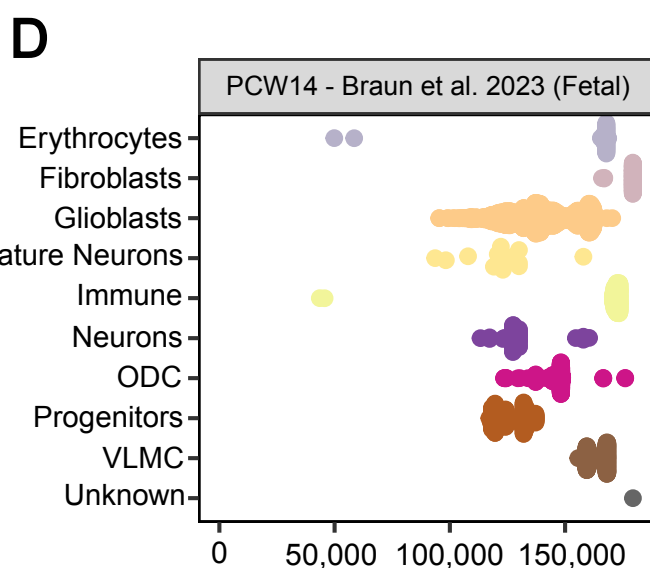
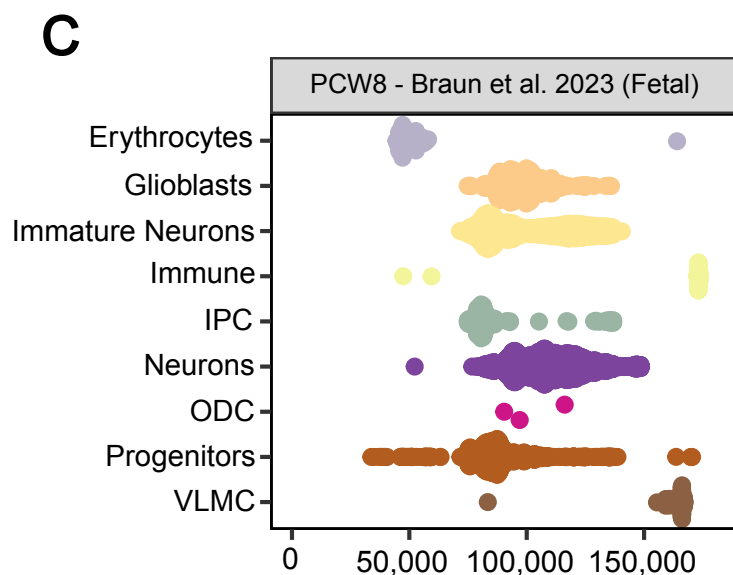
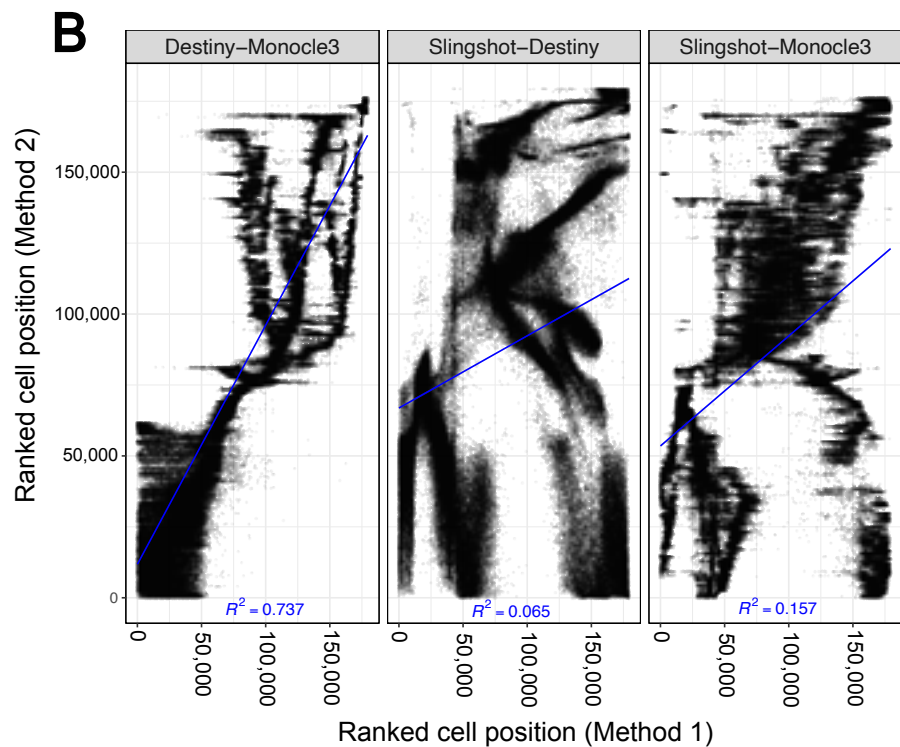
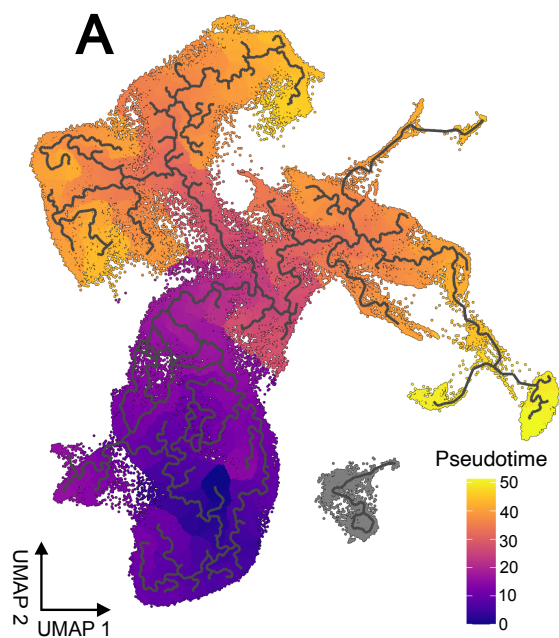


Supplementary Fig 10 Single cell RNA-seq profiling of in vitro and in vivo fetal samples. **A**, Dataset overview (this study) showing donor/model combinations for fetal (left) and in vitro (2D/3D, right) samples. Each column represents a donor/model with 2-9 replicates, totaling 1,346-9,394 cells. **B**, UMAP visualizations by high-resolution cell type annotation, with each facet showing one cell type: astrocytes (Astro), erythrocytes (Eryth), dopaminergic neurons (hDA1a, hDA1b, hDA2), mixed group of dopaminergic, GABAergic-related and serotonergic neurons (hDA3/hGABA/hSer), endothelial cells (hEndo), microglia (hMgl), midbrain precursors (hMidPre), dopaminergic neuroblasts (hNbDA), GABAergic neuroblasts (hNbGaba), neuronal progenitors (hNPro), pericytes (hPeric), dopaminergic precursors (hPreDA), progenitors medial floorplate (hProgFPM), progenitor midline (hProgM), radial glia 1 (hRgl1), radial glia 2 / immature astrocytes (hRgl2/immAstro), radial glia 3 (hRgl3caudal), radial glia 4/ependymal cells (hRgl4/MultiEpend), oligodendrocytes progenitor cells (OPC1, OPC2), vascular leptomeningeal cells (VLMC) and unknown cells (Unk). **C**, Upregulation of synaptic-related pathways in fetal vs 3D hDA2 cells. Only the top-10 upregulated biological processes are shown (gene ontology enrichment parameters: $pval < 0.05$, $minSize = 25$, $maxSize = 500$, $minCount = 10$). **D**, Downregulation of transcription and translation-related pathways in fetal vs 3D hDA2 cells. Only the top-10 downregulated biological processes are shown (gene ontology enrichment parameters: $pval < 0.05$, $minSize = 25$, $maxSize = 500$, $minCount = 10$). **E**, Differential abundance analysis between early and late fetal cell types. Microglia is enriched in second trimester samples, while immature neurons (hNbGaba, hNbDA) and dopaminergic neurons (hDA2, hDA1b) are more abundant in the first trimester. Some cell types (ie, OPC1, hRgl2/immAstro) show enrichment at both stages, reflecting transitional states. **F**, Immunofluorescence analysis of fetal midbrain at 19 and 22 PCW and MLOs at 70 and 120 days of differentiation for TH and the potassium channel GIRK2. Nuclei are staining with DAPI. Scale bar = 50 μm .



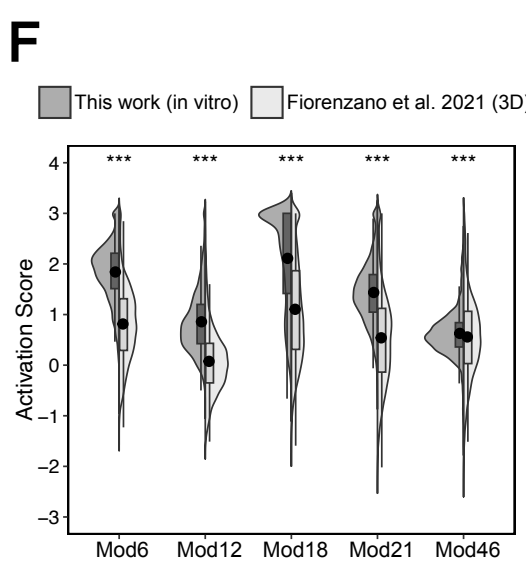
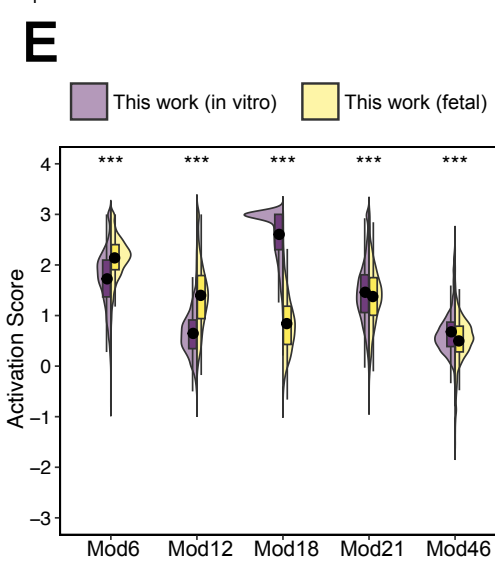
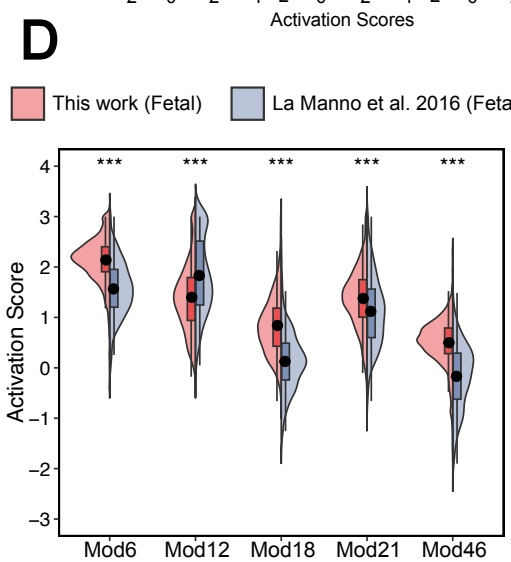
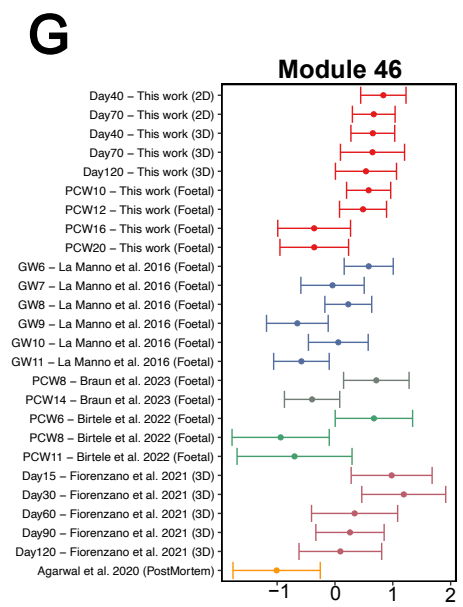
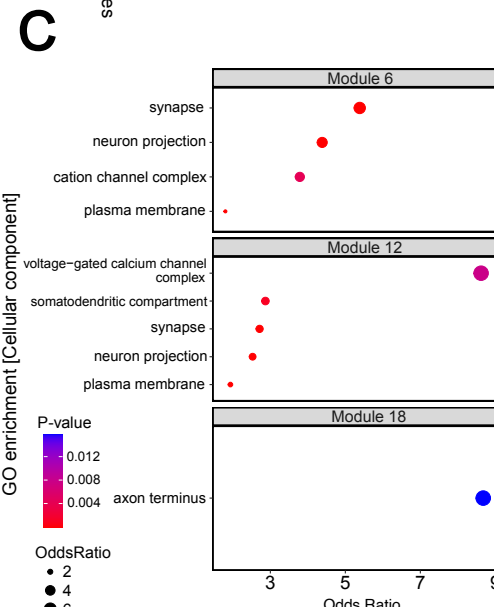
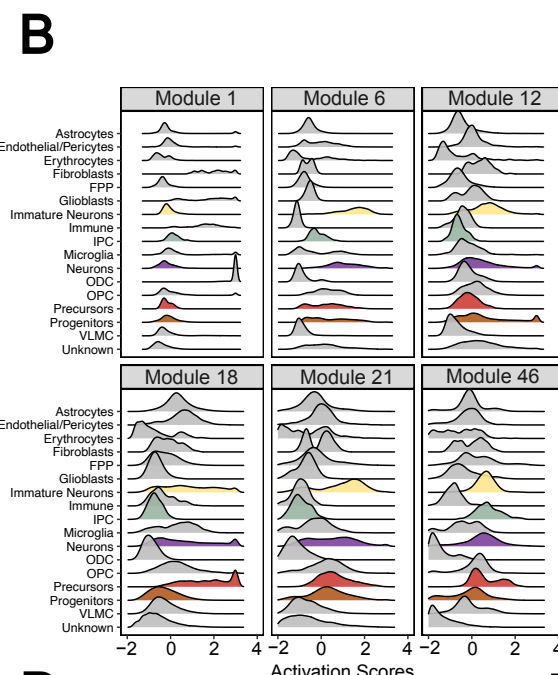
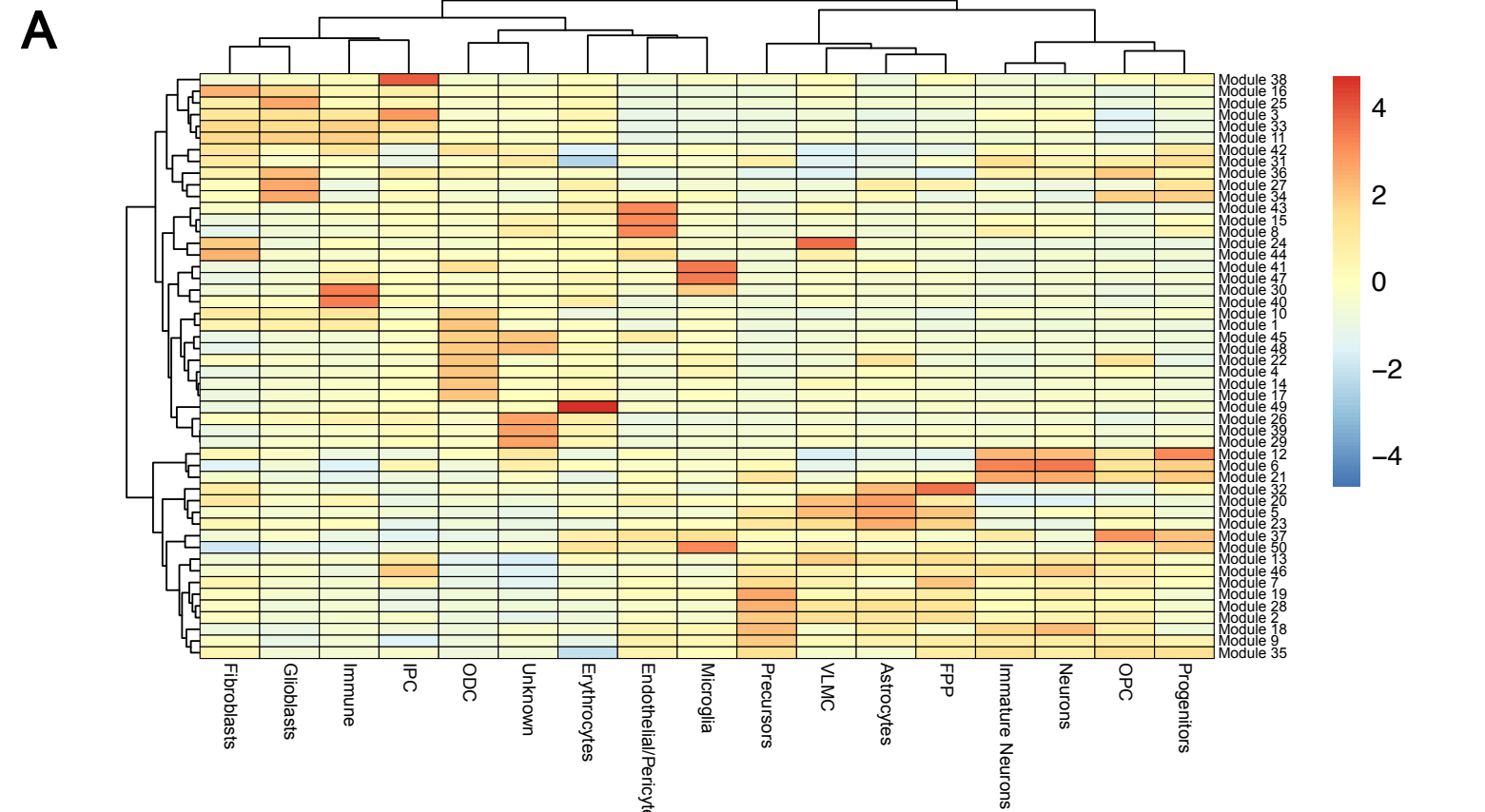
Supplementary Fig 11 Trajectory analysis of neuronal and astrocyte lineages.

A, UMAP visualization of fetal cells (PCW 10,12, 16, 20), coloured by low-resolution cell type annotation. **B**, Force-directed graph (FDG) representation of the same fetal cells. **C**, Partition-based graph abstraction (PAGA) showing the connectivity between Leiden clusters (resolution=1), with low-weight ($w < 0.05$) edges pruned. **D-E**, Inferred neuronal trajectory with cells highlighted in yellow and pseudotime direction indicated with the red arrow (d), built from the shortest path connectivity (e) between the starting point (cluster 3, early midbrain progenitors) and the ending point (cluster 20, mature neurons). Path indicated with red edges. **F**, Principal component analysis (PCA) of the neuronal lineage cells, coloured by cell type. **G-H**, Correlation between PC1 (g) or PC2 (h) variance with the annotated neuronal cell types (adjusted r -square, $p < 0.05$). Only PC1 captures the progression along the differentiation path. **I**, Top-20 and bottom-20 PC1 gene loadings, showing gene expression correlation with neuronal maturation (in green) or with progenitor-like state (in red). **J-K**, Inferred astrocyte trajectory with cells highlighted in yellow and pseudotime direction indicated with the red arrow (j), built from the path connectivity (k) between the starting point (cluster 3, early midbrain progenitors), the intermediate point (cluster 0, late midbrain progenitors) and the ending point (cluster 12, astrocytes). Path indicated with red edges. **L**, PCA of the astrocyte lineage cells, coloured by cell type. **M**, PC1 captures the progression along the astrocyte differentiation (adjusted R^2 correlation, $p < 0.05$). **N**, Pseudotime density across fetal samples: solid lines show the replicate means ($n=2$); shaded area indicates to ± 1 SD among replicate densities. **O**, Top-20 and bottom-20 PC1 gene loadings, showing gene expression correlation with astrocyte maturation (in green). **P**, Smoothed expression profiles of a curated set of marker genes tracking the astrocyte lineage, across five evenly spaced pseudotime bins. Asterisks indicate significant start-to-end gene expression changes, as evaluated per tradeSeq. The color scheme of the 10 cell types (from the low-clustering resolution) is shared among panels **A-C**, **E-H** and **K-M**.



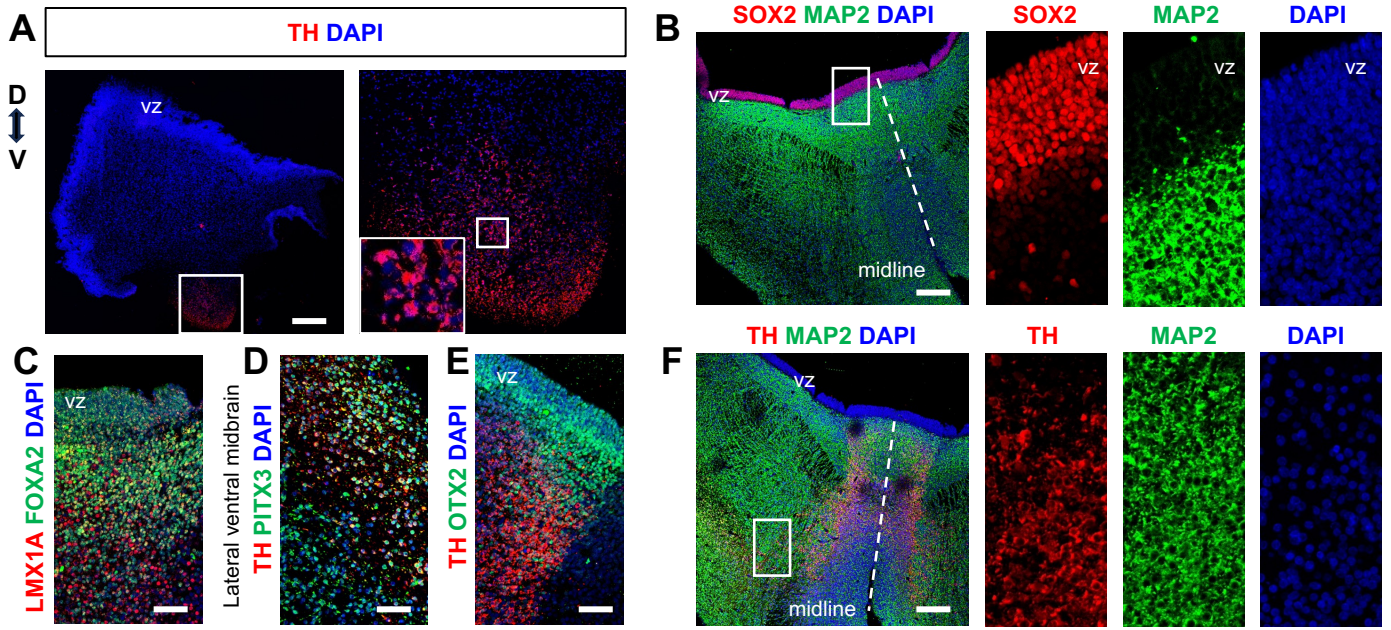
Ranked cell position (Monocle3)

Supplementary Fig 12. Meta-integration of multiple dopaminergic scRNA-seq datasets and pseudotemporal dynamics analysis. **A**, Pseudotime trajectory inferred by Monocle3 on the UMAP projection. The gradient ranges from 0 (dark blue, rooted at floor plate progenitors, FPP) to 50 (yellow, corresponding to oligodendrocytes), capturing the trajectory of cells undergoing dopaminergic differentiation. **B**, Comparison of pseudotime trajectories: pairwise correlation of ranked cell position inferred by Monocle3, Slingshot and Destiny. Only Monocle3 and Destiny show a high degree of agreement ($R^2=0.737$). **C-F**, Violin scatter plots depicting Monocle3-inferred pseudotime distribution per cell type, based on the unified annotation for the integration. Panels correspond to post-conceptional week 8 (c) and 14 (d) from Braun et al. 2023; or to the 3D-MLO in vitro model (e) and the fetal post-conceptional week 10 (f) from this study. The x axis reflects the ranked cell position across the integrated dataset, used here as a pseudotime proxy.

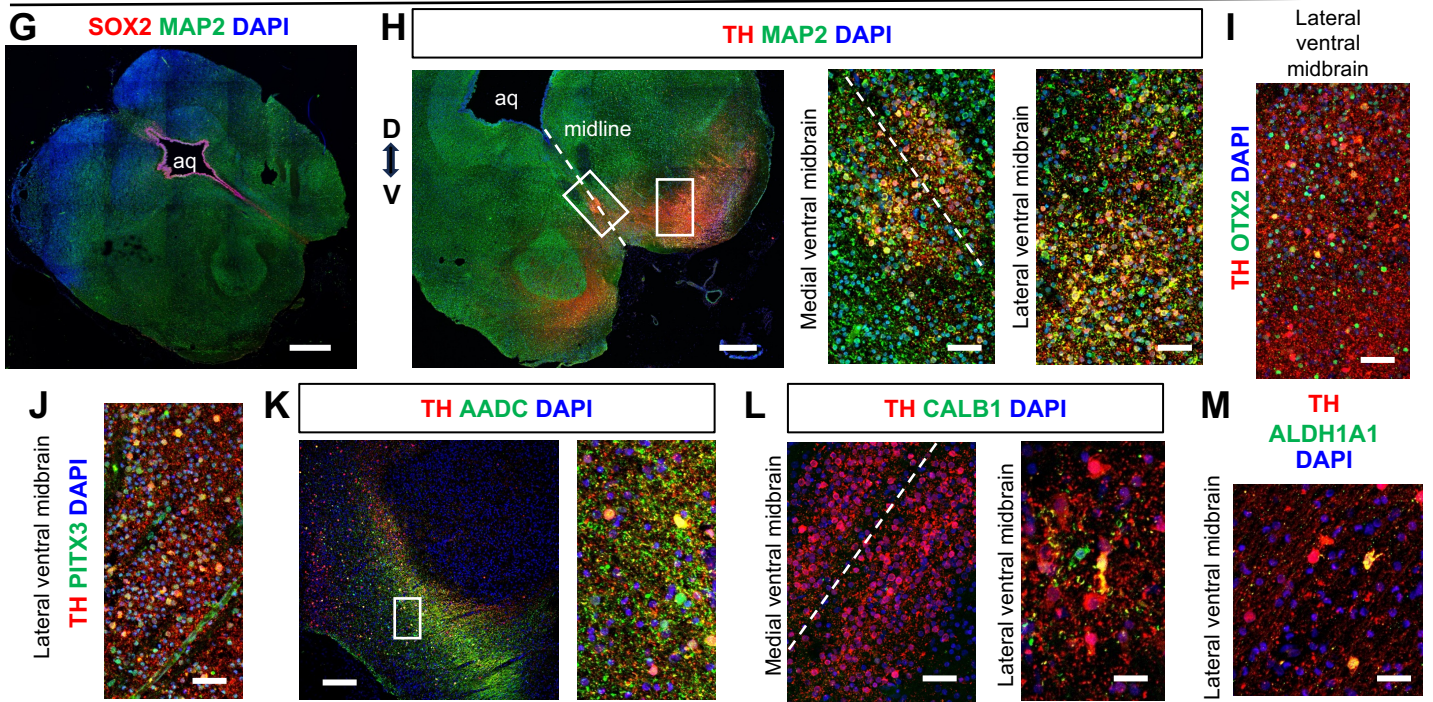


Supplementary Fig 13. Modules of co-regulated genes that change as a function of pseudotime. **A**, Heatmap displaying the activation scores of pseudotime-dependent gene modules across cell types. Each tile represents the average activation score (aggregated module scores) for each cell type (unified annotation in the columns). The inferred gene modules (rows, n=50) include groups of trajectory-variable genes that are co-regulated. **B**, Ridge density plot showing the activation scores across cell types for the ODC-specific module (Module 1) and the top-enriched modules in neurons (Modules 6, 12, 18, 21 and 46). Neuron-enriched modules are typically also activated in immature neurons and other cell types, such as progenitors (Modules 6 and 12), precursors (Module 18), and intermediate progenitor cells (Module 46). **C**, Gene ontology enrichment analysis for neuron-enriched modules (adjusted $p < 0.05$). Module 6 is enriched in synapse function, module 12 in voltage-gated calcium channel complexes, and module 18 in axonal structure. Modules 21 and 46 showed no significant enrichment in gene ontology (GO) or KEGG pathways. Odds ratios were calculated based on term and query sizes, effective domain size and intersection size. **D-F**, Split violin plots comparing the activation scores for five neuron-enriched modules across datasets: fetal cells from this study vs. La Manno et al. 2016 (**D**); *in vitro* cells vs. fetal cells both from this study (**E**); and *in vitro* cells from this study vs. the 3D-dopaminergic model from Fiorenzano et al. 2021 (**F**), respectively. Significance level: $p < 10^{-3}$ (***, One-way permutation test). **G**, Activation score distribution for the neuron-enriched module 46 across timepoints (rows) and datasets (colors). Mean values and standard deviation error bars shown for each timepoint combination.

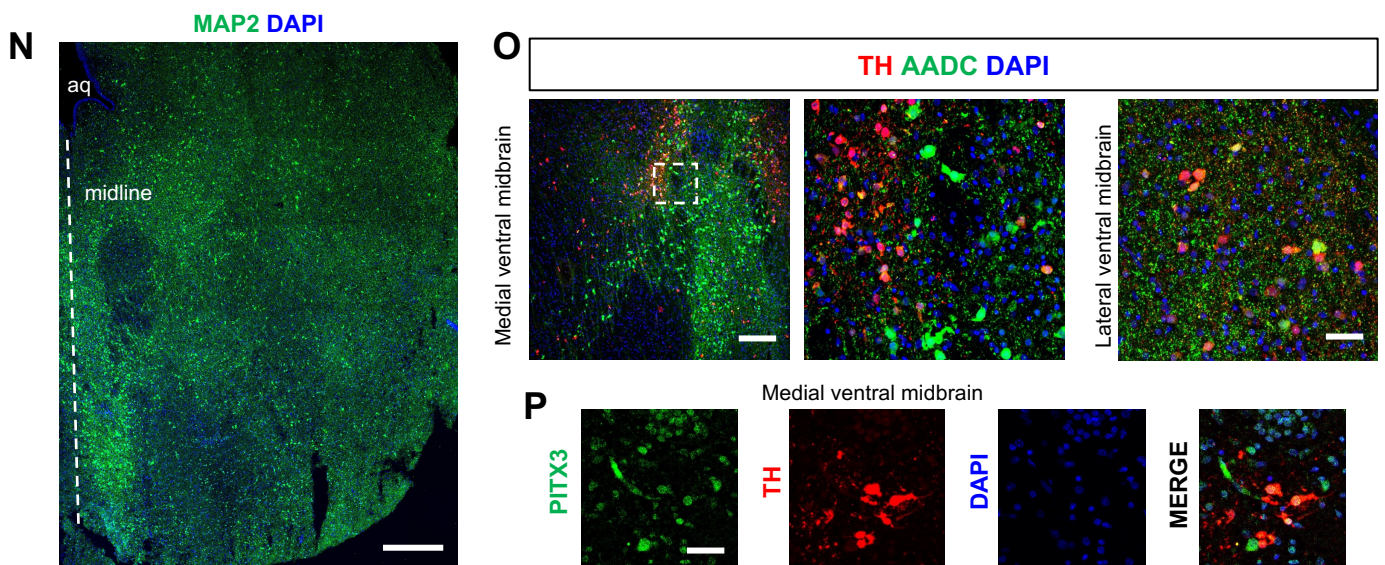
7 PCW



11 PCW

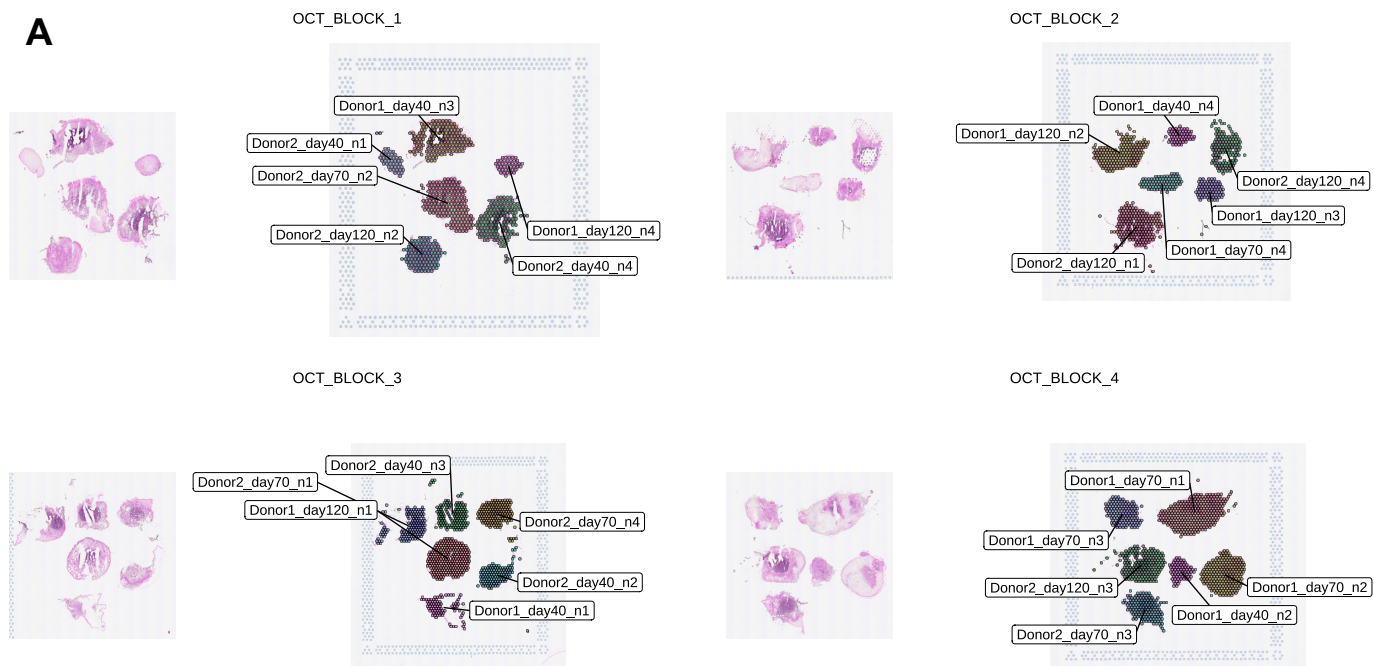


17 PCW

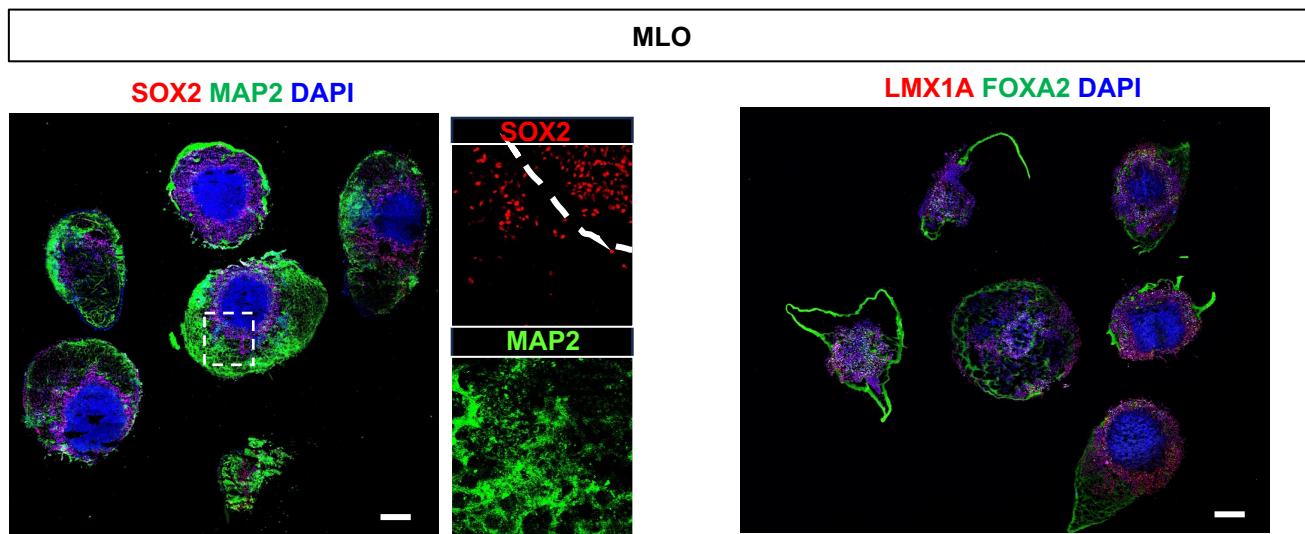


Supplementary Fig 14 Midbrain fetal systems. A-F Immunofluorescence images of 7 PCW fetal midbrain showing positive cells for TH (sample used in spatial analysis) (scale bar = 300 μ m), SOX2 and MAP2 (scale bar = 200 μ m), LMX1A and FOXA2 (scale bar = 50 μ m), TH and PITX3 (scale bar = 50 μ m), TH and OTX2 (scale bar = 50 μ m), TH and MAP2 (scale bar = 200 μ m). **G-M**, Immunofluorescence analysis of 11 PCW fetal midbrain for SOX2 and MAP2 (scale bar = 500 μ m), TH and MAP2 (scale bar = 300 μ m – *left panel*; scale bar = 100 μ m – *right panel*), TH and OTX2 (scale bar = 50 μ m), TH and PITX3 (scale bar = 50 μ m), TH and AADC (scale bar = 200 μ m), TH and CALB1 (scale bar = 100 μ m – *left panel*; scale bar = 20 μ m – *right panel*), TH and ALDH1A1 (scale bar = 20 μ m). **O-P**, Representative immunofluorescence images of the 17 PCW human midbrain showing expression of MAP2 (scale bar = 500 μ m), TH and AADC and TH (scale bar = 100 μ m – *left panel*; scale bar = 50 μ m – *right panel*) and PITX3 (scale bar = 30 μ m) Nuclei are staining with DAPI. Ventricular zone (VZ), aqueduct (aq).

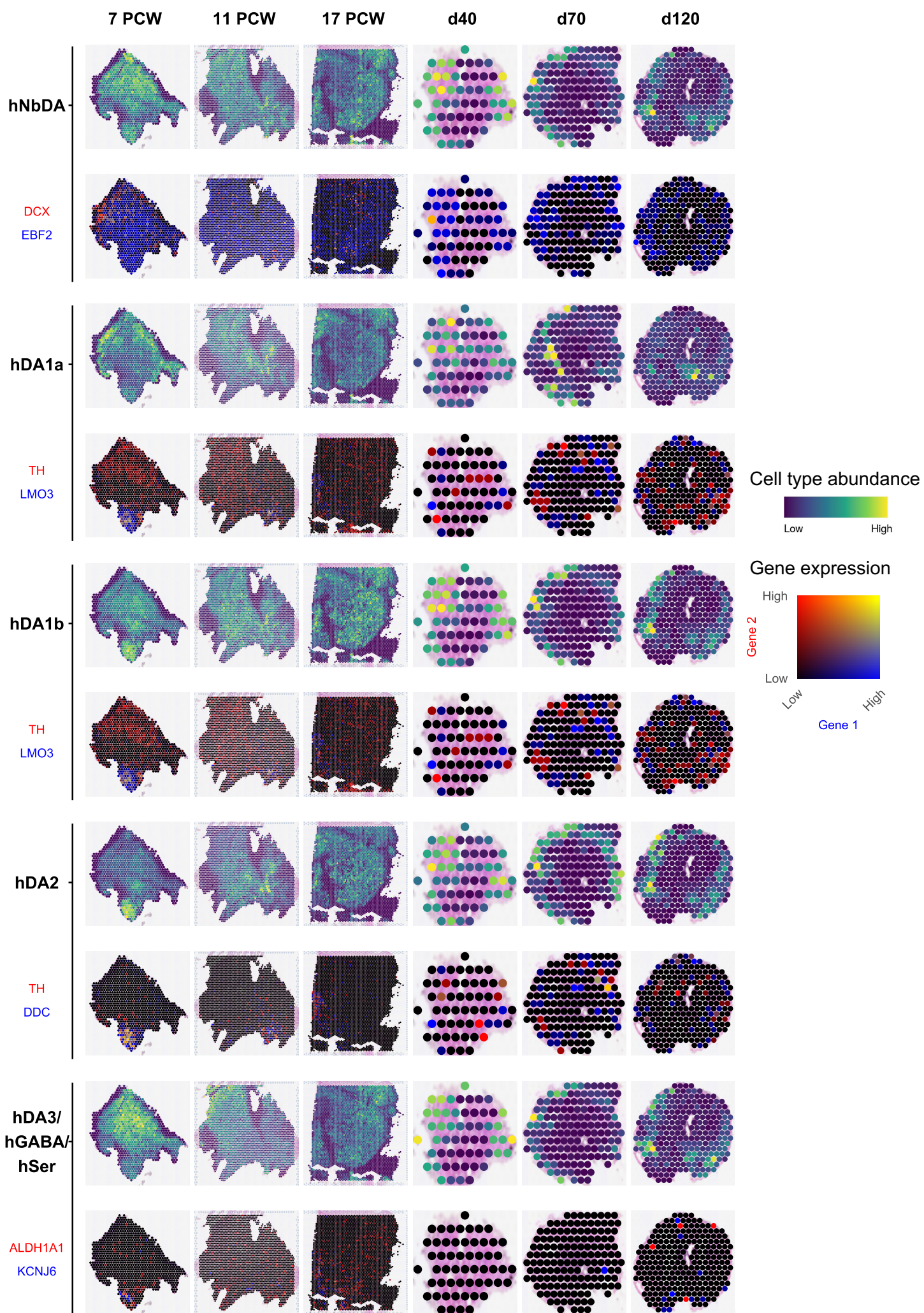
A



B

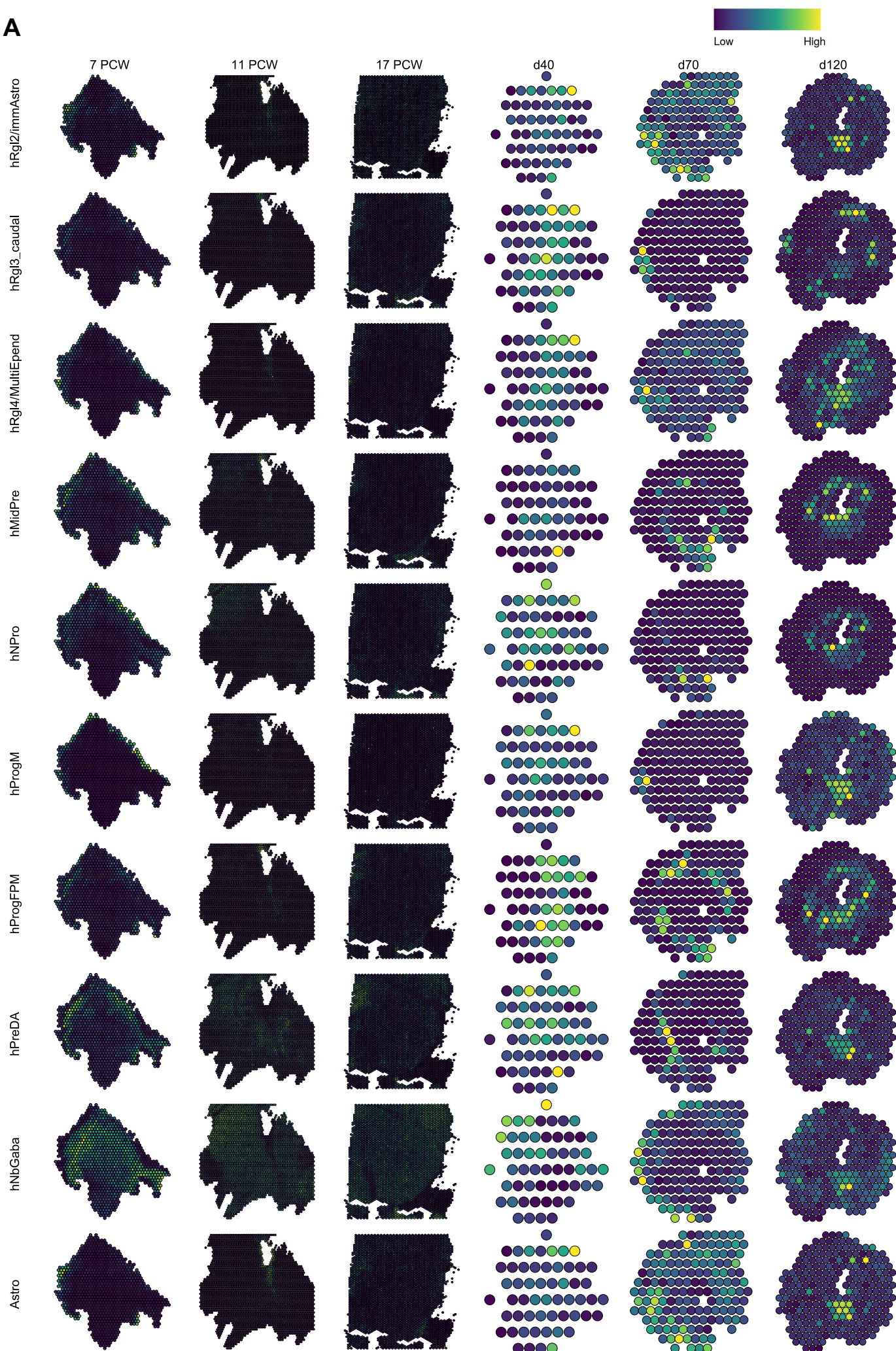


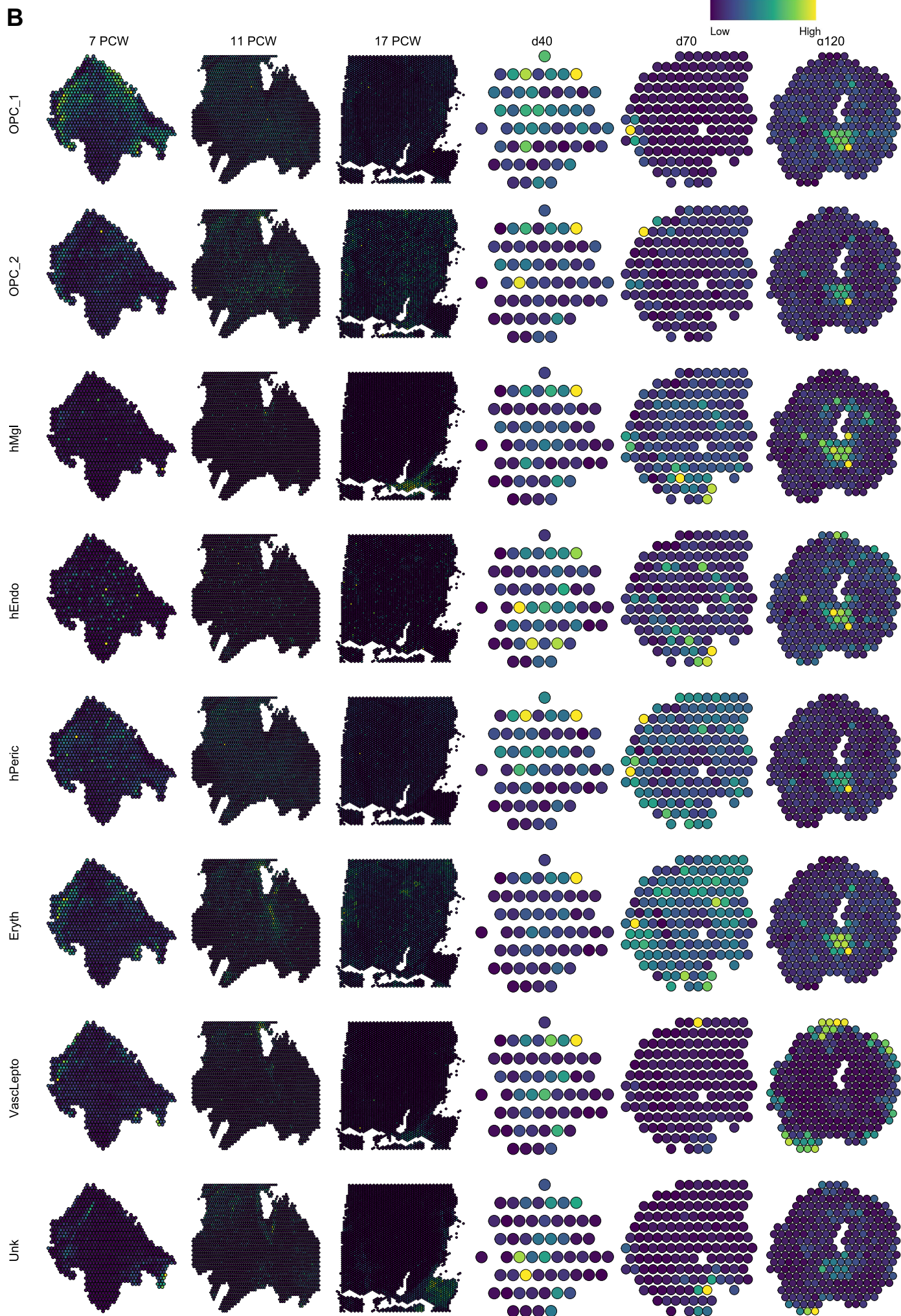
Supplementary Fig 15 MLO systems. **A**, Arrangement of MLOs in Visium capture areas. **B**, Immunofluorescence analysis for SOX2, MAP2 (*left panel*), and LMX1A and FOXA2 (*right panel*) in MLO samples analyzed with spatial transcriptomic. Nuclei are stained with DAPI. Scale bars = 500 μ m.



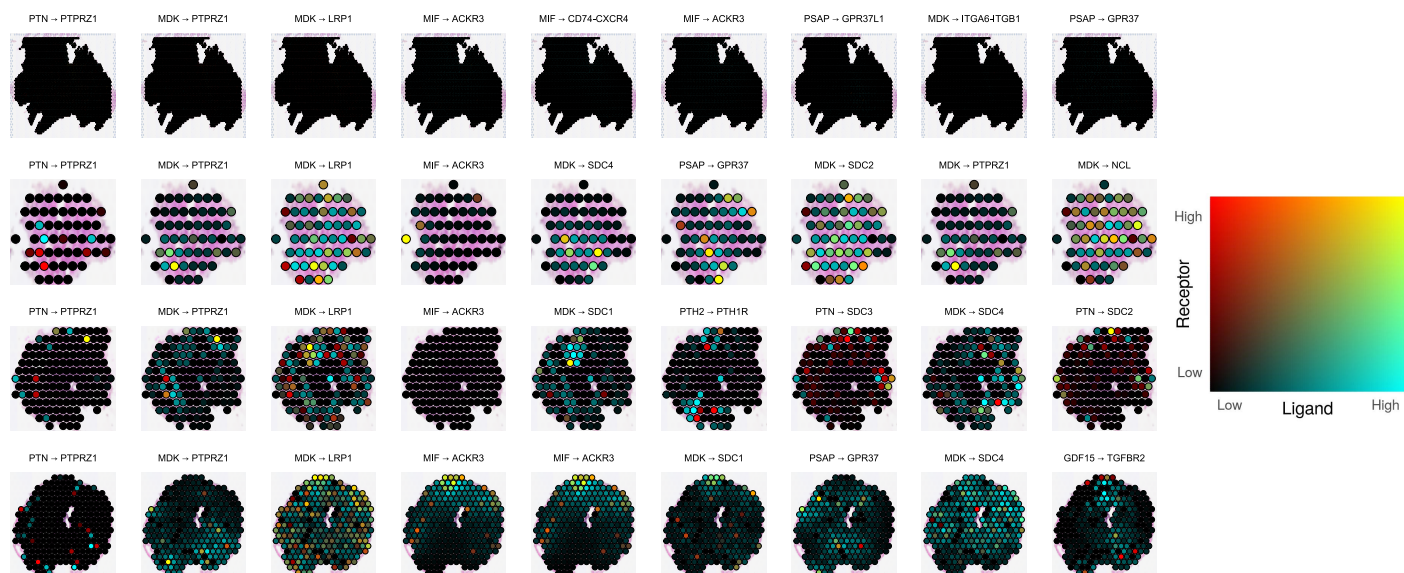
Supplementary Fig 16 Distribution of dopaminergic populations in fetal tissue and MLOs. Abundance of dopaminergic lineage cells in tissues and organoids, as estimated by the Cell2Location deconvolution algorithm. Expression of two marker genes for each cell type are displayed below.

A

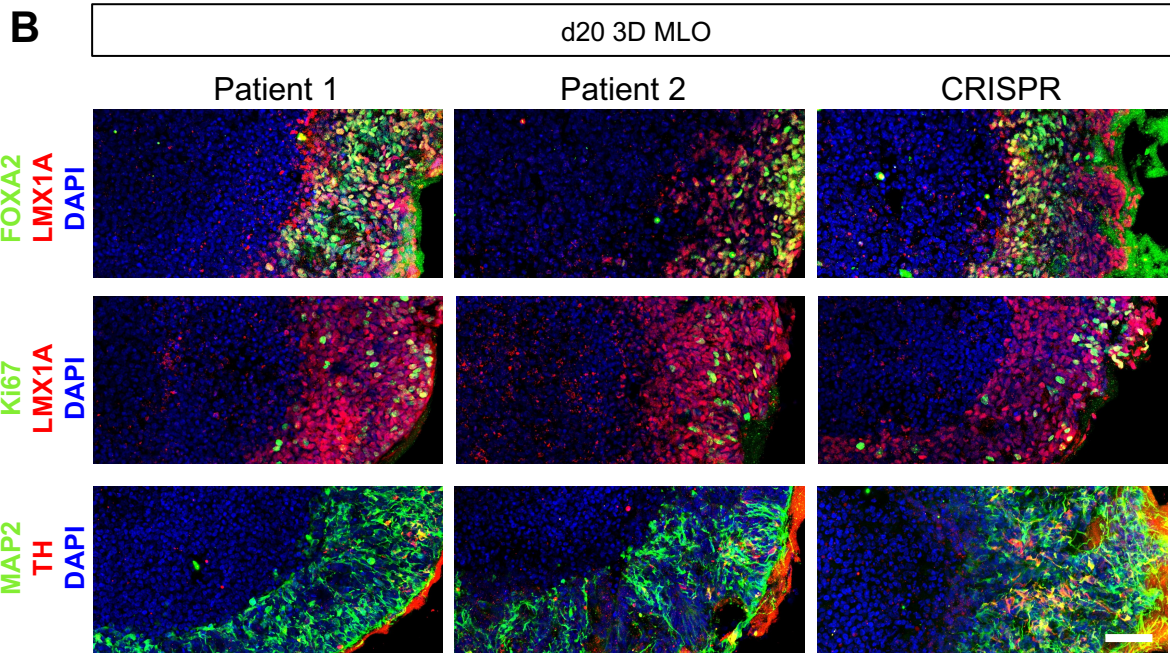
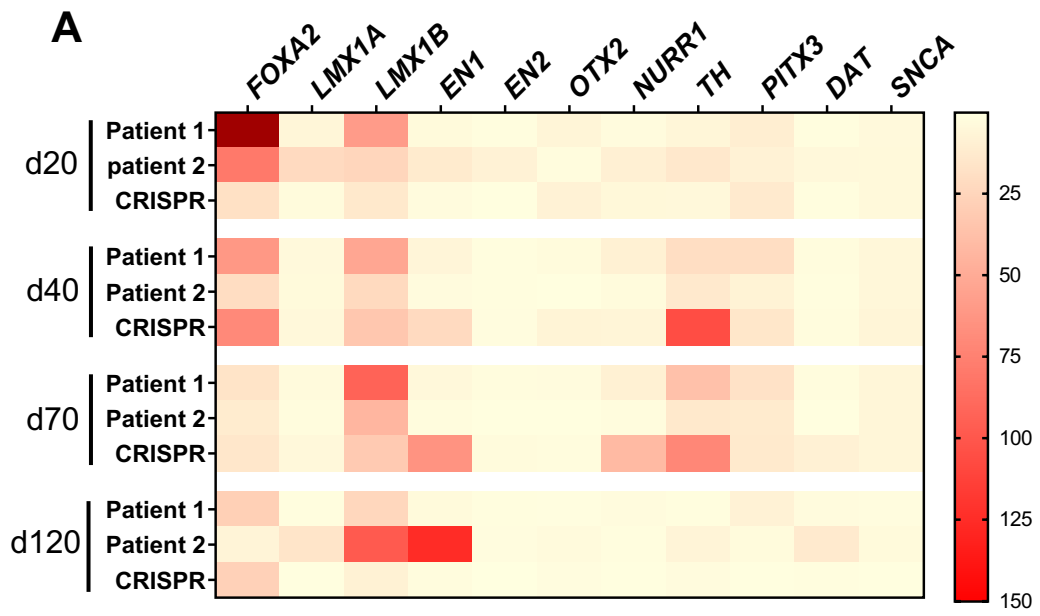




Supplementary Fig 17 Distribution of cell types in fetal tissue and MLOs. A,B, Abundance of neuronal and non-neuronal cells in tissues (left three columns) and organoids (right three columns), estimated by the Cell2Location deconvolution algorithm.



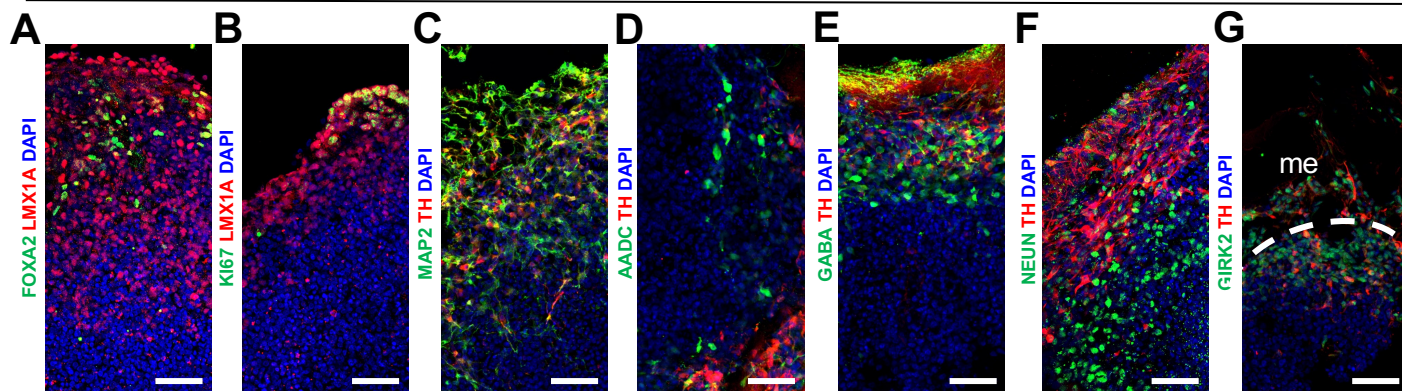
Supplementary Fig 18 Distribution of cell-cell communication pathways in fetal tissue and MLOs. A, Expression of pathways at each spot in 11 PCW fetal midbrain and MLOs at 40, 70 and 120 days of differentiation. Computed with COMMOT. Pathways selected according to spatial autocorrelation as determined by the Moran's I non-parametric test.



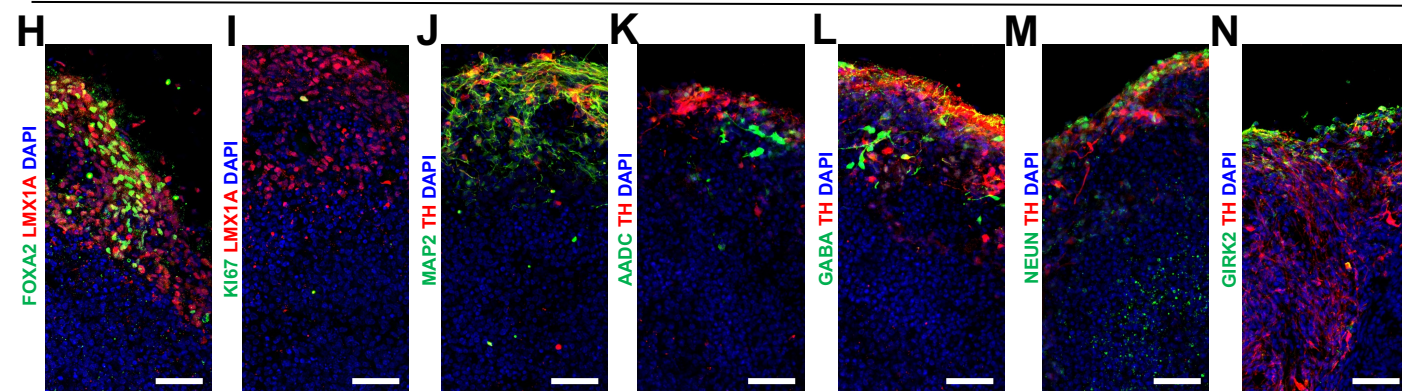
Supplementary Fig 19 Characterization of DTDS derived MLOs. **A**, qRT-PCR for midbrain related markers *FOXA2*, *LMX1A*, *LMX1B*, *EN1*, *EN2*, *OTX2*, *NURR1*, *PITX3*, *DAT*, *SNCA* relative to housekeeping gene (GAPDH) and normalized to their respective iPSCs (n=3-4 for each line D20-40-70, n=1 for each line D120). **B**, Immunofluorescence analysis for midbrain and neuronal-related proteins FOXA2, LMX1, MAP2 and TH, and Ki67 in Patient 1, Patient 2 and CRISPR corrected isogenic line-derived MLO at 20 days of differentiation. Nuclei are staining with DAPI. Scale bar = 50 μ m. Source data are provided as a Source Data file.

d40 3D MLO

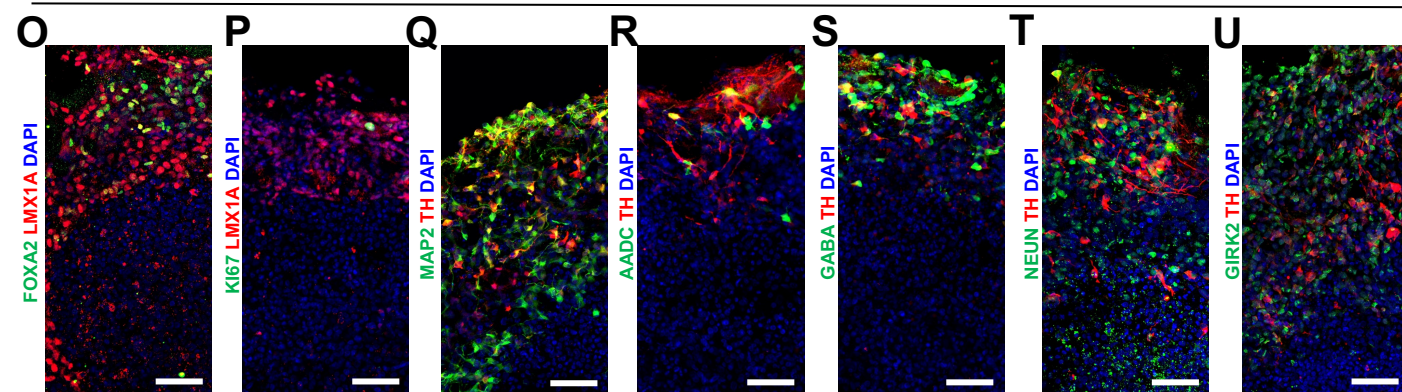
Patient 1



Patient 2



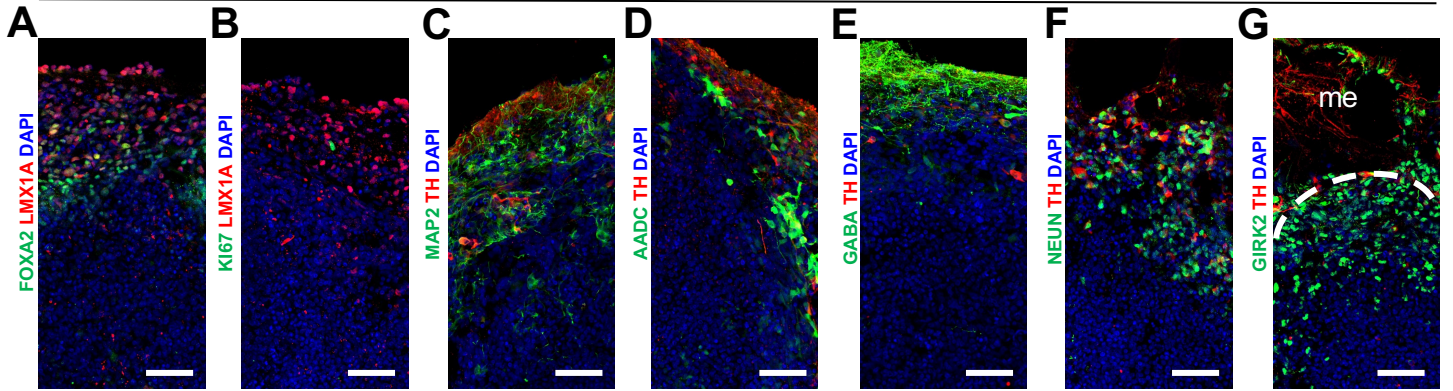
CRISPR



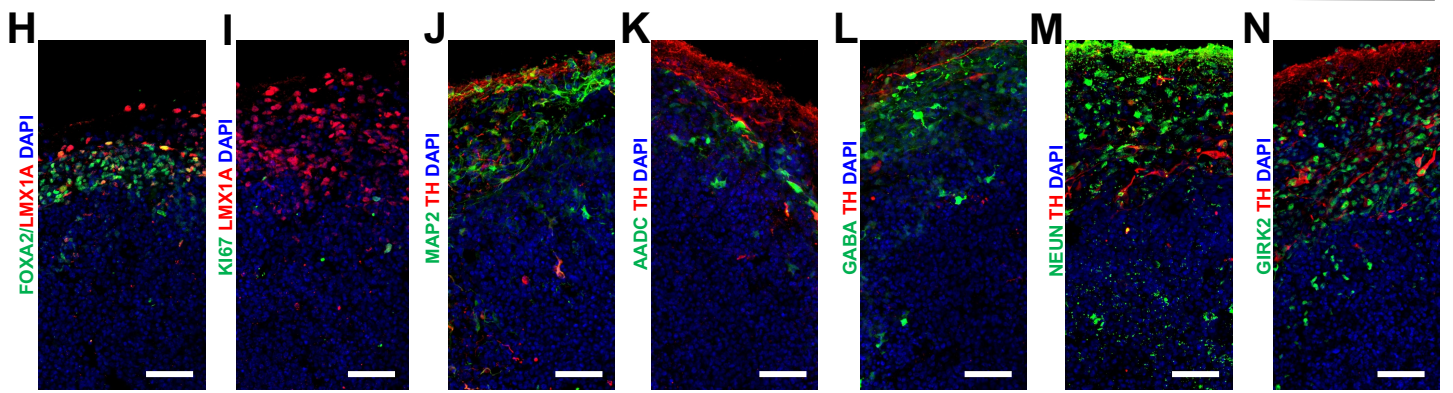
Supplementary Fig 20 Maturation of mDA neurons in DTDS-derived MLOs at 40 days of differentiation. A-U, Representative immunofluorescence images of Patient 1, Patient 2 and CRISPR isogenic lines showing expression of midbrain and neuronal-related proteins FOXA2, LMX1A, MAP2, TH, AADC, GABA, NEUN and GIRK2 and the proliferative marker Ki67. Nuclei are staining with DAPI. Scale bars = 50 μ m. Matrigel embedding (me).

d70 3D MLO

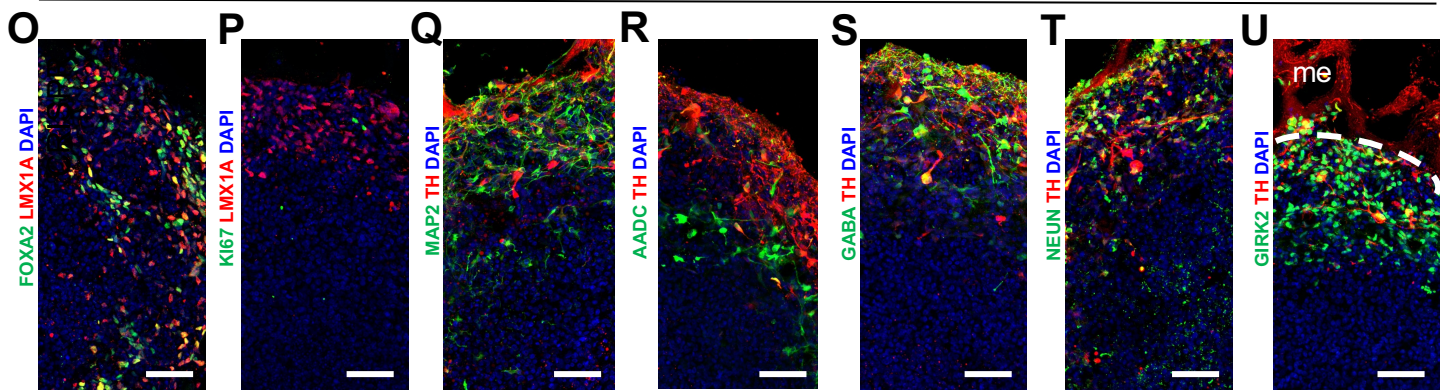
Patient 1



Patient 2



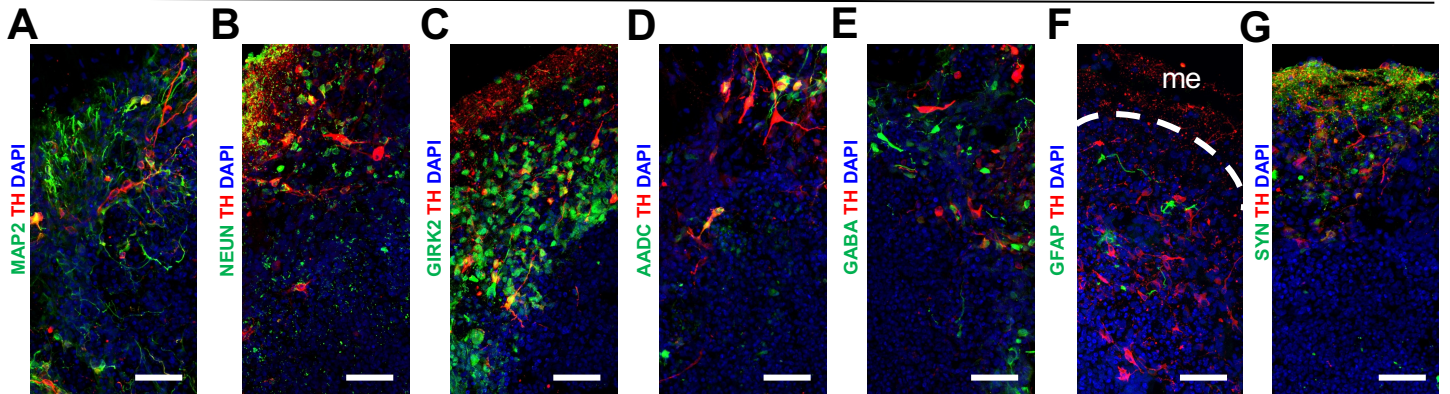
CRISPR



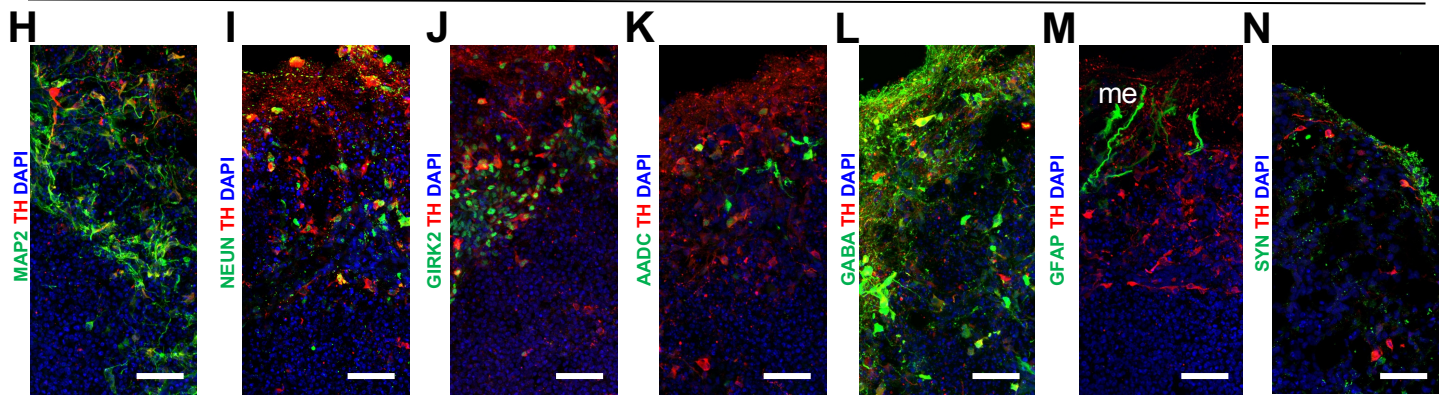
Supplementary Fig 21 Maturation of mDA neurons in DTDS-derived MLOs at 70 days of differentiation. A-U, Representative immunofluorescence images of Patient 1, Patient 2 and CRISPR isogenic lines showing expression of midbrain and neuronal-related proteins FOXA2, LMX1A, MAP2, TH, AADC, GABA, NEUN and GIRK2 and the proliferative marker Ki67. Nuclei are staining with DAPI. Scale bars = 50 μ m. Matrigel embedding (me).

d120 3D MLO

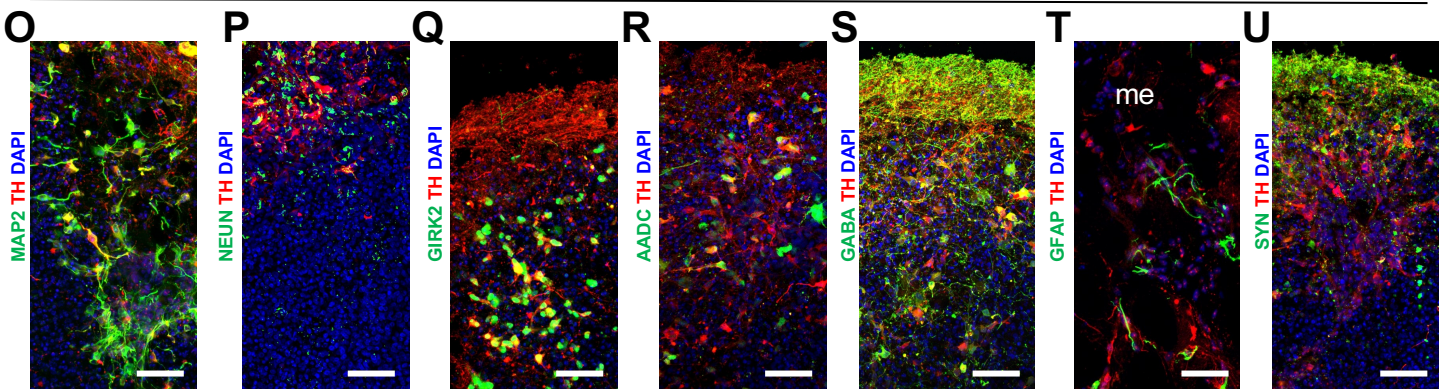
Patient 1



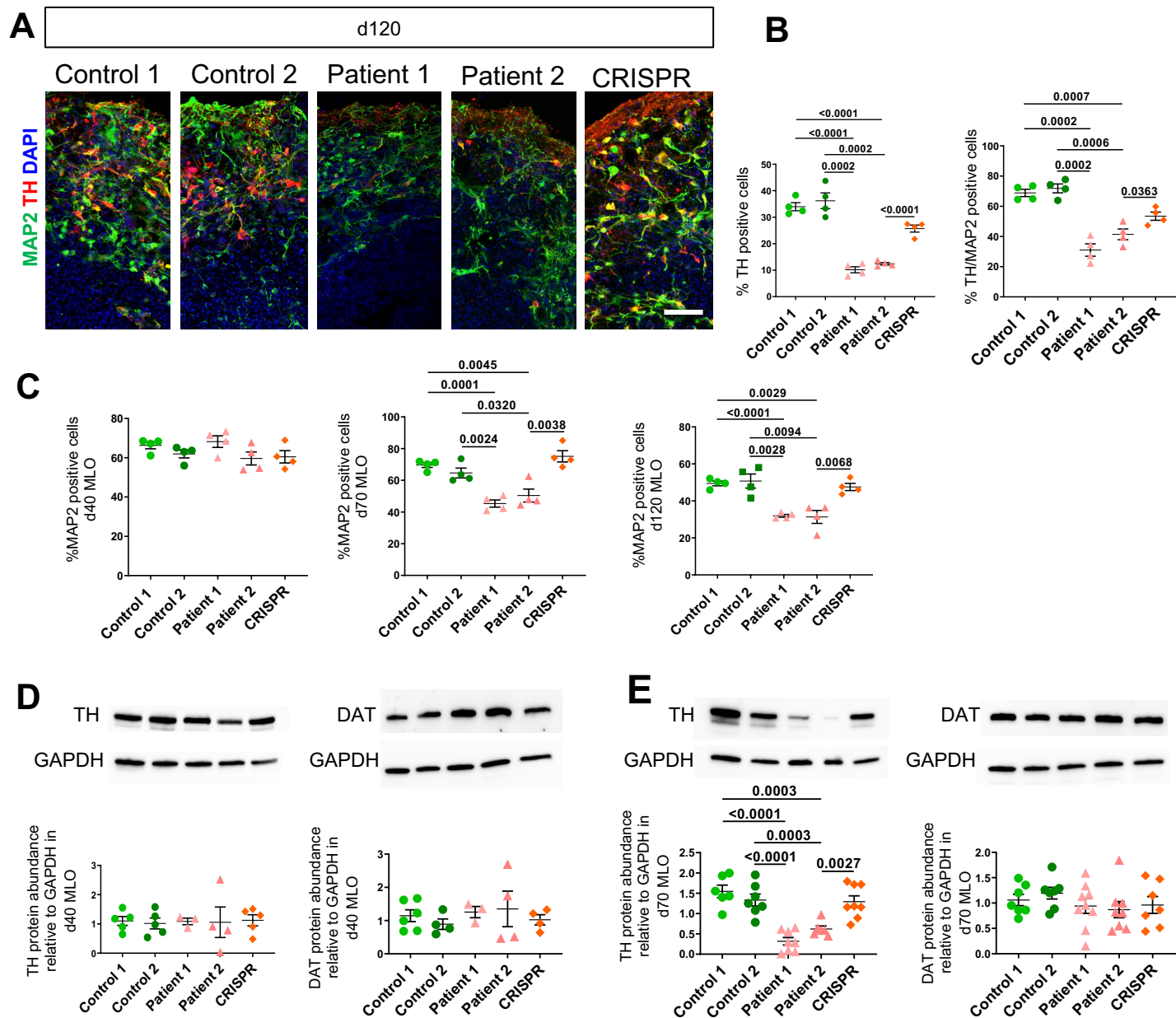
Patient 2



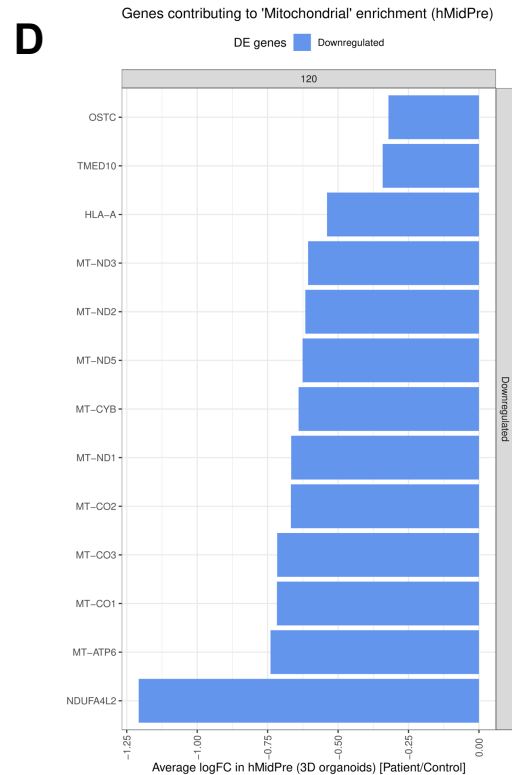
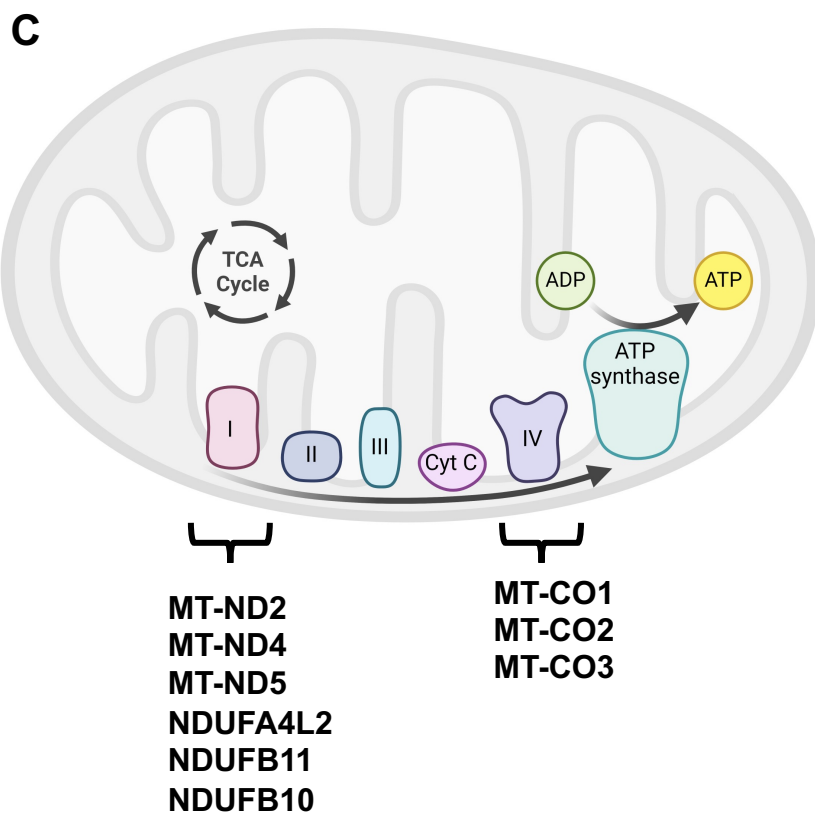
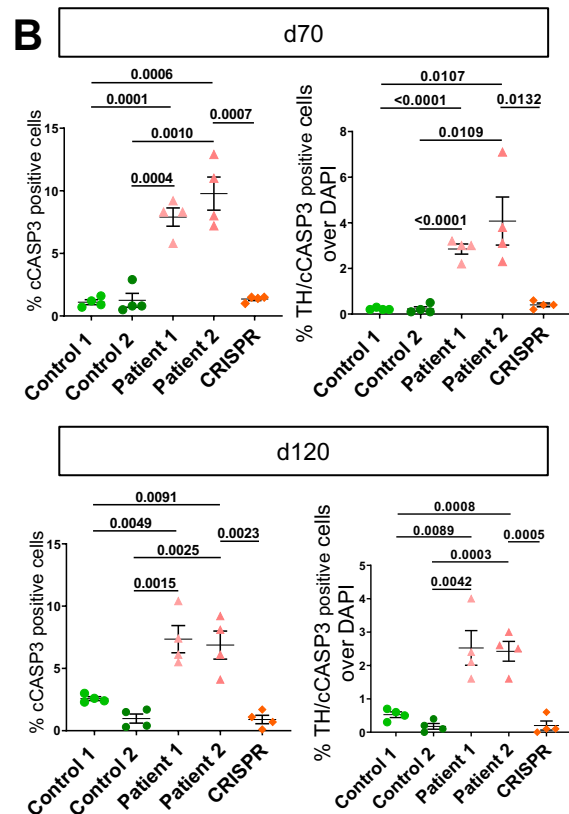
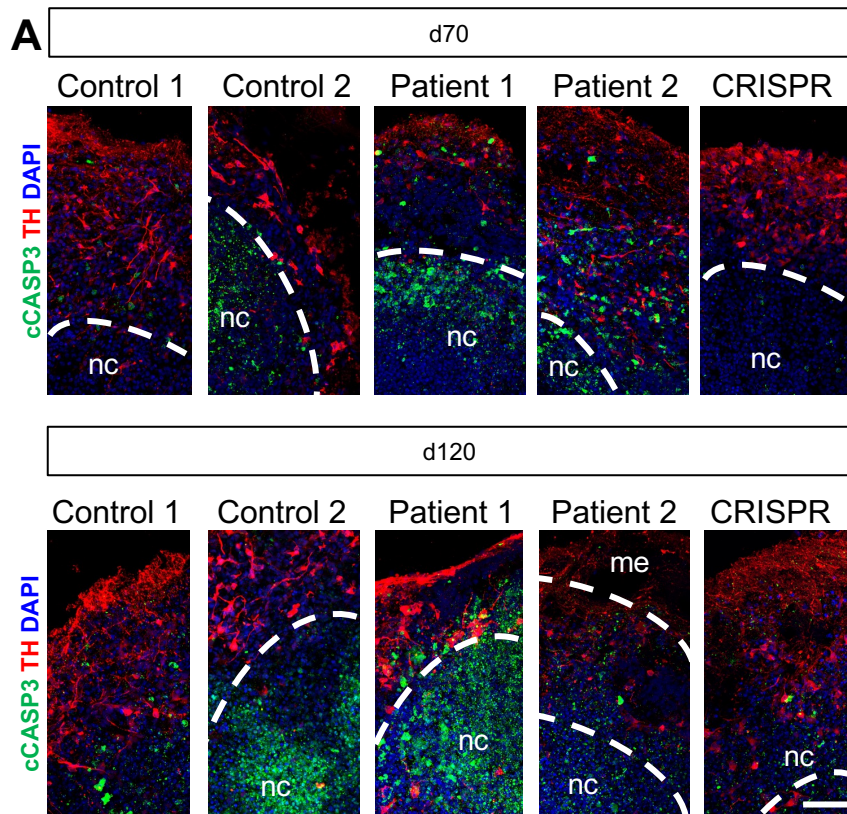
CRISPR



Supplementary Fig 22 Maturation of mDA neurons in DTDS-derived MLOs at 70 days of differentiation. A-U, Representative immunofluorescence images of Patient 1, Patient 2 and CRISPR isogenic lines showing expression of mDA neuron-related proteins TH, AADC and GIRK2, mature neuronal makers MAP2, NEUN, SYP, neural cells GABA positive cells and GFAP positive glial cells. Nuclei are staining with DAPI. Scale bars = 50 μ m. Necrotic core (nc), Matrigel embedding (me).



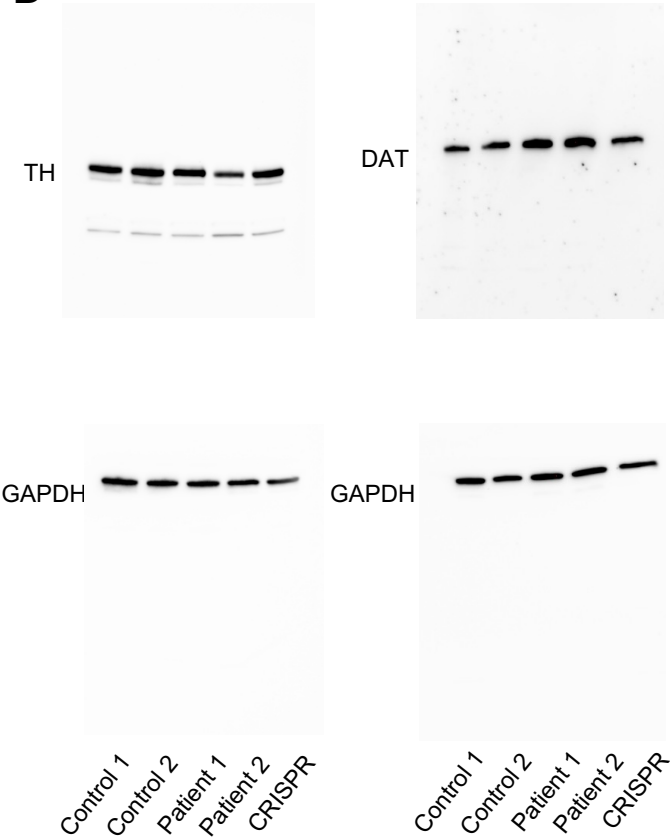
Supplementary Fig 23 DTDS MLOs show loss of mDA neurons and reduced TH protein content. **A**, Immunofluorescence analysis of control and DTDS patient-derived MLOs for TH and MAP2 at 120 days of differentiation. Nuclei are staining with DAPI. Scale bar = 50 μ m. **B**, Quantification of TH positive and TH/MAP2 double positive neurons for patients and controls lines at 120 days of differentiation. **C**, Quantification of MAP2 total positive cells in DTDS control and patient-derived MLOs at 40, 70 and 120 days of differentiation. **D-E**, Western blot analysis for TH and DAT and relative protein abundance to GAPDH in DTDS control and patient-derived MLOs at 40 and 70 days of differentiation respectively. Error bars indicate SEM. DTDS lines were independently compared to controls using two-tailed Student's *t*-test for all analyses. Statistically significant differences indicate comparison of Control 1 or 2 to Patient 1 or 2 and Patient 2 to CRISPR. Source data are provided as a Source Data file.



Supplementary Fig 24 DTDS MLOs show increased levels of cCASP3 positive cells. **A**, Immunofluorescence analysis of control and DTDS patient-derived MLOs for cleaved CASP3 (cCASP3) and TH at 40 and 70 days of differentiation. Nuclei are staining with DAPI. Positive cells located in the necrotic core (nc) were excluded from the quantification. Scale bar = 50 μ m. **B**, Quantification of total cCASP3 positive cells and TH/ cCASP3. Error bars indicate SEM. DTDS lines were independently compared to controls using two-tailed Student's *t*-test for all analyses. Statistically significant differences indicate comparison of Control 1 or 2 to Patient 1 or 2 and Patient 2 to CRISPR. Necrotic core (nc). **C**, Graphic representation of mitochondria genes dysregulated in DTDS mDA neurons and role in mitochondrial respiratory chain. Created in BioRender. BUDINGER, D. (2025) <https://BioRender.com/gddkq14>. **D**, Average LogFC for mitochondrial genes downregulated in patients MLOs compared to controls for midbrain precursors cell type (hMidPre). Matrigel embedding (me). Source data are provided as a Source Data file.

Uncropped western blot scans in supplementary figure 23

D



E

