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Modeling temporal dynamics of early embryogenesis and aging

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Abstract

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Throughout life, every individual undergoes a wide array of experiences, yet one constant remains: the passage of time across distinct life stages. In the prenatal phase, we develop from a single-celled zygote into a complex embryo through highly orchestrated cell fate decisions. After birth, our bodies continue to grow and learn, eventually entering a phase of gradual decline marked by aging. This aging process is characterized by increasingly disordered changes in cellular states, ultimately leading to dysfunction and cell death. While development and aging are often seen as opposite ends of the life spectrum, they share core biological principles: both involve dynamic gene expression programs, shifts in cellular identity, and remodeling of tissue architecture. A central question in both fields is: what genetic programs govern these transitions in cell states?

Despite significant progress, a systematic, single-cell resolution understanding of these genetic programs remains lacking. Two major challenges hinder such efforts: first, it is difficult to obtain continuous human data over time; second, sample conditions are often limited, restricting our ability to assess contributing factors to variation. However, advances in single-cell RNA sequencing (scRNA-seq) now allow us to profile millions of cells across finely resolved time courses. I hypothesize that applying scRNA-seq to in vitro models or closely-related model organisms offers a powerful approach to uncovering the temporal progression of cellular states. In this thesis, I present two projects that leverage scRNA-seq and computational analysis to address this question.

In the first project, we used scRNA-seq to study lineage specification in an in vitro model of early human embryogenesis known as gastruloids. We identified a retinoic acid signaling axis that is critical to early embryogenesis and improved the alignment between the in vitro model and the

human embryo. Ultimately, we demonstrated that this new enhanced model can be used to study genetic variation during early embryogenesis through large-scale perturbation experiments.

In the second project, we performed scRNA-seq on brain samples from rhesus macaques across the lifespan—from early infancy (5 months) to late adulthood (21 years). Through computational analysis, we constructed aging trajectories that capture changes in cell abundance and transcriptional profiles at single-cell resolution. These trajectories allowed us to identify cell subtypes vulnerable to aging and uncover gene regulatory networks driving their transitions. By aligning macaque aging trajectories to human neurodegenerative disease signatures, we observed strong convergence between the two processes.

Together, these studies examine the temporal dynamics of gene regulation during both embryogenesis and aging. By establishing a new in vitro platform to model early human development and constructing aging trajectories in the non-human primate brain, we aim to uncover the genetic drivers of cell state transitions across the human lifespan. This work lays the groundwork for a deeper understanding of developmental and aging processes and enables comparative analyses between them.

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Chapter 1: Introduction

1.1 Overview of human development and aging

Human life spans a continuum of dynamic biological processes, beginning with a single fertilized cell and culminating in the pleiotropic physiological changes of aging. Understanding this full spectrum — from the establishment of cellular diversity in early embryogenesis to its gradual erosion during aging — provides insight into the principles that maintain homeostasis and those that lead to its breakdown.

Changes in both development and aging unfold as structured, time-dependent sequences of molecular and cellular events. Recent advances in high-resolution molecular profiling have given invaluable opportunities to dissect the temporal progression of these life stages. In early development, technologies such as single-cell sequencing have revealed how cell identities were established through a series of cell fate decisions based on autonomous and non-autonomous molecular events. In aging, these same tools are uncovering how cell identities were shifted with increasing transcriptional noise, dysregulated epigenetic controls and progressive loss of cellular homeostasis. Importantly, theories have proposed that aging could be linked to a combination of antagonistic events ¹, hyperactivation of signaling pathways ^{2,3} and continuation of environmental factors ⁴ in development. These theories are supported by recent geroscience studies.⁵ The above evidence have suggested that molecular mechanisms of aging and development could be partially intersected. ⁵

Examining the temporal molecular mechanisms of development and aging in parallel offers a unique opportunity to identify shared molecular features — including chromatin dynamics, transcription factor networks, and signaling pathways. At the same time, it highlights fundamental asymmetries: the precision and reproducibility of early development stand in contrast to the stochasticity and heterogeneity that characterize aging. By anchoring this thesis in two complementary projects — one focused on early human embryogenesis, the other on brain aging in non-human primates — I aim to lay the groundwork for understanding the molecular architecture that shapes identity across the human lifespan.

1.2 Advances in study of early Development

One of the most intriguing mysteries of biology is how life develops from a single cell. The development of an embryo starts with a fertilized egg. It divides into a group of similar cells, but then the potential paths of each cell grow exponentially as more and more complex structures arise. When we look at its final product, a fetus, we will always ponder how each cell chooses its own path, and at the same time coordinate with others to build this complex and sentient being. Understanding lineage specification during embryogenesis is important because it could advance our understanding of preterm birth defects, improve reproductive technology such as In Vitro Fertilization and prenatal testing, shed light on stem cell engineering and potentially help reveal the aspects of development that are uniquely human. Recent advances in technologies have enabled researchers to trace cell lineage and delineate the determinants of cell fate decisions during lineage specifications.

GESTALT (genome editing of synthetic target arrays for lineage tracing), a method that uses CRISPR/Cas9-mediated mutations to record cell division history in an organism's DNA.⁶ In this approach, an array of CRISPR target sites ("barcode") is inserted into the genome; as development proceeds, Cas9 induces diverse insertions/deletions in the barcode in different cells, creating unique mutation patterns that are passed to descendant cells. By sequencing these barcodes in many single cells, researchers can infer a lineage tree of the entire organism. Notably, GESTALT applied in zebrafish revealed that most cells within an adult organ derive from only a small number of embryonic progenitors, with progressive restriction of progenitors to germ layers and then organs.

McDole et al. developed an adaptive light-sheet microscope that dynamically adjusts to the growing embryo, allowing direct captured of 3D positions and divisions of thousands of cells over time in a developing mouse embryo from gastrulation through early organogenesis.⁷ The authors also developed computational tools to reconstruct each cell's lineage trajectory after imaging. With this strategy, the authors built a "dynamic atlas" of post-implantation mouse development at single-cell resolution, including cell fate histories and tissue morphogenesis across the entire embryo.

Alternative to directly following cellular development over time, advances in single-cell sequencing have enabled 'indirect' lineage reconstruction by sampling cells at multiple timepoints. Compared to the genome-editing and microscopy approaches, single-cell sequencing are affordable and have less technology barriers. In this approach, embryos at different timepoints will be profiled with single-cell technology (e.g. single-cell RNA sequencing, single-cell ATAC sequencing). This strategy has taken advantage of asynchronous development of cells in embryos. Therefore, cells at

various nearby stages of development will be captured within each time point of sampling. Computational methods such as pseudotime⁸ can be used to reconstruct the cellular trajectories by ‘stitching’ a series of snapshots of cells at different developmental stages.

In 2018, two independent studies profiled tens of thousands of single cells from zebrafish embryos around the gastrula stage. Farrell et al. (2018) sequenced ~38,000 cells spanning 12 stages from blastula through early segmentation in zebrafish, reconstructing the “specification tree” of 25 distinct cell lineages spanning from high blastula stage to shortly after completion of gastrulation.⁹ They developed URD, a simulated diffusion-based computational reconstruction method, to map cellular trajectories and showed that 1) signaling perturbation of embryos (Nodal mutant) will canalize transcriptomes into a subset of wild-type trajectories but not new ones, 2) existence of intermediate cells that could trans-specify from one cell type to another. In parallel, Wagner et al. applied scRNA-seq to >92,000 zebrafish cells collected during the first day of development, producing a high-resolution cell state landscape of early zebrafish embryogenesis.¹⁰ They have also applied TracerSeq, a transposon-based barcoding strategy to link clonally related cells and learn that clonally related cells are often differentiated into restricted range of the cell state landscape.

Exploiting the invariant cell lineage of *C. elegans*, Packer et al. generated a lineage-resolved single-cell atlas of *C. elegans* embryogenesis.¹¹ They profiled the transcriptomes of ~86,000 individual cells from worm embryos, capturing essentially every cell state from early gastrulation to the onset of organogenesis. This dataset allows for interrogation of analysis of how lineage and fate specification are coupled: 1) correlation between a cell’s lineage and its transcriptome changes dynamically over developmental stages; 2) multilineage priming of sister cells contribute to lineage branching; 3) cells from different lineage could converge to the same anatomical cell type and transcriptomic state.

This strategy has been demonstrated to successfully study lineage specification in more complex mammalian species. In 2019, two independent studies have profiled mouse embryos at early embryogenesis stages. Pijuan-sala profiled 116,312 single cells from mouse embryos collected from 6.5 to 8.5 days post-fertilization.¹² Cao et al. presented the Mouse Organogenesis Cell Atlas (MOCA) by profiling approximately 2 million single cells from 61 mouse embryos covering embryonic days 9.5 to 13.5.¹³ Building on the success of these mouse development atlases, Qiu et al. have pushed towards integrating transcriptome snapshots into continuous developmental trajectories. They introduced the concept of TOME (Trajectories of Mammalian Embryogenesis), a directed acyclic graph linking cell states across 19 sequential embryonic stages, effectively stitching together a “digital fate map” from implantation to late gestation.¹⁴ In 2024, Qiu and Martin et al.,

extended the atlases by capturing mouse prenatal development from late gastrulation all the way to birth.¹⁵ They profiled ~12.4 million nuclei from 83 mouse embryos staged at 2–6 hour intervals. This dataset with high temporal resolution enabled annotation of hundreds of cell types across mouse embryonic development and could pinpoint regulators for each differentiation event.

Despite the technical breakthroughs in model organisms, studying early human development remains challenging. Ethical constraints and scarcity of samples mean that human embryonic tissue (especially around gastrulation) is extremely difficult to obtain – researchers must rely on rare donations from elective procedures or use in vitro embryo models, each with limitations. Moreover, species-specific differences in developmental timing and gene regulation caution against assuming that animal models fully recapitulate human embryogenesis. Key early processes (e.g. primitive streak formation and germ layer specification) may have human-specific aspects that necessitate direct observation of human embryos. Until recently, our knowledge of human gastrulation came mostly from historic anatomical specimens and stem-cell based models, leaving a major gap in the “black box” of human development.¹⁶

Recognizing this gap, scientists have begun assembling single-cell maps of human embryonic and fetal development, though these remain less comprehensive than those of model organisms. In 2020, two landmark studies have built genomic atlases of human embryos in the organogenesis stage. Cao et al. generated a single-cell transcriptome atlas of early human organogenesis by profiling donated embryos in the 4–8 week range (first trimester).¹⁷ Complementing this, Domcke et al. profiled chromatin accessibility in ~800,000 single cells from comparable human fetal samples, mapping over 1 million accessible DNA regions across those cell types.¹⁸ Together, these studies yielded a first draft of a Human Developmental Cell Atlas for the organogenesis period, covering both the gene expression and regulatory landscapes of developing human tissues.

Even more elusive has been the human gastrulation stage, which occurs around weeks 2–3 of development and had never been molecularly characterized from intact embryos until recently. In 2021, Tyser et al. reported the first single-cell analysis of a Carnegie Stage 7 human embryo (~16–19 days post-fertilization, early gastrulation).¹⁶ In this study, they identified the expected cell populations of the gastrulating human. In 2023, Xu et al. probed the cellular diversity of human embryos at Carnegie Stage 12–16, covering late gastrulation to early organogenesis in human embryos. They highlighted potential human-specific gene expression patterns during early organogenesis.¹⁹

In summary, pioneering studies with advanced technologies have significantly expanded our understanding of early embryogenesis in model organisms and humans. However, a substantial gap

remains in the study of human embryogenesis, where data from key stages during gastrulation are missing. Moreover, the heterogeneity of genetic backgrounds from donors and the rarity of samples complicate efforts to derive statistically robust conclusions. To address these limitations, in the next chapter, I will introduce how stem-cell based models offer a tractable and scalable platform to study human embryogenesis.

1.3 Stem Cell Models for Studying Human Embryogenesis

Embryonic stem cells (ESC) are pluripotent, meaning that they have the potential to differentiate into all the future somatic lineages and the germ line in an organism. They are derived from an early embryonic structure, called blastocyst. During the blastocyst stage, cells are segregated into trophectoderm (TE), which later differentiate into extraembryonic tissues, and primitive endoderm (PrE), and epiblast (Epi), which will give rise to embryonic tissues. ESCs are derived from inner cell mass (ICM), a structure located in the inside of the blastocyst and composed of progenitor cells biased towards PrE and Epi. These ESCs can maintain their pluripotency in vitro by self-renewing cell divisions. These ESCs are referred to as ‘naïve’ ESCs, because they share similar transcriptional and methylation signatures as the earliest pluripotent cells in mammalian embryos. Alternatively, ESCs can also be derived from later stages of embryonic development. These ESCs are more restricted in their potential to differentiate and cannot contribute to a blastocyst. Therefore, they are referred to as ‘primed’ ESCs. Compared to mouse ESCs, human ESCs are closer to ‘primed’ than ‘naïve.’

ESCs in adherence culture can be differentiated into various embryo derivatives and follow the gene expression patterns of differentiation in vivo. For example, mouse ESCs that are induced to differentiate into mesodermal derivatives will undergo a transient pulse of Brachyury expression as they exit pluripotency.^{20,21} This indicates that ESCs maintain an autonomous schedule of differentiation as in embryos, even when they are cultured in vitro.

However, ESCs in adherent culture will not develop patterning and embryo structure. Over the past decades, researchers have tried to create a three-dimensional(3D) self-organizing embryo in vitro from stem cells. In early efforts to mimic embryo development, stem cell researchers have placed large numbers of ESCs (>1000) in non-adherent conditions in the presence of serum. They have successfully generated 3D ensembles of different cell types, which they called Embryoid Bodies (EBs).²² However, EBs are unstructured and disorganized 3D ensembles, which lack embryonic structures.²³ Although localization of Brachyury expression can be observed in EBs but no further patterning happens. However, EBs can generate some cell types that are difficult to induce in

adherent culture.²⁴ These suggest that cells receive non-autonomous signals from their neighbors and extracellular environments as they differentiate in the embryo. In 2009, Hans Clever and Toshiro Sato described the first intestinal organoid where they culture stem cells from the mouse intestine in an extracellular matrix gel, called Matrigel.²⁵ The intestinal organoids show organized structures of small-intestinal crypts. Using EBs as starting point and Matrigel, researchers developed several neural organoids with neural patterned structures, including optic cups²⁶ and brain organoids.²⁷

The biggest differences between in vitro 3D culture and in vivo embryo is the lack of interactions between ESCs and the extraembryonic tissues. To introduce these interactions, several groups have combined ESCs with stem cells of extraembryonic lineages. In 2018, Revirion et al, combined ESCs and trophoblast stem cells (TSCs) in suspension culture and successfully generated structures that mimic blastocysts, which they referred to as blastoids.²⁸ However, these blastoids fail to progress beyond an early epiblast stage and implant properly. Adding extra-embryonic endoderm (XEN)^{29,30} or Gata4-induced primitive endoderm²⁹, ESCs developed epiblast-like structure with anteroposterior (AP) polarity and initiate gastrulation-like movement, but fail to progress into gastrulation and form the body plan.

ESCs receive various signals from extraembryonic tissues. The limitations of the above models indicate potential differences between signaling in vitro and in vivo. An interesting observation has been made on mouse P19 embryo carcinoma cells. P19 aggregates displayed elongation morphogenesis with a distinct anterior–posterior body axis as in the embryo, regulated by Wnt signaling.³¹ Building on this observation, Turner et al, cultured a defined number of mouse ESCs in small volumes of basal medium to form spherical aggregates. They added a Wnt agonist (e.g. Chiron) to the aggregate on the third day of culture and observed 80% of the aggregates elongate and with cell expression Brachyury sorting to the leading end. The aggregates can be further developed to resemble mammalian body plan after five days of culture that they are referred to as ‘gastruloids’.³² However, the development of gastruloids do not resemble those in the embryo because there’s no formation of a primitive streak.³³ The formation of gastruloids suggests ESCs might have an intrinsic program for symmetry-breaking in 3D that could be initiated without presence of extraembryonic tissues and formation primitive streak. In the embryo, extraembryonic tissues might provide more refining cues to further control the body plan formation.³⁴ Tracking gene expression over time shows that gastruloids undergo transcriptional changes in the same order as embryonic gastrulation, and they develop gene expression patterns equivalent to those in E8.5

mouse embryos after 120h in culture.^{35,3633,37} This suggest that Wnt signaling might be required during embryonic gastrulation for symmetry breaking by coordinating the expression of Brachyury.

Since the invention of synthetic embryo and gastruloids, researchers have developed various stem-cell based models recapitulating different aspects of early embryogenesis. All of these models are referred to as ‘stembryo.’

In 2020, Moris et al., developed a human counterpart of gastuloids. After 4 days of culture, these gastruloids elongate to form cell types in the three germ layers. However, they did not generate embryonic structure as their mouse counterparts.³⁸ Based on the protocol of human gastruloids, somitoid³⁹, axioid⁴⁰, segmentoid⁴¹ are developed to model formation of somites (somitogenesis) which occurs throughout gastrulation in embryos. In addition to CHIR, the protocol of somitoid³⁹ includes FGF, TGFbeta inhibitors, and BMP inhibitors to potentially restrict development of other mesodermal lineage than presomitic mesoderm. They have successfully obtained epithelial somite-like structures and the segmentation clock. They have identified matrigel is essential for epithelization of the somites but not their differentiation. Interestingly, modulation of Wnt signaling concentration at the beginning of axioid formation would bias neural and somitic fate depending on the concentration. The protocol of axioid⁴⁰ also relies on FGF and TGFbeta inhibitors and Matrigel. They have also identified the potential role of retinoic acid in generating better somite epithelialization by potentially regulating mesenchymal-to-epithelial transition factors.

Elongating Multi-Lineage Organized (EMLO) gastruloids models human trunk formation, including neuromesodermal progenitor-derived central nervous system, the neural crest cell-derived peripheral nervous system, the endoderm-derived primitive gut tube, and the mesoderm-derived splanchnic mesenchyme. They are induced by CHIR and FGF, and then transferred to a shaking culture without supplied extracellular matrix. Hepatocyte growth factors (HGF) and insulin-like growth factors (IGF-1) are supplied⁴²

While the models above only recapitulate aspects of embryos gastrulating embryos, researchers have made advances of generating synthetic whole embryos ex utero, progressing to the stage of early-mid gastrulation. In 2022, Tarazi et al. generated generated a mouse synthetic embryo model.⁴³ They differentiated mouse naïve ESCs into embryonic and extra-embryonic lineages by transiently expressing key transcription factors: doxycycline-inducible Cdx2 drives a subset of ESCs toward trophoblast (placental) fate, and inducible Gata4 primes another subset toward primitive endoderm (yolk sac) fate. Mixed populations of wild-type, iCdx2, and iGata4 ESCs are co-aggregated to form blastocyst-like spheroids. They then applied a doxycycline pulse from 24 h to 48 h after aggregation to establish lineage specification of ESCs. After the pulse, the aggregates

composing a mix of ESCs biased towards different fates are then cultured in a two-phase, rotating incubation system with controlled medium and oxygen conditions. The aggregates developed egg-cylinder-shaped that contain structures like somites and neural tubes.⁴³

Building on the success of synthetic mouse embryos⁴³, Oldak et al., has set out to create a human synthetic embryo. They applied overexpression of key transcription factors (i.e. GATA4, GATA6, CDX2 and GATA3) and media conditions to induce three extra embryonic lineages (i.e. hypoblast, ExEM and trophoblast) constituting the post-implantation human embryo and mix them after induction. When aggregated in 3D, these mixtures of induced naive cells form structures containing an epiblast domain (OCT4+), a surrounding amnion-like and trophoblast cavity (SDC1+), and a disk of hypoblast (SOX17+) and mesoderm, achieving a embryo structure close to post-implantation embryo by ~day 13 of culture.⁴⁴

With single cell transcriptomics and spatial transcriptomics, these stembryo models were characterized to contain a subset of cell types from the three germ layers. Their distinct spatial gene expression patterns along the anteroposterior and dorsal-ventral axis are reminiscent of those in the embryos.^{37–41,45} Most of these stembryo models mimic mid-to-posterior trunks in the embryo due to the posterior-bias of Wnt signaling.

It is important to establish relationships between these stembryo models and in vivo embryos in terms of developmental stages. In mouse gastruloids, the starting state of ESCs resembles the pluripotent cells of an E4.0 embryo. At 48 hours of culture, their global transcriptional profile corresponds to approximately E6.0, at the onset of gastrulation.³⁵ This explains the role of Wnt signaling in driving ESCs to posterior mesendoderm in gastruloids at this stage. After 48 hours, the gene expression and cell type composition changes reflect that gastruloids are undergoing gastrulation and formation of the body plan, including somites and neural tubes. Combining standards from both cell type composition and embryonic structures, mouse gastruloids at the terminal state are mapped to mouse embryos at E8.5. However, different stembryo models recapitulate different aspects of embryos and some of them lack embryonic structures, so creating a systematic way to stage among different models remains a challenge.

1.4 Morphological and Molecular Changes in the Aging Brain

Brain aging is a pleiotropic process leading to changes that span transcriptional shifts, morphological remodeling, and epigenetic modifications. The changes vary across brain regions and cell types. Comparing brain aging in humans and other species revealed conserved and

species-specific patterns. Normal brain aging is associated with pathologies and cognitive decline and shares similar molecular signatures with neurodegenerative diseases.

Aging in the brain was associated with remodeling of brain structures. A series of studies have characterized changes in brain volume over the lifespan of non-human primates^{46,47}. Longitudinal studies in cognitively normal older adults have documented a generalized reduction in grey matter volume⁴⁸ including pronounced atrophy in the frontal cortex,^{49,50} which correlates with cognitive decline⁵¹, and is further accelerated in Alzheimer's disease⁵². Dash et al., surveyed structural T1-weighted MRI scans of the brains of 66 animals across the lifespan and found regional differences in volume changes. They have observed enlargement of the lateral ventricles and a smaller volume in the frontal cortex, caudate, putamen, hypothalamus, and thalamus in older animals. Unexpectedly, a greater volume was measured in hippocampus, amygdala, and globus pallidus. This line of evidence was supported by a previous study that no noticeable size reduction was observed in hippocampal volume in rhesus macaque, and the size does not correlated with cognitive memory impairment.^{46,53} This is contrary to human aging, where the decrease in hippocampal volumes is a hallmark, suggesting species-differences.⁵⁴

At the cellular level, neurons and glia also undergo characteristic age-related morphological alterations. During aging, it is evident that neurons undergo morphological alterations, including a reduction in the complexity of dendrite arborization and dendritic length and a decrease in spine numbers as well as the excitatory synapses within spines. Other studies provided supporting evidence that glutamate receptor-mediated excitatory responses and the levels of glutamate neurotransmitters and receptor expression decreases with age.⁵⁵ Aged microglia display enlarged, rounded cell bodies and less complex and shortened processes, with a more frequent presence of 'dystrophic' microglia. 'Dystrophic' microglia in aged human brains show accumulation of intracellular lipofuscin and iron deposits as a result of oxidative stress and reduced housekeeping efficiency. Characteristics of aged microglia are both reminiscent of activated microglia (i.e. increased expression of inflammatory markers and cytokines), and associated with decline of functions (i.e. changed cytoplasmic structure, reduced ability to phagocytose and to migrate, decreased process motility, and changes in receptor expression).⁵⁶ In humans, aged microglia was associated with 'adipose saccules', which is exacerbated in Alzheimer's disease(AD) in an APOE variant dependent manner.⁵⁷ Lipid processing and innate immunity are both enriched categories of genetic risk factor for AD⁵⁸, where lipid droplet synthesis could be triggered by innate immune response.⁵⁹ In a recent single cell study of mouse aging, Allen et al., has compared immune

activation between aging and LPS-induced innate immunity in the brain, and identified both similarities and notable differences in the activation patterns.

Over the past decade, a growing number of studies have revealed that progressive changes to epigenetic information accompany aging in both dividing and nondividing cells.^{60,61} In 1967, whole-genome DNA methylation was found to be related to the age of spawning salmon.⁶² In 1987, the nucleosome occupancy in human skin fibroblasts was shown to decrease during aging, suggesting that chromatin configuration may change in the aging process.⁶³ In 2010, histone methylation was first linked with life extension, and it was demonstrated that H3K4me3 demethylation in the germline boosted the lifespan of *C. elegans*.^{63,64} In 2013, the concept of the “epigenetic clock” was proposed to link DNA methylation with biological age.⁶⁵ In the brain, epigenetic remodeling, such as DNA methylation, histone modifications, was observed to be associated with aging. DNA methylation shows global decrease in the brain, with age and gene-specific hypo- or hyper-methylation. Want et al., identified age-associated decrease of lysine KDM6B in hippocampus. KDM6B prevents methylation of vesicular glutamate transporter 1 and 2 in glutamatergic neurons to maintain synaptic plasticity. On the other hand, histone acetylation could modulate expression of senescence genes⁶⁶ and aging-related synaptic plasticity genes⁶⁷ during aging by occupying promoter regions of these genes. Recent studies also demonstrated that aging-mediated changes in H3K27ac and H3K9ac in the human cerebral cortex are associated with AD.^{68,69} However, the epigenetic landscape and regulation of other less common epigenetic marks and the expression dynamics of their regulators are largely unexplored.

With the emergence of sequencing technology, age-associated transcriptional shifts in the brain were identified. Genome-wide transcriptome studies (microarrays, RNA-seq) in humans first showed that synaptic plasticity and neuronal signaling genes are broadly downregulated with age, while stress and immune-response genes are upregulated⁷⁰. Many genes involved in neurotransmission (e.g. glutamate receptors, vesicle proteins, Ca²⁺ signaling molecules) decline in expression by old age^{70,71}, whereas genes related to antioxidant defenses, DNA repair, and inflammatory pathways show increased expression.⁷¹ These transcriptomic shifts likely underlie age-related reductions in synaptic function and memory, as well as a pro-inflammatory milieu in the aged brain.⁷⁰ Beside brain-wide changes, multi-regional studies have revealed region-specific aging signatures. Large-scale studies in rhesus macaques found that while many aging gene-expression changes are convergent across regions, each region also has a subset of unique differentially expressed genes.^{72,73} One study noted that gene expression becomes more heterogeneous in older adults, likely due to accumulating stochastic damage and somatic mutations.⁷⁰ It is also now clear

that transcriptional variability increases with age. Interestingly, the increased noise is most pronounced among genes that are normally downregulated with age.⁷⁴

1.5 Single cell technology revolutionized study of brain aging

The advances in single-cell technology has illuminated the study of aging in the brain. Researchers were able to identify cell type specific molecular signatures^{75,76,77} and cellular communities and interactions that are changed.^{78,79} Ximerakis et al. profiled tens of thousands of individual cells from young vs. old mouse brains.⁷⁷ They found that aging does not induce one uniform genetic program across the brain, but rather drives a distinct transcriptional trajectory in each cell type. Some gene expression changes were coordinated across many cell types (e.g. upregulation of ribosome biogenesis and protein folding genes, hinting at a response to proteostatic stress), whereas other changes were cell-type-specific or even opposite between cell types.

Employing a latent factor analysis on concerted changes of transcriptional programs among cell types, Ling et al., has identified a synaptic neuron and astrocyte program.⁷⁹ Genes in cell types involved in this program show reduced expression in schizophrenia and aging, underlying potential determinants of declines in cognitive flexibility and plasticity in these conditions. Overall, single-cell data highlights that aging changes are mosaic in the brain in a cell type specific manner. On the other hand, single cell studies shed light on changes in cell types that are sparse and hard to isolate. Hammond et al. performed single-cell RNA sequencing of microglia from mice across the lifespan. They discovered that microglia in aged mice bifurcate into diverse states: alongside “homeostatic” microglia, new subsets emerge in old brains expressing higher levels of pro-inflammatory and chemokine genes.⁸⁰ Chien et al., profiled the human frontal cortex by single-nucleus multiomic DNA and transcriptome sequencing and identified a specific upper layer excitatory neuron subtype in the human cortex that is particularly vulnerable in aging.^{75,76}

In summary, single-cell technology enables detections of cell type and region specific changes during aging in the brain while minimizing batch effects. However, single cell data spanning multiple regions and timepoints from the human brain is hard to obtain and could suffer from autopsy biases and long postmortem intervals in the clinical setting. In the next section, I will go through the model organisms for studying brain aging.

1.6 Brain Aging and the Need for Primate Models

Understanding the molecular and cellular mechanisms of brain aging requires experimental models that balance biological relevance and practical accessibility. While postmortem human samples are commonly used, they are usually subjected to confounding factors such as their agonal states and unnatural aging process. On the other hand, genetic heterogeneity, autopsy biases and varying postmortem intervals could further complicate interpretation. To address these gaps, various model organisms and in vivo models are established. Among them, the most widely used systems include in vitro stem cell-based models, rodents, and non-human primates (NHPs) — each offering distinct advantages and limitations.

Stem cell-based models, including human iPSC-derived neurons and cerebral organoids, allow for mechanistic interrogation of aging-related pathways in a human genetic background. These systems are amenable for genetic manipulation and anti-aging interventions testing. Aging signatures were induced in iPSC-derived cells via 1) long term culture ⁸¹, 2) telomere shortening ⁸², 3) progerin expression ⁸³. Critically, many iPSC-based neurons lost their original aging signatures after a few passages following reprogramming, which complicates their use in studying late-onset disorders. Direct transdifferentiation of aged somatic cell type to the target neurons or glia could preserve endogenous aging features.⁸⁴ However, they remain limited in their capacity to model the full complexity of brain aging, with the lack of vascularization, multicellular interactions, and the environmental context that shapes aging in vivo.

Rodents offer powerful genetic tools with large sample size and short lifespans, which have been instrumental in identifying conserved aging hallmarks and validating anti-aging interventions. Studies of brain aging in mice reveal conserved aging pathways among vertebrates, such as age-related loss of synaptic plasticity and neuronal network function, myelin loss and immune activation of glial cells.^{76,85} Nonetheless, rodents differ significantly from humans in brain neuroanatomy, lifespan, and social behavior, and their cognitive aging phenotypes may not fully capture those seen in humans. Importantly, rodents lack a gyrencephalic cortex and exhibit less complex prefrontal organization — features critical to understanding higher-order cognitive decline. At the microscopic level, glial cells in mice behave differently in the context of neurodegenerative aging. For example, the classical gene sets for determining ‘disease-associated microglia’ (DAM) in the mouse AD model were not found in human microglia.⁸⁶

Non-human primates occupy a key middle ground. Rhesus macaques are phylogenetically close to humans with similar neuroanatomy. Although their lifespans are 3–4 times shorter than that of

humans, rhesus macaques traverse a remarkably human-like aging trajectory—manifesting declines in cognition, physiology, and motor function that mirror those observed in humans.⁸⁷ Chiou et al. demonstrated that the survival curves of wild rhesus macaques resemble those of humans, particularly in showing late-life mortality acceleration — a feature absent in most laboratory animals. Wild macaques also experience environmental and social stressors, such as food competition, hierarchy instability, and disease exposure, which shape brain aging in ways more comparable to humans. Moreover, populations like those on Cayo Santiago island⁸⁸ exhibit relatively homogeneous genetic backgrounds due to geographic isolation, offering unique opportunities to control for genetic variability while studying aging in naturalistic conditions.⁸⁹

Despite these advantages, macaques do not fully recapitulate human-specific neurodegenerative changes, such as the hallmarks of Alzheimer's disease. Human Alzheimer's disease (AD) typically initiates in the entorhinal cortex and spreads to the hippocampus via synaptic circuits, accompanied by substantial neuronal loss and accumulation of tau and amyloid pathology. While recent studies have demonstrated that rhesus monkeys can naturally develop hyperphosphorylated tau (p-Tau), filamentous structures, Neurofibrillary tangles (NFTs) are rarely detected in the brains of aged NHPs.^{90,91} Moreover, most rhesus macaque in the wild only live up to early stage of aging (~20 years) where Tauopathy have not been fully propagated and developed (Tau deposition similar to Braak stage I/II). Notably, despite rhesus macaque brains exhibit age-associated amyloid plaques burden, the plaques are not correlated with cognitive decline.⁹² These differences likely reflect species-specific aging trajectories and underscore the importance of cautious interpretation when extrapolating macaque findings to human late-onset neuropathologies.

Thus, while macaques provide a biologically relevant system to explore the molecular and cellular correlates of cognitive aging, caution is warranted in extrapolating findings to late-stage neurodegeneration. Understanding both the convergent and divergent hallmarks between macaque and human brain aging will help clarify which aspects of aging are conserved vs. human-specific — and where additional models or complementary approaches are needed.

1.7 Computationally characterize aging signatures

To identify age-related changes, statistical tests were performed to identify age-related changes in cellular composition and gene expression. Given the overdispersion of count data of cell composition and single cell gene expression, a negative binomial model⁹³ or zero-inflated negative binomial model⁷⁶ was employed to model gene expression. Gene expression will be modeled using a generalized linear model with age and other confounding factors (e.g. sex, UMI) as covariates

when donor effects are assumed to be minimal (e.g., in laboratory mice)⁹³, or using a linear mixed-effects model to account for donor variability when such effects are present (e.g., in human or macaque samples)^{94,95}. In the events of small sample size, a likelihood test could be employed to enable robust detection of age effects.⁹³

On the other hand, variable amounts of cells are harvested from different donors due to technical factors (e.g. dissection error) especially for cell type with small number of cells recovered. Therefore, variability of total cell count across samples of different ages could be a challenge to detect age-related changes in cellular composition. A new package called Hooke (ref) applies a poisson log-normal model to capture extra variability by incorporating total count as an offset term. This tool could be employed in analyzing human and macaque samples where cell recovery could be highly variable among donors.

With the current limitations on cell recovery and sequencing powers, we will run into the problem of limited statistical power of small sample size if we want to test multiple regions and cell subtypes at the same time. Lack of statistical power by chance in some conditions might confound the comparison of age effects among regions and cell subtypes. Expression quantitative trait loci (eQTLs) are often performed on multiple cell types or tissues. Statistical tools have been developed for joint analysis of multiple conditions, such as multivariate adaptive shrinkage (MASH). MASH learns patterns of sparsity, sharing and correlations among effects of individual conditions and uses the learned patterns to improve effect estimates and measures of significance. This method could theoretically be applied to differential abundance and gene expression analysis across multiple conditions for more robust comparison among conditions.

Modern analytic tools used to infer gene regulatory networks in single cells can be applied to identify master regulators of aging. For example, pseudotime reveals transcriptional trajectory of aging and orchestrated changes of genes. SCENIC and motif analysis identify key transcription factors driving age-related expression changes. Co-expression/network analyses (e.g. WGCNA-like) pinpoint modules of genes that rise or fall together in aging.

In summary, statistical approaches in modern single cell analytics tools can dissect the transcriptional change and gene regulation of the brain aging. However, a limitation in the field is that most single cell data are descriptive. We can only infer correlations among genes but not causality. Testing causality in the macaque brain is challenging but could be feasible via stem cell model or laboratory mice mentioned above.

1.8 Thesis outline

In summary, current efforts to build single cell atlas of early embryogenesis have expanded our knowledge of temporal molecular events during lineage specification. However, coverage of human embryogenesis is largely limited, especially during the period of gastrulation. Recent advances in stem cell engineering have enabled symmetry breaking of stem cells in vitro to form embryo-like structures. These stembryo models recapitulate different aspects of lineage development and structure formation in gastrulation and could serve as reliable tools for large scale screens. Even with these advances, a stembryo model recapitulating both the embryo structure and the complex lineage makeup of gastrulating human embryos are still absent.

To address this limitation, **in Chapter 2**, I described ‘Human RA gastruloids’, a model developed by my colleagues and I to model both the posterior embryo structure and lineage development of the three germ layers during human gastrulation. We identified discontinuous retinoic acid pulses that could induce formation of neural tube and somites as those in in vivo human embryos.

Moreover, it enabled further development of lineages from three germ layers from the symmetry breaking aggregates. To establish a relationship between stembryo models and in vivo embryos, we developed an in silico staging method to align stembryo models with in vivo embryo stages. With this in silico staging method, we were able to characterize our ‘Human RA gastruloids’ to be at a stage close to mouse E9.5 embryos and CS11 macaque embryos, developed to the most advanced stage in gastrulation compared to those reported before.

On the other hand, mammalian brains have been shown to undergo pleiotropic changes in morphological and molecular studies. Single-cell analysis revolutionizes our understanding of these changes by pinpointing cell types responsible for these changes and the underlying molecular network. Most of these studies are performed in postmortem human brains, laboratory mice, and stem-cell based models which each have their own limitations in studying healthy human brain aging. **In Chapter 3**, we highlight rhesus macaques with similar genetic background from an isolated island as robust examples to study brain aging. My college and I have performed single cell transcriptomic profiling to study the temporal molecular events in these rhesus macaques. The profiling covers multiple brain regions across the lifespan of wild macaques, enabling systematic dissection of regional heterogeneity. We identified aging changes that are specific to cell types and regions, and uncovered the relationship of healthy aging and neurodegeneration.

Chapter 2: Retinoic acid induces human gastruloids with posterior embryo-like structures

This Chapter is adopted from published work with minimum changes.

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ABSTRACT

Gastruloids are a powerful in vitro model of early human development. However, although elongated and composed of all three germ layers, human gastruloids do not morphologically resemble post-implantation human embryos. Here we show that an early pulse of retinoic acid (RA), together with later Matrigel, robustly induces human gastruloids with posterior embryo-like morphological structures, including a neural tube flanked by segmented somites, and diverse cell types including neural crest, neural progenitors, renal progenitors, and myocytes. Through silico staging based on single-cell RNA-seq (scRNA-seq), we find that human RA-gastruloids progress further than other human or mouse embryo models, aligning to E9.5 mouse and CS11 cynomolgus monkey embryos. We leverage chemical and genetic perturbations of RA-gastruloids to confirm that WNT and BMP signalling regulate somite formation and neural tube length in the human context, while transcription factors TBX6 and PAX3 underpin presomitic mesoderm and neural crest, respectively. Looking forward, RA-gastruloids are a robust, scalable model for decoding early human embryogenesis.

2.1 Introduction

The molecular, cellular and developmental biology of early human embryogenesis is of fundamental interest. However, studying in vivo post-implantation early human development, directly or by ex vivo culture^{96–98}, is challenging. In vitro embryo models, which derive from pluripotent stem cells (PSCs)^{30,99–103}, are a promising alternative for accessing early human development. For example, PSC-derived gastruloids^{32,33,35,38,45} bear derivatives of all three germ layers and specify an anteroposterior (A-P) axis³³.

The addition of Matrigel during mouse gastruloid induction results in morphological features even more characteristic of *in vivo* development, including a neural tube and segmented somites^{37,45}.

However, these structures are not observed in conventional human gastruloids. Although derivative protocols yield human embryo models with segmented somites^{39–41,104,105}, spinal cord neurons and skeletal muscle cells¹⁰⁶, or elongated multi-lineage structures with peripheral neurons⁴², a human embryo model with balanced representation of the neural and somitic lineages remains elusive.

We investigated differences between mouse and human gastruloids, which led us to hypothesise that neuromesodermal progenitors (NMPs) in conventional human gastruloids are mesodermally biased. In seeking to correct this, we discovered that an early pulse of retinoic acid (RA), together with later Matrigel, induces human gastruloids with posterior embryo-like morphological structures, more advanced cell types, and less inter-individual variation, than observed in conventional gastruloids. Here we extensively characterise these human RA-gastruloids, including leveraging scRNA-seq data to computationally stage their progression relative to other mammalian embryo models and *in vivo* development. Finally, we perform chemical and genetic perturbations of human RA-gastruloids to showcase their potential as a model for advancing our understanding of early human embryogenesis.

2.2 Comparative analysis of human vs. mouse gastruloids

We sought to induce human gastruloids with more advanced morphological features than observed in conventional human gastruloids³⁸. To this end, we initially attempted Matrigel supplementation, previously shown to induce mouse gastruloids with trunk-like structures (TLS)³⁷. However, although Matrigel supplementation substantially increased the extent and success rate of human gastruloid elongation, it did not alter their morphology (Extended Data Fig. 1; Table S1).

To investigate this difference, we performed scRNA-seq on conventional human gastruloids¹⁰⁷, without Matrigel supplementation, at 24, 48, 72 and 96 hrs after induction (Fig. 1a-b). After filtering low-quality cells and doublets, we performed dimensionality reduction, unsupervised clustering, and cell type annotation on the transcriptional profiles of ~44,000 cells from 4 timepoints (Fig. 1c; Table S2). In these data, we can follow the emergence of key cell populations over time (Fig. 1d; Supplementary Fig. 1-4). At 24 hrs after seeding, we observe a continuum of molecular profiles with heterogeneous expression of transcription factors with established roles in early development (Fig. 1d; Supplementary Fig. 1; Table S3). Distinct populations of FOXA2+ cells were either NOTO+ or SOX17+, and presumably correspond to axial mesoderm and definitive endoderm equivalents, respectively (Supplementary Fig. 1). For annotation, integration with

scRNA-seq data from Carnegie Stage (CS7) human embryos¹⁶ was informative, and suggest that early stage human gastruloids model the primitive streak, nascent mesoderm and emergent mesoderm (Supplementary Fig. 5). However, extraembryonic, hematopoietic endothelial, and primordial germ cell (PGC) equivalents were missing, as were epiblast and non-neural ectoderm equivalents.

Through integration with scRNA-seq data from conventional⁴⁵ and TLS³⁷ mouse gastruloids (Supplementary Fig. 6), we annotated cell types in 48-96 hr human gastruloids, including a continuum from NMPs (TBXT+; SOX2+; NKX1-2+; CDX2+) to presomitic mesoderm (TBX6+) to a differentiation front (MESP2+; RIPPLY2+; RIPPLY1+) giving rise to differentiated somites (FST+; PAX3+); and additionally, advanced (OSR1+; WT1-) and cardiac mesoderm (HAND1+; NKX2-5+; TNNT2+) (Fig. 1d; Supplementary Fig. 2-4). Axial mesoderm or notochord-like cells (NOTO+) were also detected at later timepoints. Through integration with mouse E6.5-E8.5 data¹², we could also track a gut/endoderm-like subpopulation through 96 hrs (APELA+, FOXA2+, KRT8+, SOX17+) (Supplementary Fig. 7a-h).

Bipotential NMPs are the source of both presomitic mesoderm (PSM) and posterior neural tube¹⁰⁸⁻¹¹². To compare the differentiation potential of NMPs in mouse vs. human gastruloids, we performed a focused analysis of NMPs, PSM and neural tube cells in scRNA-seq data from mouse gastruloids⁴⁵, mouse TLS³⁷, and human gastruloids³⁸. Although NMPs and PSM were detected in all three models, neural tube cells (IRX3+, SOX1+, PAX6+) were identifiable only in mouse gastruloids and mouse TLS (Fig. 1d-e; Table S2). Based on this observation, we hypothesised that a bias in the differentiation potential of NMPs towards mesodermal fates might underlie the failure of human gastruloids to generate an elongated neural tube.

Retinoic acid (RA) is a signalling molecule that can induce neural cell fates from NMPs, both in vivo and in vitro¹¹²⁻¹¹⁹. We compared the expression of transcripts encoding enzymes involved in RA synthesis or degradation between gastruloid models (Extended Data Fig. 2a-b). Human gastruloids exhibit much lower expression of ALDH genes, which encode enzymes that convert retinal to retinoic acid (Extended Data Fig. 2a-b). The contrast is particularly stark for ALDH1A2, which encodes the enzyme that catalyses RA synthesis from retinaldehyde; its expression remains low in human gastruloids, but is acutely upregulated then downregulated at 96-120 hrs in mouse gastruloids and TLS, suggesting a critical time window for receptivity to RA signalling in this model (Extended Data Fig. 2c). We also observed higher expression of CYP26 genes, which counteract RA signalling, in human gastruloids (Extended Data Fig. 2a-e). Conversely, human gastruloids exhibit higher expression of some WNT genes at 0 and 24 hrs (Extended Data Fig. 2b).

Taken together, these observations suggest that if NMPs in conventional human gastruloids are biased towards mesodermal fates, it may be due to insufficient RA and/or excess WNT signalling.

2.3 An early pulse of retinoic acid induces trunk-like structures

To test this hypothesis, we sought to restore the bipotential state of NMPs by supplementing human gastruloids with RA. In addition to Matrigel (which, on its own, failed to induce a neural tube or segmented somites; Extended Data Fig. 1; Table S1), we explored adding various concentrations of RA to the gastruloid induction medium (Supplementary Fig. 8a). Encouragingly, SOX2-mCit intensities increased at 24 or 96 hrs in a dose-dependent manner (Supplementary Fig. 8b-c). However, although gastruloid elongation was enhanced at RA concentrations ranging from 100 nM to 1 μ M, we observed neither neural tube formation nor somite segmentation.

We speculated that continuous RA exposure might perturb the differentiation of other cell types between 24 and 48 hrs. We therefore attempted a similar experiment, but withdrew RA at 24 hrs and then added it back at 48 hrs together with 10% Matrigel (Extended Data Fig. 3a). Remarkably, this temporally discontinuous RA regimen induced the elongation of SOX2-mCit-positive neural tube-like structures as well as the formation of primitive segmentations of apparent somites (Extended Data Fig. 3b). Building on this finding, we optimised the number of cells used in the initial seeding of gastruloids (Extended Data Fig. 3c). With a larger seeding, discontinuous RA, and Matrigel supplementation, gastruloids exhibited multiple, segmented somites, together with a neural tube-like structure along an A-P axis (Fig. 2a-b). These structures formed robustly under these conditions, with 89% of elongated gastruloids exhibiting both segmented somite and neural tube-like structures across five independent experiments (Fig. 2c). We hereafter refer to these as ‘human RA-gastruloids’.

Interestingly, while the first pulse of RA (0-24 hrs), together with Matrigel starting at 48 hrs, was sufficient to induce structures resembling a neural tube flanked by somites, the second pulse of RA (48-120 hrs) was not (Fig. 2d), suggesting the early pulse of RA underlies the maintenance of bipotentiality in early NMPs. Consistent with the lower expression of ALDH genes in human gastruloids (Extended Data Fig. 2), neither retinol or retinal, which are metabolic precursors of RA, could substitute for RA in this role (Supplementary Fig. 9). In contrast, as shown by Yamanaka et al. (2022), retinal, retinol and RA are interchangeable in facilitating somite epithelialization⁴⁰.

We also assessed modulation of the concentration of CHIR99021 (hereafter CHIR; an agonist of WNT signalling) during pre-treatment of human RA-gastruloids. Lower CHIR resulted in elongated gut tube-like structures with expression of SOX17-tdTomato and FOXA2 alongside neural tube

extension, albeit with greater disorganisation of gastruloid structures (Extended Data Fig. 4). The CHIR dose-dependent appearance of gut tube-like structures suggests different WNT signalling requirements for endoderm and mesoderm differentiation from the primitive streak/mesendoderm-like state.

Immunostaining confirmed that the neural tube-like structures are SOX2+/SOX1+ (Fig. 2e-f), while the somite-like structures are PAX3+¹²⁰. Both neural tube and somite-like structures exhibited asymmetrical accumulation of F-actin (stained by Phalloidin) and CDH2 on their apical sides (Fig. 2f; Extended Data Fig. 5a-b), similar to *in vivo* embryonic somites and neural tubes^{121–123}.

We next quantified the morphological properties of human RA-gastruloids (Extended Data Fig. 5c-k). Overall, human RA-gastruloids elongate as a function of time and reach a length (1.5-2.0 mm; Extended Data Fig. 5d) similar to other human somite model systems (segmentoids⁴¹, 1.2-1.6 mm; axioids⁴⁰, 1.0-1.4 mm). Somite counts increased with time, reaching 8-9 pairs in human RA-gastruloids by 120 hrs (Extended Data Fig. 5j). Somite lengths, widths, and areas are comparable (~100 μ m) to the axioid⁴⁰ and somitoid³⁹ models (Extended Data Fig. 5k).

Interestingly, most somites in human RA-gastruloids are paired and epithelialized even without the second pulse of RA (Extended Data Fig. 5l-o). This contrasts with axioids⁴⁰, which mostly depend on a late pulse of retinoid signalling for somite epithelialization. Possible explanations for this difference include residual effects from the first RA pulse, residual RA molecules from the first RA pulse, or additional signalling from cell types present in RA-gastruloids but not in axioids. Upon live imaging, human RA-gastruloids exhibit periodic generation of segmented somite pairs in approximately 5-hour intervals, similar to human embryos^{124,125} and somitogenesis models^{39,40} (Extended Data Fig. 6a-c).

Taken together, these results show that an early pulse of RA, together with Matrigel, robustly induces human gastruloids with trunk-like morphological structures, i.e. a neural tube flanked by segmented somites. Importantly, although the human RA-gastruloids characterised above were generated from RUES2-GLR¹²⁶ ESC cell line, the same induction conditions robustly gave rise to these morphological structures when starting with another human ESC (H9) cell line or a human iPSC (WTC11) cell line (Supplementary Fig. 10).

2.4 Transcriptional profiling of human RA-gastruloids

We applied scRNA-seq to human RA-gastruloids. Clustering and annotation of 5,347 and 18,324 single cell profiles identified 9 and 12 cell types at 96 and 120 hrs, respectively (Fig. 3a-d). In addition to cell types observed in conventional human gastruloids (NMPs, cardiac mesoderm,

differentiation front, somites, gut), we identified cell types resembling neural tube (PAX6+; SOX1+), neural crest (FOXD3+; SOX10+), neural progenitors (ONECUT1+; ONECUT2+), intermediate mesoderm (IMM; WT1+; OSR1+), and renal epithelium (LHX1+; PAX2+) at 96 hrs, as well as myocytes (NEB+), cardiomyocytes (TNNT2+) and endothelium (PLVAP+) at 120 hrs (Fig. 3a-d; Supplementary Fig. 11; Extended Data Fig. 7a-c). Posterior HOX gene expression was enhanced at 120 hrs relative to 96 hrs (Extended Data Fig. 7d-e).

We were surprised to observe many of these additional cell types, e.g. IMM and renal epithelium, because they are absent not only from conventional human gastruloids, but also from mouse gastruloid and TLS^{37,38,45}. During *in vivo* development, IMM arises between the somites and lateral plate mesoderm along the mediolateral axis. To investigate the spatial distribution of the IMM-annotated cells, we immunostained human RA-gastruloids with anti-WT1 antibody or anti-PAX8. Consistent with expectation, both the WT1+ cells (annotated as IMM) and PAX8+ cells (annotated as renal epithelium) were located lateral to somite structures (Fig. 3e; Supplementary Fig. 11c-d).

We also identified neural crest-like cells (SOX10+, FOXD3+), which are absent from conventional mammalian gastruloids, although observed in the EMLO model^{42,127}. In mammalian development, multipotent neural crest cells arise at the dorsal aspect of the neural tube and migrate throughout the embryo¹²⁸. To visualise the spatial distribution of these neural crest-like cells, we immunostained human RA-gastruloids for SOX10. Remarkably, the neural crest-like cells were asymmetrically localised to one side of the neural tube, potentially the dorsal equivalent, suggesting that the spatial patterning of neural crest cells may be recapitulated in human RA-gastruloids (Fig. 3f-j).

To investigate the possibility of dorsal-ventral spatial patterning of the neuroectoderm of human RA-gastruloids more deeply, we isolated and reanalyzed scRNA-seq data from annotated neural tube, neural crest, and neural progenitor cells (Extended Data Fig. 8a-d; Table S4). A large proportion of neural tube cells expressed dorsal neural tube or roof plate markers, including PAX3. In contrast, ventral neural tube or floor plate markers were not expressed. These results suggest that human RA-gastruloids are dorsally biased, similar to mouse TLS and other embryo models^{37,129–132}. We speculate that the incomplete establishment of the D-V axis is due to the lack of a Sonic hedgehog (SHH)-secreting notochord¹³³.

Is any aspect of neural differentiation ongoing in human RA-gastruloids? We compared neural differentiation trajectories in human RA-gastruloids to those in cynomolgus monkey embryos at CS11¹²⁹. We ordered the transcriptomes of neural tube and neural progenitor cells by pseudotime to obtain neural differentiation trajectories for each species. We then identified differentially expressed

genes (DEGs) whose expression showed significant changes ($\text{FDR} < 0.05$) along a pseudotime axis corresponding to neural differentiation, and compared their dynamics (Extended Data Fig. 8e-i, see Methods). We observed 86% of the DEGs in human RA-gastruloids showed conserved dynamics with monkey embryos (Extended Data Fig. 8f-g). Similarly, 83% of the neural differentiation DEGs in monkey embryos show conserved dynamics in human RA-gastruloids (Extended Data Fig. 8h-i). To the extent that differential dynamics are observed, they likely arise from the limitations of this model (e.g. dorsal bias, lack of anterior neural tube) and/or species differences. Overall, however, our results suggest that human RA-gastruloids may recapitulate at least some aspects of early neural differentiation.

We also performed a more detailed analysis of somites, by reanalyzing scRNA-seq data from paraxial mesoderm derivatives (i.e. somites, differentiation front, and myocytes) for spatial markers of somites' rostrocaudal (UNCX; TBX18) and dorsoventral (PAX3; PAX1) organisation (Extended Data Fig. 9a-b). Although PAX3 (dorsal) was strongly expressed in somites, PAX1 (ventral) was not (Extended Data Fig. 9c-d; Table S5), suggesting that like neural tube, somites in human RA-gastruloids are dorsally biased. Additionally, as seen in both vertebrate embryos¹³⁴ and mouse gastruloid and TLS models^{37,45}, both UNCX (caudal) and TBX18 (rostral) were expressed in a mutually exclusive manner (Extended Data Fig. 9e-h), suggesting that we are reconstituting the rostral-caudal axis of somites. Consistent with this, we identified subsets of somitic cells that may correspond to migratory muscle precursors (PAX7+; MET+), myotome (MYF5+; MET+), syndetome (SCX+), sclerotome (PAX9+, NKX3-2+), endotome (KDR+) and an unknown cell type (DNAJC5B+) (Extended Data Fig. 9d; Table S5). It is noteworthy that we observe a subset resembling sclerotome, a ventral somite cell type, in the absence of SHH expression, since it has been reported that its differentiation is promoted by SHH signaling^{135,136}.

Overall, these scRNA-seq analyses confirm that we are inducing neural tube and segmented somite-like structures in human gastruloids through a discontinuous regime of RA. Although the dorsal-ventral axis is not fully established, human RA-gastruloids contain more advanced cell types than previously achieved in either human or mouse gastruloid models, suggestive of operational signalling gradients along both the dorsoventral (neural crest) and mediolateral (intermediate mesoderm) axes. Particularly in the 120 hrs human RA-gastruloids, we also observe progression towards even more differentiated cell types (e.g. neural progenitors, cardiomyocytes, myocytes, renal epithelium) and the appearance of endothelium.

2.5 Computational staging of human and mouse embryo models

The original report of human gastruloids assessed, based on morphological features and Tomo-seq¹⁰⁷, that they model late CS8 to early CS9 in human development³⁸, which corresponds roughly to embryonic day (E) 7.5 to E8.0 of mouse development. Because we observed more advanced cell types, we hypothesised that human RA-gastruloids might model more advanced stages of human development. However, staging based on morphology, individual cell types, or marker gene expression patterns is somewhat ad hoc. We therefore sought to develop a more systematic framework^{137,138} for benchmarking the progression of mammalian embryo models relative to in vivo development.

As a first step, we performed principal components analysis (PCA) of pseudobulk RNA-seq profiles of human gastruloids and RA-gastruloids (96-120 hrs) along with two available datasets of human embryos at CS7¹⁶, CS12 and CS13¹⁹ (Fig. 4a; extraembryonic cell types excluded). While the first principal component (PC1) appeared to capture differences in scRNA-seq technologies, the second principal component (PC2) correlated with developmental time (Fig. 4b-d). The genes most correlated with PC2 included pluripotency related genes (e.g. POU5F1), heterochronic genes (e.g. LIN28A), molecular markers of heart and neural lineages (e.g. HES1, ZIC1, TNNT2), and Hox genes (e.g. HOXC9, HOXC6, HOXB5) (Supplementary Fig. 12a-b). Gene Ontology (GO) enrichment found positively correlated genes enriched for biological processes such as anterior-posterior pattern specification, epithelial and mesenchyme development, and embryonic organ development (Supplementary Fig. 12c-d).

To validate the apparent correlation of this human-derived PC2 with developmental progression, we sought to relate it to in vivo mouse development, where we have access to fine-scale temporal sampling of embryonic development. For mouse E7-E10.5, roughly equivalent to human CS7-CS13 (Table S6), scRNA-seq data is available from several sources, staged in either 6 hr¹², 24 hr¹³, or single somite¹⁴ increments. We therefore generated pseudobulk RNA-seq profiles for staged mouse embryos, as well as mouse gastruloid and TLS models^{37,45} (once again excluding extraembryonic cell types) and then projected these onto the human-derived PC space (Fig. 4a-b). Despite differences with respect to species and technology, mouse embryos staged at 6 or 24 hr intervals were ordered by PC2 in a stage-congruent manner (Fig. 4b-d). Human-derived PC2 was also informative at a finer temporal scale, as it was highly correlated with somite counts of E8.5 mouse embryos¹⁴ (Spearman's correlation = 0.83; Fig. 4d). Overall, these results suggest that PC2 is capturing an aspect of developmental progression that generalises from human to mouse, at least for the developmental window considered.

Although CS7 human embryos aligned with E7.0 mouse embryos, consistent with previous stage assessments, CS12-CS13 embryos were placed beyond E10.5 mouse embryos (Fig. 4e), suggesting that anatomical vs. molecular alignments of mouse and human developmental stages may not be fully concordant (Table S6). Conventional human gastruloids³⁸ at 72 hrs mapped to E7.75-E8 mouse embryos, corresponding to CS8-CS9, consistent with the original staging³⁸ (Fig. 4e). Both mouse gastruloids⁴⁵ and TLS³⁷ models at 120 hrs mapped to E8.5 mouse embryos (Fig. 4e). Finally, human RA-gastruloids at 120 hrs mapped to E9.5 mouse and CS11 macaque embryos (Fig. 4e).

We next sought to stage individual cell types within a given model. To test this concept, we took major cell types from mouse embryos (E8.5 to E10.5) and projected each onto the human-derived PC2 axis. Encouragingly, the computational staging of nearly all of these lineages was reasonably correlated with ground-truth developmental progression, despite the species difference (Supplementary Fig. 13a-d). We therefore extended this approach to various individual cell types from human gastruloids³⁷. The results generally agreed with those obtained at the level of pseudobulked gastruloids, with cell types from conventional human gastruloids³⁸ at 96 hrs mapping to earlier than E8.5, and cell types from human RA-gastruloids at 120 hrs mostly mapping to around E9.5 (Supplementary Fig. 13e).

Overall, these results show how "computational staging" based on scRNA-seq data can be used to benchmark the developmental progression of mammalian embryo models.

2.6 Comparison of human RA-gastruloids to other embryo models

The number of human embryo models is increasing, with some more somitic³⁹⁻⁴¹ and others more neural^{42,127} in character. While this paper was in review, two additional models were reported (axial¹⁰⁵ and trunk¹⁰⁴ organoids), which like RA-gastruloids exhibit more balanced somitic vs. neural representation. We leveraged published and newly generated scRNA-seq data to compare these and other models with human RA-gastruloids with respect to developmental progression (Fig. 4e) and the cell types represented (Extended Data Fig. 10). Somitic embryo models, including d7 human somitoids, d5 axioids, d4 segmentoids, mapped to E8.5, E8.5, and E8-E8.25 mouse embryos, respectively, with cell types limited to mainly somitic lineages. Although neural embryo models took significantly longer to culture, d16 EMLO and d10 EO models mapped to E9.5 and E8.25-E8.5 mouse embryos, respectively, with cell types limited to mainly neural lineages. The recently reported human models, d5 axial organoids¹⁰⁵ and d7 trunk-like organoids¹⁰⁴, mapped to E8.0-E8.25 and E8.5 mouse embryos, respectively, and lacked many of the more advanced cell types present in d5 human RA-gastruloids (e.g. renal epithelium, myocyte, gut). The d5 human

RA-gastruloids mapped to E9.5 under the same framework, and consistent with that, contained the greatest diversity of cell types (somites, neural tube, NMP, neural crest, neural progenitors, myocytes, cardiac myocytes, intermediate mesoderm, renal epithelium, epithelium, and gut endoderm) (Fig. 4e; Extended Data Fig. 10).

Embryo models can also differ with respect to the efficiency and consistency with which they form. From a morphological perspective, human RA-gastruloids form embryo-like morphological structures with greater efficiency than other embryo models (89% for human RA-gastruloids [both neural tube and segmented somites] (Fig. 2c) vs. 50% for mouse TLS³⁷ [segmented somites] vs. 4% for Matrigel-embedded mouse gastruloids⁴⁵ [striped *Uncx4.1* expression] vs. 20-50% for EMLO⁴² [elongation] vs. 40% for human trunk-like organoids [clustered somites]¹⁰⁴). To further evaluate consistency in terms of cell type compositional variance, we “hashed” individual human RA-gastruloids prior to scRNA-seq with sci-Plex¹³⁹ (Fig. 5a-f). The resulting data shows that individual human RA-gastruloids exhibit less individual-to-individual variation than mouse gastruloids¹⁴⁰, particularly with respect to the balance of the somitic vs. neural tube lineages (Fig. 5g-h). Looking forward, we anticipate that inter-individual consistency will be important for arrayed screens, wherein substantial technical variation in the rate of gastruloid formation and/or cell type composition would compromise statistical power and the interpretation of results.

Overall, these results show that human RA-gastruloids exhibit more advanced developmental progression (Fig. 4e) and greater cell type diversity (Extended Data Fig. 10) than contemporary embryo models. They also give rise to posterior embryo-like morphological structures more robustly (Fig. 2c) and exhibit less inter-individual variation with respect to cell type composition (Fig. 5).

2.7 Perturbation of WNT and BMP signalling in RA-gastruloids

We next sought to evaluate human RA-gastruloids as a system for perturbing canonical signalling pathways. WNT signalling is known to play a key role in somite formation¹⁴¹. To explore the role of WNT signalling in RA-gastruloids, we reintroduced CHIR at 48 hrs (Supplementary Fig. 14a). Consistent with mouse TLS and human somitoids^{37,39}, this resulted in an excess of somite-like structures along the entire anterior-posterior axis (Supplementary Fig. 14b) and gastruloids nearly twice as long (Supplementary Fig. 14c). The result supports a role for WNT signalling in somite segmentation and tail elongation^{142,143} that is broadly conserved in vertebrate embryogenesis, through to humans.

BMP signalling plays important roles in lineage segregation during early development. To perturb BMP signalling in RA-gastruloids, we added either LDN193189 (LDN; a BMP inhibitor) or BMP4 at 48 hrs onwards (Fig. 6a). With LDN, the neural tube-like structure was consistently longer, while with BMP4, it was shorter and posteriorly confined (Fig. 6b-c). Of note, early LDN treatment (0-24 hrs) had a greater effect in terms of boosting neural tube length (Supplementary Fig. 15).

To evaluate the role of BMP signalling more broadly, we performed scRNA-seq on LDN-treated RA-gastruloids. We detected vastly more NMPs and neural tube cells than in the untreated condition (Fig. 6d-f; Supplementary Fig. 16a). These observations support previous reports that BMPs may restrict the area within which NMPs can arise to the tailbud region^{109,110,144,145}. With LDN-mediated BMP inhibition, this restriction is removed, potentially allowing for expansion of the NMP pool. On the other hand, LDN treatment also resulted in a paucity of other advanced cell types, including IMM, renal epithelium, cardiac cells, neural crest cells, and myocytes (Fig. 6d-f; Supplementary Fig. 16a), suggesting a positive role for BMP signalling in their induction. Consistent with this, these same cell types expressed ID genes (direct targets of BMP-SMAD signalling) in untreated human RA-gastruloids (Supplementary Fig. 16b).

To investigate these effects further, we separately examined the consequences of BMP inhibition on neural or somitic lineages. In neural lineages, we observed increases in not only neural tube and NMP markers, but also tailbud markers (FGF17, WNT5a)^{146,147}, supporting the possibility that LDN-mediated BMP inhibition may promote the generation of tailbud NMPs (Fig. 6g-i). However, in addition to a decrease in neural crest marker, we also observed marked decreases in PAX3 (dorsal) and PAX7 (dorsal) expression, consistent with the established role of BMP in dorsalizing neural cells¹⁴⁵, as LDN may be counteracting the dorsal bias of untreated human RA-gastruloids. A similar phenomenon is observed in the somitic lineages, where we observe a modest decrease in PAX3 (dorsal) expression and a marked increase in PAX1 (ventral) expression (Fig. 6j-l).

In the somitic lineages, marker gene reductions also confirm the marked depletion of myocytes upon BMP inhibition (Fig. 6j-l). We also observe a decrease in recently characterised markers of the endotome (KDR1, EBF2, ETV2)^{148,149} (Fig. 6m). In normal development, the endotome is thought to migrate to the dorsal aorta and specify hematopoietic stem cells, but its developmental regulation is poorly understood¹⁵⁰. Interestingly, MEOX1, an essential homeobox transcription factor for the specification of the endotome¹⁴⁸, remains unaffected by BMP inhibition (Fig. 6k-l). This suggests BMP signalling may be required for the regulation of endotome development downstream of MEOX1; alternatively, these may be secondary effects of other alterations to somites upon BMP inhibition.

2.8 Perturbation of key transcription factors in RA-gastruloids

We next sought to evaluate human RA-gastruloids as a system for genetic perturbations. For proof-of-concept, we chose the transcription factors TBX6 and PAX3, since their roles in early development are well characterised in mouse models^{151,152}. To knock out these genes, we introduced CRISPR-Cas9 RNA-protein complexes (RNPs) to human PSCs and subsequently induced RA-gastruloids (Fig. 7a; Supplementary Fig. 17).

In mice, *Tbx6* is an established master regulator of NMP fate, promoting somitic differentiation and suppressing neuronal lineages via repression of a *Sox2* enhancer^{152,153}. Relative to non-targeting-control (NTC)-RNP RA-gastruloids, TBX6-KO RA-gastruloids exhibited a shorter body length (Fig. 7b; Supplementary Fig. 18). Upon performing scRNA-seq, we observed a gross excess of SOX2+ neural cells (including neural crest) in TBX6-KO RA-gastruloids (Fig. 7c-e; Supplementary Fig. 19). In contrast, presomitic mesoderm was almost entirely absent. We speculate that this is a secondary effect of the loss of presomitic mesoderm, which may contribute to the appropriate posterior environment for definitive endoderm in the tailbud.

To further characterise the regulatory programs underlying the shift of NMPs toward neural fates in TBX6-KO RA-gastruloids, we examined DEGs in neural lineages (Supplementary Fig. 20a-d,f,h). As expected, many genes involved in neural development were upregulated. Perhaps less expectedly, additional transcription factors normally expressed in the posterior tailbud, including *CDX2* and various *HOX* genes (*HOXA7*, *HOXA9*, *HOXB9*, *HOXC6*, *HOXC8*), were among the most downregulated genes in the neural lineages of TBX6-KO RA-gastruloids, relative to the same lineages in controls. Whether the expansion of neural lineages in TBX6-KO RA-gastruloids is contributed to by transdifferentiation from somitic lineages as seen in mouse models^{37,109} is an outstanding question, potentially addressable with chimeric WT/KO RA-gastruloids, lineage tracing¹⁵⁴, or by a combined knockout-reporter assay³⁷.

Turning to the PAX3-KO RA-gastruloid, although morphologically similar to controls, clear differences were observed upon scRNA-seq profiling. This included an increased proportion of neural cells but a decreased proportion of neural crest (Fig. 7c-g; Supplementary Fig. 19). This is consistent with a previous finding reporting dysregulation of neural crest development in *Pax3*-deficient or *Sploch* mutant mice^{155,156}. Again focusing on neural lineages, we examined DEGs in PAX3-KO RA-gastruloids (Supplementary Fig. 20e,g,i). Consistent with the loss of neural crest, sharply down-regulated genes included neural crest markers (e.g. *SOX9*) as well as drivers of the epithelial-to-mesenchymal transition (e.g. *SNAI2*). Upregulated genes included *PAX7*, *PAX6* and

SFRP2, normally expressed in dorsal progenitor domains 3-6 of the embryonic caudal neural tube^{157,158}, suggesting that the dorsal bias of human RA-gastruloids may be mitigated by knockout of PAX3. Altogether, these observations are consistent with pleiotropic roles for PAX3 in early human development, e.g. including in neural crest cell development, EMT and dorsal-ventral patterning.

Overall, we conclude that RA-gastruloids may serve as a versatile model for studying early human post-implantation development, via both chemical and genetic perturbations.

2.9 Discussion

Here we show that early but limited RA supplementation, together with Matrigel, robustly induces human gastruloids with embryo-like morphological structures, including a neural tube and segmented somites. The early RA pulse appears to balance human NMPs, such that they will later adopt both mesodermal and neural fates. Human RA-gastruloids also progress to a more advanced developmental stage than conventional gastruloids and consistent with that, contain more advanced cell types, including neural crest, neural progenitors, renal progenitors, and myocytes.

Why do conventional human gastruloids, which possess both NMPs and presomitic mesoderm (Fig. 1), fail to form segmented somites even in Matrigel, while Matrigel alone is sufficient to support somitogenesis in mouse gastruloids^{37,45}? We speculate there is a minimum threshold on the size of the axial progenitor pool at the onset of Matrigel-induced somitogenesis. Consistent with this hypothesis, the number of seeded cells is a critical factor in human RA-gastruloid morphogenesis (Fig. 2d, Extended Data Fig. 3c). To assess this further, we examined the cell type composition of conventional human gastruloids vs. axiolooids, somitoids, and segmentoids, based on scRNA-seq data³⁹⁻⁴¹. At 48 hrs, axiolooid, somitoid, and segmentoid models were entirely composed of PSMs (91%, 92%, 82%, respectively) and NMPs (9%, 8%, 18%, respectively), a trend conserved in mouse gastruloid/TLS^{37,159} (Supplementary Fig. 21a-c). However, conventional human gastruloids had far fewer PSMs (~9%) or NMPs (~2%), and a high proportion of cardiac mesoderm-like cells (38%). The latter expressed several BMPs (Supplementary Fig. 21d), which could negatively impact axial progenitor maintenance, including of NMPs^{109,110}, as also observed in the LDN-treated RA-gastruloids (Fig. 6e).

Additional indirect support for our hypothesis can be found in the means by which various groups achieve in vitro human somitogenesis by adding BMP and/or NODAL inhibitors to the pre-treatment medium, such as SB431542 and DMH1 (somitoid³⁹), SB431542 (axiolooid⁴⁰), or LDN183189 (segmentoid⁴¹) (Table S7). As BMP and NODAL signals promote differentiation toward extraembryonic lineages and endoderm^{102,160}, suppressing these signals during pre-treatment

may protect the axial progenitor pool from depletion or exhaustion before the onset of somitogenesis. However, the early inhibition of these signalling pathways might also restrict progenitors' future potential. In contrast, we found an early RA pulse actively induces bipotent NMPs¹¹⁹ during pre-treatment. By avoiding BMP or NODAL inhibitors, we might have inadvertently facilitated the retention of progenitors' potential to later diversify into more advanced cell types, e.g. lateral plate mesoderm¹⁶¹, intermediate mesoderm¹⁶², neural crest¹⁶³, and myocytes¹⁶⁴. Overall, our results suggest that initial/early conditions, including the number of seeded stem cells as well as their signalling state, strongly shape the self-organisation potential of gastruloids and thus what structures and cell types will ultimately develop even at much later timepoints¹⁶⁵. Of note, it remains possible that the dependence on seeding size is due to the impact of gastruloid size on morphogen concentrations and/or gradations, in addition to (or rather than) the absolute number of cells.

While we found "computational staging" to be a powerful approach for benchmarking mammalian embryo models against one another as well as *in vivo* development, there are limitations. First, this approach may be restricted to the specific developmental window from which the human PC2 was constructed (roughly corresponding to E7-E10.5 of mouse development). It is possible that this window could be extended, both earlier and later, with an improved reference dataset. Second, since we used human gastruloids to "fill" temporal windows for which human embryo data was not available while constructing PC space, cell types absent from gastruloids might be problematic to computationally stage. For example, the expression of the anteriormost HOX genes are weaker than posterior HOX genes in gastruloids (Extended Data Fig. 7d), although the contribution of HOX genes to PC staging appears minimal (Supplementary Fig. 12e-f). Altogether, we conclude that computational staging offers a robust and standardised framework for assessing the relative developmental progression of mammalian embryo models, including across cell types and species. Although scRNA-seq does not capture the spatial patterning of embryos/embryo models; we can imagine future versions of computational staging that incorporate spatial transcriptomic information.

There are tremendous possibilities on the horizon for leveraging embryo models to decode early human embryogenesis and to model certain developmental disorders (e.g. neural tube defects). Our results show that compared with alternative embryo models, human RA-gastruloids exhibit more advanced developmental progression (Fig. 4e), greater cell type diversity (Extended Data Fig. 10), more robust formation of embryo-like morphological structures (Fig. 2c; Extended Data Fig. 5-6; Supplementary Fig. 10) and less inter-individual variation (Fig. 5). As we consider how best to

scale reverse genetic approaches to study the phenotypic consequences of thousands of specific coding and regulatory mutations on early human development, these attributes of human RA-gastruloids are attractive.

2.10 Supplementary Materials

Ethics Statement

Our research on the induction and molecular analysis of human conventional and RA-gastruloids was reviewed and approved by the Embryonic Stem Cell Research Oversight (ESCRO) of the University of Washington (E0047-001), and is in compliance with the principles laid out by the ISSCR Guidelines for Stem Cell Research and Clinical Applications of Stem Cells¹⁶⁶. No new experiments involving human embryos were performed in this study, and all natural human embryo data analysed were obtained from publicly available datasets.

Statistics & Reproducibility

For box plots in this study, Upper whisker, upper box edge, bar, and lower box edge represent $1.5 \times$ IQR, third quartile (Q3), median, and first quartile (Q1), respectively. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications^{37,38}. No experimental data were excluded from the analyses. Sequencing data exclusion criteria is outlined in the method section, including filtering out the substandard data in single-cell measurements, following the general practice in the field. Human conventional gastruloids and RA-gastruloids used in experiments were randomly selected from each timepoint before sample preparation. The Investigators were not blinded to allocation during experiments and outcome assessment. Data distribution was assumed to be normal but this was not formally tested.

Human pluripotent stem cell (hPSC) culture

RUES2-GLR hESCs were gifted by Dr. Ali Brivanlou (Rockefeller University). H9 hESCs were obtained from WiCell. WTC11 hiPSCs were gifted by Dr. Bruce Conklin (Gladstone Institutes). RUES2-GLR hESCs were maintained in StemFlex medium (Thermo, A3349401) on Geltrex (Thermo, A1413201). H9 hESCs and hiPSCs were maintained in Essential 8 Flex medium (Thermo, A2858501). hPSCs were routinely passaged using StemPro Accutase (Thermo, A1110501) to new Geltrex-coated wells as recommended by the manufacturer. For the first 24 hrs after passaging, hPSCs were cultured in the medium with 10 μ M Y-27632 (Sellek, S1049) to prevent apoptosis¹⁶⁷.

Conventional human gastruloid induction

For conventional human gastruloid induction, we carefully followed the method described in previous reports^{38,168}. 4×10^4 hESCs were seeded on $0.5 \mu\text{g}/\text{cm}^2$ Vitronectin-coated 12-well plate (Gibco, A14700) in Nutristem hPSC XF medium (Biological Industries, 05-100-1A) with $10 \mu\text{M}$ Y-27632. On day 1, the medium was replaced with Nutristem containing $5 \mu\text{M}$ Y-27632. On day 2, the medium was replaced with Nutristem. On day 3, the medium was replaced with Nutristem containing $3.25 \mu\text{M}$ CHIR99021 (CHIR, Millipore, SML1046). Since CHIR is relatively unstable and can exhibit batch-to-batch inconsistency, we aliquoted it into $10 \mu\text{l}$ volumes, kept at -20°C for up to 3 months, and used freshly thawed CHIR for each gastruloid induction. 24 hrs later, cells were detached by StemPro Accutase and dissociated into single cells. 400 cells were seeded to 96 wells with $40 \mu\text{l}$ Essential 6 medium (Thermo, A1516401) containing $0.5 \mu\text{M}$ CHIR and $5 \mu\text{M}$ Y-27632. At 24 hrs, $150 \mu\text{l}$ of Essential 6 medium was added to each well. At 48 hrs, $150 \mu\text{l}$ of the medium was removed with a multi-channel pipette, and $150 \mu\text{l}$ of Essential 6 medium was added. This medium change was repeated at 72 hrs. Human conventional gastruloids were collected or discarded by 96 hrs.

Human RA-gastruloid induction

A detailed step-by-step protocols can be found here;

<https://dx.doi.org/10.17504/protocols.io.261ge5epog47/v1>. 2×10^4 hESCs were seeded on $0.5 \mu\text{g}/\text{cm}^2$ Vitronectin-coated 12-well plate (Gibco, A14700) in Nutristem hPSC XF medium (Biological Industries, 05-100-1A) with $10 \mu\text{M}$ Y-27632. On day 1, the medium was replaced with Nutristem containing $5 \mu\text{M}$ Y-27632. On day 2, the medium was replaced with Nutristem. On day 3, the medium was replaced with Nutristem containing $3.25 \mu\text{M}$ CHIR (Millipore, SML1046). On day 4, cells were detached by StemPro Accutase and dissociated into single cells. 4,000-5,000 cells were seeded to 96 wells with $50 \mu\text{l}$ Essential 6 medium (Thermo, A1516401) containing $1 \mu\text{M}$ CHIR, 500 nM RA (Millipore Sigma, R2625), and $5 \mu\text{M}$ Y-27632. At 24 hrs, $150 \mu\text{l}$ of Essential 6 medium was added to each well. At 48 hrs, $150 \mu\text{l}$ of the medium was removed with a multi-channel pipette, and $150 \mu\text{l}$ of Essential 6 medium containing 5% Matrigel and 100 nM RA was added and maintained in 37°C , 5% CO_2 incubator until 120 hrs. As with conventional gastruloids, we used freshly thawed CHIR for every RA-gastruloid induction.

For RA-gastruloid induction from the H9 cell line, 4×10^4 H9 hESCs were seeded on $0.5 \mu\text{g}/\text{cm}^2$ Vitronectin-coated 12-well plate in Essential 8 medium with $10 \mu\text{M}$ Y-27632. On day 1, the medium was replaced with Essential 8 containing $5 \mu\text{M}$ Y-27632. On day 2, the medium was replaced with Essential 8 containing $5 \mu\text{M}$ CHIR. On day 3, the medium was replaced with Essential 8 containing 500 nM RA, $5 \mu\text{M}$ CHIR and $10 \mu\text{M}$ SB431542 (Selleck chemicals, S1067).

On day 4, cells were detached by StemPro Accutase and dissociated into single cells. 4,000-5,000 cells were seeded to 96 wells with 50 μ l NDiff 227 medium (Takara, Y40002) containing 1 μ M CHIR and 5 μ M Y-27632. At 24 hrs, 150 μ l of NDiff 227 medium was added to each well. At 48 hrs, 150 μ l of the medium was removed with a multi-channel pipette, and 150 μ l of NDiff 227 medium containing 5% Matrigel and 100 nM RA was added and maintained in 37C, 5% CO₂ incubator until 120 hrs. Human RA gastruloids were collected or discarded by 120 hrs.

For RA-gastruloid induction from the WTC11 cell line, 2×10^4 WTC11 hiPSCs were seeded on 0.5 μ g/cm² Vitronectin-coated 12-well plate in Essential 8 medium with 10 μ M Y-27632. On day 1, the medium was replaced with Essential 8 containing 5 μ M Y-27632. On day 2, the medium was replaced with Essential 8 containing 5 μ M CHIR. On day 3, the medium was replaced with Essential 8 containing 500 nM RA, 5 μ M CHIR and 10 μ M SB431542 (Selleck chemicals, S1067). On day 4, cells were detached by StemPro Accutase and dissociated into single cells. 4,000-5,000 cells were seeded to 96 wells with 50 μ l NDiff 227 medium containing 1 μ M CHIR and 5 μ M Y-27632. At 24 hrs, 150 μ l of NDiff 227 medium was added to each well. At 48 hrs, 150 μ l of the medium was removed with a multi-channel pipette, and 150 μ l of NDiff 227 medium containing 5% Matrigel and 100 nM RA was added and maintained in 37C, 5% CO₂ incubator until 120 hrs.

Chemical perturbation

To perturb signalling pathways in human RA-gastruloids, we added 1 μ M LDN-193189 (STEMCELL Technologies, 72147), 100 ng/ml BMP4 (R&D, 314-BP-010), or 3 μ M CHIR to the medium at 48 hrs along with 5% Matrigel and 100 nM RA.

Genetic perturbation with CRISPR/Cas9 RNPs

RNP complexes were prepared according to the manufacturer's procedures. Briefly, equal molar amounts of crRNA and tracrRNA (IDT, 1072532, listed in Table S8) were mixed and heated at 95C for 5 minutes in a thermal cycler and kept at room temperature for 10-20 min for hybridization. AltR-Cas9 protein (IDT, 1081058) was added to the crRNA and tracrRNA mixture to assemble Cas9-RNPs. RUES2-GLR hESCs were trypsinized with StemPro Accutase and the reaction was quenched with StemFlex media supplemented with 10 mM Y-27632. 2×10^5 cells were transferred to a new tube and centrifuged at 250g for 5 min. Cells were resuspended in 20 μ l nucleofection buffer (16.4 μ l Nucleofector® Solution + 3.6 μ l Supplement) provided in P3 Primary Cell 4D-Nucleofector™ X Kit S (Lonza, V4XP-3032). After the addition of 3 μ l RNP and 0.5 μ l of Alt-R Cas9 Electroporation Enhancer (IDT, 1075915), cells were transferred to 16-well Nucleocuvette™ Strips and nucleofected with the CA-137 program. The nucleofected cells were

transferred to the 12-well plate which contains Nutristem with 10 mM Y-27632 and after 24 hrs, the medium was replaced with Nutristem without Y-27632. Cells were maintained until they reached 50-70% confluence. Then, RNP-introduced cells were transferred onto 0.5 $\mu\text{g}/\text{cm}^2$ Vitronectin-coated 12-well plates. We then proceeded to the RA-gastruloid induction steps described above and collected at 120 hrs for sc-RNA-seq analysis.

Immunostaining of gastruloids

For whole-mount immunostaining, gastruloids were fixed in 4% paraformaldehyde overnight at 4 °C, washed with PBST (0.2% Tween20), soaked in blocking buffer (PBS containing 0.1% BSA and 0.3% Triton X-100) overnight at 4 °C and then incubated with primary antibodies diluted with blocking buffer overnight at 4 °C. The samples were washed with washing buffer (PBS containing 0.3% Triton X-100), incubated with secondary antibodies and DAPI overnight at 4 °C, and washed and mounted in Fluoro-KEEPER antifade reagent (Nacalai USA). For phalloidin staining, Alexa Fluor™ 647 Phalloidin (A22287; Thermo) was added to the secondary antibody at a dilution of 1:400. All samples were analysed with an LSM710 (Zeiss) or LEICA SP8X (Leica) confocal microscope with negative control samples where the primary antibodies were not added. The antibodies used in this study are listed in Table S9.

In situ hybridization chain reaction (HCR)

HCR and following measurements of UNCX4.1 and TBX18 were performed as described previously^{39,40,45,169}. RA-gastruloid was fixed with 4% PFA at 4C overnight. After the wash with PBST (PBS + 0.1% Tween 20), RA-gastruloid are dehydrated with MeOH and kept in -20C for longer storage. Probes are designed by Molecular Instruments Inc. on TBX18 (Accession NM_001080508.3, hairpin B1), UNCX4.1 (Accession NM_001080461.3, hairpin B2), HOXA1 (Accession NM_005522.5, hairpin B1), HOXA5 (Accession NM_019102.4, hairpin B2), and HOXA10 (Accession NM_018951.4, hairpin B3). Hairpin B1 was labelled with Alexa 488, B2 was labelled with Alexa 546, and B3 was labelled with Alexa 647. Images were taken with the Nikon A1R system. For the quantification of the TBX18/UNCX4.1 staining, Fiji with ImageJ v2.14.0 package was used. Intensities of each channel were quantified along the longitudinal axis of RA-gastruloids with the segmented line tool, with a 50 line width. Subsequently, a LOESS smoothing function was applied to smooth the intensity data with the parameter $f=0.075$ in R. Intensity data were normalised to their respective means to facilitate direct comparisons. Differences between TBX18 and UNCX4.1 intensities were then calculated, plotted and used for the peak detection using findpeaks function from the pracma package with a minimum peak distance of 60. Plots were made with the ggplot package.

Morphometric quantification

Imaging of both conventional and RA-gastruloids was carried out using a DMI8 inverted microscope (Leica). Subsequent image analysis was performed with the ImageJ software package, specifically the Fiji distribution. Prior to quantification, images underwent level and contrast adjustments to enhance the visibility of structural features. For each experimental condition and time point, at least three gastruloids ($N \geq 3$) were subjected to analysis. Data from the morphometric quantification were visualised via custom R code, available on our GitHub site (<https://github.com/shendurelab/Human-RA-Gastruloid/>).

Neural tube quantification

For neural tube analysis, the segmented line tool in Fiji was used to measure the length of the SOX2-positive area. The centre of this area was also determined with the same tool. Neural tube width was quantified at three distinct positions along the length of the SOX2-positive area: 10%, 50%, and 90%, from the posterior to the anterior end, using the straight-line tool. The widths obtained at these positions were averaged to calculate the average neural tube width for each gastruloid. The Length-to-Width (L/W) ratio of the neural tube was calculated by dividing the length by the average width.

Whole body quantification

For whole-body morphometric analysis, the segmented line tool was employed to measure the total length of the area captured in the bright field. Whole-body width was measured at three standardised positions (10%, 50%, 90%) along the length from posterior to anterior ends, and these measurements were averaged to calculate the average whole body width. The L/W ratio for the whole body was determined similarly to the neural tube L/W ratio.

Somite quantification

For the morphometric analysis of somites, the length, width, and area were measured on one side of the somites based on their order from the posterior end. Length and width were quantified using the straight-line tool, and area was measured with the polygon selection tool. To assess the frequency of somite pairing in RA-gastruloids, first, we checked whether there are two rows of somite structures within a single RA-gastruloid. Then, we defined putative somite pairs based on their positional order (e.g. the third somite from the posterior end on both sides) along the posterior-to-anterior axis. Next, we checked whether members of each putative somite pair have similar sizes (area). Somites with areas exhibiting 70% to 130% similarity were classified as "paired somites". This comparison was made for 3 randomly chosen putative somite pairs within each gastruloid. A gastruloid was

subsequently designated as "paired gastruloid" if at least 2 out of 3 putative somite pairs were classified as "paired somites".

Quantification of epithelialization

120 hrs RA-gastruloids were immunostained with Phalloidin with anti-SOX1 antibody and anti-PAX3 antibody (listed in Table S9). We defined a "gastruloid with epithelialized somite or neural tube" as ones in which at least one epithelialized somite or neural tube that exhibited accumulation of Phalloidin staining at the apical side of its SOX1 or PAX3-positive cells. N = 11 and 10 for gastruloids with 2nd RA pulse and without 2nd RA pulse, respectively.

Live imaging of human RA-gastruloids

After supplementation with Matrigel at 48 hrs, RA-gastruloids were promptly placed under a microscope (DMi8, Leica). They were subsequently stored in a humidified CO₂-chamber for an additional 48 hrs. Images were taken at consistent 30-minute intervals. To achieve a comprehensive representation of the whole RA-gastruloid structure, tiled images were later combined and converted to movies using a custom macro. The custom macro is available on GitHub (<https://github.com/shendurelab/Human-RA-Gastruloid/>). Based on the movie, segmentations were manually determined based on the emergence of the boundaries of somites at the posterior region.

Single-cell RNA-seq of gastruloids

Gastruloids (N = 48-96) were collected from 96 wells using a micropipette, transferred to PBS (-) to wash out the medium and incubated with 0.05% Trypsin-EDTA for 8 min at 37°C. After quenching the reaction by the addition of 1/10 volume of FBS, gastruloids were dissociated into single cells by repeated pipetting. For the 0 hrs sample, pre-treated PSCs were washed with PBS (-) once and dissociated into single cells with StemPro Accutase. Dissociated single-cell suspensions were loaded in Chromium Next GEM Chip G Single Cell Kit (Chromium, PN-1000120) on a 10X Chromium controller according to the manufacturer's instructions. Single-cell RNA-seq libraries were generated with Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1 (Chromium, PN-1000121) and Dual Index Kit TT Set A, 96 reactions (Chromium, PN-1000215). Library concentrations were determined by Qubit (Invitrogen) and the libraries were visualised by TapeStation (Agilent). All libraries were sequenced on NextSeq 2000 (Illumina) (Read 1: 28 cycles, Read 2: 85 cycles, Index 1: 10 cycles, Index 2: 10 cycles).

Processing of sequencing reads

Base calls were converted to fastq format using the cellranger v6.0.0¹⁷⁰ mkfastq function. The sequencing reads are then demultiplexed based on i5 and i7 barcodes, mapped to hg38 reference

genome and assigned to GRCh38 (GENCODE v32/Ensembl 98) genes with cellranger v6.0.0 count function with default settings.

sc-RNA-seq analysis

The filtered feature barcode matrices generated by cellranger were loaded into R and converted to Seurat objects with Seurat v4.1.1¹⁷¹. The thresholds for UMI counts and feature counts were determined by the valley in the bimodal distribution of UMI counts and feature counts, respectively. Cells showing upper outlier values in the violin plot of UMI counts and feature counts are also excluded to remove potential doublets. To further ensure doublets were removed, Scrublet was used to calculate a doublet score for each cell. Cells with doublet scores higher than the simulated threshold value by Scrublet are potential doublets with two different cell types and were excluded from the dataset. Parameters for filtration of low quality cells and doublets are described in Table S10. As we observed strong cell cycle effects in our dataset after initial dimensionality reduction, we decided to remove cell-cycle related genes¹⁷² (specifically, genes with prefix HIST, MT, TOP, CDK, CCN, CDC, CCDC, MKI; genes containing MALAT, AUR and genes with suffix NUSAP, SMC, CENP, UBE, SGO, ASPM, PLK, KPN, RP, PTTG, SNHG, CK, BUB, KIF, KCNQ, SMO, HMG, S100, LINC, ATP, IGFBP, HSP, FOS, JUN). After quality control, each dataset was log-normalised, and heterogeneity associated with cell cycle stage and mitochondrial contamination were regressed out with SCTranform. The top 3000 variable genes identified by SCTranform were then used for dimensionality reduction by PCA. The first 30 PCs were used for dimensionality reduction by UMAP. Cells were clustered by graph-based clustering algorithms. In particular, 30 PCs were used to compute the 20-nearest neighbours of each cell to construct the shared nearest neighbour (SNN) graph. Then clusters were identified with the Louvain algorithm with a resolution of 0.8. SNN construction and clustering are implemented through the FindNeighbors and FindClusters functions in the Seurat package with default settings for all other parameters. Samples with two batches (e.g. 72 hrs conventional gastruloids, 120 hrs RA-gastruloids) were integrated with Harmony with the top 2,000 highly variable genes. Time-course sc-RNA-seq datasets were integrated with Harmony as implemented in Seurat. Briefly, datasets from each timepoint were merged together. The merged dataset was log-normalised and the top 2,000 variable genes were selected for downstream integration. Then, the merged dataset was z-transformed and heterogeneity associated with cell cycle stage and mitochondrial contamination were regressed out. Dimensionality reduction by PCA was implemented to generate a low-dimensional embedding of cells in the merged dataset. The low-dimensional embedding was then input into Harmony to correct for batch effects among samples collected from different experiments.

To compare conventional human gastruloid cell types with mouse gastruloids⁴⁵/mouse TLS³⁷ cell types, a lift-over list of mouse and human genes was downloaded from BioMart-Ensembl (Ensembl Genes 102). The gene features in the mouse and human datasets were converted to a list of human and mouse orthologous pairs. The relationship of multiple homologs to one gene in the other species were retained in this case. After feature conversion, human and mouse gastruloid datasets were integrated with Seurat V3 integration algorithm¹⁷³. Specifically, human and mouse datasets were log-normalised and z-transformed separately. The top 3,000 variable genes in both datasets were selected for integration. 30 CCA PCs were used to search for integration anchors. The integrated dataset was then subjected to dimensionality reduction by PCA. The top 30 PCs were used for UMAP visualisation with min.dist of 0.3. We used default settings for all the other parameters. We used the same strategy for integration of conventional human gastruloids and mouse embryos (E6.5-E8.5)¹², except we used reciprocal PCA for dimensionality reduction (reduction method = rpca) for FindIntegrationAnchors to reduce the computing time.

To compare cell types between conventional human gastruloids vs. human CS7 embryos¹⁶, we first downsampled each cell type in conventional human gastruloids to 63 cells/cell type because the large discrepancy of cell numbers between gastruloid (8,000-19,000 cells in each dataset) vs. embryo (1,195 cells) datasets. We noticed that the large discrepancy of cell numbers could lead to overfitting of cell types between two datasets because dimensionality reduction will be largely driven by the datasets with many more cells. After downsampling, the gastruloid and embryo datasets were merged. The merged dataset was log-normalised and the top 3,000 variable genes selected for downstream integration. Then, the merged dataset was scaled and heterogeneity associated with cell cycle stage and the mitochondrial contamination were regressed out. Dimensionality reduction by PCA was implemented to generate a low-dimensional embedding of cells in the merged dataset. The low-dimensional embedding was then inputted into Harmony to correct for batch effects among samples. Top 30 PCs from Harmony were used for UMAP visualisation with min.dist of 0.3.

Cell type assignment with non-negative least squares (NNLS)

Cell types in gastruloids were compared with cell types from corresponding lineages in mouse embryos (E8.5-E10.5). Orthologous genes were identified in both datasets and converted to orthologous pairs as described above. Nonnegative least-squares regression (NNLS) was applied to predict gene expression in target cell type (T_a) in dataset A based on the gene expression of all cell types (M_b) in dataset B, $T_a = \beta_{0a} + \beta_{1a}M_b$, based on the union of the 200 most highly expressed genes and 200 most highly specific genes in the target cell type. Datasets A and B were then switched for

prediction; that is, predicting the gene expression of target cell type (T_b) in dataset B from the gene expression of all cell types (M_a) in dataset A, $T_b = \beta_{0b} + \beta_{1b}M_a$. Finally, for each cell type a in dataset A and each cell type b in dataset B, two correlation coefficients, $\beta = 2(\beta_{ab} + 0.001)(\beta_{ba} + 0.001)$, were combined to obtain a statistic for which high values reflect reciprocal, specific predictivity. The β scores were then subjected to a z-score scaling across all selected cell types.

Comparative analysis of neural differentiation trajectories

Cynomolgus monkey sc-RNA-seq data was downloaded from GEO (GSE193007). Spinal cord cells from CS11 in the first batch were selected for later comparison. To accommodate the fact that the neural tube in human RA-gastruloids are dorsally biased, as reported in other gastruloid models (e.g. TLS³⁷), we excluded neural tube cells of monkey embryos with ventral markers (PHOX2B+, FOX2A+, TFF3+) from this analysis. Highly variable genes for trajectory analysis were calculated following methods in the original study. After log-normalisation, we selected 2,000 highly variably expressed genes by the FindVariableFeatures function in Seurat with 'mean.var.plot'. Spinal cord cells were then sub-clustered with default settings of Seurat and annotated using marker genes from the original study. The raw count matrix was then imported into Monocle2 for pseudotime analysis. The raw count matrix was normalised by size factors. The 2,000 highly variable genes were selected for dimensionality reduction with DDRtree. The cells were then ordered by orderCells in Monocle2 and each assigned a pseudotime value. By examining distribution of cell types along the resulting trajectory, only the trajectory that leads to neural progenitor cells were selected for differentially expressed gene analysis (DEG). The DEG analysis was performed with the VGAM package by modelling gene expression as a smooth, non-linear function of pseudotime. Genes with q-value less than 0.05 were defined as DEGs along the neural differentiation trajectory of cynomolgus monkeys.

Neural differentiation trajectories were inferred for human RA-gastruloids in the first batch with the same methods as cynomolgus monkeys using the 2,000 highly variable genes calculated from the sc-RNA-seq analysis step. DEGs of neural differentiation trajectories in human RA-gastruloids were computed in the same way as for cynomolgus monkey embryos.

The dynamics of DEGs in cynomolgus monkey embryos embryo and human RA-gastruloids were compared between species. In particular, DEGs from one species were clustered by hierarchical clustering (ward.D2) into 2 groups (up-regulated, down-regulated) according to their dynamics in cynomolgus monkey embryos and human RA-gastruloids. Based on their group assignments in both species, DEGs were then classified into 6 categories: up-up, down-down,

up-down, down-up, up-unexpressed, down-unexpressed. Unexpressed means transcripts of this DEG are not detected in the other species.

Staging of in vitro embryo models

We downloaded the following data; 10x Genomics sc-RNA-seq data from CS12-16 human embryos (GSE157329)¹⁹, Smart-seq2 sc-RNA-seq data from a CS7 human embryo (<http://www.human-gastrula.net>)¹⁶, 10x Genomics sc-RNA-seq data from CS8-11 cynomolgus monkey embryo (GSE193007)¹²⁹, 10x Genomics sc-RNA-seq data from mouse embryos E6.5-8.5 (E-MTAB-6967)¹⁷⁴, sci-RNA-seq data from mouse embryos E8.5-13.5 (GSE186069 and GSE186068)^{13,14}, 10x Genomics sc-RNA-seq data from mouse gastruloids (GSE123187)⁴⁵, TLS (GSE141175)³⁷, somitoids (ERX7494538)³⁹, axioloids (GSE199576)⁴⁰, segmentoids (GSE195467)⁴¹, EMLO (GSE166603)⁴² and EO (GSE155383)¹²⁷. As human gastruloids are composed of only embryonic lineages, we excluded cells derived from extra-embryonic tissues from these analyses. Extraembryonic tissues were excluded from all embryo datasets. As described above, cell cycle and mitochondrial genes were removed. Since CS7 data, which was generated with the Smart-seq2 technology¹⁶, had significant variations in library depth between single cells and didn't have the UMI counts, additional normalisation with Seurat V4.1¹⁷¹ NormalizeData function was performed. After this normalisation, the read distribution for each cell approximated a Poisson distribution with a similar library size range as UMI counts from other datasets.

Where possible, all embryo datasets were pseudobulked by embryo. Specifically, all UMI counts connected to a particular embryo sample (or embryo sample, in the case of pools) were added together for each gene. Human and mouse gastruloids were pseudobulked by sample (as these derive from multiple embryos). Specifically, all UMI counts connected to a particular gastruloid sample were added together for each gene.

Since we are mainly focusing on human early embryonic development, highly variable genes from human embryo developmental datasets^{16,19} were used for downstream analyses. For CS12-16 embryos, highly variable genes were calculated based on Poisson distribution¹⁷⁵ and a total of 1,361 highly variable genes were obtained. We then performed an intersection of CS12-16 embryos' highly variable genes with features in CS7 embryos, human and mouse gastruloid samples. The intersection resulted in a total of 447 human genes for dimensionality reduction with PCA. The pseudobulk transcriptomes of human embryos CS12-13, human gastruloids (0-96 hrs) and human RA-gastruloids (96-120 hrs) were used to construct a PC space with these 447 human genes. Specifically, the pseudobulk matrix was normalised by sequencing depth, log-transformed and z-transformed by Seurat V4.1¹⁷¹. PCA analysis was performed on the normalised matrix. To

accomplish cross-species comparison, we then converted the 447 human genes to human and mouse orthologous pairs as described above. The raw count pseudobulk matrix of mouse embryos, cynomolgus macaque embryos, human embryo models and mouse embryo models were combined with human raw count matrix. The resulting matrix was normalised as described above and projected onto the human-derived PC space by multiplying the loadings of the human-defined PCs to the normalised matrix. We then computed the Spearman's correlation between ground truth developmental time and PC values of mouse embryo samples for the top 7 PCs. Based on the highest correlation of mouse developmental time (i.e. somite counts and embryonic days) and PC values, PC2 was selected to represent developmental time. The normalised expression of genes in each sample were subjected to Pearson's correlation with the PC2 embeddings of the sample in order to obtain top genes highly correlated or anti-correlated with PC2.

Lineage-specific staging of in vitro models

All embryo datasets were separated into cell types based on annotations from the original papers. Where possible, each cell type were pseudobulked by embryo sample (or embryo samples, in the case of pools). Specifically, all UMI counts connected to a particular cell type in a particular embryo sample were aggregated for each gene. Human and mouse gastruloids were also separated by annotated cell types. Each cell type is pseudobulked by gastruloid samples (as these derive from multiple embryos). Specifically, all UMI counts connected to a particular cell type in a particular gastruloid sample were aggregated for each gene.

We then projected mouse embryo cell types at E8.5-10.5 onto the PC space constructed from human whole embryo samples and human gastruloids, described above. Human or mouse gastruloid cell types were projected to the same PC space to evaluate their progression relative to related cell types.

Evaluation of inter-variation of cell type composition of human RA-gastruloids and mouse gastruloids based on single-cell RNA-seq from individual gastruloids

sc-RNA-seq data for individual 120 hrs human RA-gastruloid were obtained by sciPlex sc-RNA-seq method¹³⁹. The 120 hrs mouse gastruloid data were downloaded from the public dataset GSE212050. To account for differences in the total number of cells per individual gastruloid, we performed random sampling. Specifically, 100 cells were randomly sampled from each individual gastruloid in both the human and mouse datasets. This sampling was performed independently ten times per gastruloid to generate pseudo-replicates. For each cell type, within each replicate and species, the standard deviation (S.D.) of the frequencies was calculated. An Analysis

of Variance (ANOVA) was subsequently performed for each cell type to test for significant differences in S.D. values between human and mouse samples. If the ANOVA was significant ($p < 0.05$), a post-hoc Tukey's Honest Significant Difference (HSD) test was conducted to further evaluate pairwise differences between the species. All statistical analyses were executed using a native stats package in R.

Data availability

Raw sequencing data and processed single cell datasets that support the findings in this paper are available in the Gene Expression Omnibus (GEO) under accession GSE208369. The hg38 reference genome was used for mapping human-derived sequencing reads. Reanalyses of previously published datasets were also deposited to GEO, again under accession GSE208369. These published datasets were originally downloaded from the following sources: 10x Genomics scRNA-seq data from CS12-16 human embryos (GSE157329)¹⁹, Smart-seq2 scRNA-seq data from a CS7 human embryo (<http://www.human-gastrula.net>)¹⁶, 10x Genomics scRNA-seq data from CS8-11 cynomolgus monkey embryos (GSE193007)¹²⁹, 10x Genomics scRNA-seq data from mouse embryos E6.5-8.5 (E-MTAB-6967)¹⁷⁴, sci-RNA-seq3 data from mouse embryos E8.5-13.5 (GSE186069 and GSE186068)^{13,14}, 10x Genomics scRNA-seq data from mouse gastruloids (GSE123187)⁴⁵, TLS (GSE141175)³⁷, somitoids (ERX7494538)³⁹, axioids (GSE199576)⁴⁰, segmentoids (GSE195467)⁴¹, EMLO (GSE166603)⁴² and EO (GSE155383)¹²⁷. Source data are provided with this study. All other data supporting the findings of this study are available from the corresponding author on reasonable request

Code availability

Code for analysing the single cell datasets and performing morphometric measures is available at <https://github.com/shendurelab/Human-RA-Gastruloid/>.

2.11 Figures

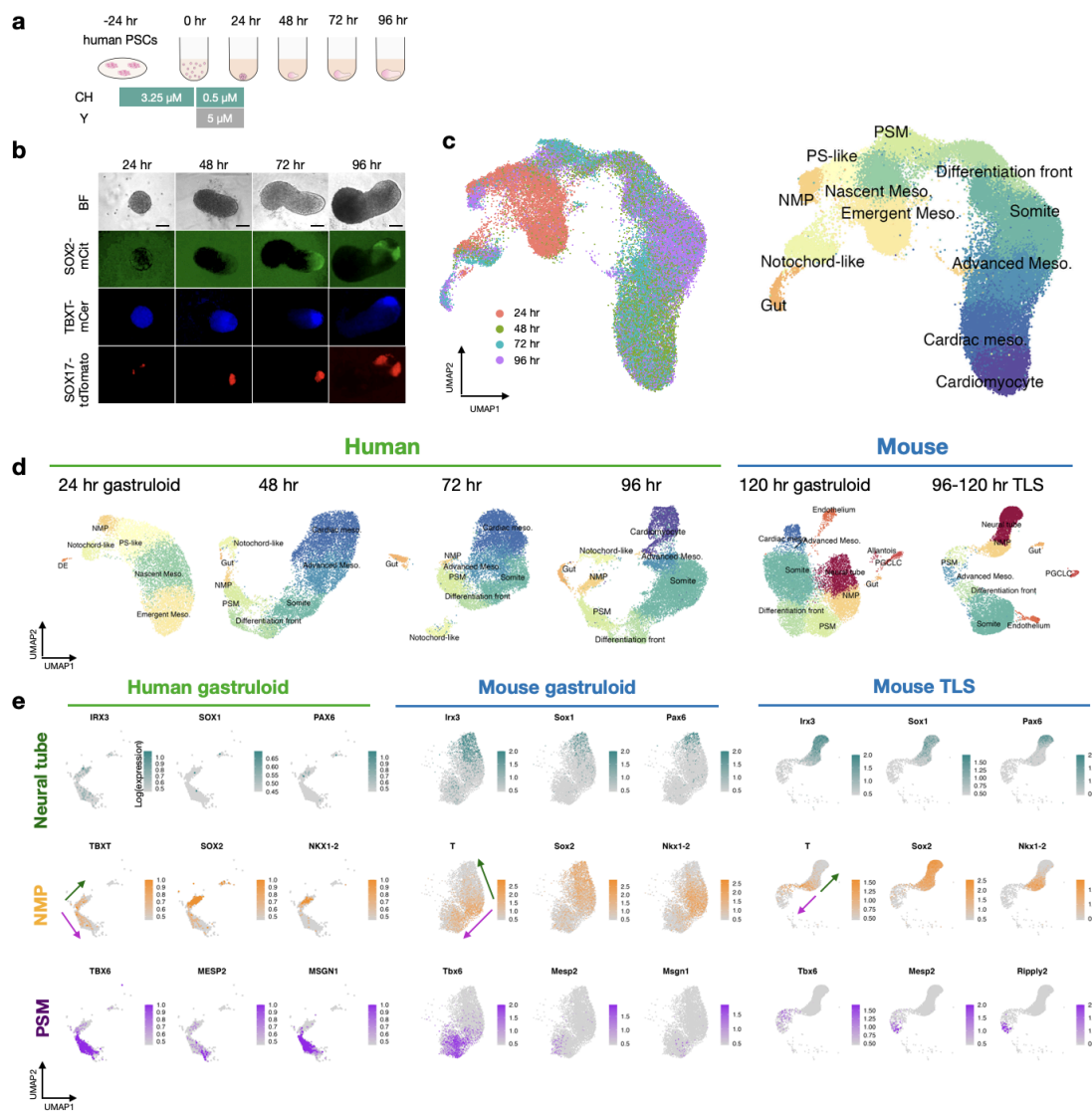


Figure 2.1: Figure 1. Single cell transcriptional profiling of a time-series of conventional human gastruloids.

a, Schematic of conventional human gastruloid protocol³⁸. CH, CHIR99021; Y, Y-27632. b,

Representative images of RUES2-GLR¹²⁶ hPSC-derived human gastruloids. The experiments were

repeated independently six times with similar results. SOX2-mCit, pluripotent and ectoderm marker; TBXT-mCer, mesoderm marker; SOX17-tdTomato, endoderm marker. Scale bar, 100 μ m. c, Integrated UMAP of ~44,000 scRNA-seq profiles from four timepoints of human gastruloid development, coloured by timepoint (left) or cell type annotation (right). 48 to 96 gastruloids were sequenced per time point. NMP, neural mesodermal progenitor; PSM, presomitic mesoderm; PS, primitive streak. d, UMAP projection of scRNA-seq profiles from individual timepoints of human gastruloids (generated by this study based on published protocols³⁸) or published data from mouse gastruloids⁴⁵ or mouse TLS³⁷. PGCLC, primordial germ cell-like cell; DE, definitive endoderm. e, Normalised expression of marker genes for neural tube (upper row), NMPs (middle row), or PSM (lower row) in UMAP projections of scRNA-seq profiles of extracted cell types (neural tube, NMP and PSM cells) from human gastruloids at 96 hrs, mouse gastruloids at 120 hrs⁴⁵, or mouse TLS at 120 hrs³⁷. Arrows represent putative differentiation of NMPs towards neural tube (green) and PSM (purple) fates, respectively. The key point is that NMP-like and PSM-like cells are detected in all three models, but neural tube-like cells are detected only in the two mouse models.

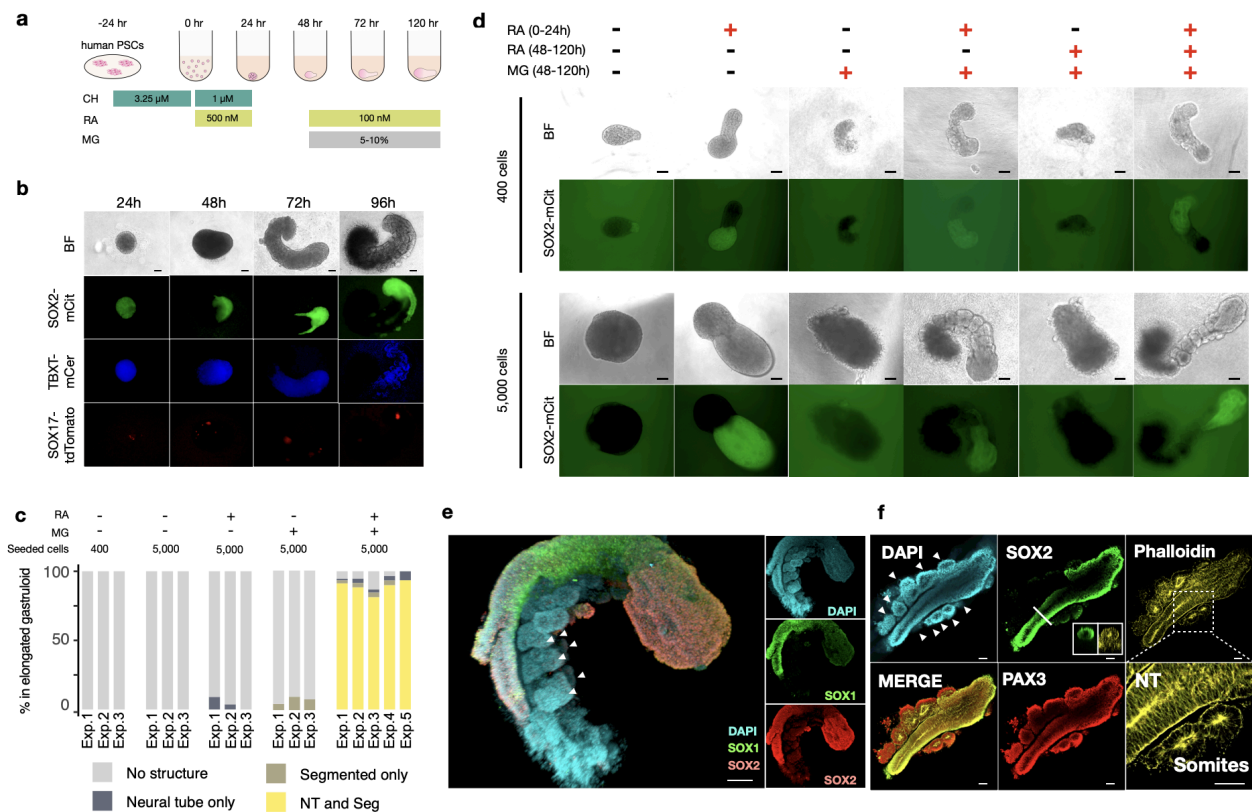


Figure 2. Robust induction of human gastruloids with both a neural tube and segmented somites via a discontinuous regimen of retinoic acid.

a, Schematic of human RA-gastruloid protocol. RA, retinoic acid; MG, Matrigel; CH; CHIR99021. RA was applied for the first 24 hrs after induction, then withdrawn, then added back at 48 hrs along with 5-10% Matrigel. b, Representative images of developing human RA-gastruloids. Scale bar, 100 μ m. N = 768 (96 x 8 plates) human RA-gastruloids showed similar morphology (elongated gastruloid with flanking somites) and patterns of marker gene expression (asymmetric, elongated SOX2-mCit⁺ signal flanked by non-overlapping weak TBXT-mCerulean signal overlaying somites). c, Quantification of the frequency of neural tube elongation and somite segmentation under various experimental conditions. NT, Neural tube; Seg, segmented somites. d, Representative images of gastruloids with or without RA/Matrigel from 400 cells (Top) or 5,000 cells (Bottom). The concentration of RA at 0-24 hrs and 48-120 hrs was 500 nM and 100 nM, respectively. N = 24-48 per condition. The concentration of Matrigel was 5%. Scale bar, 100 μ m. e, 3D projections of immunostained 120 hrs RA-gastruloids. The SOX1⁺, SOX2⁺ region corresponds to the neural tube-like structure, flanked by somite-like structures. Arrowheads indicate paired somites. N = 12/13 human RA-gastruloids showed similar morphology (elongated gastruloid with flanking somites) and patterns of marker gene expression (asymmetric, elongated, coincident SOX1 and SOX2 staining that did not extend to flanking somites). Scale bar, 100 μ m. f, Confocal section of immunostained 120 hrs RA-gastruloid. Phalloidin staining shows the apical accumulation of F-actin in SOX2⁺, PAX3⁺ neural tube and PAX3⁺ somites. Slice images of the area indicated in the bold line are shown in the SOX2 staining image. The magnified region in the Phalloidin staining image is indicated by a dotted square. Arrowheads indicate paired somites. Scale bar, 100 μ m. N = 9/11 human RA-gastruloids showed similar morphology (elongated gastruloid with flanking somites) and patterns of marker gene expression (asymmetric, elongated SOX2 staining flanked by PAX3 staining of flanking somites).

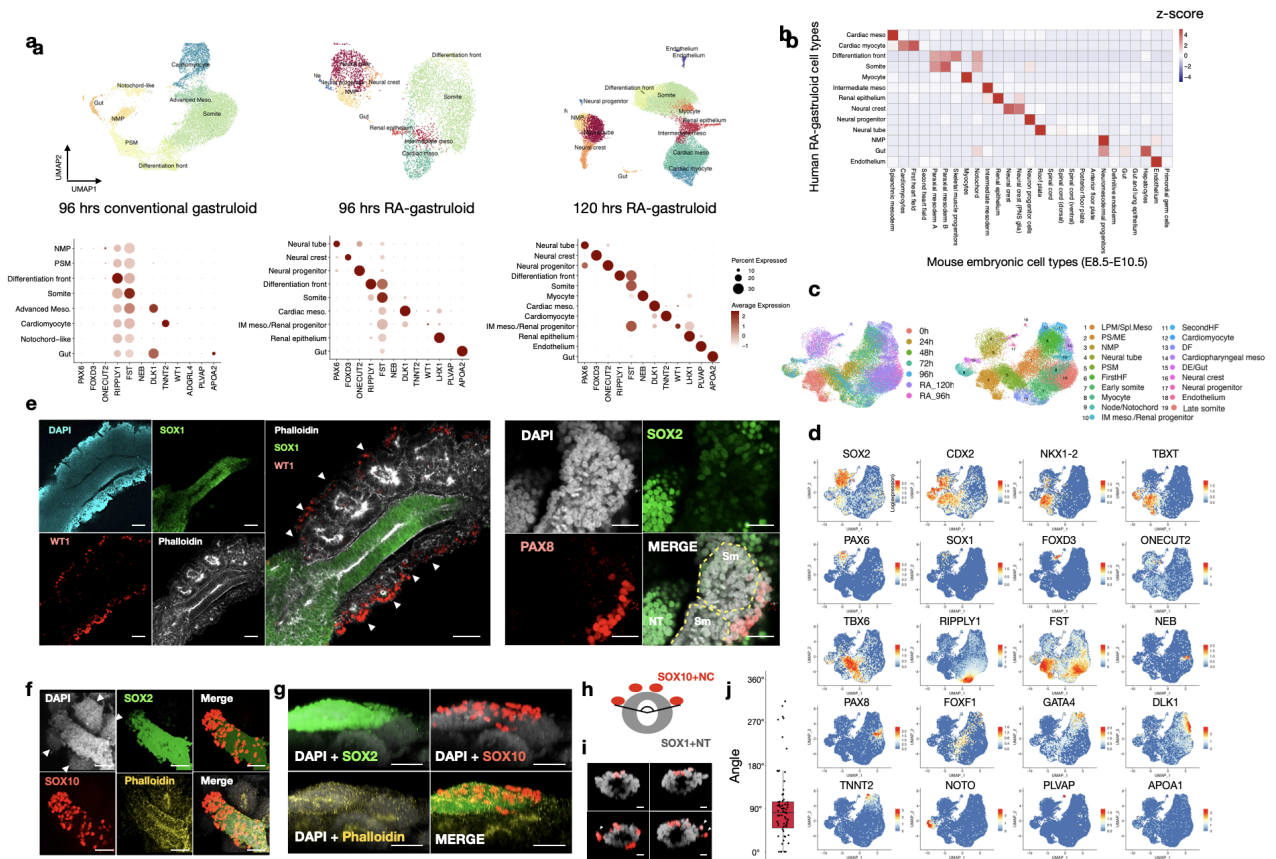


Figure 3. Induction of neural crest, IMM and other advanced cell types in human RA-gastruloids.

a, (upper) Annotated UMAP of scRNA-seq profiles from conventional (96 hrs) or RA (96 or 120 hrs) human gastruloids. 48 to 96 gastruloids were sequenced per time point. PSM, presomitic mesoderm; DF, differentiation front; NMP, neuromesodermal progenitor; IM meso., intermediate mesoderm. (lower) Marker gene expression. b, Cell type mapping of 120 hrs human RA-gastruloids against mouse embryonic datasets^{13,14} via non-negative least squares regression. c, Integrated UMAP of scRNA-seq data from conventional (0-96 hrs) and RA (96-120 hrs) human gastruloids. LPM/Spl.meso, lateral plate mesoderm/splanchnic mesoderm; PS/ME, primitive streak/mesoendoderm; FirstHF, first heart field; IM meso/renal progenitor, intermediate mesoderm/renal progenitor; SecondHF, second heart field; DF, differentiation front; DE/Gut, definitive endoderm/gut. d, Marker gene expression. e, Immunostaining of IMM and renal epithelium in 120 hrs human RA-gastruloids. (left), Anti-WT1 (red, IMM), anti-SOX1 (green, neural tube), phalloidin (white, F-actin) or DAPI (cyan, nuclear) staining. WT1+ IMM-like cells appear lateral to the phalloidin-stained somites. Scale bar, 100 μ m. (right) Anti-PAX8 (red, renal epithelium), anti-SOX2 (green, neural tube) or DAPI (cyan, nuclear) staining. Scale bar, 100 μ m. f, Immunostaining of IMM and renal epithelium in 120 hrs human RA-gastruloids. g, Immunostaining of IMM and renal epithelium in 120 hrs human RA-gastruloids. h, Immunostaining of IMM and renal epithelium in 120 hrs human RA-gastruloids. i, Immunostaining of IMM and renal epithelium in 120 hrs human RA-gastruloids. j, Scatter plot of Angle vs. SOX10+NC.

Arrowheads indicate paired somites. N = 4/4 and N = 3/4 gastruloids showed similar patterns of marker gene expression, respectively (punctate WT1 or PAX8 staining at the lateral border of somites). Sm, somite; NT, neural tube. f-j, Immunostaining of neural crest-like cells in 120 hrs RA-gastruloids. f, 3D projection of somite and neural tube with anti-SOX2 (green, neural cells), anti-SOX10 (red, neural crest), phalloidin (yellow, F-actin) or DAPI (white, nuclear) staining. Arrowheads indicate paired somites. g, Lateral views of human RA-gastruloids. Scale bar, 50 μ m. N = 11/12 gastruloids showed similar patterns of marker gene expression (punctate SOX10 staining asymmetrically localised on one surface of putative neural tube). h, Quantification of distribution of SOX10⁺ neural crest cells on neural tubes. Two straight lines were drawn from the centre point of a neural tube toward the outermost SOX10⁺ cells. NT, neural tube; NC, neural crest. i, Representative images of sliced neural tube images. Arrowhead indicates migrating SOX10⁺ cells. Scale bars, 10 μ m. The experiments were repeated independently three times with similar results. j, Boxplot showing the distribution of the angle encompassing all SOX10⁺ cells observed on the surface of a given gastruloid. N = 71.

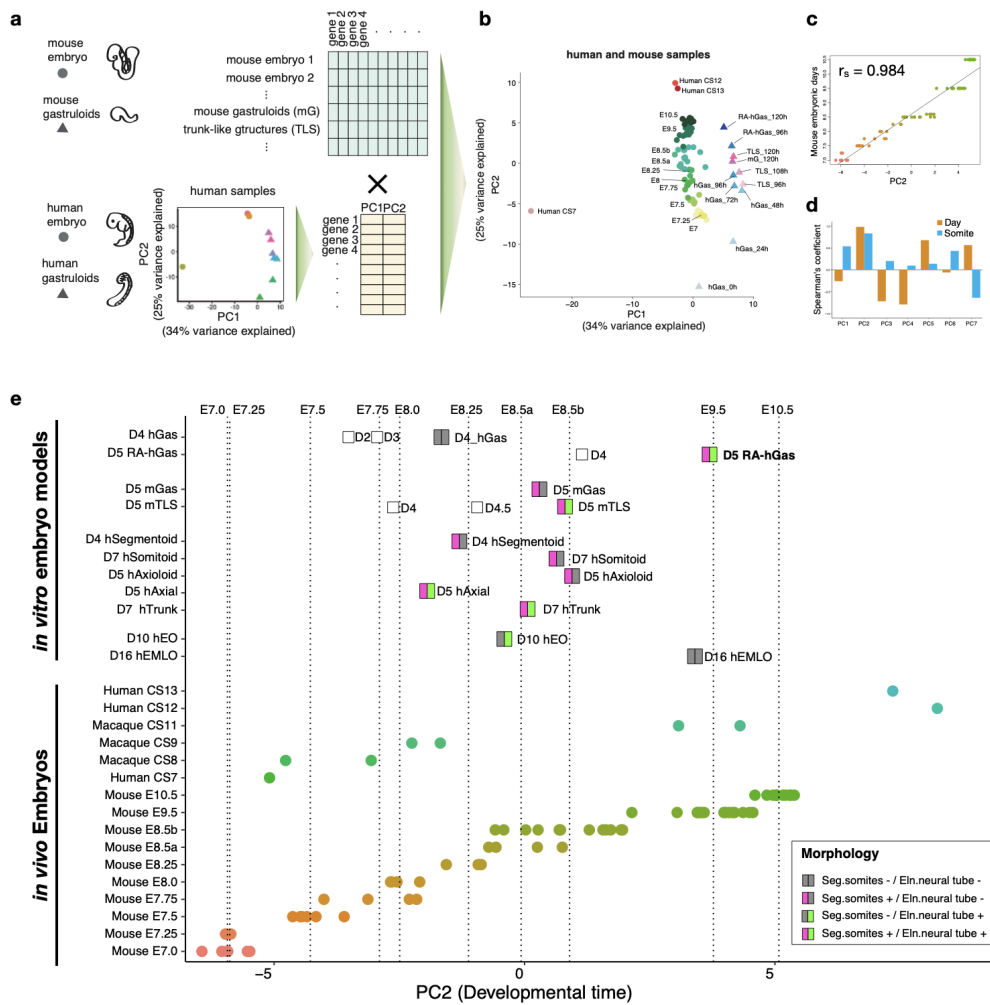


Figure 4. Computational staging of human RA-gastruloids and other mammalian synthetic embryo models.

a, Schematic of strategy for computational staging. In brief, PCA on human samples defines a principal component (PC) correlated with developmental progression (PC2). Projection of data from tightly staged mouse embryo data onto this human-defined PC enables staging of the relative progression of synthetic embryo models across species and systems. b, Projection of data from pooled mouse gastruloids (mG, 120 hrs), pooled mouse TLS (96, 108 and 120 hrs), and individual^{13,14} or pooled¹⁷⁴ mouse embryos (E7.0-E10.5) onto PC space defined by the analysis of human data (CS, carnegie stage human embryos; hGas, conventional human gastruloids; RA-hGas, human RA-gastruloids). c, Scatterplot and Spearman's correlation of mouse embryos' PC2 values (x-axis) and their embryonic stage (y-axis; E7.0-10.5). A fitted regression line from a linear model is plotted. r_s , Spearman's correlation. d, Spearman's correlation of mouse embryos' PC values for various human-defined PCs, focusing either on mouse embryo day (E7.0-10.5) or somite count

(0-12 somites). e, Pseudo-bulk transcriptomes of pooled human embryo models including RA-gastruloids (RA-hGas, 96, 120 hrs), conventional human gastruloids (hGas, 48, 72, 96 hrs)³⁸, human somitoids (hSomitoid, day 7)³⁹, human segmentoids (hSegmentoid, day 4)⁴¹, human axioids (hAxioid, day 5)⁴⁰, human axial organoids (hAxial, day 5)¹⁰⁵, human trunk-like organoids (hTrunk, day 7)¹⁰⁴, EMLO gastruloids (hEMLO, day 16)⁴², human EOs (hEO, day 10)¹²⁷; pooled mouse embryo models including gastruloids (mGas, day 5)⁴⁵ and TLS (mTLS, day 5)³⁷; individual human embryos at CS7, CS12, and CS13^{16,19}; individual macaque embryos at CS8, CS9 and CS11¹²⁹, and individual^{13,14} or pooled¹⁷⁴ mouse embryos, projected onto the human-derived PC2. For mouse embryos, E8.5 and earlier samples are pooled embryos profiled with 10X Genomics scRNA-seq¹⁷⁴, while E8.5b and later samples are individual mouse embryos profiled with sci-RNA-seq3^{14,176}. The dotted lines indicate the median PC2 values of mouse embryos at each embryonic day. Embryo models are coloured by the presence/absence of the morphologies of segmented somites and/or an elongated neural tube structure.

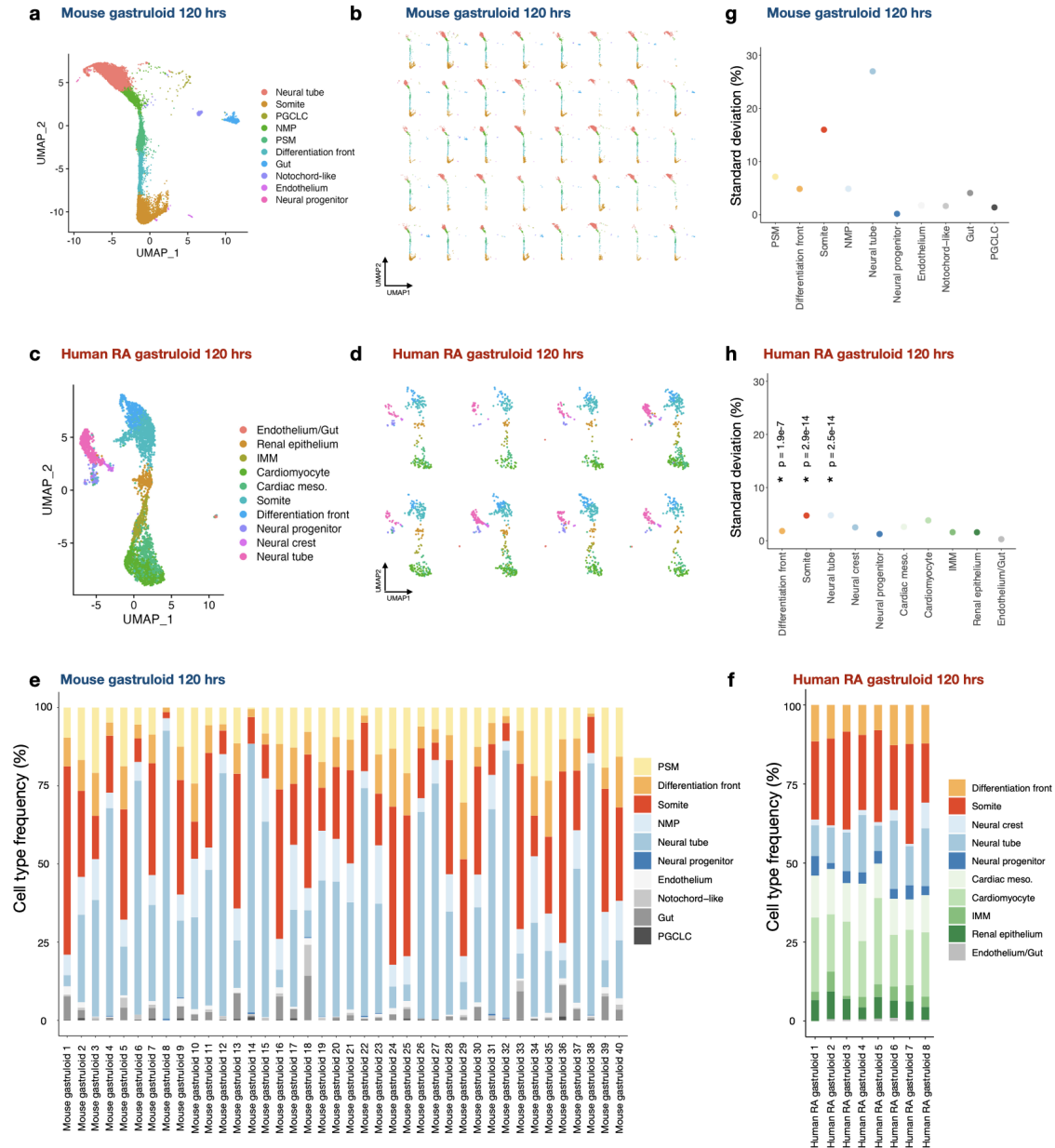


Figure 5. Inter-individual variation in cell type composition in mouse gastruloids vs. human RA-gastruloids.

a-b, UMAP visualisation of cell types observed in mouse gastruloids at 120 hrs, based on data from GSE212050¹⁴⁰. In panel (a), all individuals are represented, whereas in panel (b), the same UMAP projection shows the contribution of individual gastruloids. c-d, same as panels (a-b), but based on individual human RA-gastruloids with scRNA-seq data obtained by sci-Plex¹³⁹. e-f, Frequency with which individual cell types are observed in individual mouse gastruloids at 120 hrs (e) or individual human RA-gastruloids at 120 hrs (f). g-h, Standard deviation of cell type proportions in individual mouse gastruloids at 120 hrs (g) or individual human RA-gastruloids at 120 hrs (h). In brief, to account for differences in the total number of cells per individual gastruloid, we randomly sampled

100 cells from each individual gastruloid, ten times per individual, to generate pseudo-replicates. For three cell types abundantly present in both models (differentiation front, somites, neural tube), we performed ANOVA to test for significant differences in S.D. values between human and mouse samples. Neural progenitor cells (NPCs) were excluded from the analysis because although present in both models, on average less than one NPC per individual mouse gastruloid was detected, precluding variance analysis. If the ANOVA was significant ($p < 0.05$), a post-hoc Tukey's Honest Significant Difference (HSD) test was conducted to further evaluate pairwise differences between the species. For all three cell types compared, variation across individuals was significantly lower in human RA-gastruloids than mouse gastruloids (differentiation front: $p = 1.89\text{e-}7$; somites: $p = 2.9\text{e-}14$; neural tube: $p = 2.5\text{e-}14$).

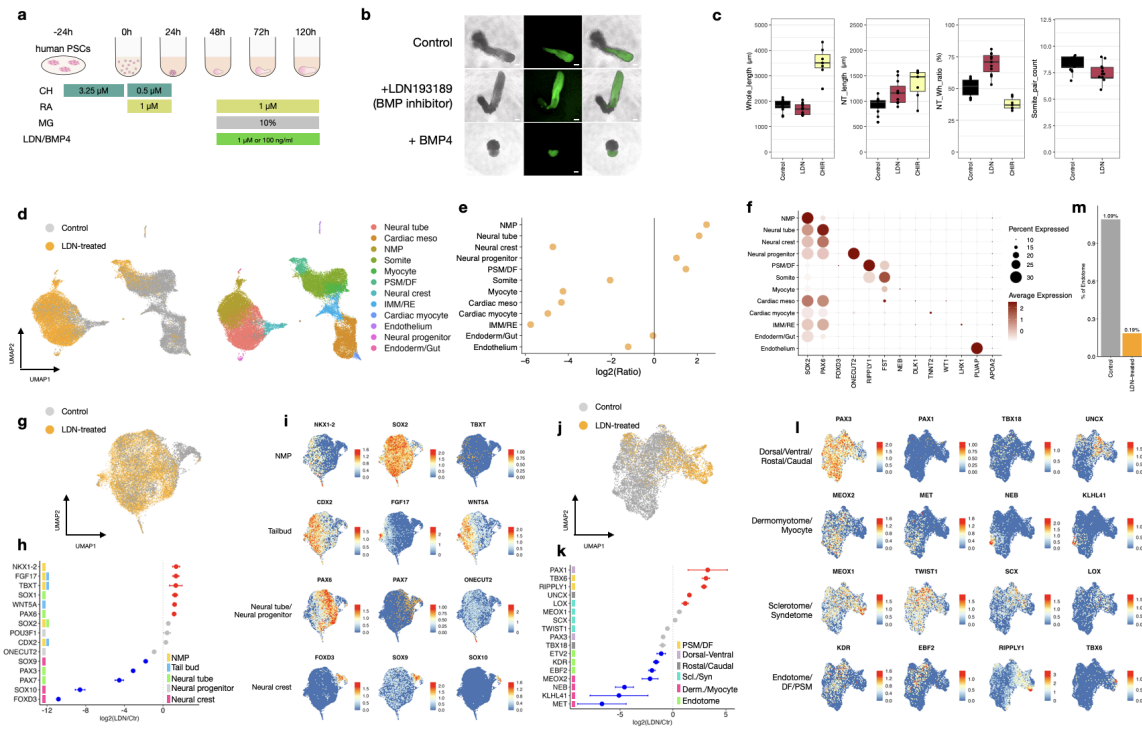


Figure 6. Effects of perturbing BMP signalling on human RA-gastruloids.

a, Schematic of perturbation of BMP signalling in human RA-gastruloids. CHIR, CHIR99021; LDN/BMP4, LDN193189 or BMP4. b, Representative images of untreated control (Control), LDN-treated, and BMP4-treated RA-gastruloids. The experiments were repeated independently three times with similar results. Scale bar, 100 μm . c, Morphometrics. From left to right: full length (μm) of 120 hrs RA-gastruloids, neural tube (NT) length (μm) measured with SOX2-mCit signal, neural-tube/full-length ratio, and somite pair counts. $N = 6$ and 11 for untreated and LDN treatment, respectively. d, (left) UMAP visualisation of co-embedded scRNA-seq data from untreated (grey) and LDN-treated (yellow) 120 hrs human RA-gastruloids. (right) Same UMAP labelled by cell type

annotation. e, Cell type composition changes upon LDN treatment of human RA-gastruloids. f, Marker gene expression in each cell type. g-i, The effects of BMP inhibition on neural lineages (NMP, neural tube, neural crest, and neural progenitor cells). g, UMAP visualisation of co-embedded scRNA-seq data (neural lineages only) from untreated (grey) and LDN-treated (yellow) 120 hrs human RA-gastruloids. h, Changes in marker gene expression with LDN treatment in neural lineages. Colour bars at left indicate the cell type(s) for which each gene is a marker. Dots indicate the ratio of average expression in LDN-treated vs. control gastruloids. Data are presented as mean values $\pm$ SEM. across pseudo-replicates (n=3). Dots corresponding to increases or decreases larger than 2-fold are coloured red and blue, respectively. i, Same UMAP projection as panel (g). Gene expression of three marker genes for each neural cell type are shown in each row. j-l, Same as panels (g-i), but restricting instead to somitic lineages with pseudo-replicates (n=3). Data are presented as mean values $\pm$ SEM. m, Effects of LDN treatment on the proportion of endotome cells. Endotome cells are defined as the subset of somitic cells that are both *KDR2*⁺ and *EBF2*⁺. The bar chart shows the proportion of endotome cells, out of all somitic cells, in two experimental conditions.

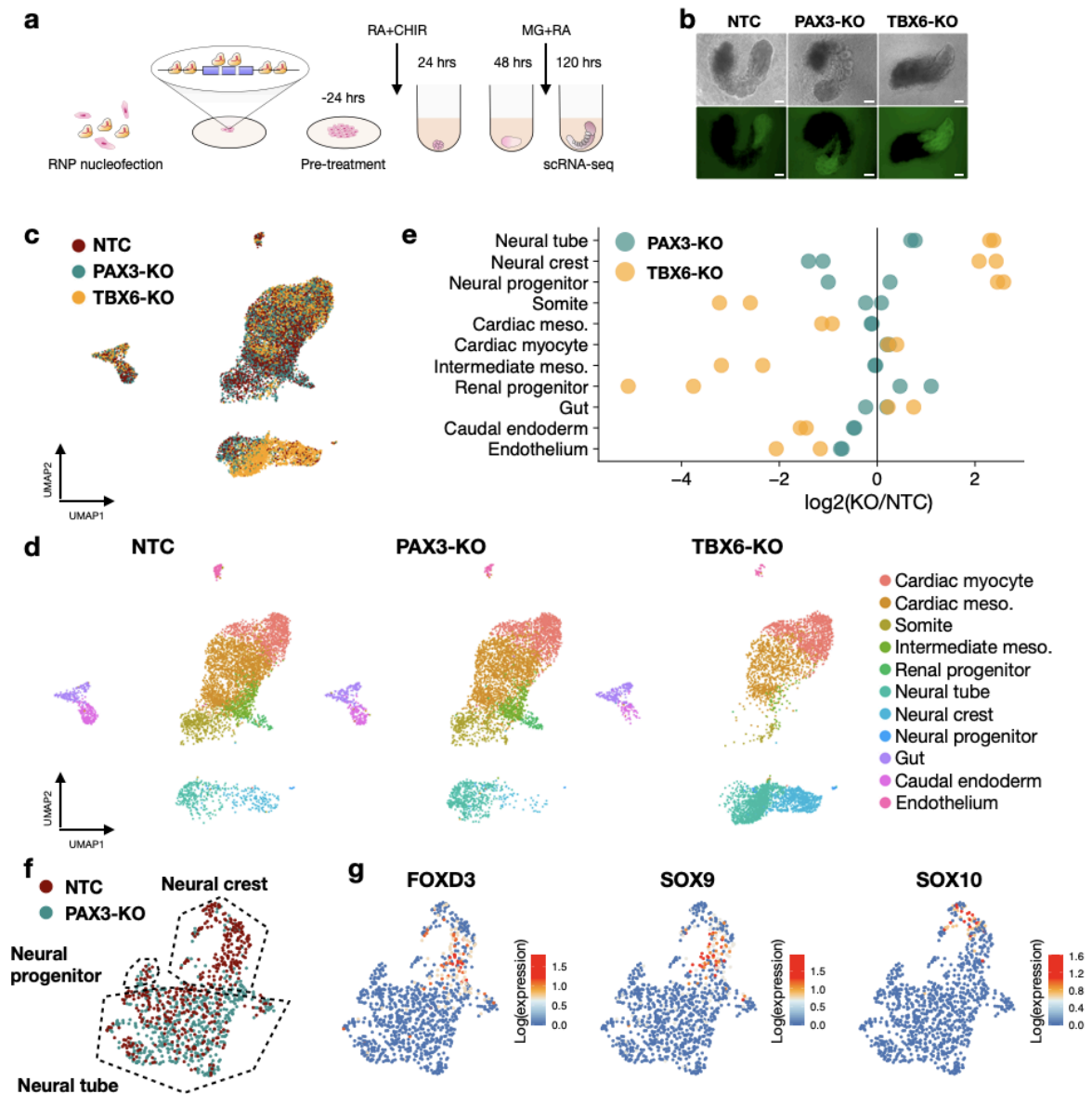
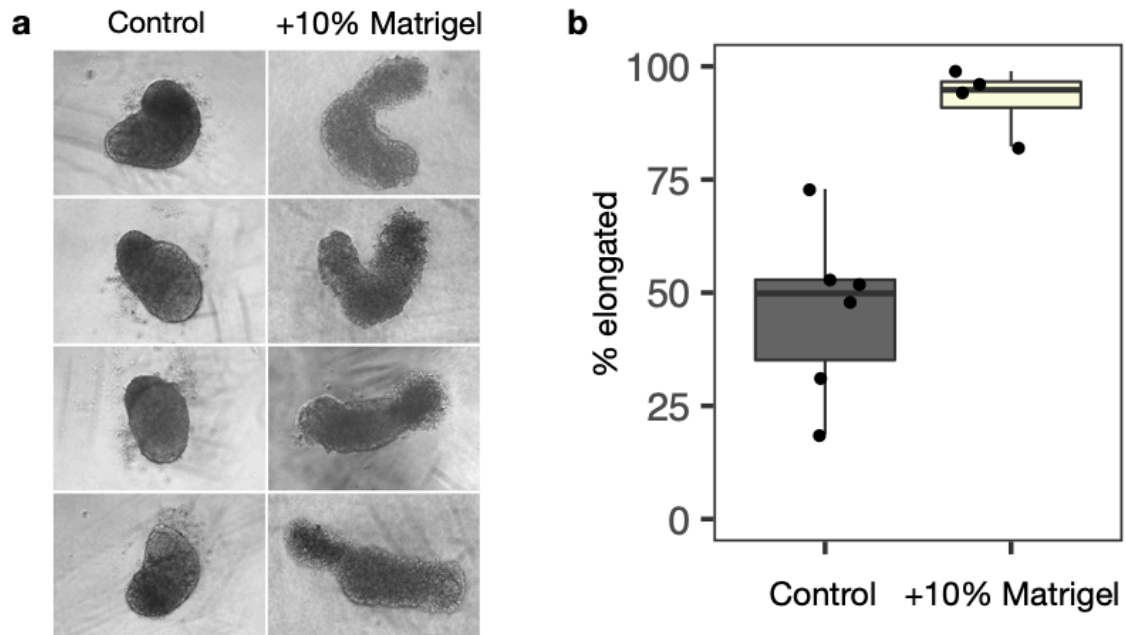


Figure 7. Genetic perturbation of transcription factors in human RA-gastruloids.

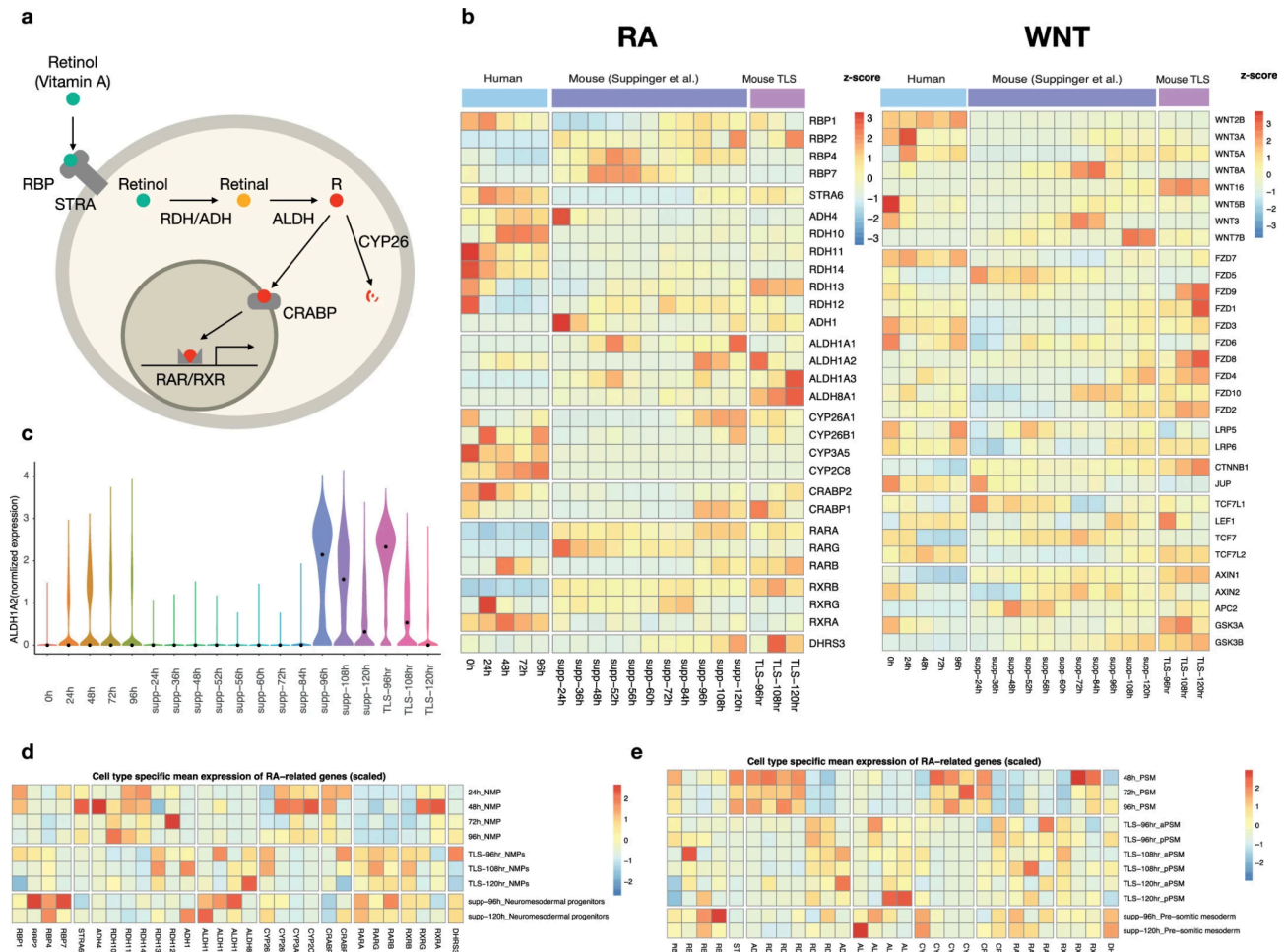
a, Schematic of transcription factor knockouts in human RA-gastruloids using CRISPR/Cas9 RNPs. Six Cas9-gRNA RNPs were nucleofected into PSCs, inducing indels or full deletion of exons of PAX3 or TBX6. Nucleofected PSCs were subjected to RA-gastruloid induction protocol. RA-gastruloids were collected at 120 hrs after cell aggregation. b, Representative images of non-targeting-control (NTC), PAX3-KO, TBX6-KO RA-gastruloids. N = 96 gastruloids for each condition. Scale bar, 100 μ m. c-d, UMAP of scRNA-seq data from NTC, PAX3-KO, and TBX6-KO RA-gastruloids, labelled by genotype (c) or cell type (d). e, Cell type composition changes upon knockout of each transcription factor. Log₂ (KO/NTC) fold changes for each cell type are shown as dots. Vertical black line corresponds to no change in the proportion of the cell type between KO and NTCs. f, Annotated UMAP embedding of neural cell types only (neural tube,

neural crest, and neural progenitor cells) from PAX3-KO RA-gastruloid and controls. g, Same embedding as panel (e), but showing gene expression (log-scaled) of neural crest marker genes.



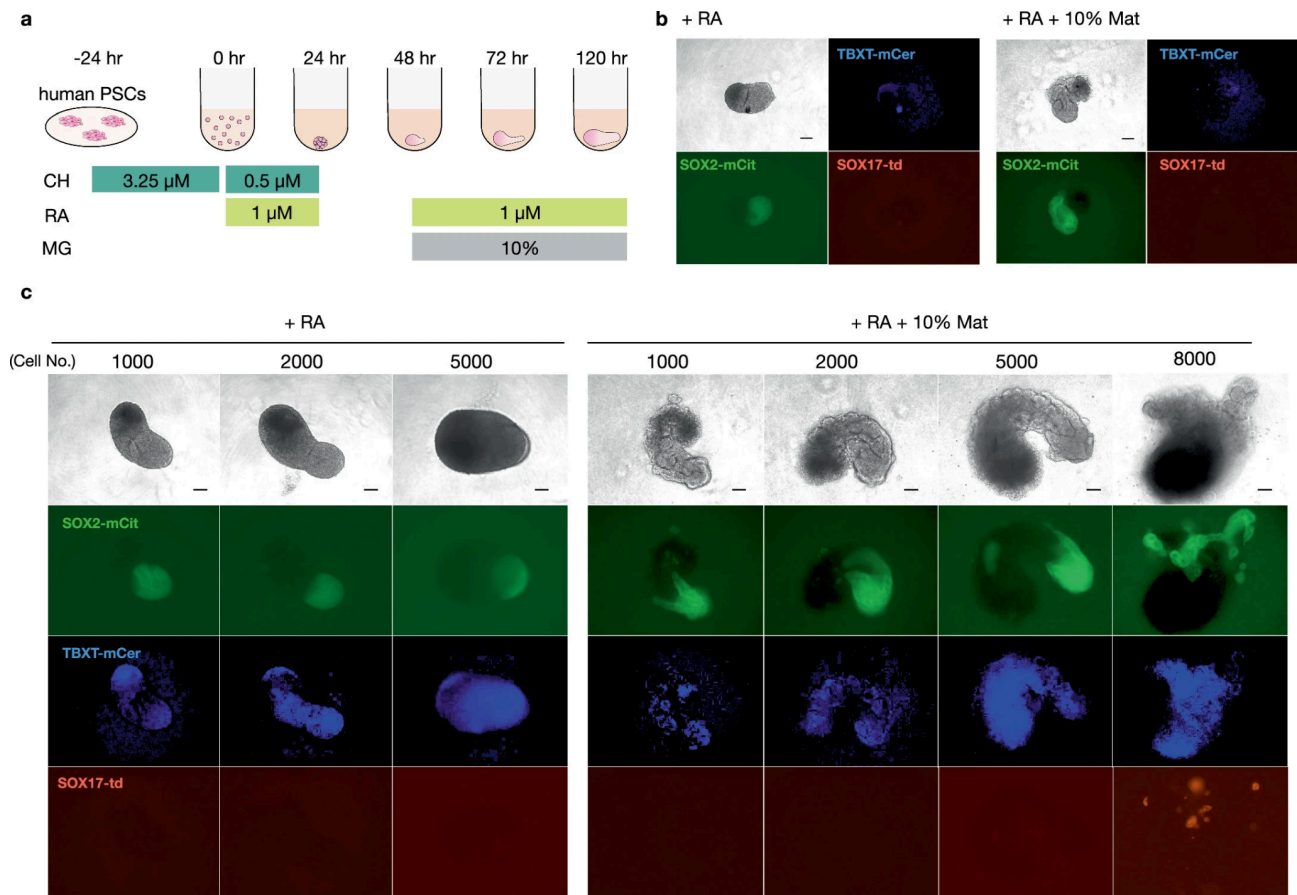
Extended Data Fig. 1: Effects of Matrigel on human gastruloid morphology.

a, Representative images of human gastruloids without (left) vs. with (right) 10% Matrigel. The addition of 10% Matrigel enhances the extent of human gastruloid elongation. b, Boxplot showing the proportion of elongated gastruloids observed in the absence (left) vs. presence (right) of 10% Matrigel across a total of ten experiments. Raw counts are provided in Supplementary Table [1](#). The addition of 10% Matrigel enhances the robustness of human gastruloid elongation.



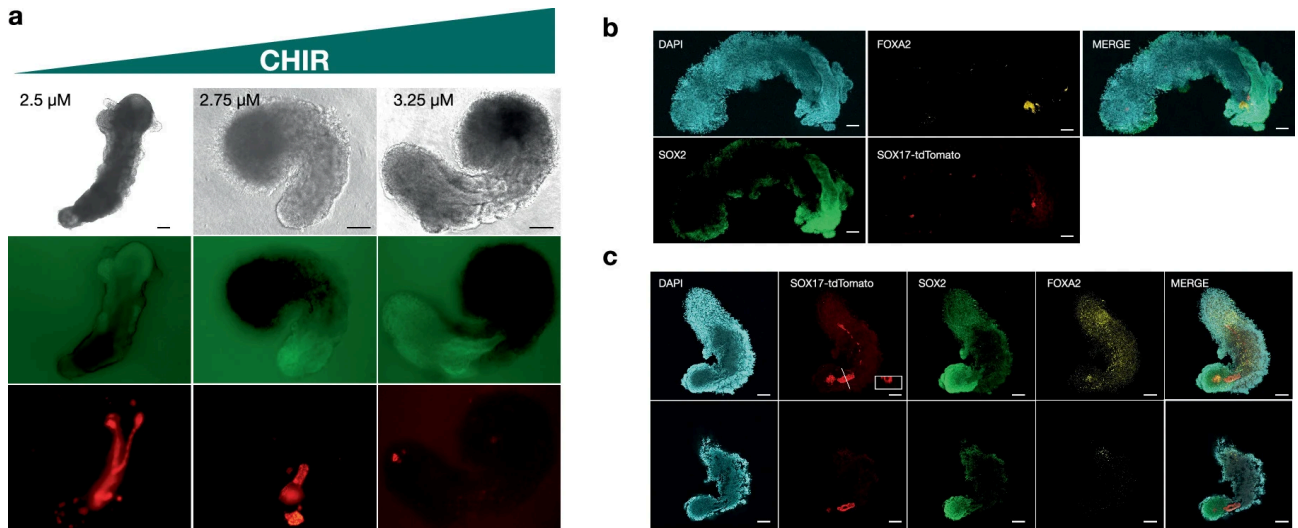
Extended Data Fig. 2: Differential expression of genes related to RA/Retinol/Retinal pathways in mouse vs. human gastruloids.

a, Schematic of RA metabolism pathways. b, Heatmaps showing expression levels of genes involved in RA (left panel) and WNT (right panel) signalling pathways in conventional human gastruloids at 0-96 hrs from Moris et al.³⁸, mouse gastruloids at 24-120 hrs from Suppinger et al.¹⁵⁹ and mouse TLS 96-120 hrs from Veenvliet et al.³⁷ Gene expression values were scaled by z-score across samples. c, Normalised expression of ALDH1A2 in conventional human gastruloids at 0-96 hrs, mouse gastruloids at 24-120 hrs¹⁵⁹, and mouse TLS at 96-120 hrs³⁷. d-e, Heatmaps showing expression levels of genes involved in RA signalling pathways in NMPs (d) and PSMs (e) of conventional human gastruloids at 0-96 hrs, mouse TLS at 96-120 hrs¹⁵⁹ and mouse gastruloids at 96-120 hrs³⁷. Gene expression levels are shown for timepoints where NMPs and PSMs were detected in a given model.



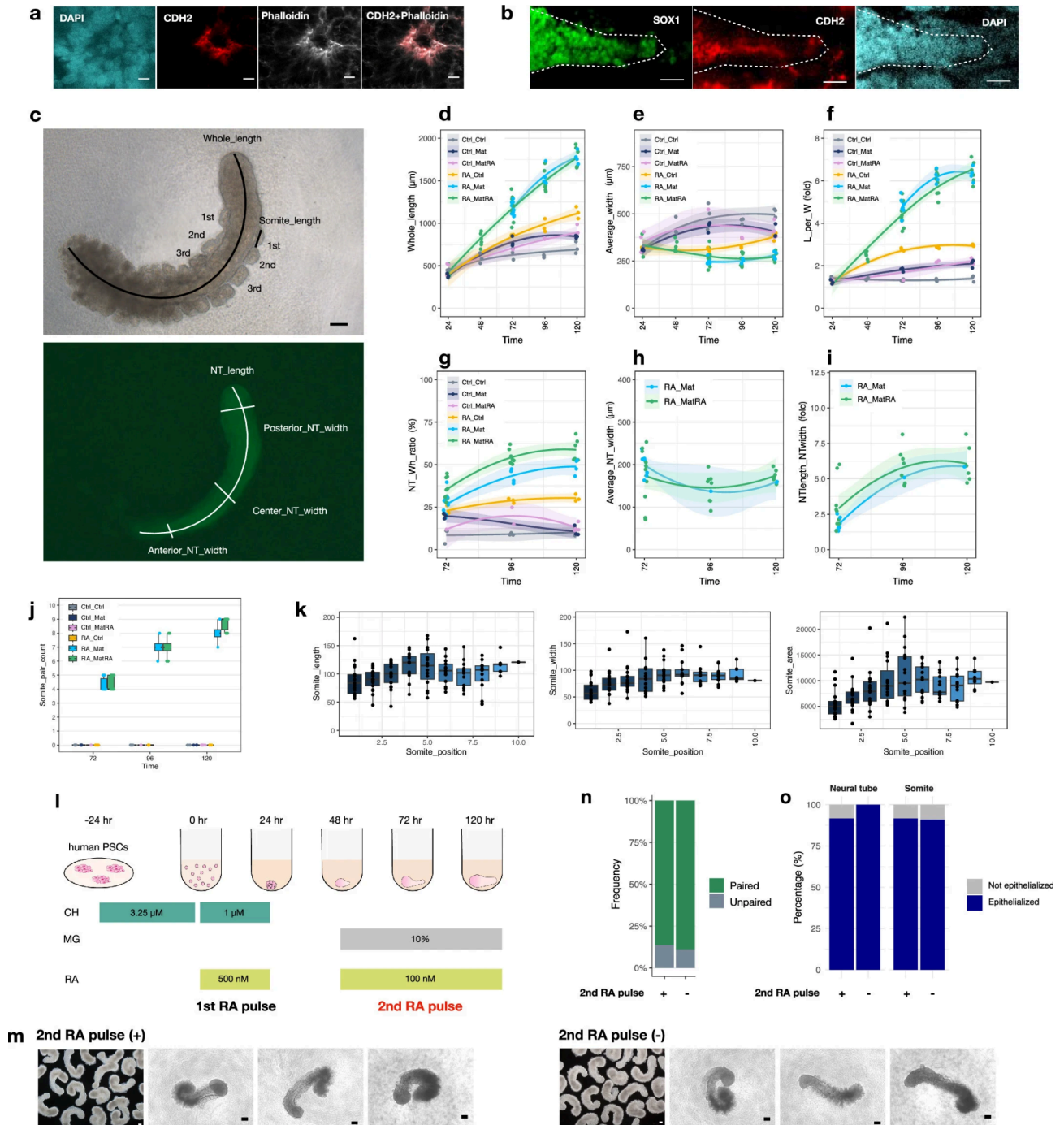
Extended Data Fig. 3: The number of seeded cells impacts human gastruloid formation in the context of a discontinuous regimen of retinoic acid.

a, Schematic of discontinuous regimen of RA and Matrigel treatment while inducing human gastruloids. RA, retinoic acid; MG, 10% Matrigel. b, Representative images of 96 h human gastruloids induced from 400 cells. 1 μ M RA (0–24 h and 48–96 h) (left) or 1 μ M RA (0–24 h and 48–96 h) + 10% Matrigel (48–96 h) (right) were added to the medium. Scale bars, 100 μ m, N = 32. c, Representative images of 96 h human RA-gastruloids while varying the number of cells used for initial seeding. Scale bars, 100 μ m, N = 48.



Extended Data Fig. 4: Lower CHIR concentrations facilitate formation of elongated gut tube-like structures in human RA-gastruloids.

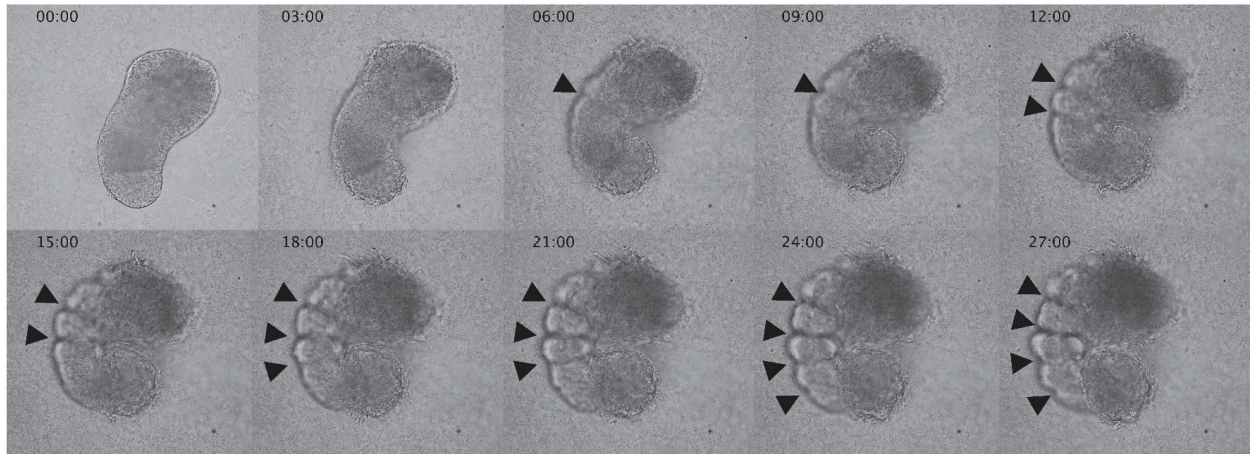
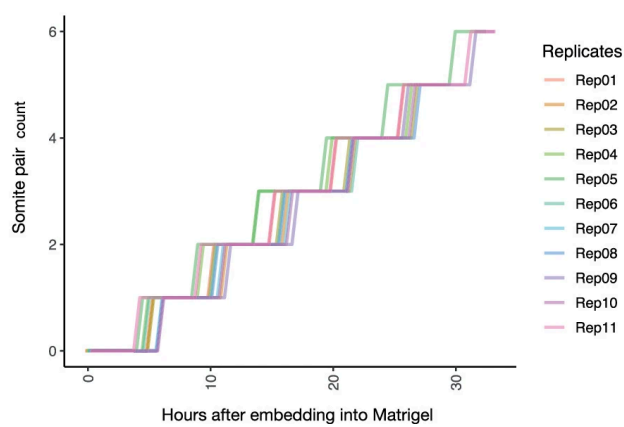
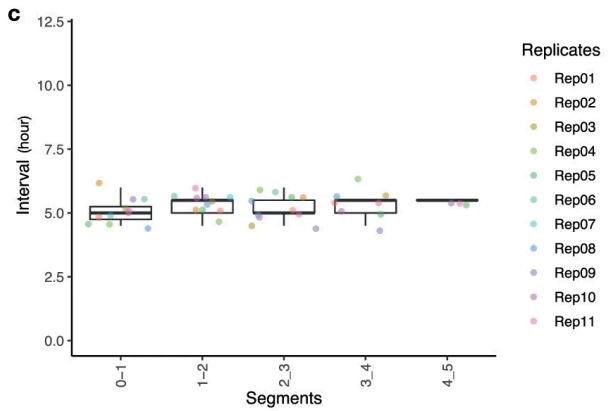
a, Effects of CHIR concentration at the pre-treatment stage on SOX17-tdTomato positive cell accumulation and elongation. Scale bars, 200 μ m N = 32, 38, and 48, respectively. b, Immunostaining of 3.25 μ M CHIR-treated 120 h human RA-gastruloids with anti-SOX2, anti-SOX17-tdTomato, and anti-FOXA2 antibodies. Scale, 100 μ m, N = 8 out of 12 showed a similar staining pattern. c, Immunostaining of 2.75 μ M CHIR-treated 120 h human RA-gastruloid with anti-SOX2, anti-SOX17-tdTomato, and anti-FOXA2 antibodies. (Top) Max projection of z-stack image. (Bottom) A slice of z-stack. Scale bars, 100 μ m. N = 5 out of 7 gastruloids showed a similar staining pattern.



Extended Data Fig. 5: Morphological properties of human conventional and RA-gastruloids.

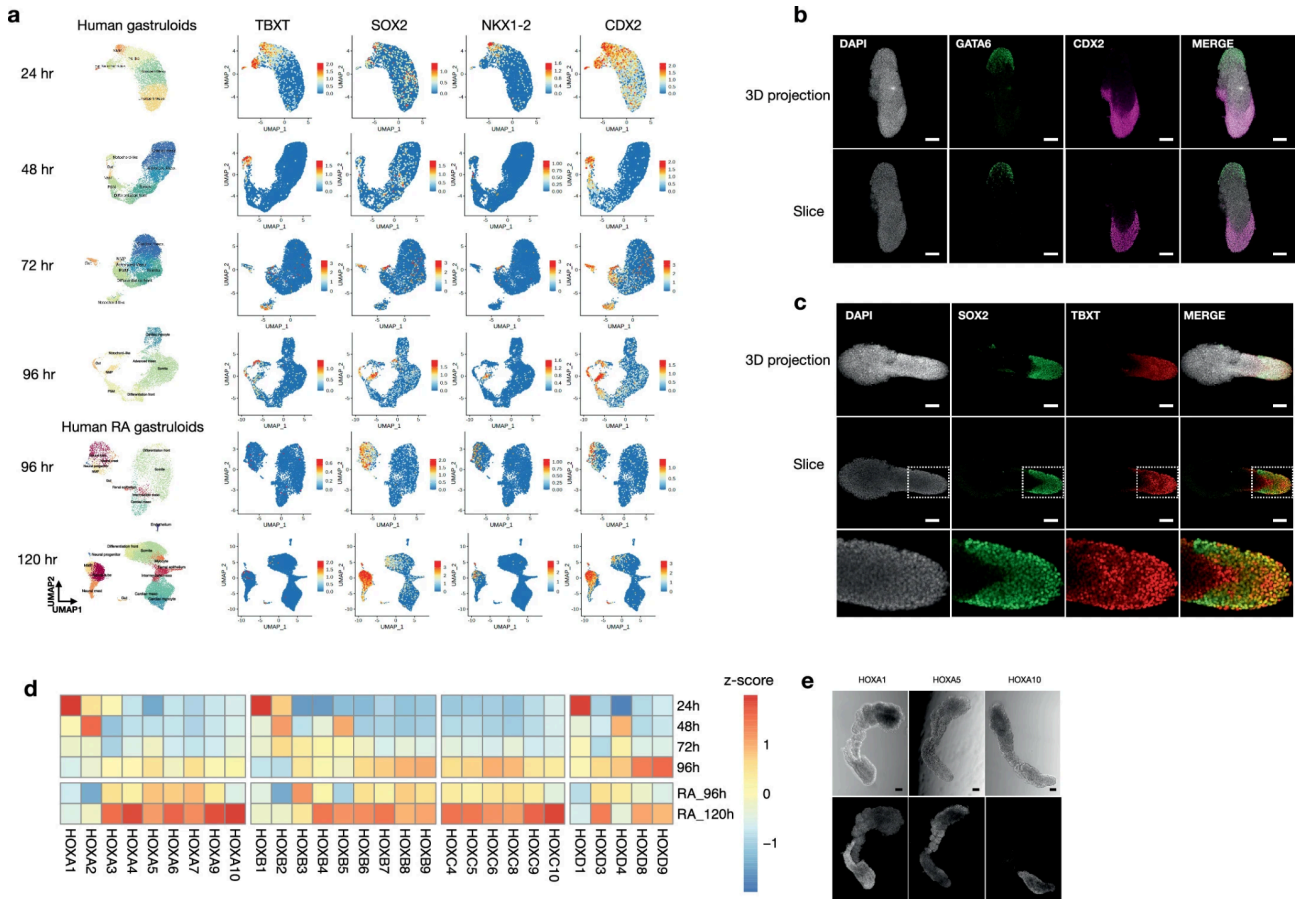
a, Immunostaining of N-cadherin (CDH2) and phalloidin in somites in an RA-gastruloid. Phalloidin-stained F-actin and CDH2 were co-localized and highly concentrated at the apical surface of somites. Scale bar, 10 μm . b, Immunostaining of N-cadherin (CDH2) and SOX1 in the neural tube in an RA-gastruloid. Scale bar, 100 μm . c, (Top) Bright-field of a human RA-gastruloid. The whole length was measured as the length of a line along the centre of the body. Each somite length was measured from the posterior end. (Bottom) SOX2-mCit view of the top picture. Neural

tube length (NT_length) was measured as the continuous SOX2+ area. The width of neural tubes was measured and averaged over several positions (10%, 50%, 90% along the full length of the structure). Scale bar, 100 μ m. d-l, Morphometric measurements of gastruloids which originated from 5,000 cells, as a function of time. Ctrl, no treatment control; RA, Retinoic acid; Mat, 5% Matrigel; MatRA, Matrigel + RA. Left and right part of each text label indicates the conditions at 0–24 h and at 48–120 h, respectively. For example, Ctrl_Ctrl indicates no treatment for both 0–24 h and 48–120 h. $N \geq 3$ for each time point and condition. d, Whole length (μ m) of gastruloids. e, Average width (μ m) of gastruloids. f, Ratio (%) of whole length to average width. g, Ratio (%) of length of neural area to the whole length. h, Average neural tube width (μ m). i, Ratio (%) of neural tube length-to-width. j, Number of somites observed as a function of time. k, Length, width, and area of somites as a function of position. $N = 16$ RA-gastruloids. l, Schematic of RA-gastruloid induction protocol, highlighting the first vs. second RA pulse. m, Bright-field images of human RA-gastruloids induced with (left column) vs. without (right column) inclusion of the second RA pulse. Scale bars = 100 μ m. n, Frequency of paired somites in RA-gastruloids with vs. without inclusion of the second RA pulse. Somites with areas within 30% of one another were classified as "paired somites". This comparison was made for 3 randomly chosen putative somite pairs within each gastruloid. A gastruloid was subsequently designated as "paired gastruloid" if at least 2 out of 3 putative somite pairs were classified as "paired somites". $N = 13/14$ (92.9%) and $N = 11/12$ (91.7%) for RA-gastruloids with vs. without inclusion of the second RA pulse, respectively. o, Frequency of neural tube (left) and somite (right) epithelialization with vs. without inclusion of the second RA pulse, respectively. Epithelialization was defined by the accumulation of phalloidin staining at the apical side of the structures upon immunostaining. The percentages indicate the frequency of gastruloids with epithelialized somite or neural tube. $N = 11$ and $N = 10$ for RA-gastruloids with vs. without inclusion of the second RA pulse, respectively.

a**b****c**

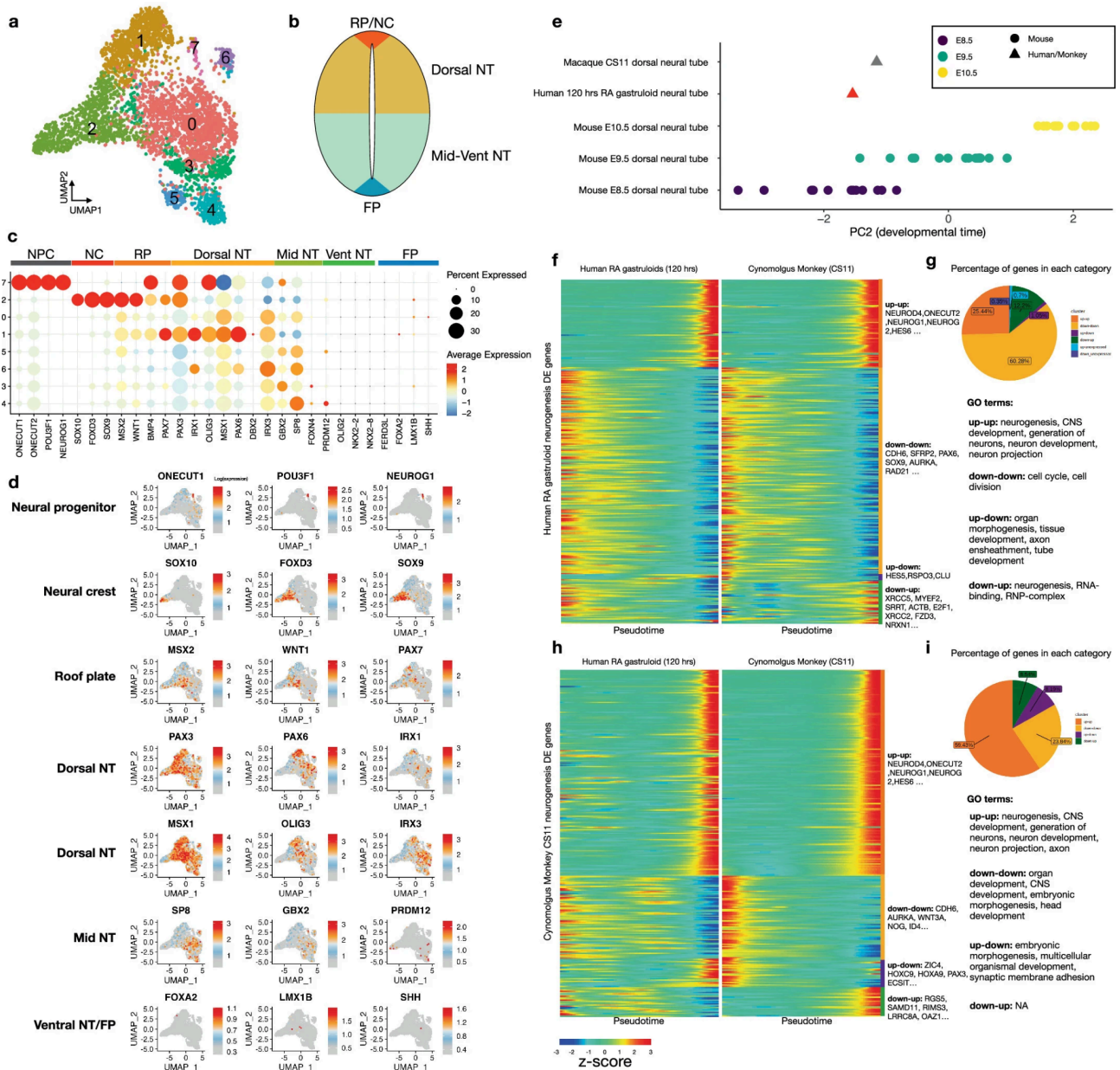
Extended Data Fig. 6: Live imaging of human RA-gastruloids.

a, Snapshots of the elongating human RA-gastruloids. After RA supplementation at 0–24 h, these gastruloids were subjected to live imaging after the addition of 5% Matrigel and RA at 48 h from the induction. Arrowheads indicate the emergence of a segmentation. The time after embedding gastruloid into Matrigel and RA is shown on the top left. b, Number of somite pairs observed in RA-gastruloid in the live imaging. The x-axis indicates the hours from embedding of the RA-gastruloid into Matrigel. c, Boxplot of the time interval between successive segmentations.



Extended Data Fig. 7: Evaluation of NMPs and anterior–posterior patterning in human RA-gastruloids.

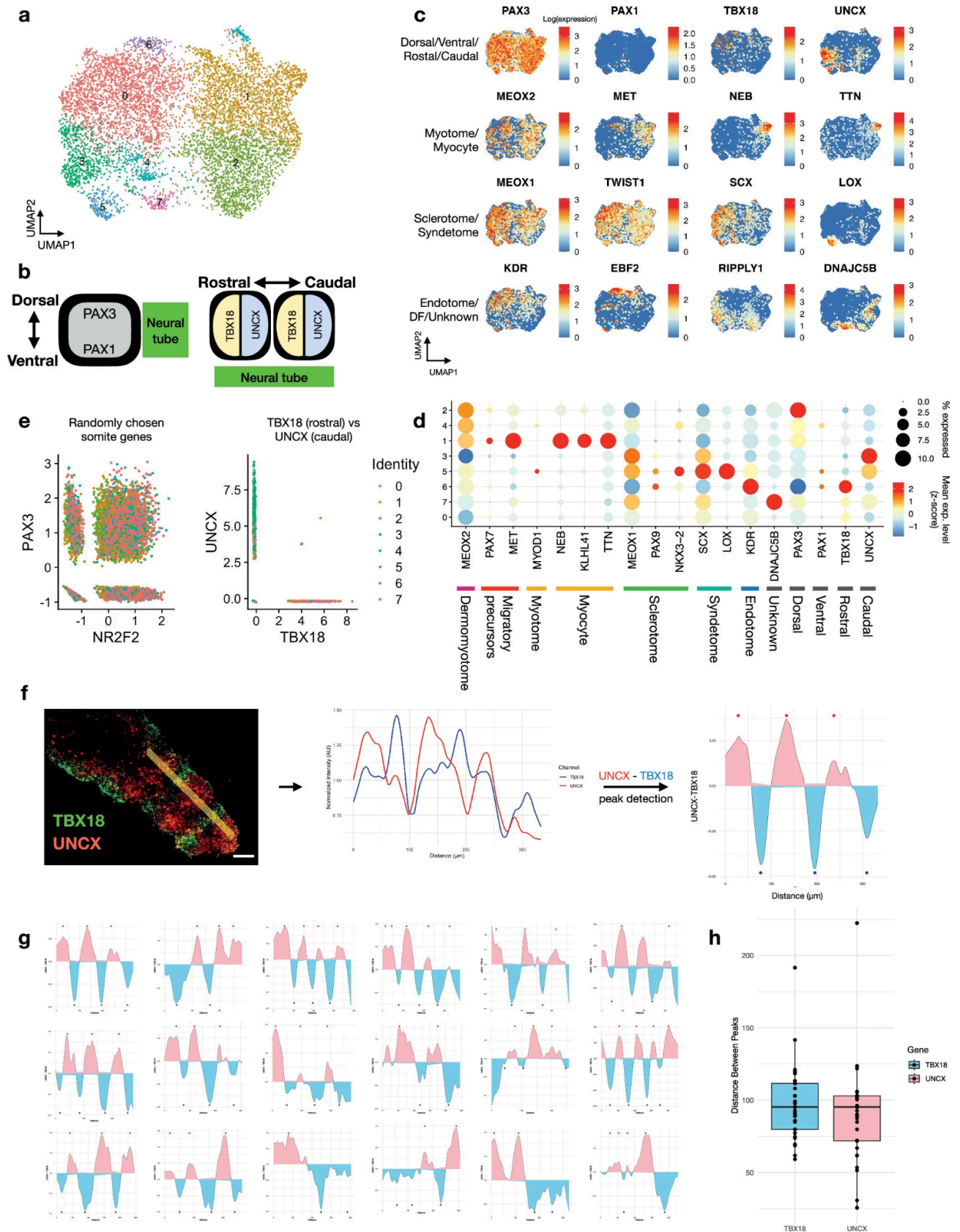
a, UMAP visualisation with cell types and normalised expression patterns of NMP marker genes reported in literature¹¹⁹ for conventional human gastruloids at 24, 48, 72, or 96 hrs, or human RA-gastruloids at 96 or 120 hrs. b, Immunostaining of GATA6 (anterior marker) and CDX2 (posterior marker) in 48 hrs human RA-gastruloid. 3D maxprojection (Top) and sliced (Bottom) view. Scale bar, 100 μ m. c, Immunostaining of SOX2 and TBXT in 48 hrs human RA-gastruloid. 3D maxprojection (Top) and sliced (Bottom) view. The dotted square in the middle row is zoomed in the bottom row. Scale bar, 100 μ m. d, Heatmap showing the relative expression of HOX genes in conventional and RA-gastruloids at various timepoints. Mean expression levels of whole cells of each sample were normalised by z-score across samples. e, Representative images of HCR of HOX genes in human RA-gastruloids at 120 hrs. (Top) Bright-field and (Bottom) HCR imaging of HOXA1, HOXA5 and HOXA10. Scale bar, 100 μ m. N = 18, 17, 21 gastruloids for HOXA1, HOXA5 and HOXA10, respectively.



Extended Data Fig. 8: Evaluation of dorsal-ventral markers and neural differentiation in human RA-gastruloids.

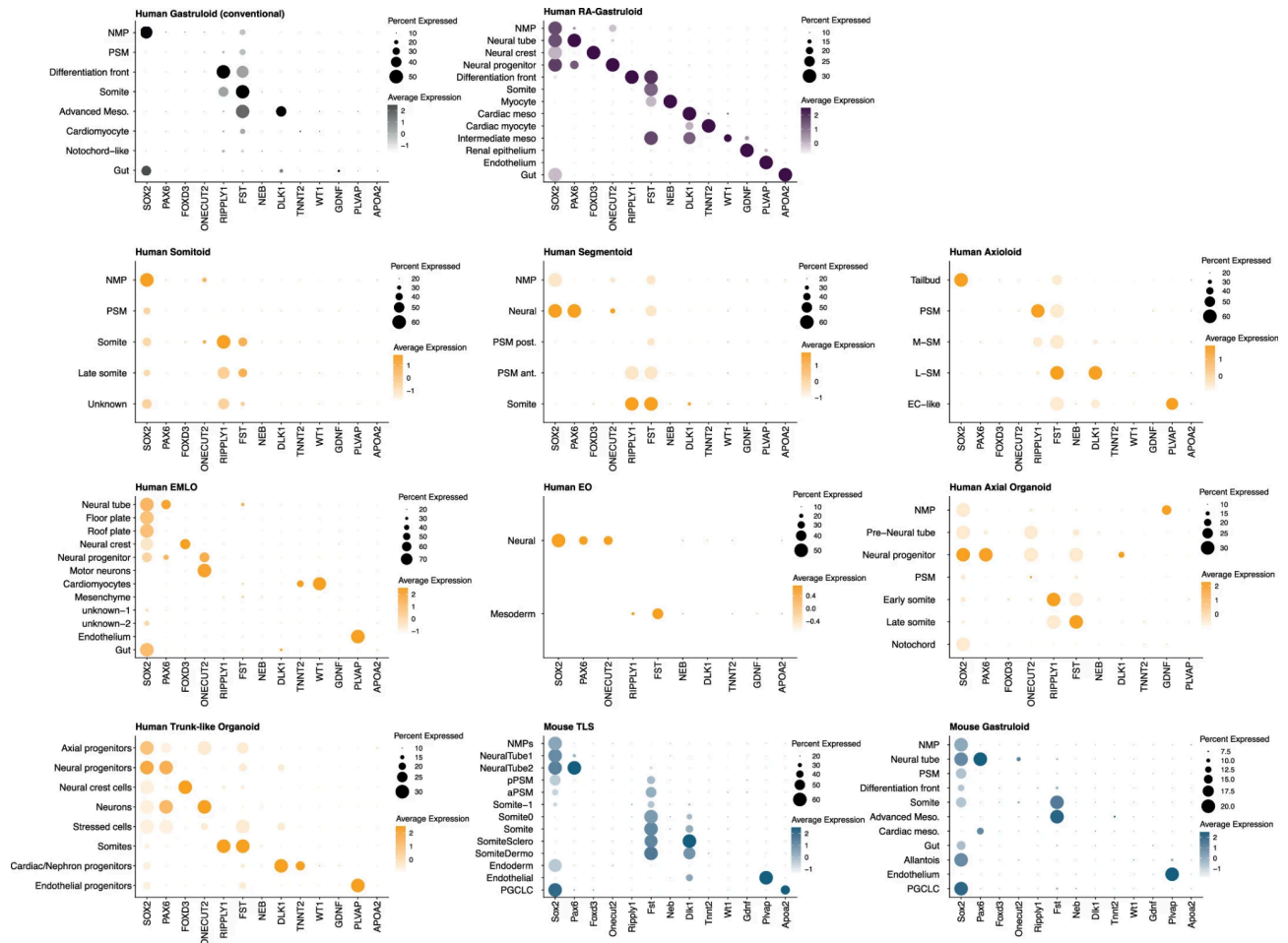
a, Clustering and UMAP visualisation of neural-related cells from sc-RNA-seq data of 120 hr human RA-gastruloids. b, Schematic of dorsal-ventral axis of neural tube, with labels corresponding to subsets of marker genes shown in panel (c). c, Bubble plot of marker gene expression patterns in each of the clusters shown in panel (a). d, Marker gene expression, projected onto UMAP shown in panel (a). NPC, neural progenitor; NC, neural crest cells; RP, roof plate; NT, neural tube; FP, floor plate. e, Staging alignment of pseudobulk profiles of neural tube cells from CS11 cynomolgus monkey embryos, neural tube cells from 120 hrs human RA-gastruloids and dorsal neural tube cells from E8.5-E10.5 mouse embryos, leveraging human-defined PC2 (see Methods, Fig. 4, Fig. S21,

Fig. S22 and corresponding sections of main text for more details). f, Developmentally differentially expressed genes (dDEGs) along the neural differentiation trajectory of human RA-gastruloids were computed. The heatmaps show side-by-side comparison of the scaled expression level of these dDEGs along neural differentiation trajectories in 120 hrs human RA-gastruloids (left) or CS11 cynomolgus monkey embryos (right). Genes are grouped into concordant (up-up: up-regulated in both species, down-down: down-regulated in both species) and discordant (up-down: up-regulated in human and down-regulated in monkey, down-up: down-regulated in human and up-regulated in monkey) categories with example genes shown for each category. Genes that are not detected in monkeys are not shown in the heatmaps. g, The percentage of dDEGs in each category and the GO terms (<http://www.gsea-msigdb.org/gsea>) associated with each category shown in panel (f). h, Same as panel (g), except that the dDEGs shown are from the neural differentiation trajectory of cynomolgus monkey. i, The percentage of dDEGs in each category and the GO terms associated with each category shown in panel (h).



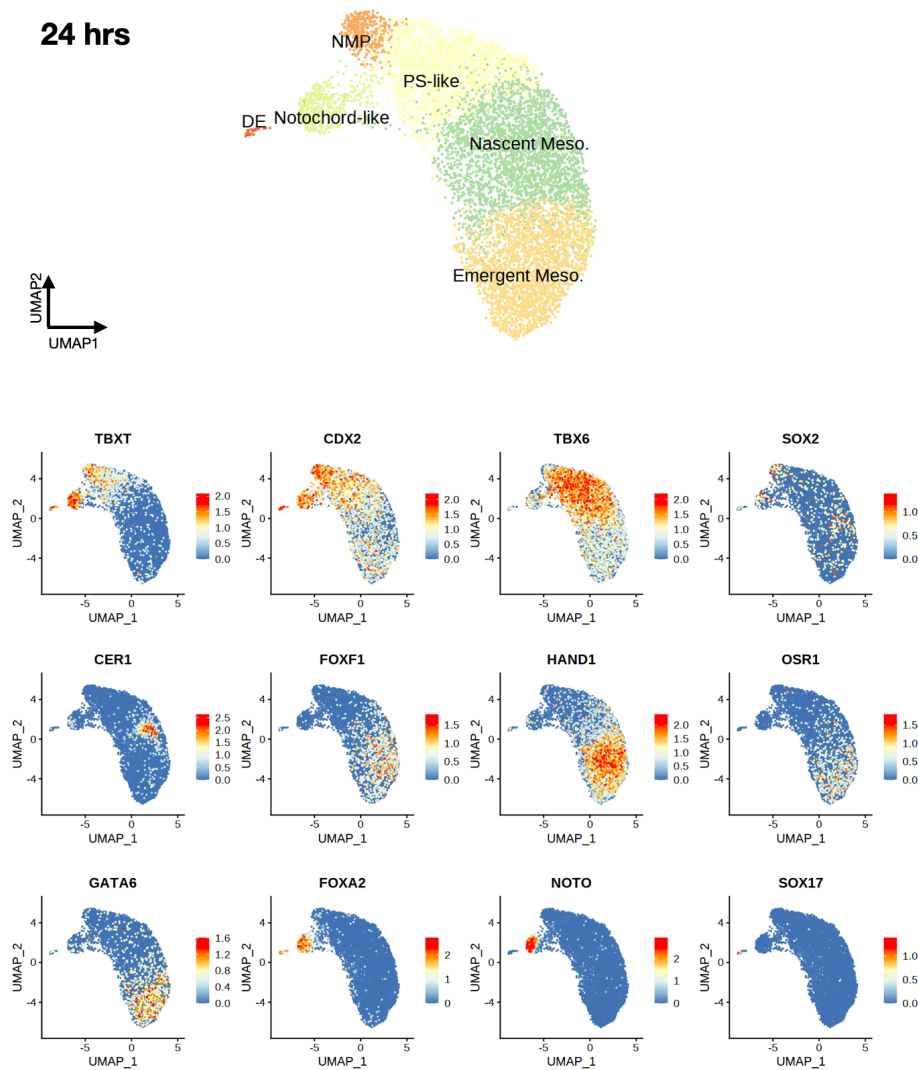
Extended Data Fig. 9: Markers of spatial patterning and differentiation in the somites of human RA-gastruloids.

a, Clustering and UMAP visualisation of somite-related cell types (somite, differentiation front, myocyte) from sc-RNA-seq data of 120 hrs human RA-gastruloids. b, Schematic of dorsal-ventral and rostral-caudal axes of somites, with expected marker genes noted. c, Same UMAP as panel (a) showing expression of selected marker genes for various subtypes of cells within somites. DF, differentiation front. d, Bubble plot showing expression patterns of selected marker genes for clusters shown in panel (a). e, Scatter plot showing scaled expression levels of randomly chosen pairs of genes (left) or TBX18 vs. UNCX (right). The expression of TBX18 and UNCX, markers of the rostral-caudal axis of somites, are mutually exclusive. f, (left) Representative image of HCR of UNCX and TBX18 in 120 hrs human RA-gastruloids, and quantification of the signal intensity along with the A-P axis. Scale bar, 50 μm . (middle) Signal intensities of TBX18 (blue) and UNCX (red) were measured on the yellow line of the left panel. Data was normalized with the mean values of each signal and processed with LOESS smoothing. (right). Line plot showing the difference between TBX18 and UNCX values. Red and blue dots indicate the peaks for UNCX-high (red) and TBX18-high (blue), respectively, detected by a findpeaks function of pracma R package. g, Similar line plots for 18 RA-gastruloids. h, Boxplot showing the distribution of the distances between successive TBX18-high (blue) or UNCX-high (red) peaks. The upper whisker, upper box edge, bar, and lower box edge represent $1.5 \times \text{IQR}$, third quartile (Q3), median, and first quartile (Q1), respectively.



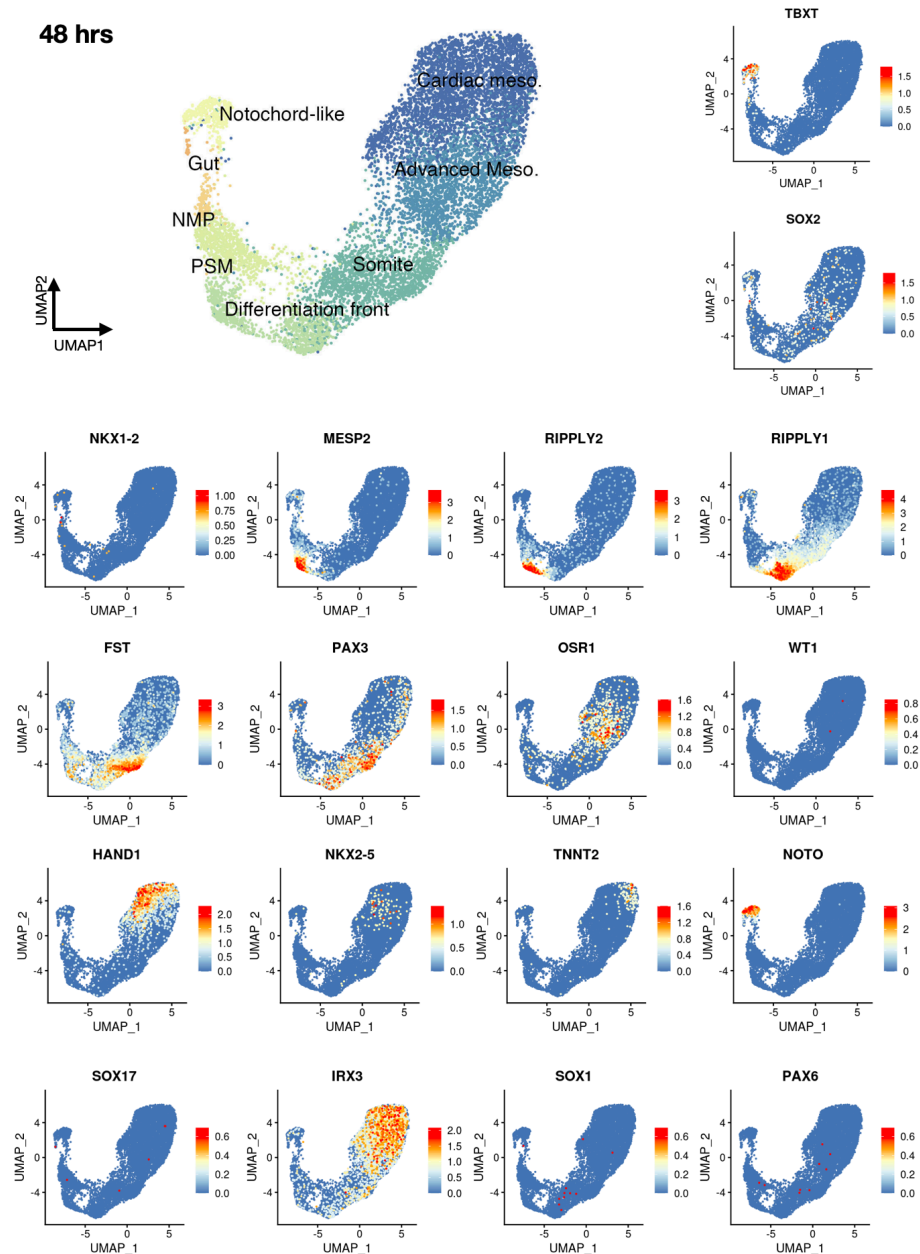
Extended Data Fig. 10: Cell types identified in various human and mouse embryo models.

Marker gene expression of annotated cell types in various embryo models are shown as bubble plots. The colours indicate the classification of embryo models; conventional human gastruloids (grey)¹¹, human RA-gastruloids (purple), other embryo models^{16,17,18,19,20,22,45} (yellow), and mouse gastruloids (blue)^{10,15}. The same set of marker genes (columns) are used in all panels.



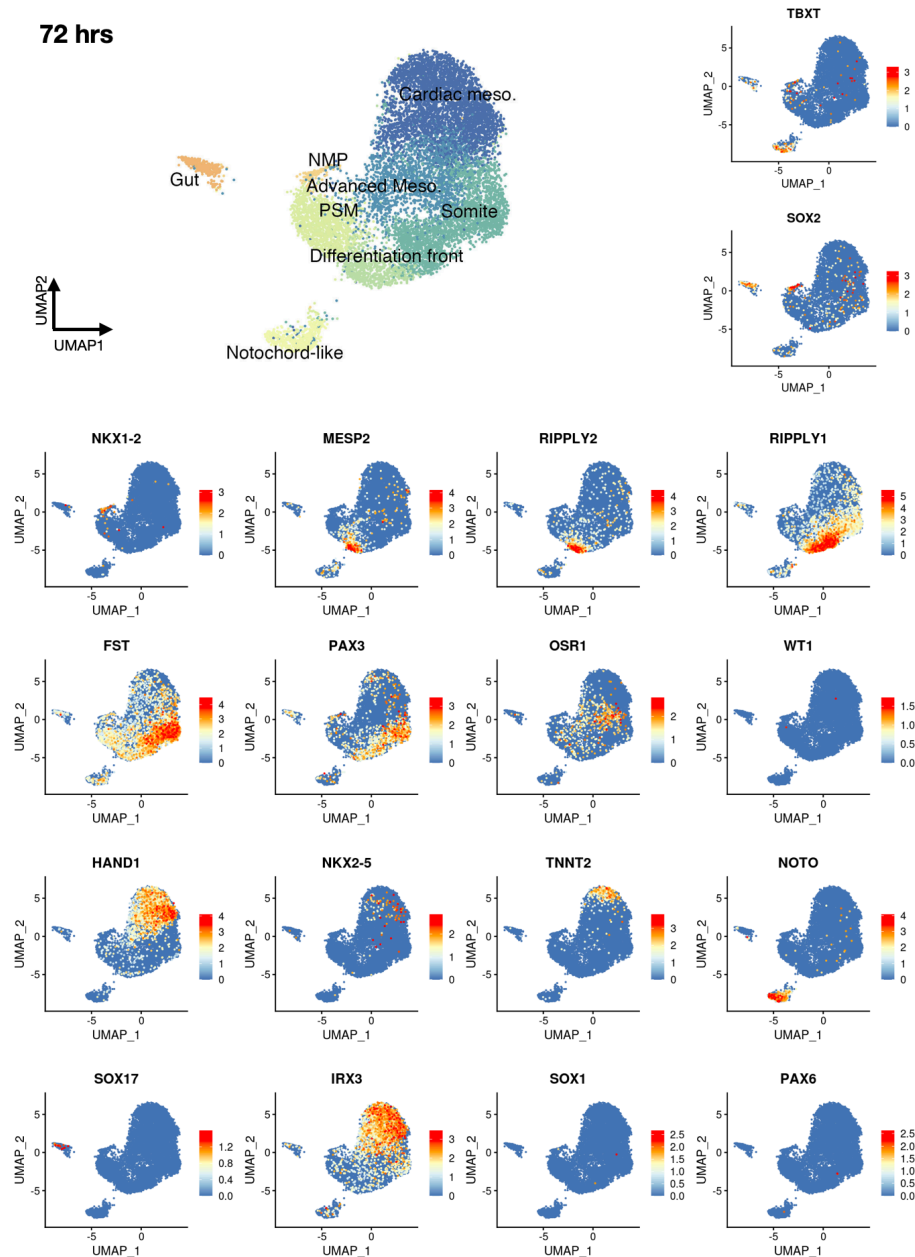
Supplementary Fig.1. Marker gene expression & cell type annotation in human gastruloids at 24 hrs after induction.

UMAP with unsupervised clustering of scRNA-seq profiles is shown, along with expression of marker genes used to annotate these clusters as a continuum of primitive streak-like, nascent mesoderm and emergent mesoderm, along with notochord-like cells and definitive endoderm. DE, definitive endoderm; PS, primitive streak.



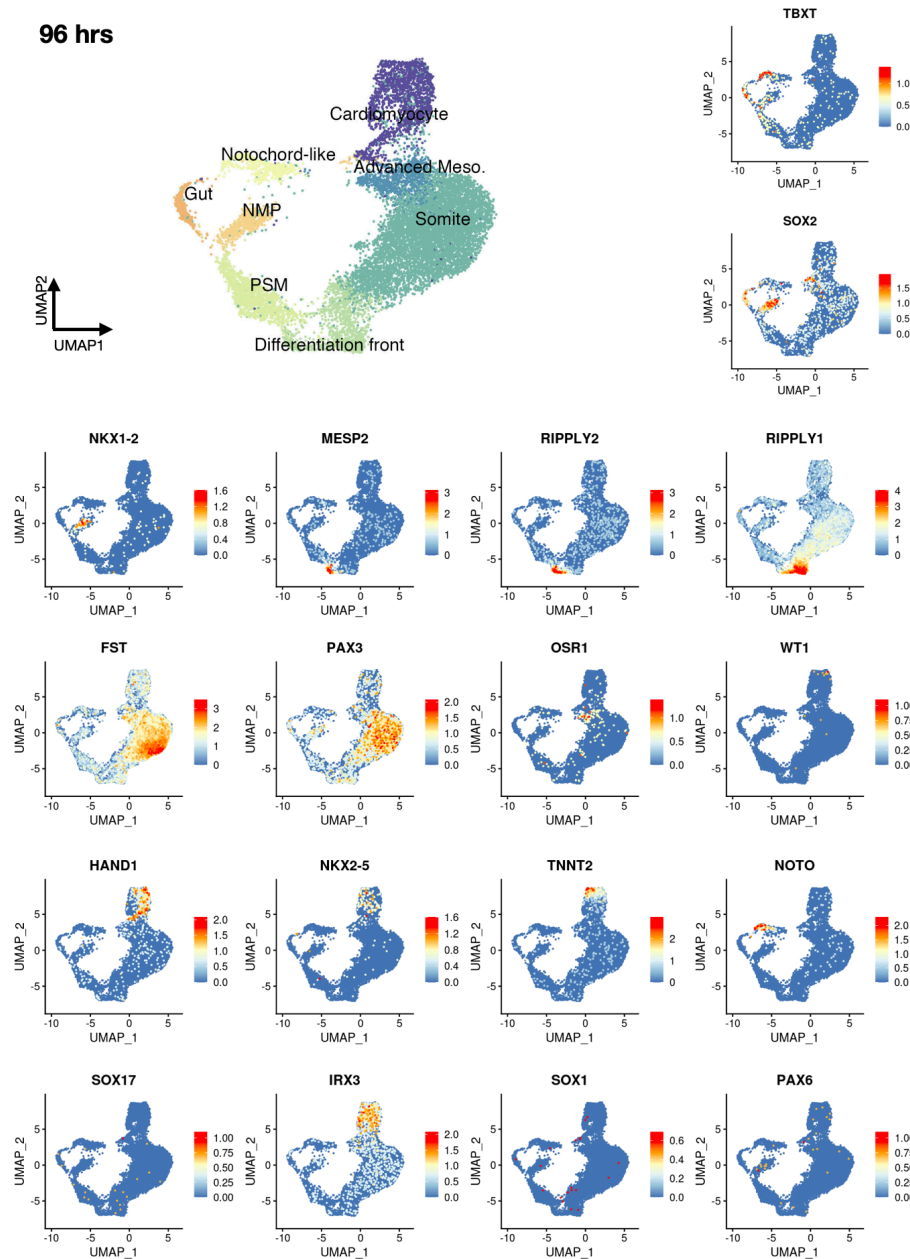
Supplementary Fig.2. Marker gene expression & cell type annotation in human gastruloids at 48 hrs after induction.

UMAP with unsupervised clustering of scRNA-seq profiles is shown, along with expression of marker genes used to annotate these clusters as a continuum of neuromesodermal progenitors, presomitic mesoderm, differentiation front, and somites; additionally, advanced mesoderm and cardiac mesoderm, as well as notochord-like and gut/endoderm-like cells, were detected. NMP, neural mesodermal progenitor; PSM, presomitic mesoderm.



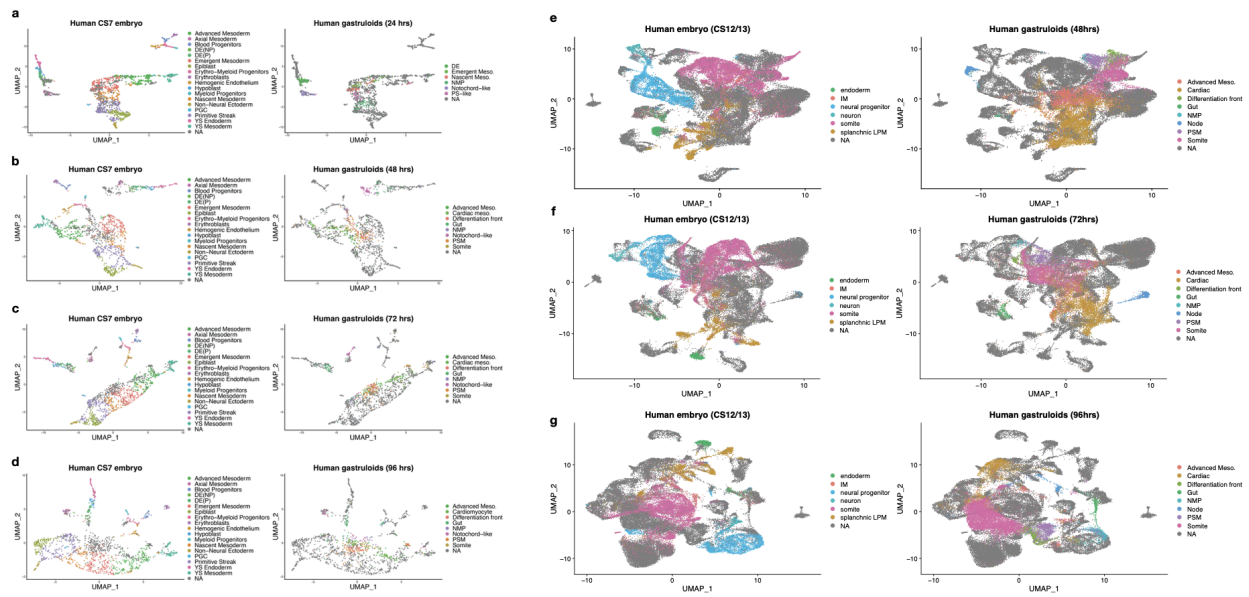
Supplementary Fig.3. Marker gene expression & cell type annotation in human gastruloids at 72 hrs after induction.

UMAP with unsupervised clustering of scRNA-seq profiles is shown, along with expression of marker genes used to annotate these clusters as a continuum of neuromesodermal progenitors, presomitic mesoderm, differentiation front, and somites; additionally, advanced mesoderm and cardiac mesoderm, as well as notochord-like cells and gut/endoderm-like cells, were detected. NMP, neural mesodermal progenitor; PSM, presomitic mesoderm.



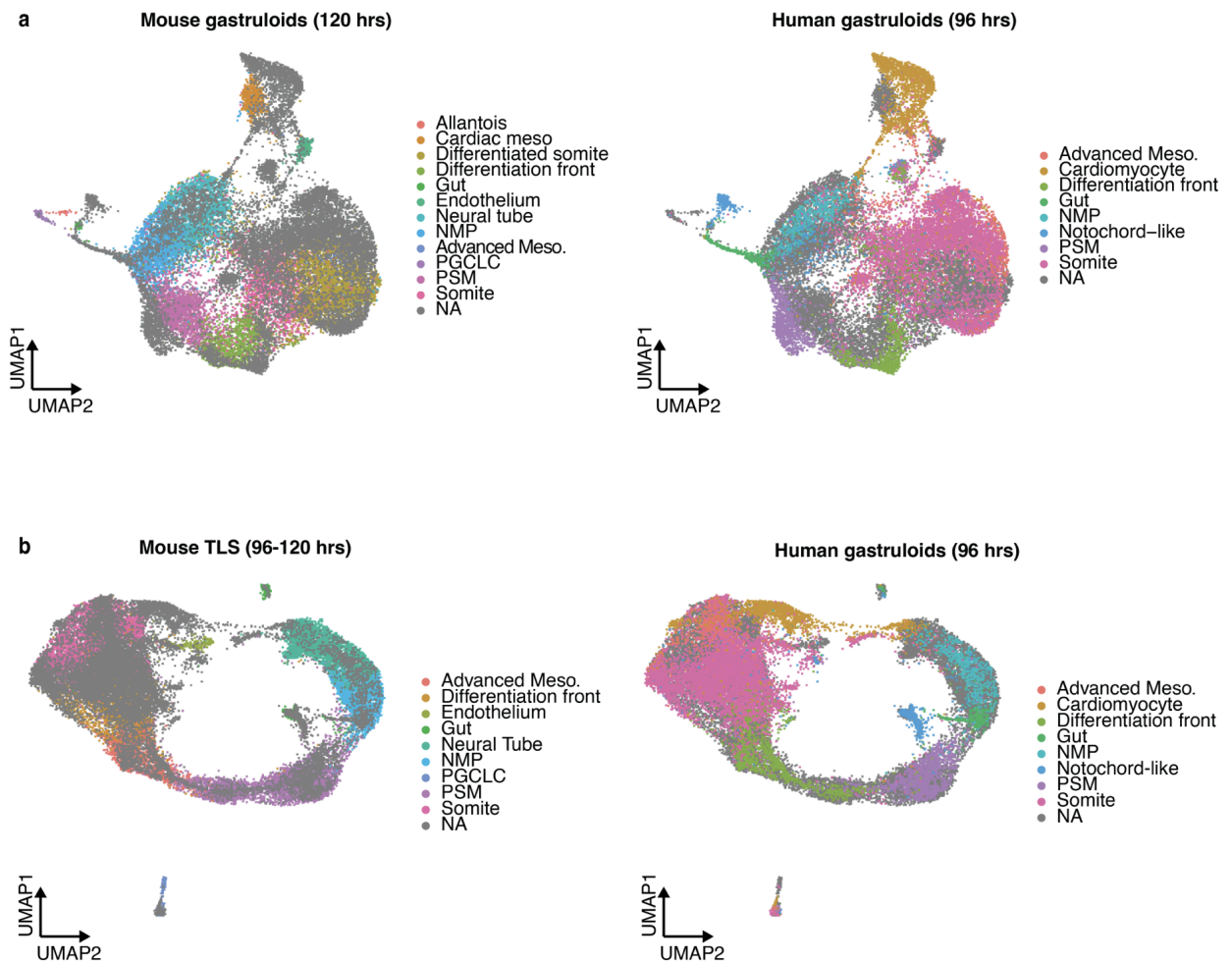
Supplementary Fig.4. Marker gene expression & cell type annotation in human gastruloids at 96 hrs after induction.

UMAP with unsupervised clustering of scRNA-seq profiles is shown, along with expression of marker genes used to annotate these clusters as a continuum of neuromesodermal progenitors, presomitic mesoderm, differentiation front, and somites; additionally, advanced mesoderm and cardiac mesoderm, as well as notochord-like cells and gut/endoderm-like cells, were detected. NMP, neural mesodermal progenitor; PSM, presomitic mesoderm.



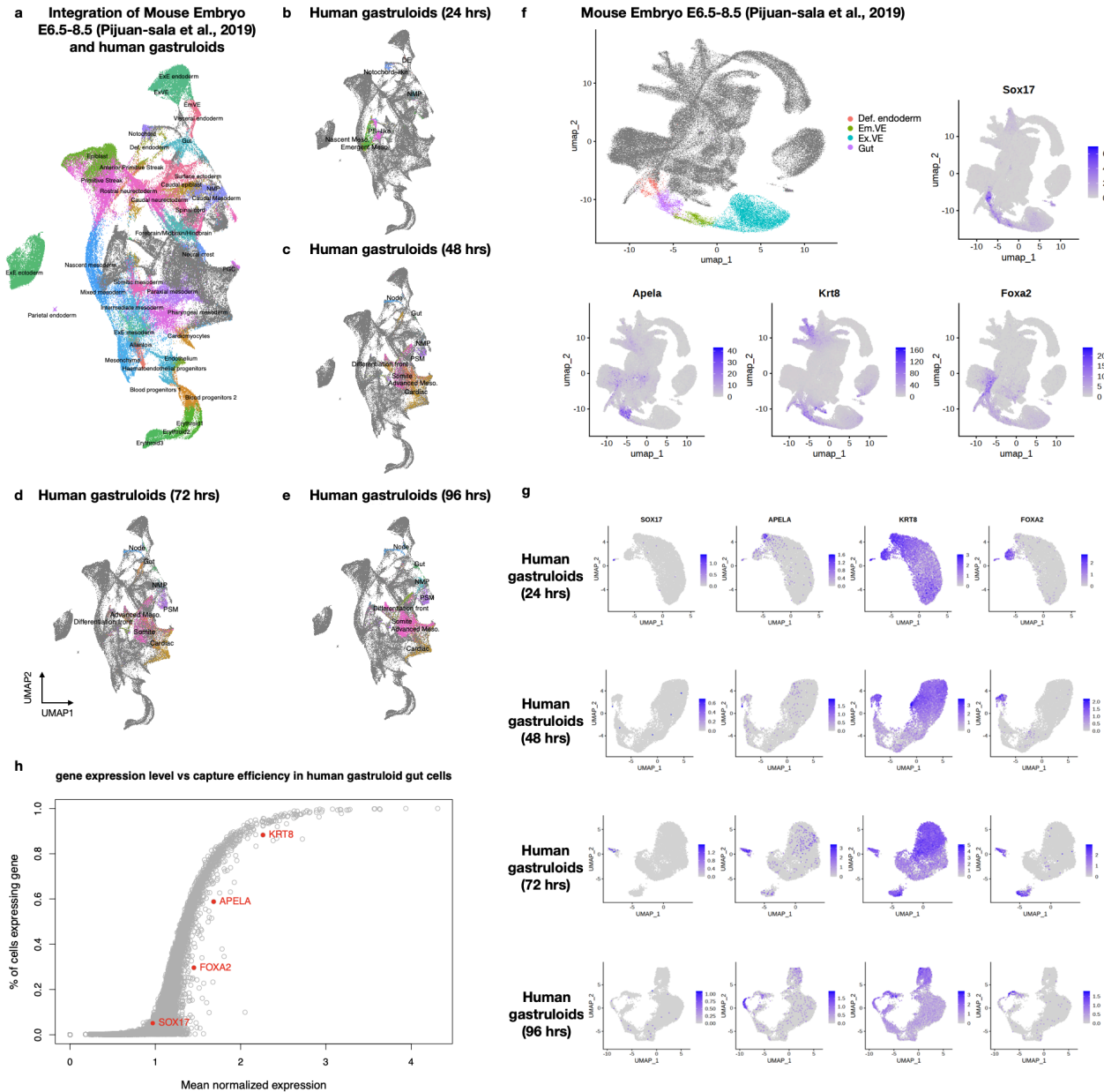
Supplementary Fig.5. Co-embedding of human gastruloid scRNA-seq profiles (24-96 hrs) with published data from either CS7 or CS12/13 human embryos.

Co-embedding and UMAP visualisation of scRNA-seq profiles of human embryos (left) with human gastruloids (right) at CS7 (a-d) or at CS12-13 (e-g) 24. In each panel, the same UMAP visualisation is shown twice, but with different subsets of cells labelled. NA refers to cells from the other sample in the co-embedding, which are instead annotated in the other column. As noted in the text, integration of scRNA-seq data from 24 hrs with CS7 data was informative with respect to cell type annotation. However, projections of 48-96 hr data with either CS7 or CS12/13 human embryo scRNA-seq profiles were not informative, potentially because the stages represented by these gastruloids fall in between CS7 and CS12/13.



Supplementary Fig.6. Co-embedding of human gastruloid scRNA-seq profiles (96 hrs) with published data from two mouse gastruloid models.

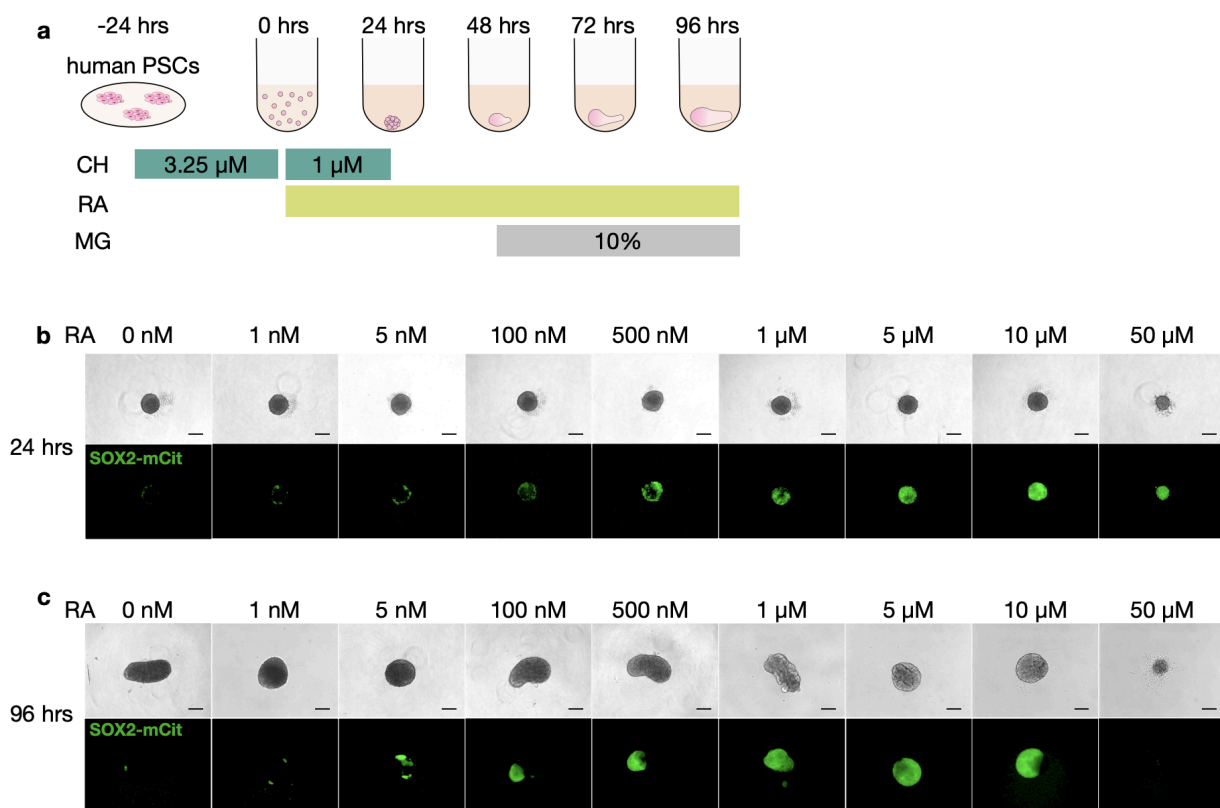
a, Co-embedding and UMAP visualisation of scRNA-seq profiles, labelled by annotations from 120 hrs mouse gastruloids¹⁰ (left) or 96 hrs human gastruloids (this study, right). NA refers to cells from the other sample in the co-embedding, which are instead annotated in the other column. b, Co-embedding and UMAP visualisation of scRNA-seq profiles, labelled by annotations from 96-120 hrs mouse TLS¹⁵ (left) or 96 hrs human gastruloids (this study, right).



Supplementary Fig.7. Mapping gut/endoderm-like cells via integration of human gastruloid and mouse embryo scRNA-seq data.

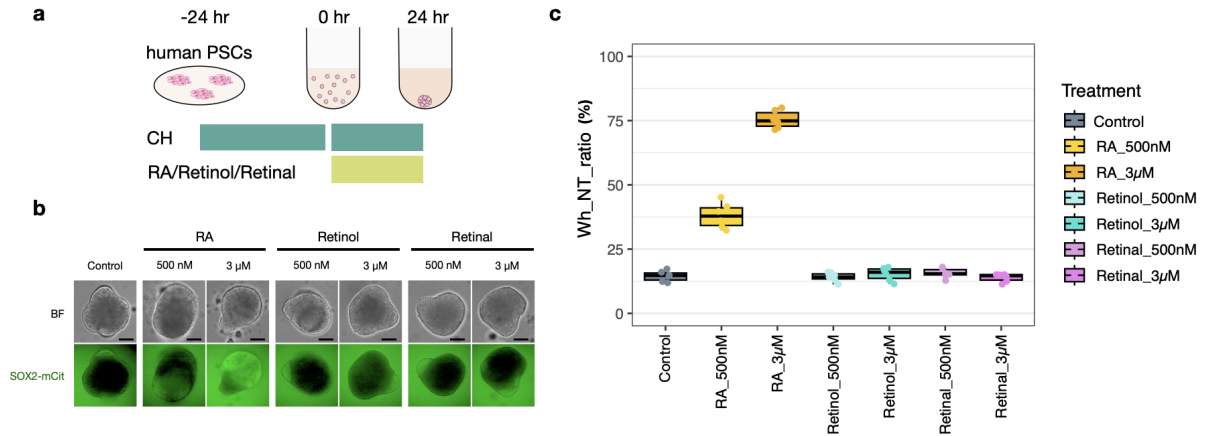
a, Co-embedding and UMAP visualisation of scRNA-seq profiles from 24-96 hrs human gastruloids (this study) and E6.5-E8.5 mouse embryos⁸⁰. Mouse embryo cells are coloured and annotated with labels from the original study. b-e, Same as panel (a), with human gastruloid cells from 24 hrs (b), 48 hrs (c), 72 hrs (d) or 96 hrs (e), coloured and annotated with labels from this study. Cells that we have annotated as resembling definitive endoderm (DE) at 24 hrs co-localize with mouse embryonic visceral endoderm (EmVE) cells. Cells that we have annotated as gut/endoderm-like at 48-96 hrs co-localize with mouse embryonic gut cells. f, Expression of gut marker genes (Sox17, Apela, Krt8, Foxa2) in mouse embryos (E6.5-E8.5) during the transition of definitive endoderm to gut, g,

Visualisation of the same gut marker genes in the development of human gastruloids. h, Variable SOX17 expression in gut/endoderm-like cell type across timepoints is possibly due to its relatively low detection in 10X Genomics data. For all endoderm/gut cells in human gastruloids (24-96 hrs) profiled with 10x Genomics scRNA-seq, the percentage of cells that expressed each gene was calculated. Among cells that express a specific gene, the average expression level (log-normalised) of that gene was further computed. The diagram shows correlation of the percentage of cells expressing a gene vs. the average expression level for that same gene. The selected gut marker genes (i.e. KRT8, APELLA, FOXA2, SOX17) are labelled in red. SOX17 transcripts are poorly captured in 10X Genomics data, relative to other gut marker genes.



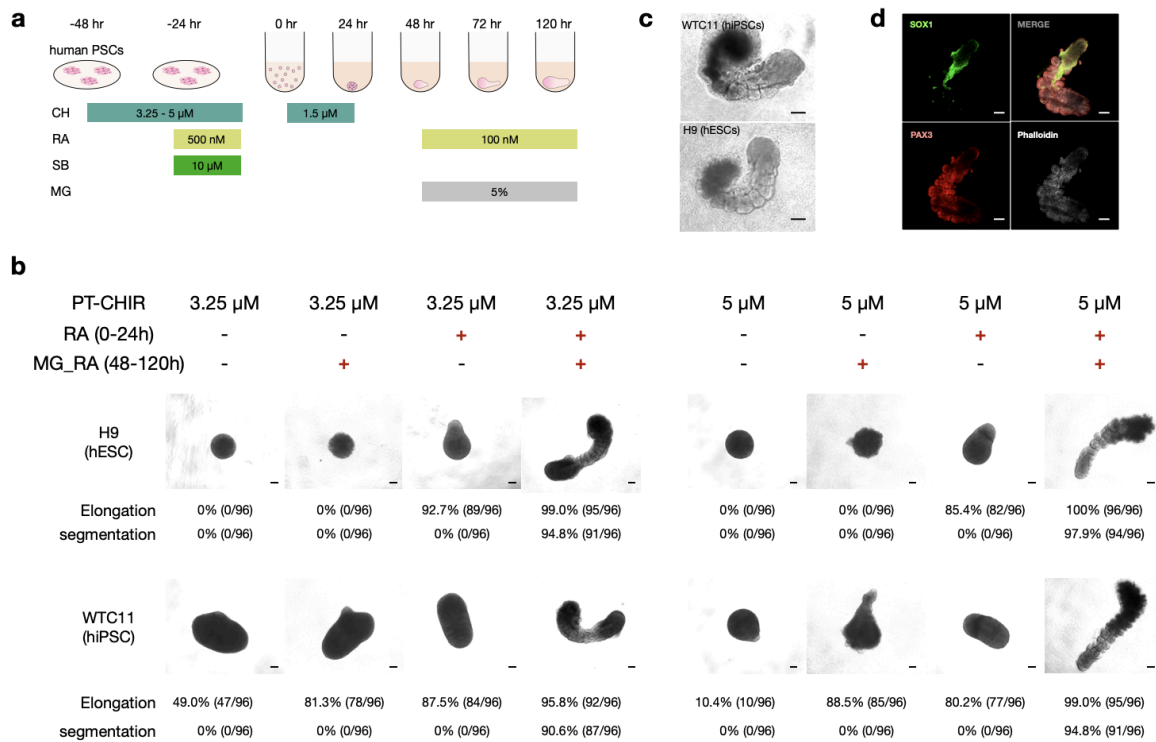
Supplementary Fig.8. Effect of continuous retinoic acid on neural cell induction in human gastruloids.

a, Schematic of RA and Matrigel treatment of human gastruloids. RA, retinoic acid; MG, 10% Matrigel. b-c, Representative images of RA and Matrigel-treated human gastruloids at 24 hrs (b) and 96 hrs (c). The intensity of SOX2-mCit correlated with the concentration of RA. Scale bars, 100 μ m, N = 48 for each condition.



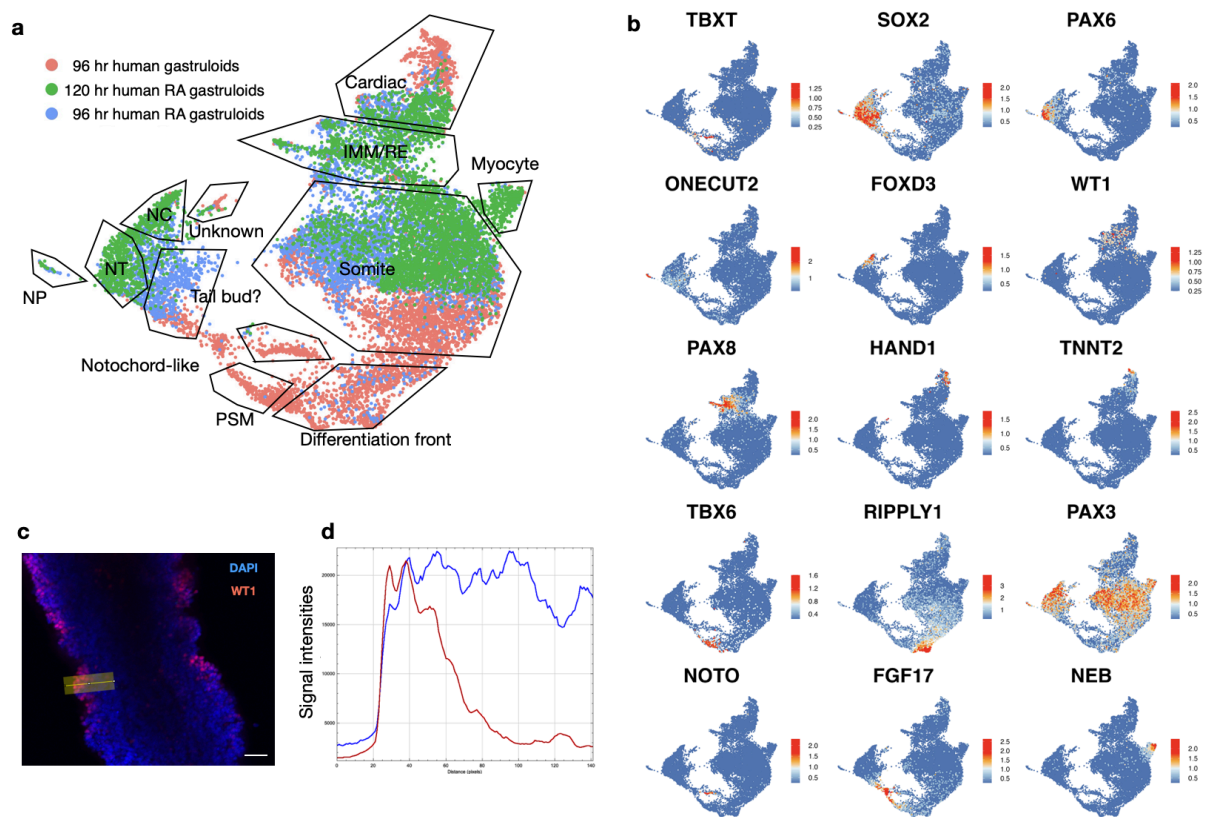
Supplementary Fig.9. The effects of RA/Retinol/Retinal on neural fate induction in human gastruloids.

a, Schematic for the induction of human gastruloids with RA, retinol or retinal supplementation. RA, retinol or retinal were added to the medium at 0-24 hrs. b, Brightfield and mCit fluorescent images of 48 hrs human gastruloids after addition of retinol, retinal or RA at 0-24 hrs. In this cell line, mCit fluorescence is indicative of SOX2 expression in human gastruloids. Scale bar, 100 μ m. c, Quantification of the SOX2-mCit signals. $N \geq 6$ gastruloids per condition.



Supplementary Fig.10. RA-gastruloid induction from human iPSCs (WTC11) and human ESCs (H9).

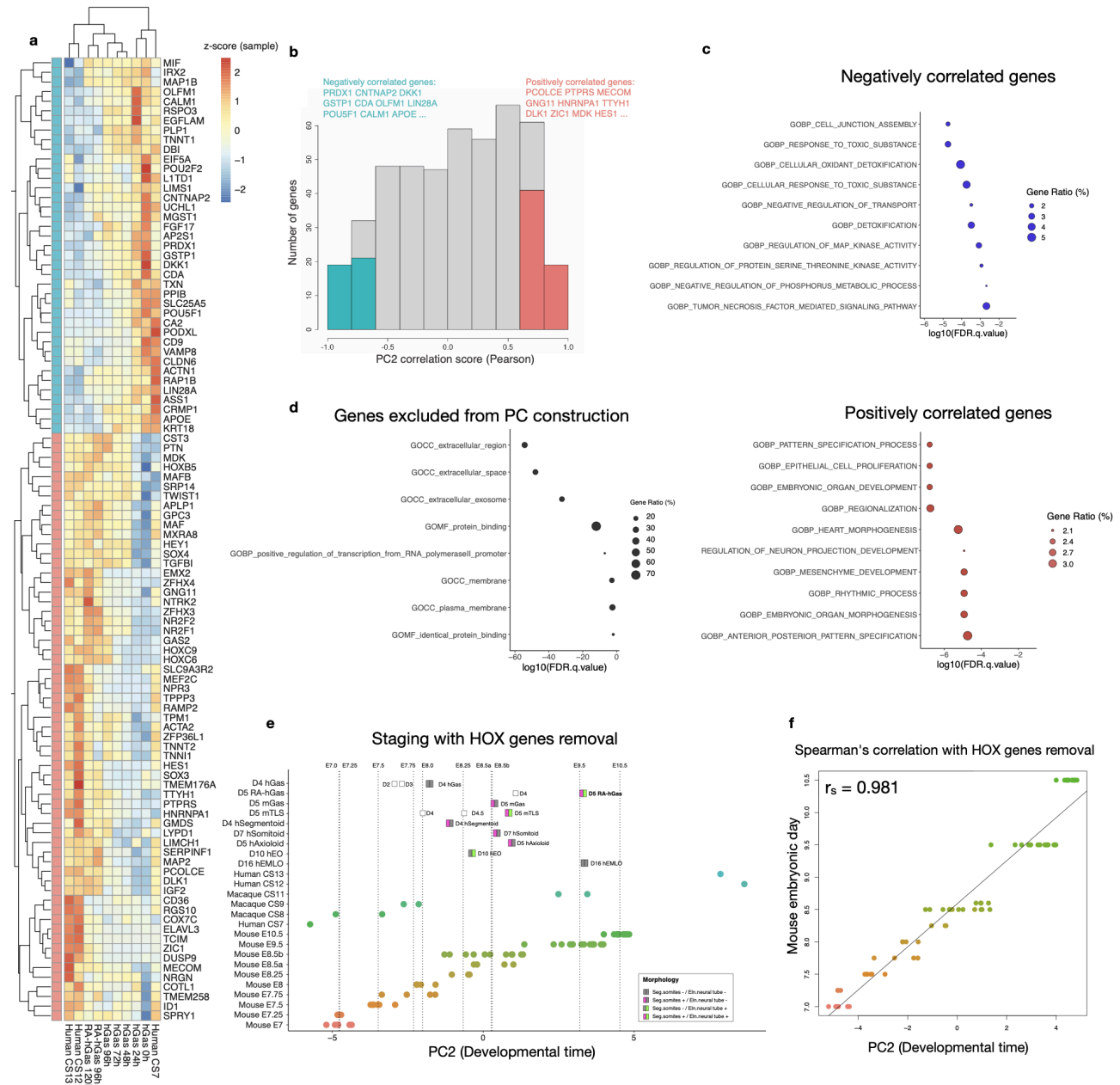
a, Scheme for pre-treatment and human RA-gastruloid induction. RA, retinoic acid; MG, Matrigel; CH; CHIR99021; SB, SB-431542. b, Effects of concentration of CHIR at pre-treatment (PT-CH), supplemented with RA at 0-24 hrs and RA and 5% Matrigel at 48-120 hrs. Scale bar, 100 μ m. c, Representative images of RA-gastruloids derived from human iPSCs (WTC11, top) and another human ESC line (H9). d, Immunostaining of WTC11-derived RA-gastruloids with SOX1-, PAX3-antibodies and Phalloidin. Scale bar, 100 μ m. N = 14 out of 17 RA-gastruloids showed similar marker gene expression patterns.



Supplementary Fig.11. Comparison of cell types detected in human conventional vs. RA-gastruloids.

a, Co-embedded UMAP of scRNA-seq data coloured by the sample of origin. Red: 96 hrs conventional gastruloid (N = 5,000 cells). Blue: 96 hrs RA-gastruloid (N = 5,000 cells). Green: 120 hrs RA-gastruloid (N = 5,000). Clusters were annotated based on marker gene expression patterns. NP, neural progenitors; NC, neural crest; NT, neural tube; PSM, presomitic mesoderm; IMM, intermediate mesoderm; RE, renal epithelium; NMP, neuromesodermal progenitor. b, Marker gene expression patterns in co-embedded UMAP. c, Immunostaining of WT1 in 120 hrs RA-gastruloids.

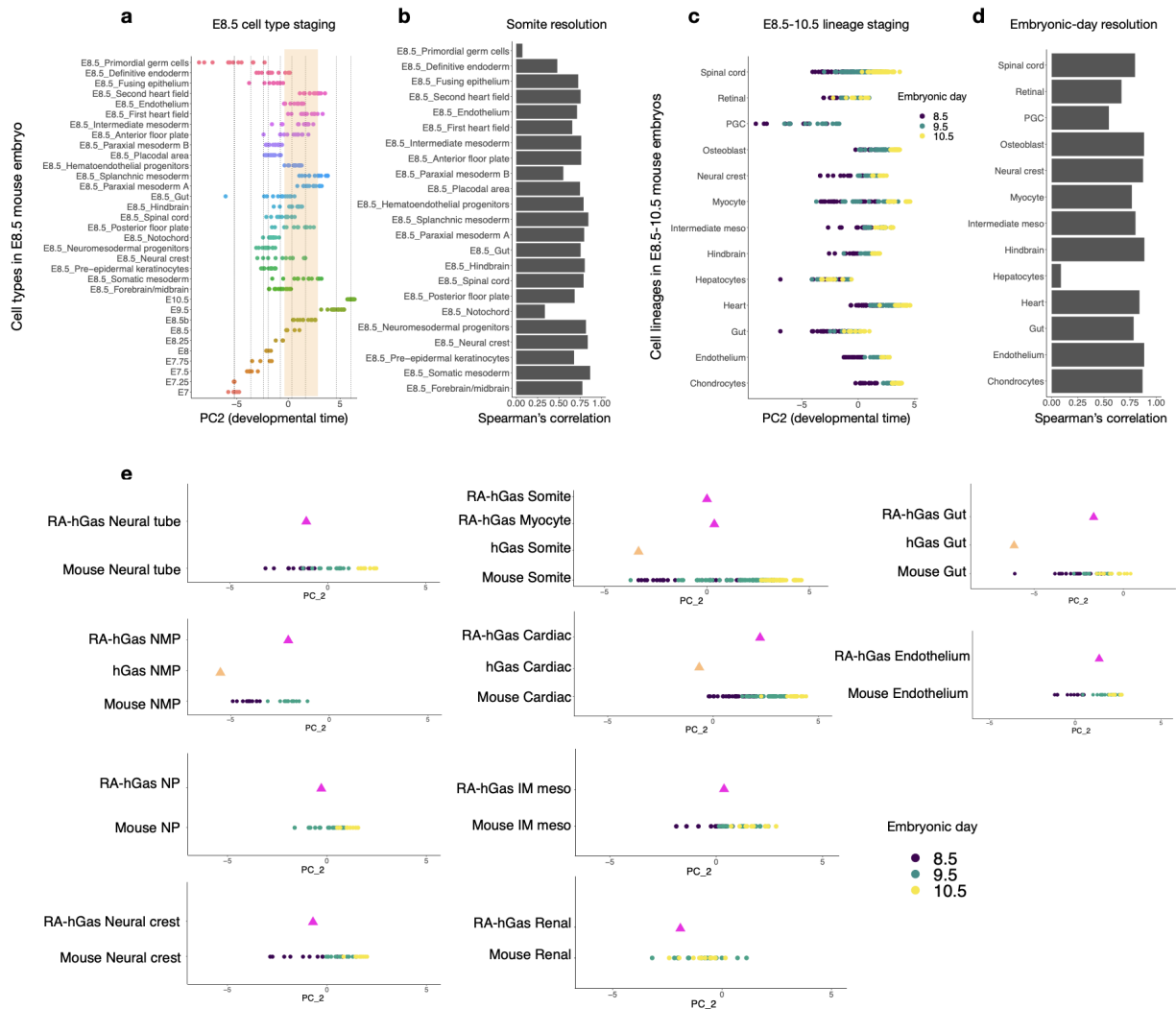
Signal intensities of DAPI (blue) and WT1 (red) were measured on the area indicated by the yellow line. Scale bar, 100 μ m. d, Measured intensities of DAPI (blue) and WT1 (red) signals. Although there may be lower DAPI staining at more internal positions, a very sharp dropoff in WT1 staining is observed at depths where DAPI signal is entirely maintained.



Supplementary Fig.12. Genes and pathways that are correlated with developmental progression.

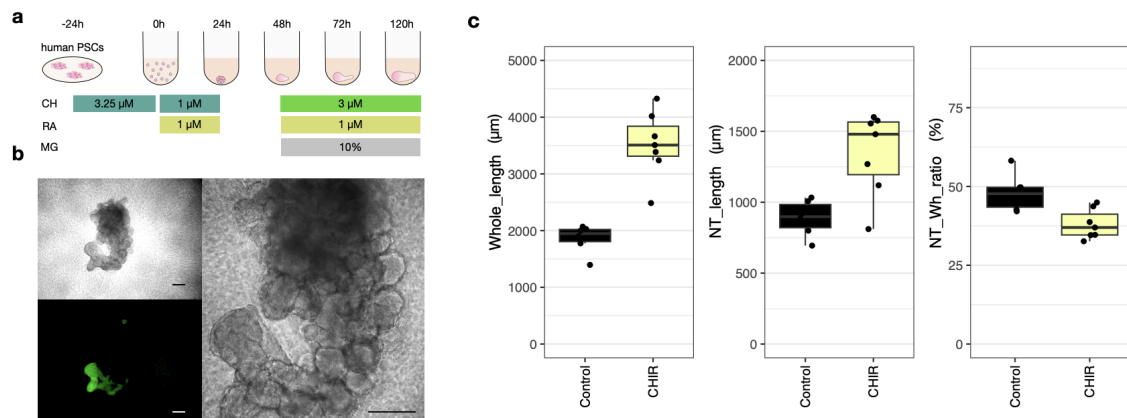
a, Biclustered heatmap shows the z-transformed expression levels of each of these top 100 genes (rows) positively (pink in column at left) or negatively (blue in column at left) correlated with PC2 in various human samples (columns). Colours correspond to z-scores. b, The top 100 genes with the

highest absolute correlation scores with PC2 were selected from all 447 genes used for PCA of human samples^{24,57}. c, Gene Ontology (GO) enrichment analysis (MSigDB v7.5.1) of the genes negatively (Top) or positively (Bottom) correlated with PC2^{83,84}. Gene Ratio: percentage of query genes in the matched GSEA gene sets. d, GO term enrichment computed by DAVID Bioinformatics Resources (<https://david.ncifcrf.gov/>) on all the human embryo variable genes excluded from the final PCA analysis. e, Similar to Fig. 4e, but after removing HOX genes. f, Scatterplot and Spearman's correlation of mouse embryos' PC2 values (x-axis) and their embryonic stage (y-axis; E7.0-10.5), after removing HOX genes. A fitted regression line from a linear model is plotted. rs, Spearman's correlation.



Supplementary Fig.13. Developmental staging of individual cell types within human RA-gastruloids against in vivo mouse development.

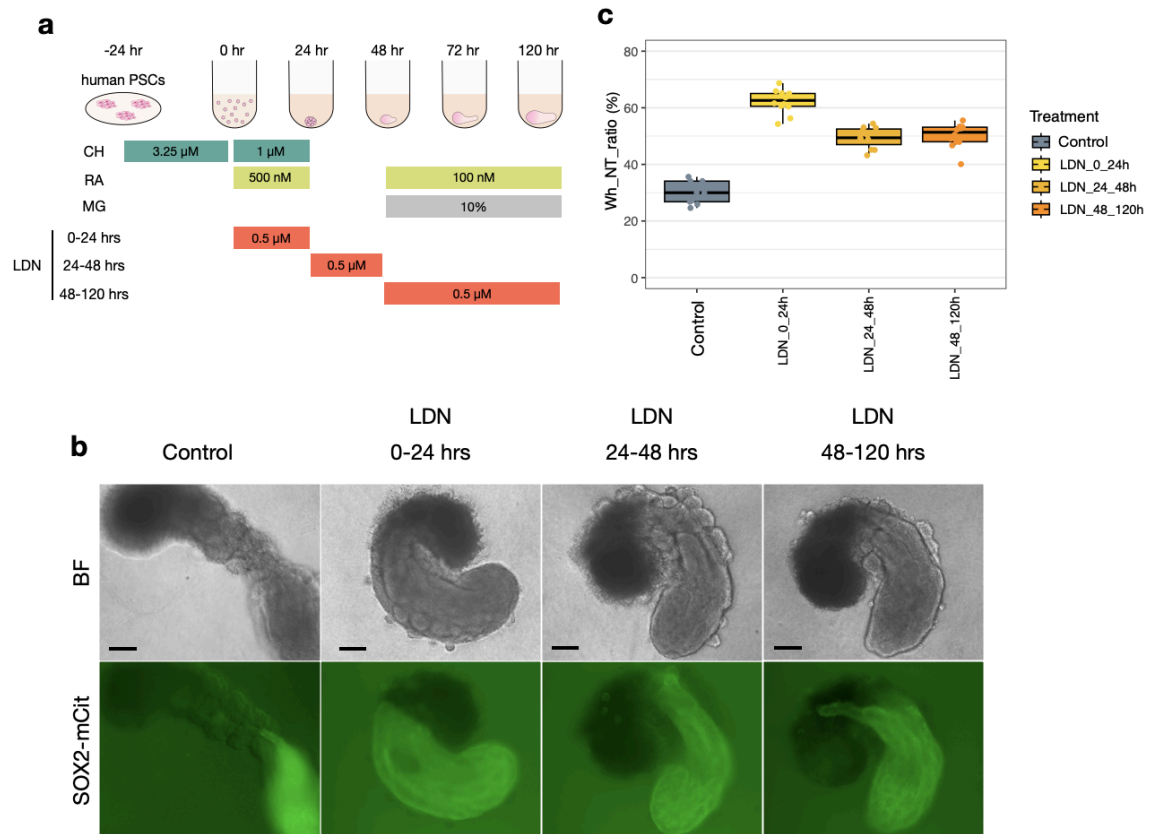
a, Major cell types from E8.5 mouse embryos^{13,14} (y-axis) were mapped onto the human-derived PC2 axis (x-axis). For top rows, each datapoint represents a pseudo-bulk transcriptome of the cell type in an individual embryo (E8.5b data generated by sci-RNA-seq3), while bottom rows correspond to pseudobulk embryos (E8.5b-E10.5) or embryo pools (E7-E8.5). Orange shaded area corresponds to PC2 range spanned by E8.5 pseudobulk embryos. b, Spearman's correlation of the PC2 values of individual mouse cell types vs. somite count. c, Major cell types from E8.5-E10.5 mouse embryos^{13,14} (y-axis) were mapped onto the human-derived PC2 axis (x-axis). Although they are not fully aligned to one another, within any given cell type, we observe that the PC2 values of E9.5 mice are generally higher than E8.5 mice, and E10.5 mice generally higher than E9.5 mice. d, Spearman's correlation of the PC2 values of individual mouse cell types vs. embryonic day (E8.5-E10.5). e, For various cell types observed in 120 hrs human RA-gastruloids, we plot the PC2 values of either the pseudobulked cell type from pooled human RA-gastruloids (top rows) or individual mouse embryos (E8.5-E10.5, bottom rows). NMP, neural-mesodermal progenitor; NP, neural progenitor; IM meso, intermediate mesoderm.



Supplementary Fig.14. Hyperactivation of WNT signalling in human RA-gastruloids. a, Schematic of experimental hyperactivation of WNT signalling in human RA-gastruloids.

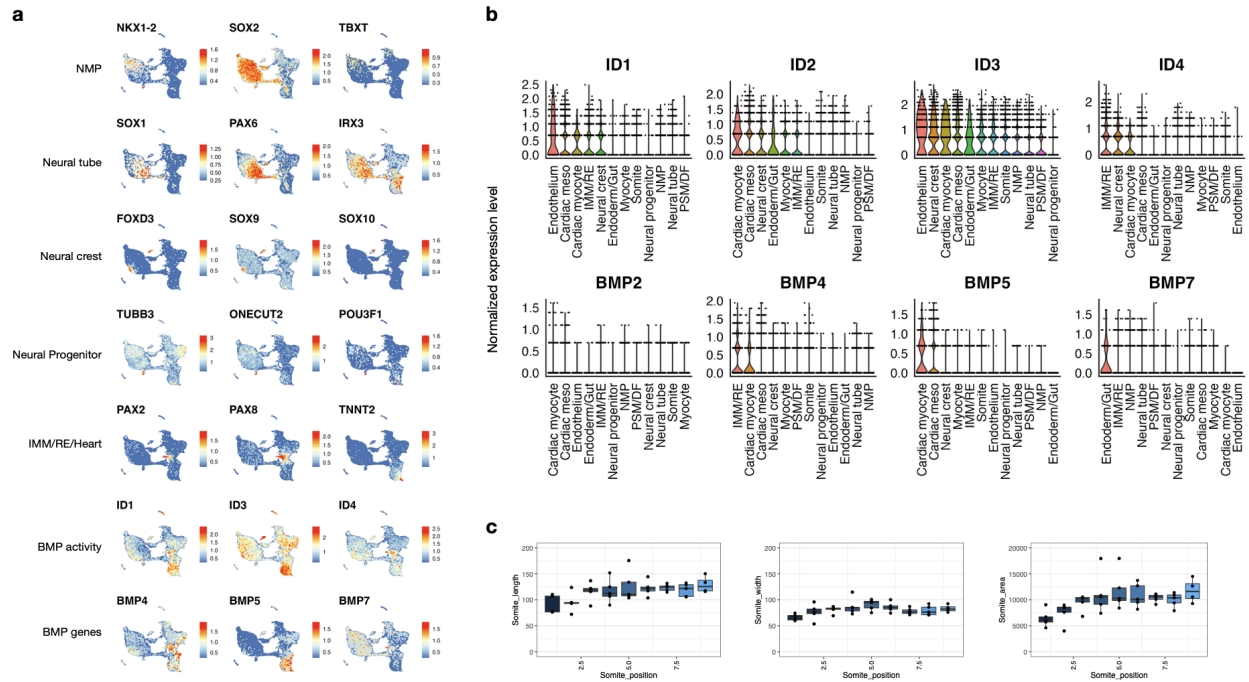
In brief, the protocol was modified to add CHIR back into the system at 48 hrs. CHIR, CHIR99021; MG, Matrigel; RA, Retinoic acid. b, Representative morphology of CHIR-treated 120 hrs RA-gastruloids. Bar, 100 μ m. N =192 human RA-gastruloids generated under the modified protocol showed similar morphology (elongated gastruloid with a high density of somite-like structures appearing along the entire anterior-posterior axis) (Top left) Bright field, (bottom left)

SOX2-mCit, and (right) magnified view of top left panel. Scale bar, 100 μ m. c, Morphometrics as described in Fig. S14. (from left to right) Full length (μ m), neural tube length (μ m) measured with SOX2-mCit signal, and neural-tube/full-length ratio of 120 hrs human RA-gastruloids. N = 6 and 7 for 120 hrs controls and CHIR treatment, respectively.



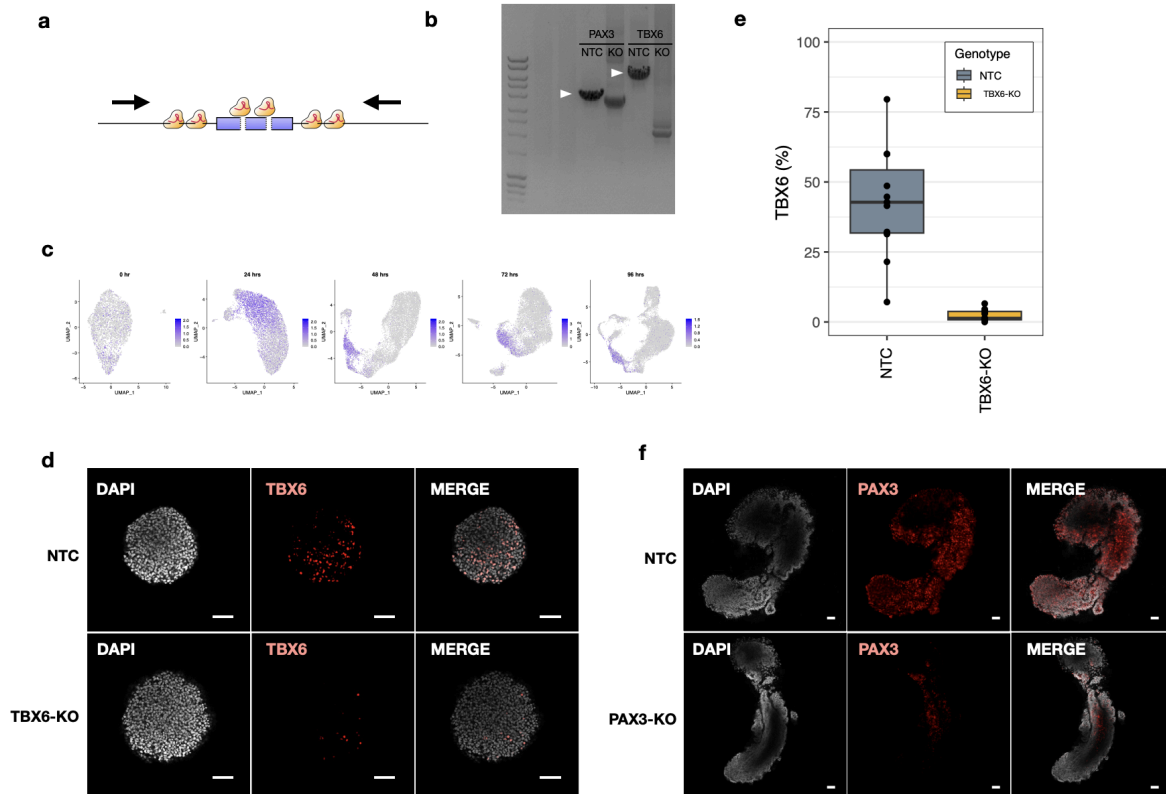
Supplementary Fig.15. Temporal inhibition of BMP signalling. a, Schematic of temporal inhibition of BMP signalling in human RA-gastruloids.

RA, retinoic acid; MG, Matrigel; CH; CHIR, CHIR99021; LDN, LDN183189. b, Representative bright-field (top) or SOX2-mCit (bottom) images of 120 hrs human RA-gastruloids with no LDN treatment (control) vs. treatment at 0-24 hrs, 24-48 hrs or 48-120 hrs. Scale bar, 100 μ m. c, Quantification of SOX2-mCit area in human RA-gastruloids upon LDN treatment. N = 9, 12, 11, 12 for controls or 0-24 hr, 24-48 hr, 48-120 hrs LDN treatment, respectively.



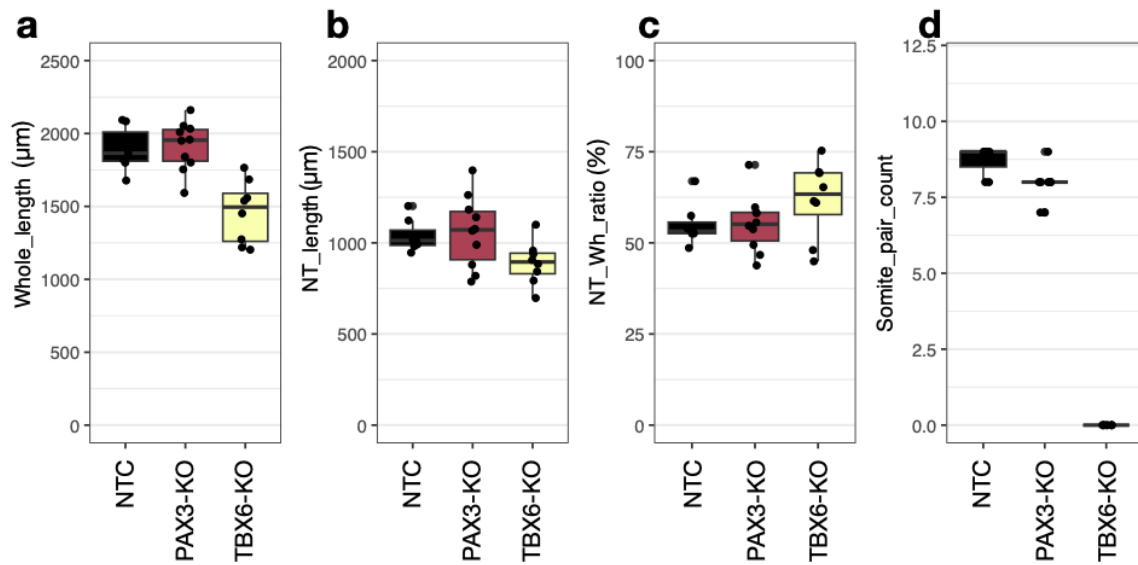
Supplementary Fig.16. Effects of LDN treatment on gene expression and somite morphology in human RA-gastruloids.

a, UMAP visualisation of co-embedded scRNA-seq data from untreated and LDN-treated human RA-gastruloids. The same projection is shown in Fig. 5d. Gene expression of three marker genes for each cell type are shown in each row, with two additional rows for markers of BMP activity (second to last row) or BMP genes (last row). b, Violin plots showing ID and BMP gene expression patterns for various cell types. Cell types along x-axes are sorted by the mean expression levels of the gene, in descending order. c, Quantification of somites in LDN-treated RA-gastruloids. Length, width and area of somites in LDN-treated RA-gastruloids as a function of position. N = 5 gastruloids.



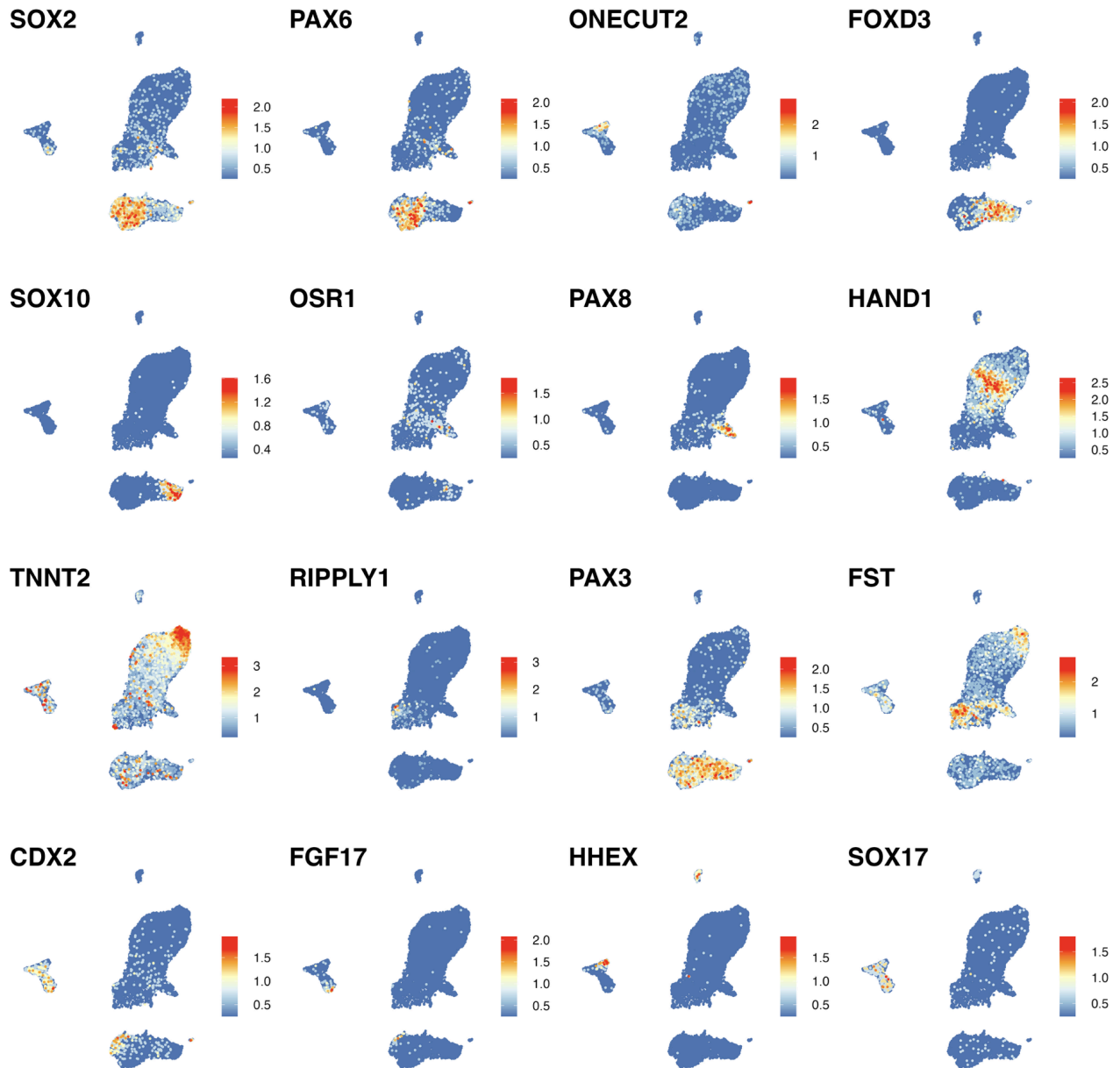
Supplementary Fig.17. Validation of knockouts in TBX6-KO or PAX3-KO human RA-gastruloids. a, Schematic for genotyping PCR.

Forward and reverse primers are indicated as arrows. Primers were set outside of the multiple Cas9-targeting sites. b, Genotyping PCR on wildtype and Cas9-RNP induced hPSCs. Transfected hPSCs were harvested and lysed at day 7 from the transfection. The predicted wildtype band sizes are shown in arrowheads. c, TBX6 expression patterns in scRNA-seq data from conventional human gastruloids, 0-96 hrs. d, Representative images of immunostaining of TBX6 protein in NTC control and TBX6-KO RA-gastruloids at 24 hrs. Scale bar, 100 μ m. e, Estimated proportion of TBX6⁺ cells in NTC controls or TBX6-KO RA-gastruloids. For panels (d-e), N = 11 and 14 for control and TBX6-KO gastruloids, respectively. f, Representative images of immunostaining of PAX3 protein in NTC control and PAX3-KO RA-gastruloids at 120 hrs. Scale bar, 100 μ m. N = 5 and 6 for control and PAX3-KO gastruloids, respectively.



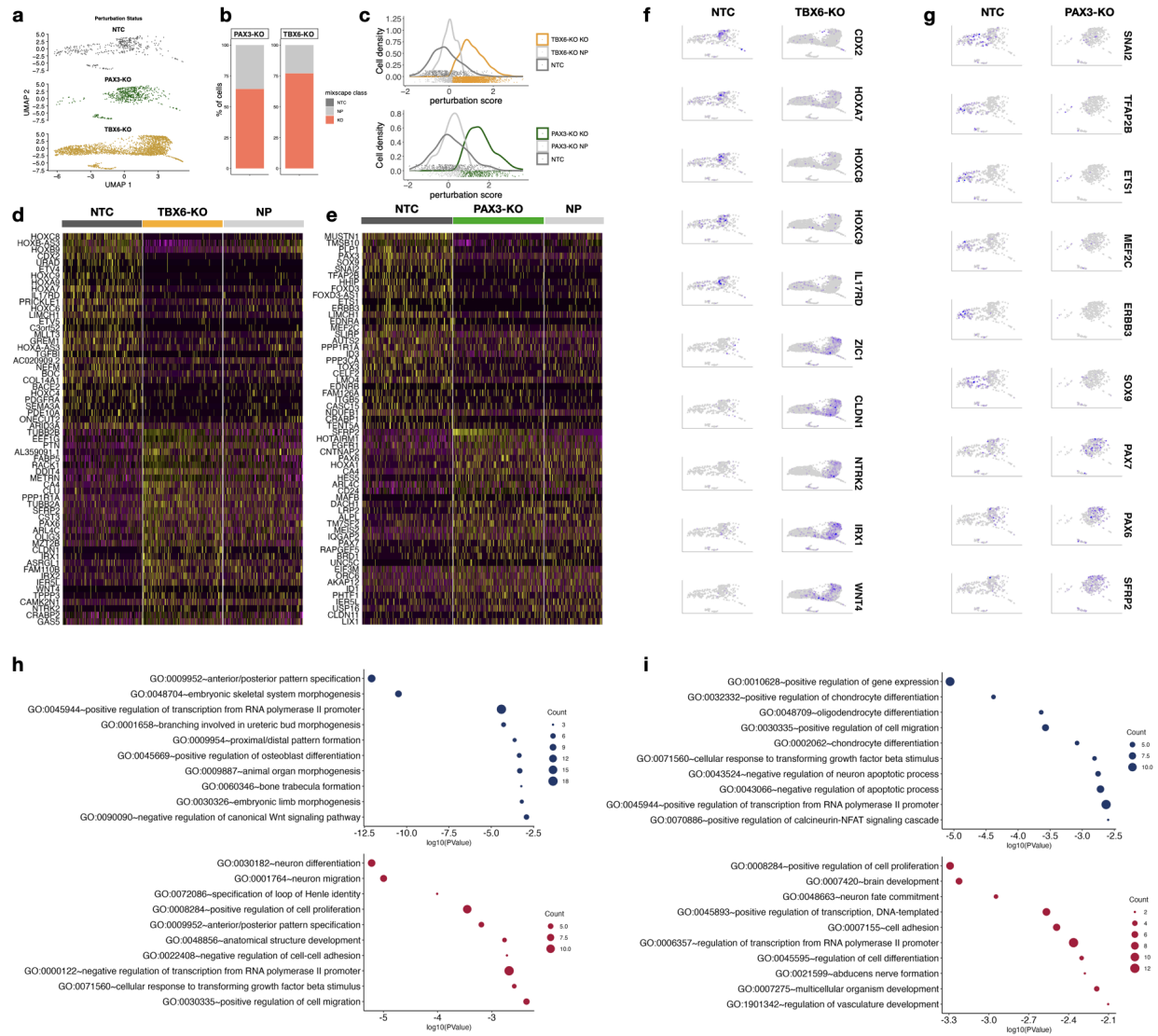
Supplementary Fig.18. Morphometrics of PAX3-KO and TBX6-KO human RA-gastruloids.

Morphological characteristics were measured as in Fig. S14. a, Full length (μm) of 120 hrs human RA-gastruloids, stratified by genotype. b, Neural tube length (μm) measured with SOX2-mCit signals, stratified by genotype. c, Neural-tube/full-length ratio, stratified by genotype. d, Somite pair counts, stratified by genotype. N = 7, 10, and 8 for non-targeting control (NTC), PAX3-KO, and TBX6-KO RA-gastruloids, respectively.



Supplementary Fig.19. Marker gene expression in NTC and genetically-perturbed human RA-gastruloids

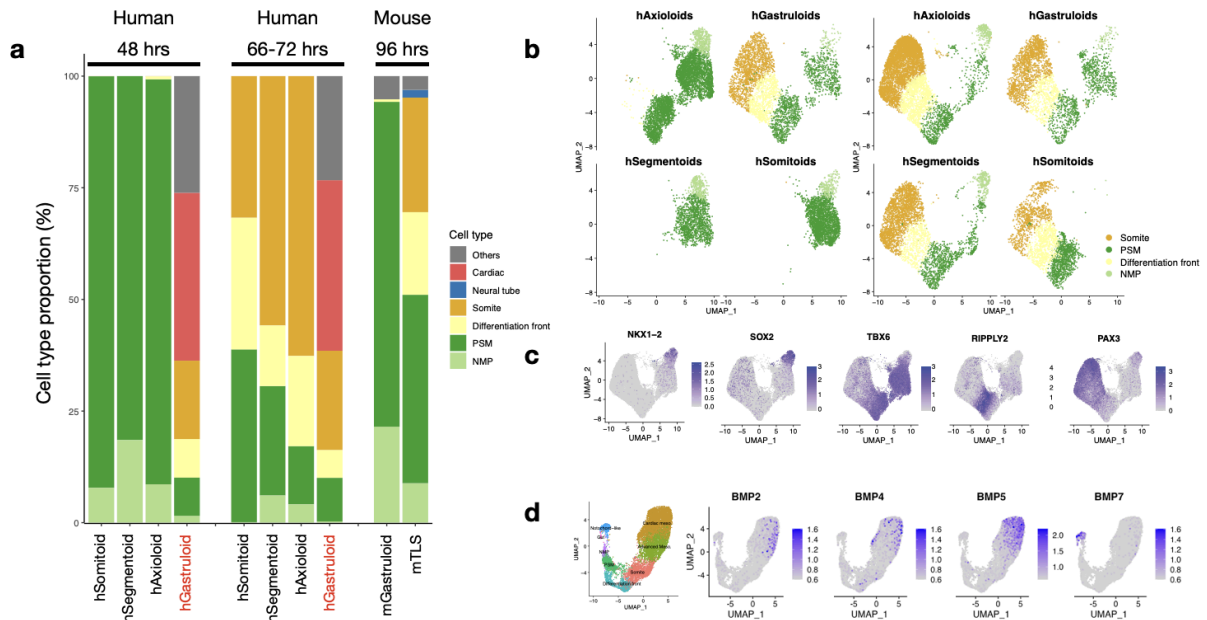
Marker gene expression in NTC and genetically-perturbed human RA-gastruloids on the co-embedded UMAP also shown in Fig. 6d. Marker genes are for the following cell types: SOX2, neural cells; PAX6, neural tube; ONECUT2, neural progenitor; FOXD3 and SOX10, neural crest; OSR1, intermediate mesoderm; PAX8, renal epithelium; HAND1, cardiac mesoderm; TNNT2, cardiac myocyte; RIPPLY1, differentiation front; PAX3 and FST, somite; CDX2, caudal cell; FGF17, definitive endoderm; HHEX, gut; SOX17, pan-endoderm.



Supplementary Fig.20. Effects of genetic perturbation on human RA-gastruloids.

a, UMAP visualisation of neural cell types (NMP, neural tube, neural crest, and neural progenitor) of NTC, TBX6-KO, and PAX3-KO human RA-gastruloids. b, Bar plots representing the results of Mixscape classification of TBX6-KO and PAX3-KO RA-gastruloids. Cells were classified as knocked out (KO) or non-perturbed (NP), based on the perturbation scores calculated by Mixscape. c, Distribution of Mixscape perturbation scores. d-e, single cell RNA heatmaps of DEGs in TBX6-KO and PAX3-KO RA-gastruloids. The top DEGs (30 up, 30 down) for TBX6-KO (d) and PAX3-KO (e) RA-gastruloids are shown. f-g, Expression patterns of representative DEGs of TBX6-KO (f) and PAX3-KO (g) projected onto the same UMAP shown in panel (a). h-i, GO

enrichment analysis on (Top) down-regulated and (Bottom) up-regulated DEGs in TBX6-KO (h) and PAX3-KO RA-gastruloids (i).



Supplementary Fig.21. Cell type composition of embryo models.

a, Barplots showing the percentage of each cell type in various embryo models at 48 to 72 hrs (human) or 96 hrs (mouse). "Others" includes advanced mesoderm, node and gut for hGastruloids; ectopic pluripotency and gut for mGastruloids; and PCGLC, endothelial, and gut for mTLS. Models shown include conventional human gastruloids (hGastruloid, 48 and 72 hrs) 11, human somitoids (hSomitoid, 48 and 66 hrs) 16, human segmentoids (hSegmentoid, 48 and 72 hrs) 17, human axioloids (hAxioloid, 48 and 72 hrs) 18, mouse gastruloids (mGastruloid, 96 hrs) 82 and mouse TLS (mTLS, 96 hrs) 15. b, Co-embedding of scRNA-seq data from several human embryo models (axioloids, gastruloids, segmentoids, somitoids) at 48 hrs (left panel) or 72 hrs (right panel), with cells from specific models highlighted in each subpanel, coloured by cell type. c, Same co-embedded UMAPs as panel (b) with gene expression for markers of NMP (NKX1-2, SOX2), PSM (TBX6), differentiation front (RIPPLY2), and somite (PAX3) cell types. d, UMAP of 48 hrs conventional human gastruloids (left-most panel, same as Fig. S3) highlighting gene expression of various BMP transcripts (remaining panels).

Chapter 3 Single cell multi-region dissection of healthy aging in macaque brain

This Chapter is adopted from publication in preparation with minimum changes.

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Abstract

Brain aging is a complex process with profound health and societal consequences. However, the molecular and cellular pathways that govern its temporal progression—along with any cell type-, region-, and sex-specific heterogeneity—remain poorly defined. Here, we present a transcriptomic atlas of 5.3 million cells from 11 brain regions of 55 nonhuman primates (29 female, 26 male), spanning 5 months (early life) to 21 years (late adulthood) of age (582 samples). We annotate 12 major cell classes and 226 subclusters, including region-specific subtypes of excitatory and inhibitory neurons, astrocytes, and ependymal cells. We identify a vulnerable excitatory neuron population that declines with age in the upper-layer cortex, along with brain-wide, age-dependent shifts in microglial and oligodendrocyte states. Finally, we chart convergent and divergent molecular signatures of aging—some with sex-specific patterns—across brain regions and cell types. This work provides a temporal, regional, and sex-stratified atlas of the aging primate brain, offering insights into cell type-specific vulnerabilities and regional heterogeneity.

3.1 Introduction

Aging is frequently accompanied by cognitive decline and is the strongest risk factor for many neurodegenerative diseases. Sex differences in brain aging further shape the incidence and trajectory of these disorders ([1, 2](#)). Structural and functional brain changes with age are well documented ([3–5](#)), and multiple biological processes—including mitochondrial dysfunction, oxidative stress, and DNA damage—have been implicated in brain aging ([6, 7](#)). However, the precise relationships between chronological brain aging, sex, molecular and cellular deterioration, and neurodegenerative phenotypes remain elusive.

The brain is composed of diverse regions and cell types, each of which may age differently (8). Single cell transcriptomics enables the resolution of age-associated molecular changes with cell type specificity, offering a potentially powerful lens into the early cellular events that may precede, accompany or drive functional decline. Recent single cell studies have begun to reveal aging-related vulnerabilities in specific brain cell types (9–11). However, these efforts have largely focused on case-control comparisons of diseased vs. unaffected individuals, rather than the natural course of aging in healthy brains.

Aging is a progressive process, and the molecular and cellular changes that underlie aging-associated phenotypes presumably initiate years and even decades before the onset of clinical symptoms. Thus, temporally-resolved profiling of healthy brains may be critical to understanding the normal trajectory of brain aging including early cellular and molecular signatures that may be harbingers of pathology. However, human datasets of this kind are scarce, and often limited to a single brain region (12). Given the brain's regional specialization and distinct functional domains, results from such region-specific studies may not generalize to the entire brain. Indeed, differential vulnerability among brain regions has been reported in both aging and neurodegeneration (13, 14), with sex differences in brain aging and disease adding further complexity (15).

Comprehensive, age-resolved, regionally diverse, sex-balanced molecular data from healthy human brains are exceptionally difficult to obtain. To the extent that they are obtained, clinical samples often suffer from variable postmortem intervals and disease comorbidities. The rhesus macaque (*Macaca mulatta*), a nonhuman primate species with close evolutionary proximity to humans, provides an excellent alternative (16). Macaque and human neuroanatomy exhibit extensive homology. Macaques also have a relatively long lifespan and experience naturally occurring age-related phenotypes—including sex-biased patterns—that mirror many aspects of human brain aging (12, 17, 18).

In this study, we leverage a unique cohort of free-ranging rhesus macaques, living under naturalistic conditions with minimal human interference (19), to construct an atlas of brain aging at high resolution with respect to both age and region. With extensive sampling of individuals across the macaque lifespan, we map the trajectories of molecular and cellular changes that accompany

physiological brain aging in a highly relevant primate model, and compare these changes across cell types, brain regions, and between the sexes to identify age-vulnerable and sex-biased cells and regions. By focusing on brain regions implicated in primate aging and cognitive decline (17), we are also able to intersect our results with genetic analyses of human neurodegenerative conditions, shedding light on the intersection of normal and pathological aging in an evolutionarily grounded manner.

3.2 Single nucleus, multi-region transcriptional profiling of the aging macaque brain

Towards a comprehensive view of transcriptional dynamics in the brain throughout the primate lifespan, we performed snRNA-seq on 5.3 million nuclei from 582 brain samples collected from 55 individuals that were members of a free-ranging, provisioned population of macaques living on the island of Cayo Santiago ⁸⁸. These 55 individuals included 29 females and 26 males, and ranged in age from 5 months (early life) to 21 years (late adulthood). From each animal, we dissected up to 11 distinct regions across the cortex: anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (dlPFC), inferior posterior parietal (IPP), primary motor cortex (M1), entorhinal cortex (EC), subcortex: hippocampus (HIP), nucleus accumbens (NAc), caudate nucleus (CN), mediodorsal thalamic nucleus (mdTN), brainstem: midbrain (MB), and lateral cerebellum (ICb) (**Fig. 1A, upper; Fig. S1A**; 48-55 individuals profiled per brain region). Individuals were selected to be roughly evenly spaced throughout this age range, which covers the lifespan of healthy wild macaques ^{89,190}, and also for balanced representation of sexes (Fig. 1A, lower).

Using an optimized sci-RNA-seq3 protocol ¹⁷⁶, we recovered a median of 5,726 nuclei per sample, and a median of 1,918 unique molecular identifiers (UMIs) per nucleus—a sixfold gain in molecular depth over our prior macaque brain atlas ¹⁹¹ (**Fig. S1B; Table S1**). Cerebellar "sentinel" samples were included in each experimental batch to monitor for technical variation, which we found to be minimal (**Fig. S1C**).

Unsupervised Louvain clustering, informed by marker gene expression and label transfer from existing primate, human and mouse brain atlases ^{191–194}, delineated 12 major cell classes spanning neuronal (glutamatergic, GABAergic, midbrain, medium spiny, cerebellar neurons (including projecting granule cells and purkinje cells), basket cells (cerebellar basket)) and non-neuronal (astrocytes, oligodendrocytes, oligodendrocyte precursors, microglia, vascular, ependymal) lineages (**Fig. 1B,E; Supplementary Note 1**). These classes were robustly detected across individuals,

regions and age groups (**Fig. 1C-D; Fig. S1D**). Cell class distributions reflected expected anatomical specializations, with a few exceptions likely attributable to minor dissection errors (**Table S2; Supplementary Note 1**)

As a preliminary analysis directed at understanding how aging patterns transcriptomic variance, we generated pseudobulk transcriptomes for each region, age group, and individual. Principal component analysis (PCA) of these profiles revealed that inter-regional variation dominates global expression structure, while age- and sex-related variance is more apparent within individual regions (**Fig. S1E-F; Supplementary Note 2**). Age explained a modest fraction of pseudobulk transcriptomic variance (median 1.1–2.6% across regions), with the cerebellum showing the strongest age-association (**Fig. S2A**). Sex explained even less variance overall (**Fig. S2B; Table S3**), consistent with analyses of bulk RNA-seq data from our own studies and the human GTEx project ^{72,195}.

3.3 Unsupervised identification and annotation of 226 neuronal and non-neuronal subclusters

To explore transcriptional heterogeneity further, we performed unsupervised subclustering within each of the 12 major classes, resulting in 226 transcriptionally distinct subclusters spanning neuronal and non-neuronal lineages (**Fig. 1E; Fig. S2C; Fig. S3; Table S4**). These subclusters reflected expected ontogenic relationships and were annotated using both comparison to reference datasets and manual curation (**Supplementary Note 3**). Throughout the remainder of the manuscript, we refer to clusters derived from unsupervised subclustering of our data as ‘subclusters’. These subclusters are annotated to neuronal and glial subtypes defined by external datasets, which we refer to as ‘subtypes’.

For example, glutamatergic neurons were sorted into subclusters that were either broadly distributed across neocortical regions or exhibited strong region-specificity, e.g. enriched in hippocampus, entorhinal cortex, and thalamus. The hippocampal subclusters comprised neurons from the well-organized CA1 and CA3 subfields, as well as the dentate gyrus (DG) region. The entorhinal cortex subclusters from L2/3 and L4/5 clustered separately from neocortical subclusters for the same layers, a similar organization as observed in the human brain [\(10\)](#). Subclusters in the thalamus exhibited unique molecular signatures (NXPH1+ and RNF220+) that were not found in the

neocortex—a separation that is conserved in mice and humans [\(10\)](#) (**Fig. 2A**). Subclustering of other cell classes captured both canonical and rare populations, such as LAMP5+ interneurons in the cortex (**Fig. 2B**), dopaminergic neurons in the midbrain (**Table S4**), and specialized astrocyte and immune subtypes (**Fig. S4A-E**). We were able to confidently annotate 85% of these subclusters using the following complementary approaches: (i) computationally comparing our subclusters to subtypes described in reference human, mouse, and macaque datasets [\(23–26, 28\)](#), and (ii) manually cross-referencing subcluster marker genes to known subtype-specific marker genes [\(9, 10, 25, 26, 28–32\)](#). Most subclusters showed strong concordance with known marker genes and external references from human, mouse, and macaque brains (**Fig. 2; Fig. S5; Table S4**). This high-resolution, multi-region dataset and corresponding annotations establish a foundation for investigating how age and sex contribute to the heterogeneity of the primate brain at single-cell resolution.

3.4 Age-associated dynamics of cell class and cell subcluster composition

To investigate the dynamics of cell type composition in these 11 brain regions over the course of the primate lifespan, we performed differential abundance analysis for each region. As changes between infants/juveniles and adults are much larger than those among adults, we excluded individuals <3 years of age from this analysis (**Fig. 3A; table S5**). Rather than using linear models of proportions, which ignore variation in the number of cells present in each sample, we applied a Poisson log-normal model, implemented in Hooke [\(33\)](#), to test differential abundance on cell counts.

We tested differential abundance of each of the 12 cell classes across 11 regions (85 combinations of region and cell class; 48–51 animals per region) (**Table S6**). Both GABAergic and glutamatergic neurons were significantly less abundant in older animals in the hippocampal formation, plausibly reflecting age-related atrophy linked to cognitive decline [\(34\)](#) (**Fig. 3B**). The abundance of oligodendrocytes increased with age in most regions except in the midbrain, while oligodendrocyte precursor cells decreased with age across the brain (**Fig. 3B-C**), consistent with prior studies [\(18, 35, 36\)](#).

3.5 Non-Neuronal Subclusters Exhibit Greater Age Sensitivity Than Neuronal Ones

To detect finer age-associated shifts in cellular composition that may be masked at the cell class level, we quantified differential abundance of subclusters relative to their cell class within each brain region of adult animals (>3 years). After excluding subclusters with fewer than 100 cells to ensure statistical power (**Table S5**), we retained 1,039 within-region subclusters (range: 57–113 subclusters per region). To further boost sensitivity, especially in regions with low cell counts, we applied multivariate adaptive shrinkage (MASH)[\(37\)\(38\)\(37\)](#), which leverages shared patterns across regions. This more granular analysis revealed that age significantly patterned cell subcluster composition in adult animals (**Fig. 3A,D; Fig. S6A**). Altogether, 101 of 1,039 region-subcluster combinations (10%) showed significant age-related changes in abundance (local false sign rate, LFSR < 0.05). These effects varied by region and cell type: the lateral cerebellum exhibited the most age-associated subclusters (12 subclusters, 21%), while the midbrain showed the fewest (4 subclusters, 4%). Among cell classes, basket cells were most affected by age (39% of combinations of region and subclusters), whereas midbrain neurons and ependymal cells remained largely stable across the lifespan.

Across the brain, non-neuronal subclusters exhibited more extensive age-related changes in abundance than neuronal subclusters. This trend was consistent across all regions examined, with the lateral cerebellum showing comparatively fewer subcluster-level changes, potentially due to its lower representation of non-neuronal cells (**Fig. 3E**). Similar patterns have been observed in aging mouse brains, where non-neuronal cell classes—especially glia—undergo notable shifts in cell state proportions with age, while neuronal clusters remain relatively stable, suggesting conservation of this phenomenon across mammalian lineage [\(39\)](#).

Age-related changes in non-neuronal subclusters were widespread, particularly among astrocytes, oligodendrocytes, and microglia, each showing abundance shifts in more than five regions (**Fig. 3F**). A homeostatic astrocyte subcluster (subcluster 1, *LYPD6B⁺ HRHI⁺*) was notably depleted early in life and continued to decline throughout life (**Fig. S6D**). Oligodendrocyte precursor cells (OPCs) and differentiating oligodendrocytes also declined broadly across the brain, consistent with a global reduction in progenitor pools with aging (**Fig. 3D; Fig. S6A**). In contrast, the abundance of pre-myelinating oligodendrocytes remained stable, while myelinating oligodendrocytes underwent

state transitions toward a subcluster (olig-OPC: subcluster 7) characterized by upregulation of the EL4 regulon and downregulation of ion channel and myelination genes (**Fig. 3G; Fig. S5C; Fig. S6E**). The increase in abundance of subcluster 7 is most pronounced in M1 and NAc (**Table S7**). This altered state closely resembles a damaged oligodendrocyte phenotype described in amyotrophic lateral sclerosis [\(40\)](#), suggesting a potentially conserved mechanism of oligodendrocyte dysfunction in aging and disease.

In contrast to glial cells, neuronal subclusters were largely stable in abundance across the lifespan. However, we identified a subset of neurons that were selectively vulnerable to aging. Glutamatergic neuron subcluster 5 (*KCNK13⁺C4H6orf141⁺*), corresponding to neocortical layer 2/3 intratelencephalic (L2/3 IT) neurons (**Fig. 3D**), this subcluster displayed consistent depletion during both neonatal development and aging (**Fig. S6B**). This same neuronal class has been shown to decline significantly in Alzheimer's disease patients in two independent human studies [\(10, 25\)](#). (**Fig. S6C**), suggesting its functional importance and potential fragility in the context of age-associated cognitive decline. We also observed depletion of inhibitory interneuron GABAergic neuron subcluster 13 (*SP110⁺ STK32A⁺*), corresponding to SST interneurons in the cortex (IPP, dlPFC, ACC) during aging (**Table S4**). This neuronal subtype is also identified as vulnerable in Alzheimer's disease [\(25\)](#). Depletion of these two subclusters suggest their functional importance and potential fragility in the context of age-associated cognitive decline.

3.6 Microglial Subclusters Reveal Age-Associated Transitions and Mirror Human APOEε4 Aging Signatures

Aging in rhesus macaques is associated with substantial shifts in microglial subcluster abundance. Migratory microglia (subcluster 5, *ENPEP⁺ SEMA6A⁺*) declined with age, while neuroinflammation-associated microglia (subcluster 4, *GLDN⁺ RASAL2⁺*) increased in abundance across nearly all brain regions, except the cerebellum. Microglia subcluster 4 is enriched for proinflammatory cytokine response genes (*CCL3, IL1B, CD83, SPPI, LPL, ITGAX*) typically upregulated in response to amyloid β pathology [\(41\)](#) (**Fig. S6F**), suggesting convergence between aging-related inflammation and Alzheimer's disease mechanisms. Among regions with abundance change, microglia subcluster 4 in dlPFC, M1 and EC show the largest increase in abundance (**Table S7**). Border-associated macrophages (microglia subcluster 7) were regionally restricted but showed significant age-related increases in the ACC and NAc. These subtle, region-specific shifts in microglial composition are only detectable at single-cell resolution (**Fig. 3H; Fig. S6A**).

To investigate the regulatory landscape of age-associated microglial transitions, we grouped microglial subclusters into two categories. Group 1 (subclusters 3, 4, 5, 6, 8, 10, 13) follows a trajectory from surveilling to redox-active and ultimately proinflammatory states, while Group 2 (subclusters 0, 1, 9, 12) comprises more stable surveilling microglia (Fig. S3E; Fig. S7A–B). Pseudotime analysis of Group 2 revealed a continuum of gene expression changes (Fig. S7A, C–E). Early-activated genes (cluster 1) were enriched for axon guidance pathways, consistent with microglial roles in tract development (42). Later-activated genes (clusters 2 and 4) were enriched for lipid and fatty acid metabolism, including CPM, SPP1, GPNMB, ACSL1, and APOE, which were upregulated toward the end of the trajectory (Fig. S7E–F). These metabolic pathways have been linked to disease-associated microglial states and are known to be modulated by genetic variation (43).

3.7 Lifespan Trajectories Reveal Continuity Between Development and Aging

Our densely sampled time-course dataset enabled us to capture diverse, non-linear trajectories of cell subcluster abundance across the lifespan. To model these patterns, we applied a cubic spline fit to the abundance of each subcluster in each brain region from birth to 21 years of age, and clustered the resulting trajectories into nine groups (**Fig. 3I**). We found that many subclusters followed a consistent pattern of change across both development and aging. In particular, subclusters in k-means clusters 1 and 5 showed sharp shifts during early life (0–5 years) followed by more gradual changes throughout adulthood (5–21 years). These continuous trajectories suggest that age-related abundance changes may represent an extension of developmental programs rather than entirely distinct processes. While spline models allowed us to capture nuanced non-linear trends, we observed greater standard error compared to linear models, highlighting the need for denser sampling—especially in mid-life—to improve confidence in trajectory inference and the detection of subtle inflection points in cellular aging dynamics.

3.8 Age substantially patterns gene regulation across the brain

Exploratory analysis of pseudobulked gene expression recapitulates our and others' findings from bulk RNA-seq, showing that regional heterogeneity in gene expression exceeds age-related

variation (**Fig S1E**). However, this does not mean age is unimportant—rather, it means that strong regional differences in cellular composition and gene expression mask many age effects at the cell- and region-specific level. Indeed, age-associated effects on pseudobulk expression were much more apparent when analyses were conducted within individual regions. Notably, infant samples were transcriptionally distinct from all other age groups, suggesting early developmental transitions (**Fig. S1E-F**).

We next tested for differences among cell- and region-specific age-related transcriptional programs by identifying age-related differentially expressed genes (aDEG). To do so, we modeled gene expression in a negative binomial linear mixed model framework, modeling age, sex, and sequencing depth as fixed effects and donor as a random effect in adult macaques (age > 3 years). To exclude conditions with low statistical power from this analysis, we set a heuristic threshold to filter out subclusters with fewer than 300 cells or 30 animals in a particular region (**Table S5**). We then applied multivariate adaptive shrinkage (MASH; [\(33, 37\)](#)), which improved statistical power in all subclusters and refined aDEG effect size estimates to facilitate robust detection of shared aDEGs of subcluster-region combinations within a given cell class (Methods).

Age substantially altered gene regulation across the brain: 62% of the genes we tested (9,380/15,117) were significantly differentially expressed with age ($LFSR < 0.05$) in at least one subcluster-region combination (**Fig. 4A**). We observed a greater number of subcluster-region combinations with a higher proportion of upregulated aDEGs across brain regions (**Fig. S8A**), with 72% of combinations showing more upregulated than downregulated aDEGs. Our ability to detect aDEGs in a subcluster-region was influenced by several technical factors, including: (i) sequencing depth, (ii) number of cells in a given subcluster-region combination, and (iii) number of genes tested (**Fig. S8B**). After controlling for these confounding factors, we found that oligodendrocytes showed the highest number of aDEGs, followed by basket cells (**Fig. 4B, S8C**). Within cell classes that are shared among regions, dlPFC, M1 and CN consistently showed a higher number of aDEGs than other regions, indicating that they might be hotspots of brain aging (**Fig. 4C, S8C**). These three regions are involved in executive functions and motor control [\(46–48\)](#), where transcriptional changes might be related to cognitive and mobility declines with aging. aDEG effect sizes, which are unbiased relative to the number of cells, were similar across most cell classes, hinting at some consistent and programmed age-associated changes in expression across genes in distinct cells and

regions (**Fig. 4A**). Similarly, the effect sizes of up- and down-regulated aDEGs were similar in most cell classes, except in microglia which had stronger effects in upregulated aDEG (**Fig. 4A**).

Most aDEGs are cell-class specific and found in only one or a subset of cell classes (median= 3 cell classes; median tested = 11 cell classes), indicating that aging predominantly drives cell class-specific transcriptional changes. In total, we identified 2513 cell-class-specific aDEGs—ranging from 3%-18% of aDEGs genes detected in each cell class (**Fig. 4D**). For example, P2RY12, a gene important for microglial homeostasis, declines specifically in microglia with age. This pattern mirrors expression changes seen in disease-associated and aged microglia [\(49\)](#) (**Fig. S8E**), further supporting the relevance of cell-class-specific aDEGs to functional decline in brain aging.

Only a handful of aDEGs were shared broadly across multiple cell classes. Notably, two aDEGs were identified across all cell classes, suggesting they may function as potential master regulators of brain aging (**Fig. 4D**). One such gene is YEATS2, which increases in expression with age in all neuronal cell types and most glia—except for microglia where expression is lower in older age. As a selective histone crotonylation reader, YEATS2 may play a key role in age-associated epigenetic remodeling in a cell-class-specific manner (**Fig. S8F**). YEATS2 is also a paralog of the drosophila ENL/AF9 domain-containing protein that can regulate lifespan in flies—pan-neuronal knockdown of this domain extends lifespan [\(50\)](#).

Our analysis of aging programs across cell classes and brain regions revealed that aging is primarily driven by cell identity. Aging programs—defined as the full set of genes tested in a given cell class and region—are more similar within the same cell class across regions than between different cell classes within the same region (**Fig. 4E**). This highlights that aging operates predominantly through cell-class-intrinsic programs. Among neurons, excitatory and inhibitory classes show particularly strong age-associated transcriptional similarity with one another across regions. Neurons also show correlations with oligodendrocytes and astrocytes—suggesting partially coordinated aging responses among electrically active or metabolically coupled cell classes. In contrast, other glial populations, such as microglia, vascular cells, and ependymal cells, display highly distinct and specialized aging programs, consistent with their divergent functional roles in homeostasis, immunity, and barrier integrity.

To understand the biological functions associated with these aging programs, we performed gene set enrichment analysis (GSEA) across each cell class and region. We identified both broadly conserved and regionally specialized pathways, which cluster by cell class and reflect shared and distinct mechanisms of aging (**Fig. S8I**). Among the most consistently downregulated pathways across neuronal populations, astrocytes, and oligodendrocytes were those involved in synaptic signaling. This synaptic decline is also present in rodents [\(8, 51\)](#), non-human primates [\(52, 53\)](#) and humans [\(6, 11, 54, 55\)](#), suggesting a strong evolutionary conservation that may underlie reduced plasticity and connectivity in the aging brain (**Fig. 4G**). Other broadly downregulated programs include those related to neuronal development, axon structure, cell adhesion, and cell signaling—changes consistent with known reductions in dendritic complexity and glutamate receptor activity during aging [\(56\)](#).

However, region and cell class context modulate these trends. For example, in microglia, age-associated downregulation of synaptic signaling is restricted to the entorhinal cortex (EC) and dorsolateral prefrontal cortex (dlPFC), while in the cerebellum, it is restricted to basket cells. Even among glutamatergic neurons, synaptic membrane-related genes decline more rapidly in neocortical regions than in the hippocampus or EC (**Fig. S9**). This pattern is consistent with stereological studies showing pronounced synapse loss in the prefrontal cortex but relatively preserved synapses in the hippocampus during normal aging [\(57, 58\)](#), but contrasts with Alzheimer's Disease, where early loss occurs in EC and hippocampus [\(59\)](#). In vascular and ependymal cells, the downregulation of genes involved in cell junctions suggests potential impairment of the blood-brain barrier with age (**Fig. 4G**) [\(60\)](#), further reflecting tissue-specific vulnerability to functional decline.

We have observed cell class specific enrichment of brain aging hallmarks that were previously identified [\(7, 61, 62\)](#). For example, astrocytes show upregulation of genes in DNA damage response, while glutamatergic neurons and GABAergic neurons upregulate genes in both DNA damage and repair. Astrocytes show a unique signature of cell senescence which is not enriched in other cell types (**Fig. S8G**), consistent with its decrease in abundance over age in multiple regions (**Fig. 3B**). Given the current literature revealing important roles of astrocytes in neuromodulation [\(63–65\)](#), loss of astrocytes with age might be connected to disruption of the neuronal network and synaptic functions. Immune activation and inflammatory response are upregulated in astrocytes, microglia

and ependymal cells in selective regions while GABAergic neurons show decrease in inflammatory response (**Fig. 4G, S8G**).

Within cell classes, regional context further shapes aDEG profiles. aDEG profiles are variably shaped by regional context. Oligodendrocytes display consistent aging signatures across regions, pointing to a systemic aging trajectory linked to myelin maintenance and progenitor pool exhaustion. In contrast, neurons show strong anatomical specificity. Glutamatergic and GABAergic neurons exhibit coordinated aging within cortical regions (**Fig S10A-B**), while cerebellar neurons and astrocytes demonstrate distinct transcriptional changes likely reflecting regional activity demands and metabolic constraints (**Fig. 10C**). For example, Bergmann glia—a cerebellum-specific astrocyte subtype—exhibit a unique suite of cerebellum-restricted aDEGs, indicating specialized aging responses in this region. (**Fig. S10D-E**)

In contrast, cell-class-specific and regionally restricted aDEGs point to specialized aging vulnerabilities. Cortical neurons display unique aDEGs enriched for synaptic signaling and oxidative stress pathways, which are not observed in their counterparts outside the cortex (**Fig. S10F**). These cortical-specific genes show limited overlap between excitatory and inhibitory neurons (Jaccard index = 0.04), yet likely contribute to regional susceptibility. Similarly, microglia exhibit both global and localized aging signatures. While their aDEG profiles are largely cell-class specific, some genes exhibit anatomically restricted expression changes with functional implications. For instance, microglia in the entorhinal cortex upregulate RhoGTPase (*ARHGAP27*) involved in clathrin-mediated endocytosis—a pathway implicated in amyloid- β clearance and neuroinflammation—linking regional microglial aging to mechanisms of neurodegeneration (**Fig. S10G**) ([66](#)).

Elimination of senescent cells (senolysis) was recently reported to improve normal and pathological changes associated with aging in mice ([67–69](#)). Discovery of region-specific neuronal subclusters with senescence markers could potentially help identify cells that might be most responsive to senolytic therapies. Genes related to senescence-associated secretory phenotype (SASP) are upregulated ($p\text{-value} < 0.05$, $\text{signal} > 20\%$) in subsets of neurons in specific regions, including VIP+ GABAergic in EC and dlPFC, PVALB+ GABAergic neurons in IPP and several D1 and D2 medium spiny neurons subclusters particularly in CN (**Fig. 4H**).

In summary, age-related changes show cellular and regional variation. This heterogeneity is potentially tied to 1) different cell classes, neuronal subtypes, and regional glial populations perform distinct roles in the brain, and these functions may become compromised or overactivated with age; 2) the specialized roles of certain cell classes may render them more vulnerable to specific age-related stressors; and (3) brain regions may experience varying degrees of physiological stress during aging, leading to disproportionate damage in certain cell populations.

3.10 Transcriptional changes in aging are highly convergent with Alzheimer's disease

After identifying age-associated transcriptional programs, we next asked whether neurodegenerative diseases like Alzheimer's disease (AD) represent an amplification of these same age-related pathways, or instead reflect the activation of distinct, disease-specific mechanisms. To do so, we compared aDEGs from our dataset to AD-differentially expressed genes (AD-DEGs) from three independent single-cell studies (**Fig. S11A-B**) ([10, 70, 71](#)). Enrichment analyses revealed significant overlap in both up- and downregulated aDEGs with AD-DEGs (**Fig. S11C**: >60% of aDEGs overlap with AD-DEGs to the same cell class, and 16-85% of aDEGs overlap with AD-DEGs from other cell classes), indicating highly convergent transcriptional responses. Notably, microglia exhibited directionally consistent convergence specific to its counterpart in AD, implicating this cell type in a common aging–neurodegeneration axis. Within the DEGs that are convergent between aDEGs and AD-DEGs, 7% are microglia specific (binomial p-value: 0.08), while 42% (binomial test p-value < 2.2e-16) are broadly changing (differentially expressed in 70% of cell class) in both aging and AD (**Fig. S11D**). This suggests convergence of both cell type specific and pan-cellular programs in microglia. Microglia-specific convergence is enriched in adaptive and innate immunity, suggesting similar immune responses in both aging and neurodegeneration (**Fig. S11E**). In contrast, transcriptional signatures in astrocytes, oligodendrocytes, and neurons reflected more global programs across cell classes. The convergence of these cell classes are mostly driven by shared DEGs (>98%) and the majority are broadly changing among cell classes (>60%, binomial test p-value < 2.2e-16) and enriched for synaptic functions. (**Fig. S11E**)

Having observed a strong convergence between aging-related and AD-associated programs, a more compelling question is when these convergent programs begin to shift during aging. To explore this, we divided our animals into four age intervals—early life (0–5 years), early adulthood (5–10 years), mid-life (10–15 years), and later life (15–20 years)—and clustered the average expression of AD-convergent genes (i.e., genes that are aDEGs in our dataset and AD-DEGs in external datasets) in each glial cell class or neuronal subtype across these age groups. We found that AD-convergent genes exhibited diverse temporal dynamics: (1) some showed marked changes in early life that persisted with reduced intensity over time, (2) others displayed gradual, cumulative shifts across the lifespan, (3) a subset underwent abrupt changes in later life, and (4) a smaller group exhibited a reversal of early-life changes in late life (**Fig. S12**). In astrocytes, 46% of AD convergent genes change mainly during transition of early life to adolescence (cluster 2 and 6), while 36% of them start accumulating changes from adolescence (cluster 1 and 3)(**Fig. S12A**). Variations of temporal dynamics were observed among astrocytes in different regions, with NAc enriched for upregulated genes from adolescence, EC enriched for genes peaked at adolescence and ICb enriched for genes upregulated in early life to adolescence. Among the other major glial cell classes (e.g. microglia, oligodendrocytes and OPCs), the most common patterns are accumulative changes over time (cluster 2 and 4 in microglia: 53%, oligodendrocytes: 54%, and OPCs: 49%) (**Fig. S12B-D**). The distribution of gene patterns are consistent among regions, suggesting brain-wide regulation of these AD-convergent programs in glial cells. It is notable that microglia show region specific aging programs but the genes overlapped with AD are showing similar temporal dynamics, which indicates that Alzheimer’s-related genes may be governed by shared regulatory mechanisms that override regional differences in microglial aging. In contrast, glutamatergic neuronal subtypes in different regions exhibit different trends of temporal dynamics. In EC L2/3 IT neurons, 38% of AD-convergent genes (cluster 3 and 6) exhibit abrupt declines in late life—a pattern that is less pronounced in cortical L2/3 IT neurons (9% in cluster 3 and 5) (**Fig. S12E-F**). Late life patterns are also observed in a large proportion of genes in HIP CA1 (37%, cluster 1 and 5), CA3 (51% in cluster 1 and 6), and DG glutamatergic neuron subtypes (46% in cluster 1,3 and 4)(**Fig. S12G-I**).

3.10 Discussion

Here, we generate a comprehensive single-cell atlas of aging across 11 brain regions in 55 rhesus macaques of both sexes, providing a high-resolution view of cell type– and region-specific trajectories in the aging primate brain. This resource enables systematic investigation of gene regulatory programs governing the aging process. Leveraging this dataset, we delineate

inter-regional and inter-cellular heterogeneity in aging dynamics, explore their relevance to human neurodegenerative disease, and uncover sex-specific differences in the aging process.

We observed substantial variability in the aging trajectories across brain cell classes and regions. Abundance changes were more prominent in glia and broadly observed across regions, while only observed in neurons within specific anatomical structures (e.g. neocortex, cerebellum). In terms of transcriptional changes, our findings support that aging is primarily driven by cell identity in the mouse brain(8, 72). Using this multi-region dataset, we quantified intra-class regional differences and found that regional context further modulates the aging program beyond cell identity. A high correlation of aging programs was observed in glia that are electrically active or metabolically coupled (i.e. oligodendrocytes, astrocytes). Neurons show regional stratification of aging programs based on brain structures (e.g. separation of neocortex and other brain regions). Neurons and astrocytes in the cerebellum display distinct transcriptional changes, reflecting regional specificity in cell activities and aging process. Other glial populations, such as microglia, vascular cells, and ependymal cells, also display highly distinct and specialized aging programs among regions, consistent with their spatial specialization among the brain (i.e. different ventricles in different brain regions). As a result of the inter-region and inter-cell class heterogeneity, we observed restriction of many aging hallmarks to specific combinations of region and cell class. We identified astrocytes to be related to most changes in the aging hallmark. Given the current literature revealing important roles of astrocytes in neuromodulation and resilience to Alzheimer's disease(63–65), our results support the link of astrocytes to age-related cognitive decline and point to several vulnerable pathways. At the subtype level, we observed specific neuronal subtype displaying pathological aging phenotype (i.e. senescence-associated secretory phenotype). Overall, our findings suggest heterogeneity at the cell class, region and subtype level. We identify subsets of heterogeneity as linked to aging vulnerability, providing potential targets for aging interventions. However, much of the heterogeneity remains to be explored.

This study also increased our understanding of human neurodegeneration with the context of healthy aging of non-human primates. We identified selectively vulnerable cell populations that change with abundance. Selective neuronal subtypes (i.e. L2/3 IT glutamatergic neurons and SST+ GABAergic neurons) decrease abundance with age. These subtypes are important to Alzheimer's disease (10, 25). An oligodendrocyte activation state and microglial proinflammatory state were also observed to increase their abundance with age. These states also show high similarity with glia states in neurodegenerative disorders (40,41). Interestingly, the abundance change of these states all

start very early in life (<5 years), with aging as an extension of development. This suggests some of the transcriptional changes of neurodegeneration are present early in life and prior to pathology.

By comparing the age-related shift in microglial state in our dataset to those in Alzheimer's disease, we identified a trajectory to the activation of lipid droplets-associated microglial states. This state is more common in individuals with the Alzheimer's-linked APOE ϵ 4/ ϵ 4 genotype (43). Given that all nonhuman primates sequenced to date are homozygous for APOE ϵ 4 by human classification criteria (44, 45), the trajectory of microglial state transitions observed in rhesus macaque aging offers a valuable model for studying APOE ϵ 4-driven neuroinflammatory aging processes in humans.

Importantly, we discovered highly convergent molecular programs between normal aging in non-human primates and pathological processes in the human brain by comparing our aging trajectories to transcriptomic data from human Alzheimer's diseases. The significant overlap between aging and neurodegenerative signatures indicates that many aspects of the neurodegenerative disorder may reflect accelerated or exaggerated features of normal aging. These convergent gene programs display varied temporal patterns, with some changes emerging during development and others accumulating throughout life, implying that neurodegeneration may have early origins or result from lifelong accumulative changes. Interestingly, several Alzheimer's-associated cell subtypes—including entorhinal cortex L2/3 IT and hippocampal CA1, CA3, and DG neurons—display a sizable set of genes with abrupt late-life changes, suggesting these populations may be particularly susceptible to age-related pathology. Further functional characterization of gene sets with distinct temporal dynamics, combined with integration of genome-wide association study data, may enhance our understanding of the mechanisms underlying age-related pathology.

Notably, non-human primates do not naturally develop full-blown neurodegenerative disorders such as Alzheimer's disease. While aged macaques may exhibit amyloid- β (A β) deposition and occasional tau hyperphosphorylation, neurofibrillary tangles and extensive neuronal loss are rarely observed. Yet, our transcriptomic analyses show that aging macaques activate gene programs that closely resemble those found in human Alzheimer's disease. This convergence opens a unique window to investigate protective mechanisms—genetic or environmental—that may buffer macaques from the more severe neuro-pathological outcomes seen in humans.

3.11 Figures

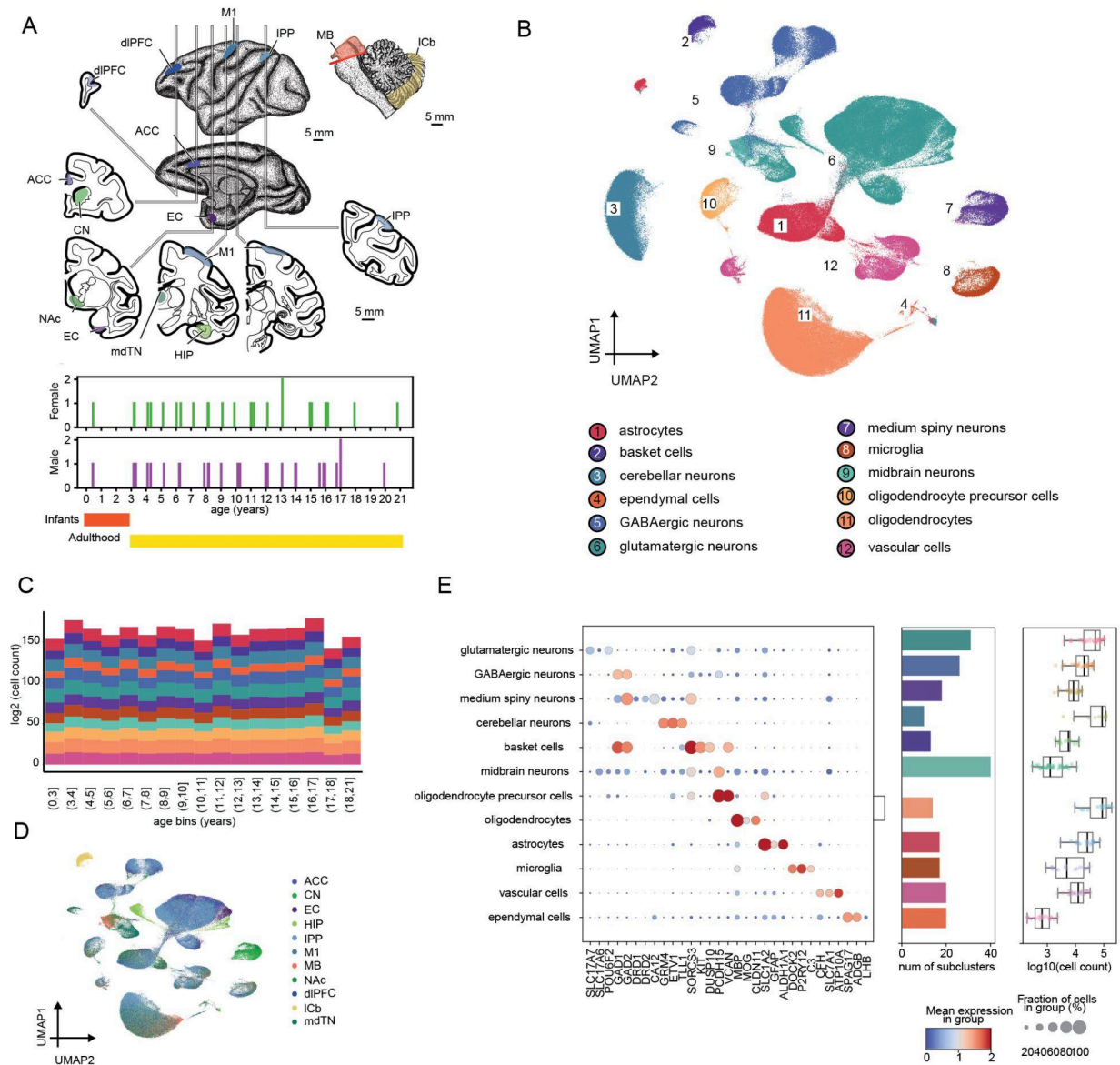


Figure 1. A multi-region single cell dataset with high temporal resolution of macaque aging.

A) Schematic of 11 brain regions profiled in this study (top), together with the age and sex distribution of the 55 individual macaques (bottom). **B)** UMAP projection of downsampled sci-RNA-seq dataset (~10% of total cells) colored by cell class assignment. **C)** Barplot showing $\log_2(\text{cell count})$ of each cell class across the 11 profiled regions in each age bin. **D)** UMAP projection of the same downsampled set of cells shown in panel B, here colored by region of origin. **E)** Marker gene expression dotplot for each cell class (left). Barplot showing number of subclusters from unsupervised Louvain clustering of each cell class (middle). Boxplot showing $\log_{10}(\text{cell count})$ for subclusters associated with each cell class (right).

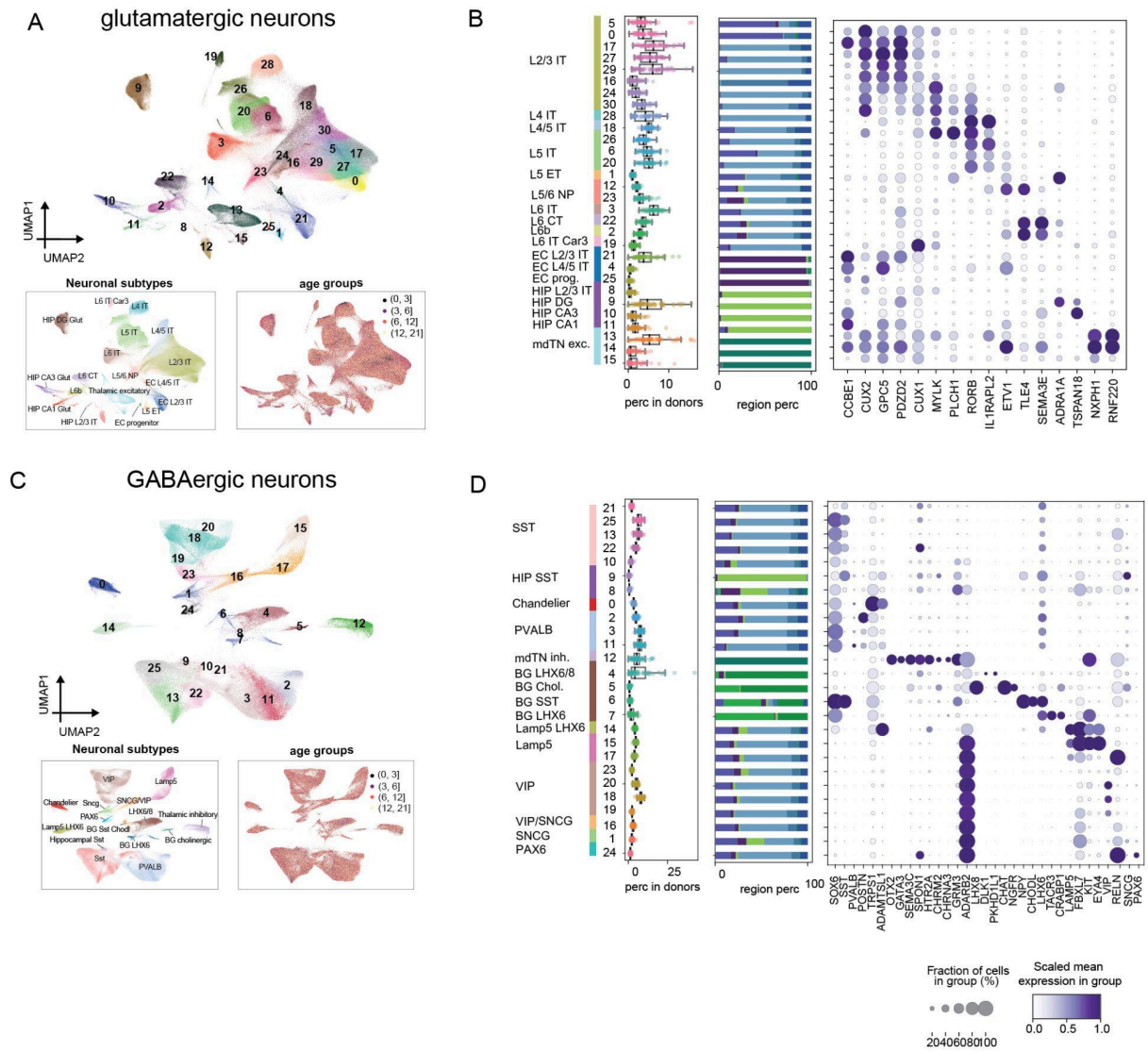


Figure 2. Heterogeneity and regional specificity of glutamatergic and GABAergic neuronal classes.

A) UMAP visualization of glutamatergic neuronal subclusters identified by unsupervised Louvain clustering (top). Insets show the same UMAP colored by annotations based on label transfer from neuronal subtypes defined by external literature (24) (bottom left) or by age group (bottom right). Subclusters are well-stratified by subtype labels, but not by age group. **B)** Each row corresponds to a glutamatergic neuronal subcluster. Boxplot showing proportion of each subcluster out of all glutamatergic neurons across individual donors, without stratification by region (left). Subclusters exhibit relatively consistent proportions across donors. Barplots showing the regional distribution of each subcluster (middle). Color assignments are identical to Fig. 1D. Many glutamatergic subclusters exhibit regional specificity. Dotplot of scaled mean expression of marker genes of glutamatergic subtypes from a list of reference datasets (9, 10, 25, 26, 28–32) in our subclusters (right). **C-D)** Same as panels A-B but for GABAergic neurons.

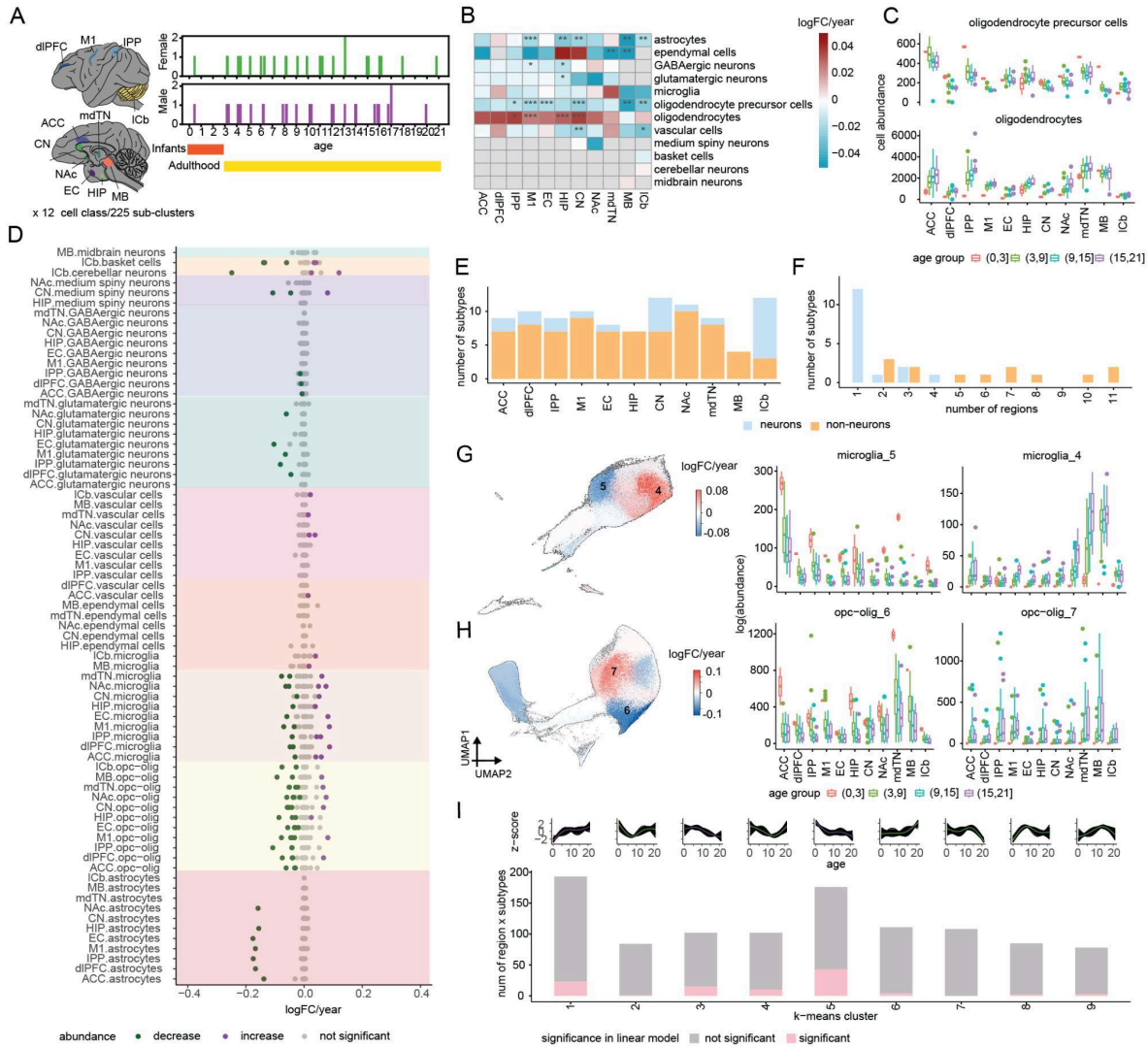


Figure 3. Cell population abundance dynamics.

A) Schematic of differential abundance test on 12 cell classes and 226 subclusters in 11 regions across the brain. **B)** Heatmap showing log-fold change of abundance change each year (coefficient output from the linear model) for each cell class-x-region combination. Adjusted p-value <0.001: ***, 0.01: **, 0.05: *. **C)** Boxplot showing cell abundance in each individual after transformation by the poisson log-normal model in Hooke (33) for each age group across regions. **D)** Dotplot showing log fold change of abundance each year (coefficient output from the linear model) for each cell subcluster and region combinations. Changes are colored by decrease in abundance (green), increase in abundance (purple), and change not statistically significant (grey). **E)** Barplot showing number of age-associated subclusters in each region colored by neuronal (orange) and non-neuronal (blue) subclusters. **F)** Barplots showing the number of regions with significant changes in abundance were detected for each subcluster. **G)** UMAP showing abundance change of microglia subclusters in log-fold change each year. Subclusters with brain-wide changes were labeled and their cell abundance were shown on the boxplots to the right. **H)** Same as panel G for oligodendrocytes subclusters. **I)** Non-linear trajectory of cell abundance changes for each subcluster and region combination (top). Barplot summarizing the number of age-associated subclusters in

each trajectory (bottom).

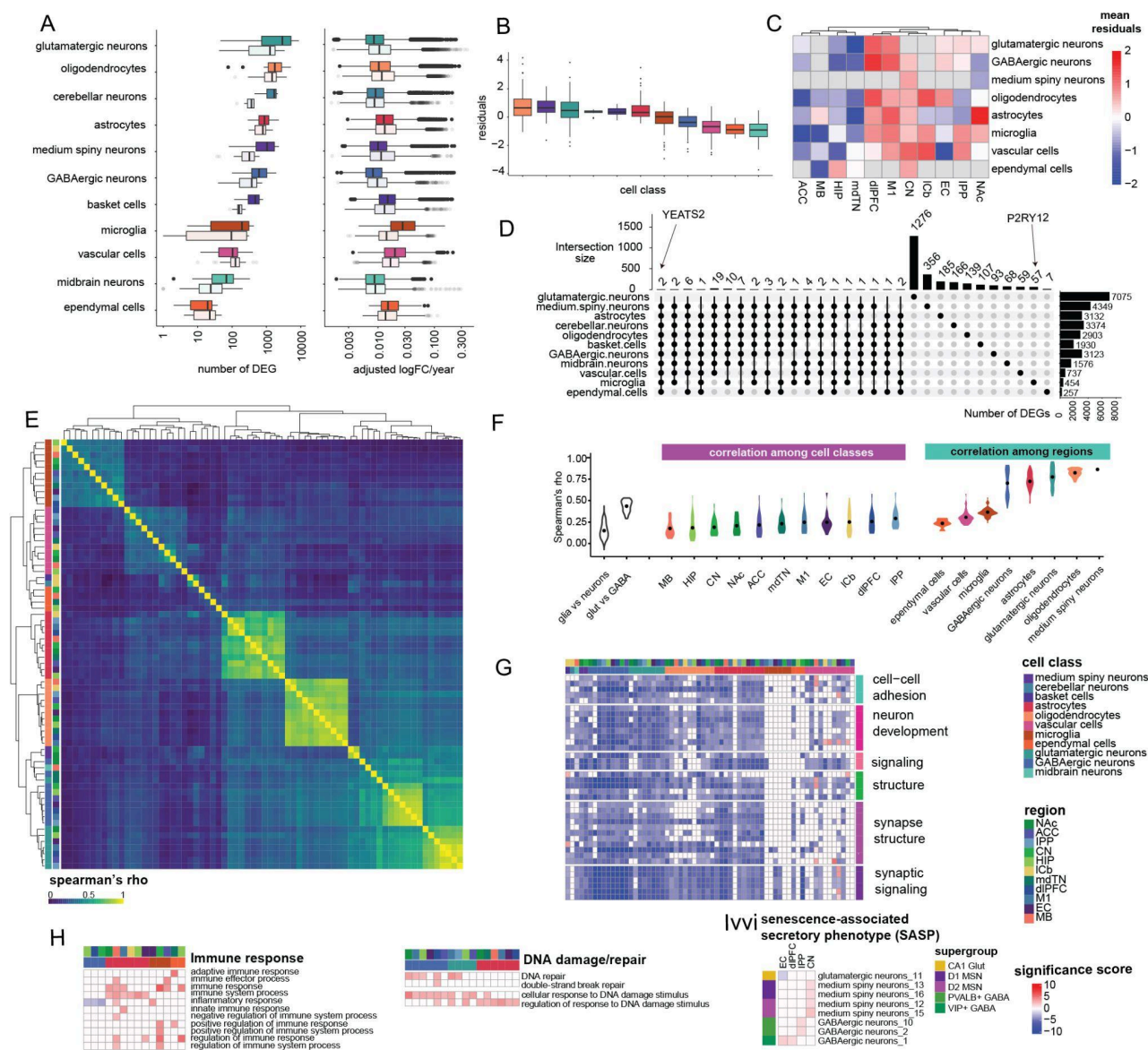


Figure 4. Age-related transcriptional changes.

A) Boxplots showing the number of aDEGs (left) with significant (LFSR<0.05) association with age and the adjusted logFC/year for these aDEGs (right) in each combination of subcluster x region. Light color indicates upregulated aDEGs and dark color indicates downregulated aDEGs. **B)** Boxplot showing the residuals after correcting number of aDEGs by cluster size and sequencing depth by a loess model (method). Positive indicates a subcluster and region combination has more aDEGs than expected, while negative indicates a subcluster x region combination has less aDEGs than expected. **C)** Heatmap showing the average residuals of subcluster x regions in each cell type. Each row is scaled by z-transformation to identify regions with higher average residuals. **D)**

Upset Plot showing the number of DEGs shared by ≥ 9 cell classes and their combinations, and number of unique aDEGs within each cell type. Examples of broadly shared aDEGs (i.e. YEATS2) and specific aDEGs (i.e. P2RY12) are highlighted. **E)** Spearman's correlation of age coefficients of all genes from the linear mixed model after MASHR correction among all combinations of cell class x region combinations. **F)** Violin plot illustrating Spearman's rho in E across four categories of comparisons: (1) glial versus neuronal classes, (2) glutamatergic versus GABAergic neurons, (3) different cell classes within the same brain region, and (4) different brain regions within the same cell class. **G)** Heatmap showing GSEA results of GO pathways broadly shared among combinations of cell types and regions. Significance score is calculated by $-\log_{10}(\text{FC sign} \times \text{p-value})$. **H)** Heatmaps showing GSEA results of immune response and DNA damage/repair pathways which show cell type and region specific enrichments. **I)** Heatmap showing specific neuronal subtypes in certain regions with enrichment in senescence-associated secretory phenotype (SASP).

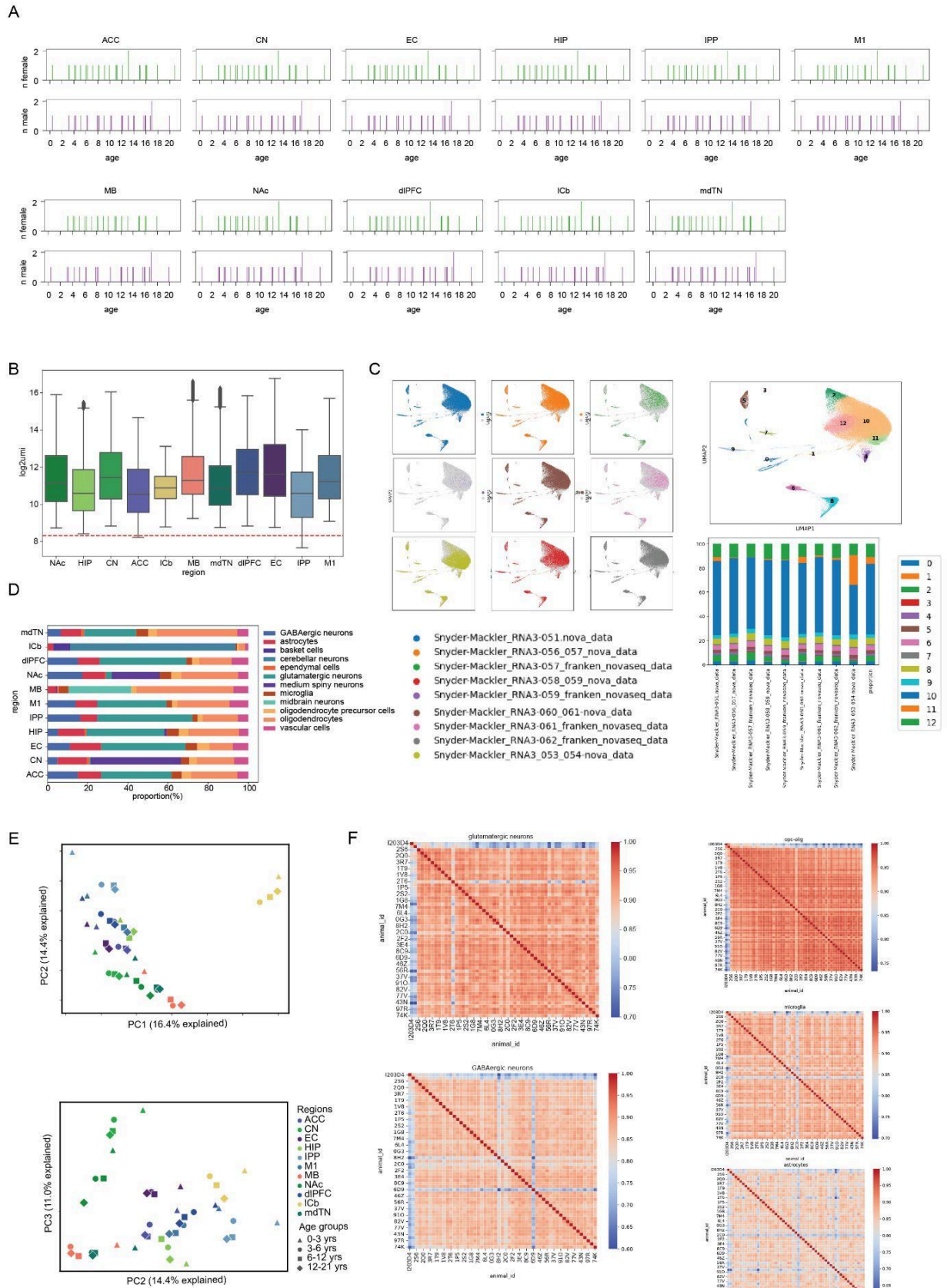


Figure S1.

A) Age and sex distribution of samples collected for each brain region. **B)** Boxplot showing distribution of number of unique molecular identifiers (UMIs, log2-transformed) recovered in each cell collected by region. The red dotted line shows median UMI recovered in previous BICCN atlas([23](#)) **C)** Jointed UMAP projections of control samples (cerebellum) from different sequencing runs, colored by the sequencing run ids where they were loaded. Unsupervised clustering was performed on the jointed dataset. Barplots showing proportion of control samples from different sequencing runs in each louvain cluster and all cells. **D)** Barplot showing proportion of cell class across regions. **E)** For each region, transcriptional profiles were aggregated to create pseudobulk transcriptomes of 4 age groups (i.e. infants: 0-3 years, adolescence: 3-6 years, adulthood: 6-12 years, aged: 12-21 years). Principal component analysis (PCA) was performed on the resulting pseudobulk transcriptomes. PCA plots showing PC1 and PC2 (upper), PC2 and PC3 showing (lower). PC1 captures differences between cerebellum and all other brain regions. **F)** Correlation of pseudobulk transcriptomes of individual samples on the top 500 genes with highest loadings in the PC most correlated with age in each cell class. Samples are ordered by chronological age.

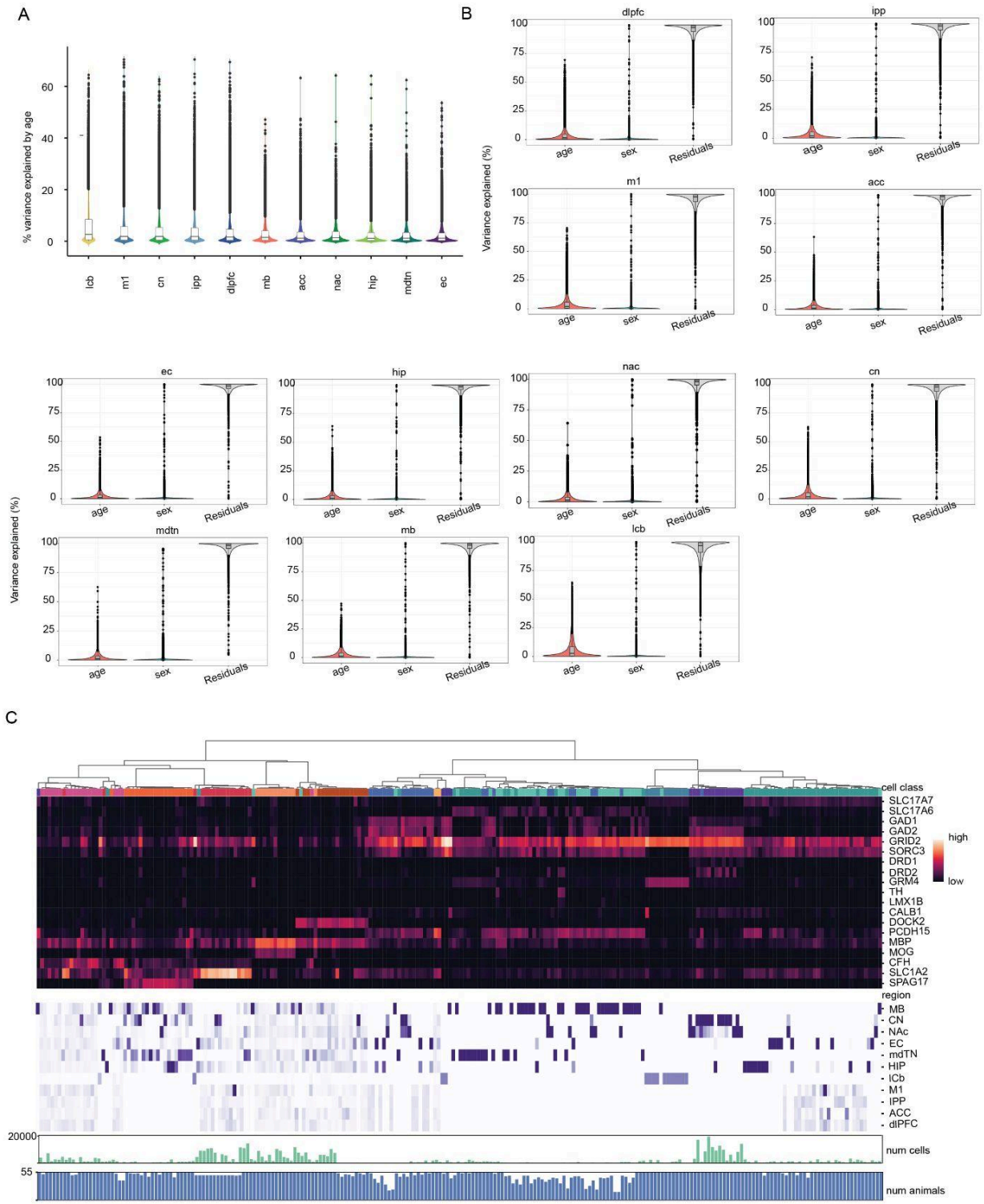


Figure S2.

A) For each region, transcriptional profiles were aggregated to create pseudobulk transcriptomes for each individual. Violin plot showing percentage variance explained by age for each gene in individual regions, ordered by highest to lowest median percentage. **B)** For each region, transcriptional profiles were aggregated to create pseudobulk transcriptomes for each individual. Violin plot showing percentage variance explained by age and sex for each region for each gene. **C)** Dendrograms showing hierarchical clustering of subclusters labeled by cell class (colors are the same as Fig 1B). Heatmap showing cell class marker gene expression among subclusters and distribution among regions. Barplots showing number of cells in each subcluster and number of donors with this subcluster in at least one region.

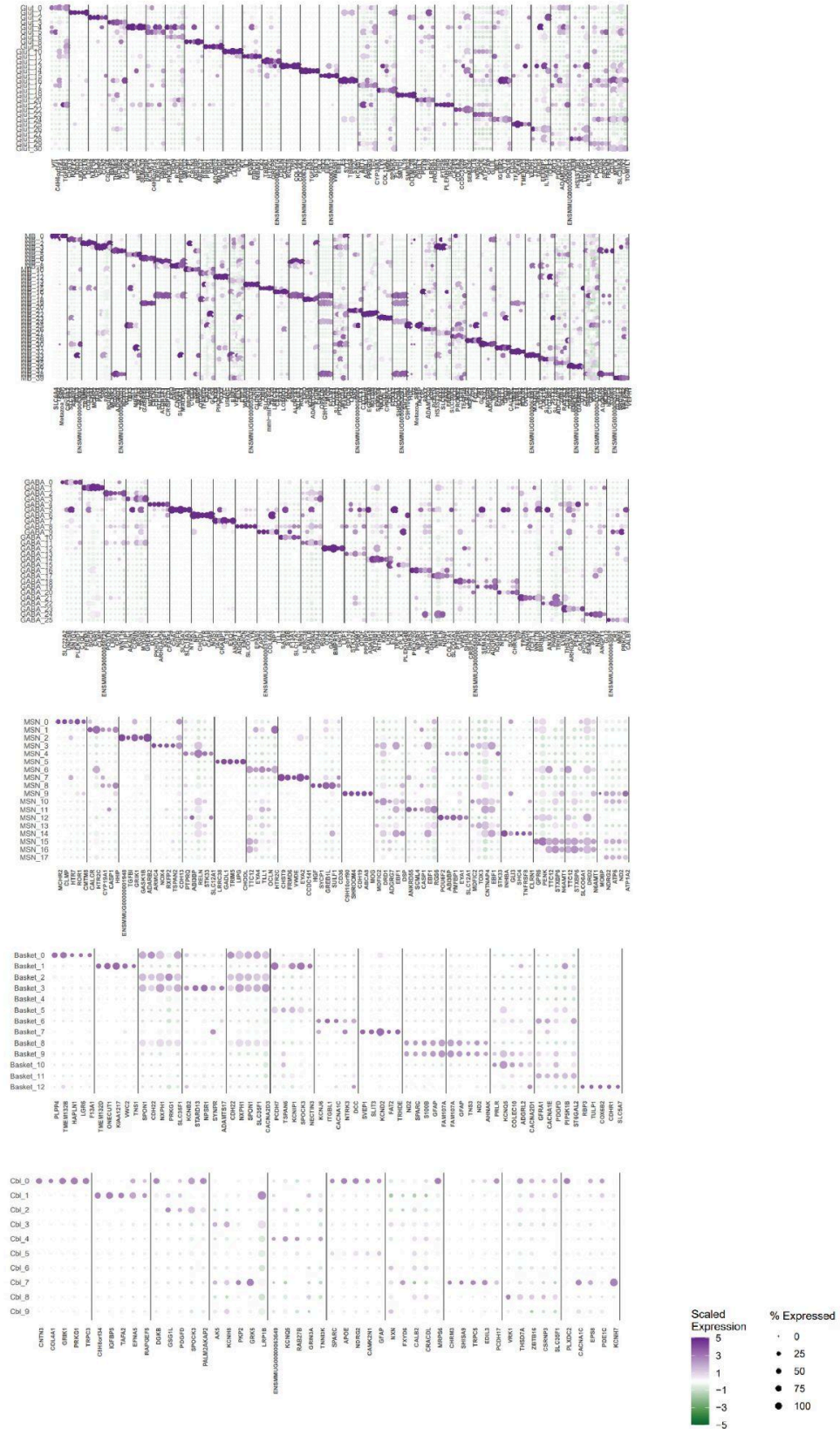


Figure S3.

Subclusters were identified by unsupervised clustering for each non-neuron cell class (OPC and oligodendrocytes were analyzed together) and for neurons from each of the 6 anatomical regions

(table S4). The top 5 marker genes with the highest z-score and log-fold change from a wilcoxon test were selected as the marker for each subcluster.

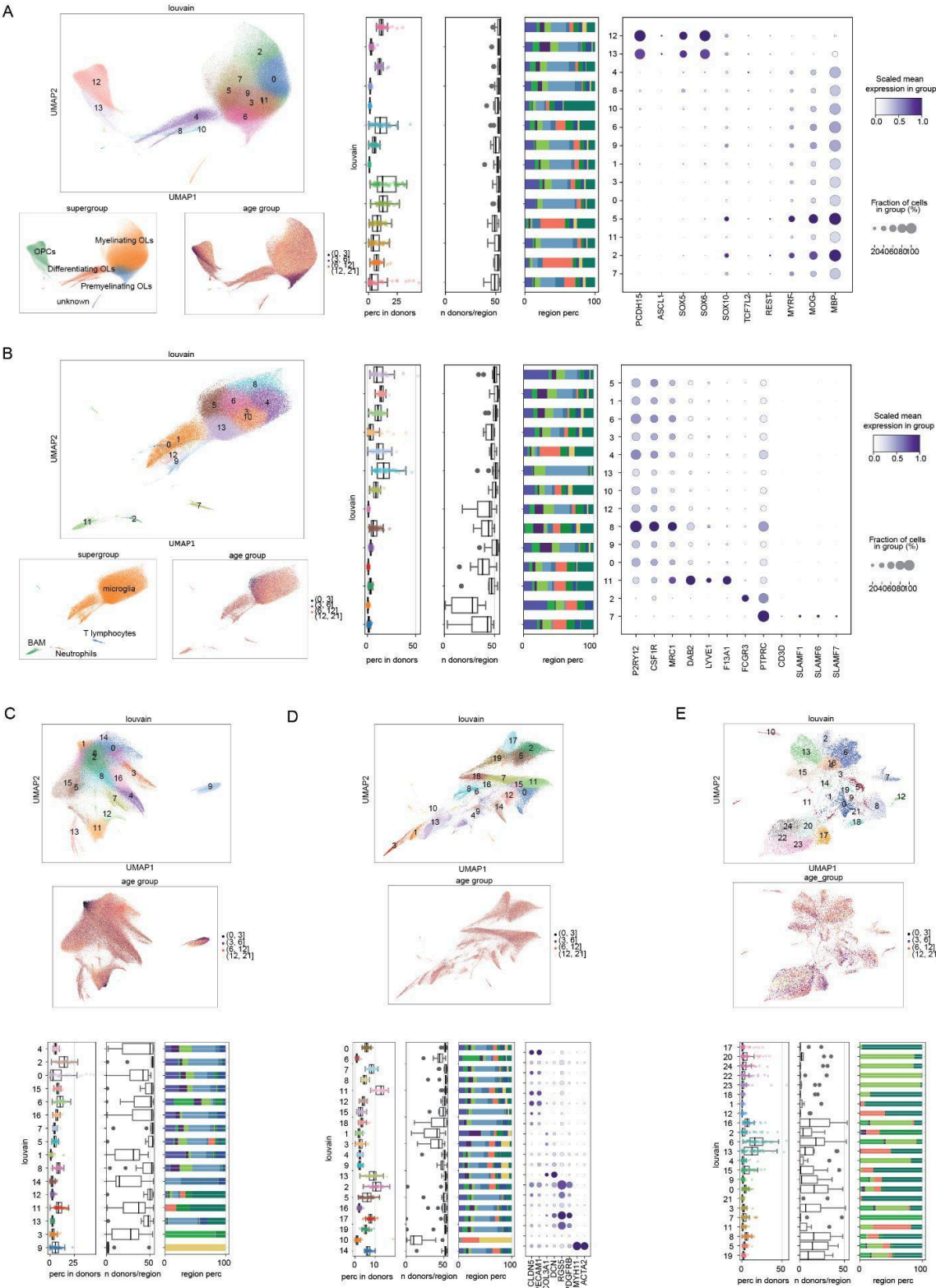
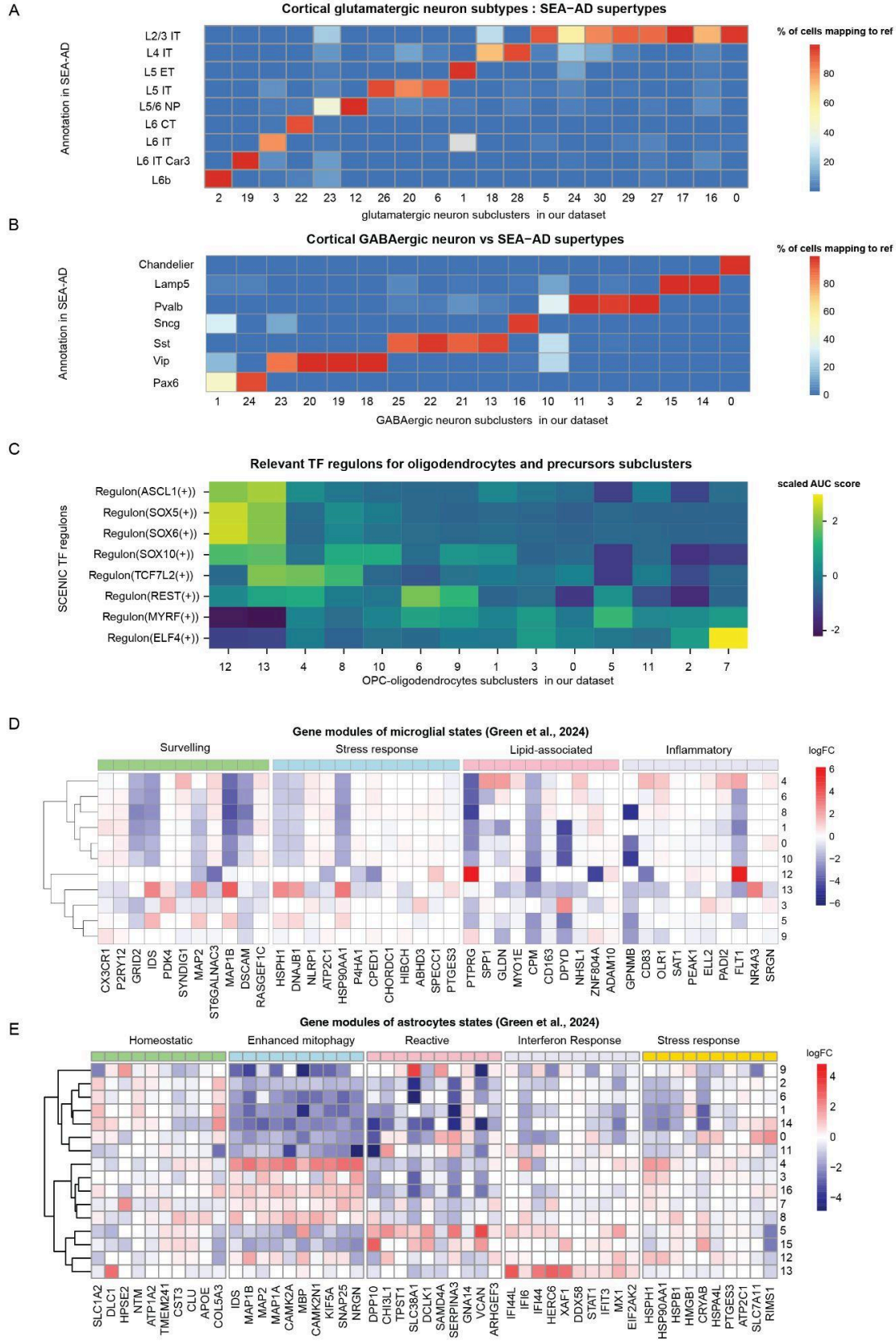


Figure S4.

UMAP visualization of oligodendrocytes (**A**), microglia (**B**), astrocytes (**C**), vascular cells (**D**), ependymal cells (**E**) subclusters identified by louvain unsupervised clustering, annotation of supergroups, and age groups. Boxplot showing proportion of each subcluster in the total population of glutamatergic neurons in each donor. Boxplots showing the number of donors with this subcluster in a specific region. Subclusters showing high differences in donor numbers among regions are region-specific subclusters. Barplots showing proportion of subclusters coming from each region. Dotplot of scaled mean expression of marker genes for supergroups in each subcluster.

**Figure S5.**

A) Heatmap showing percentage of cells in our glutamatergic neuron subclusters mapping to SEA-AD excitatory cortical subtypes with label transfer by TACCO (method). **B)** Heatmap

showing percentage of cells in our GABAergic neuron subclusters mapping to SEA-AD inhibitory cortical subtypes. **C)** TF regulons and their AUC scores computed by SCENIC for each subcluster in oligodendrocyte precursors and oligodendrocytes, scaled by row. **D)** Heatmap of log-fold change of genes related to different microglia states [78](#) in each subcluster compared to the rest. **E)** Same as D but with genes related to different astrocyte states.

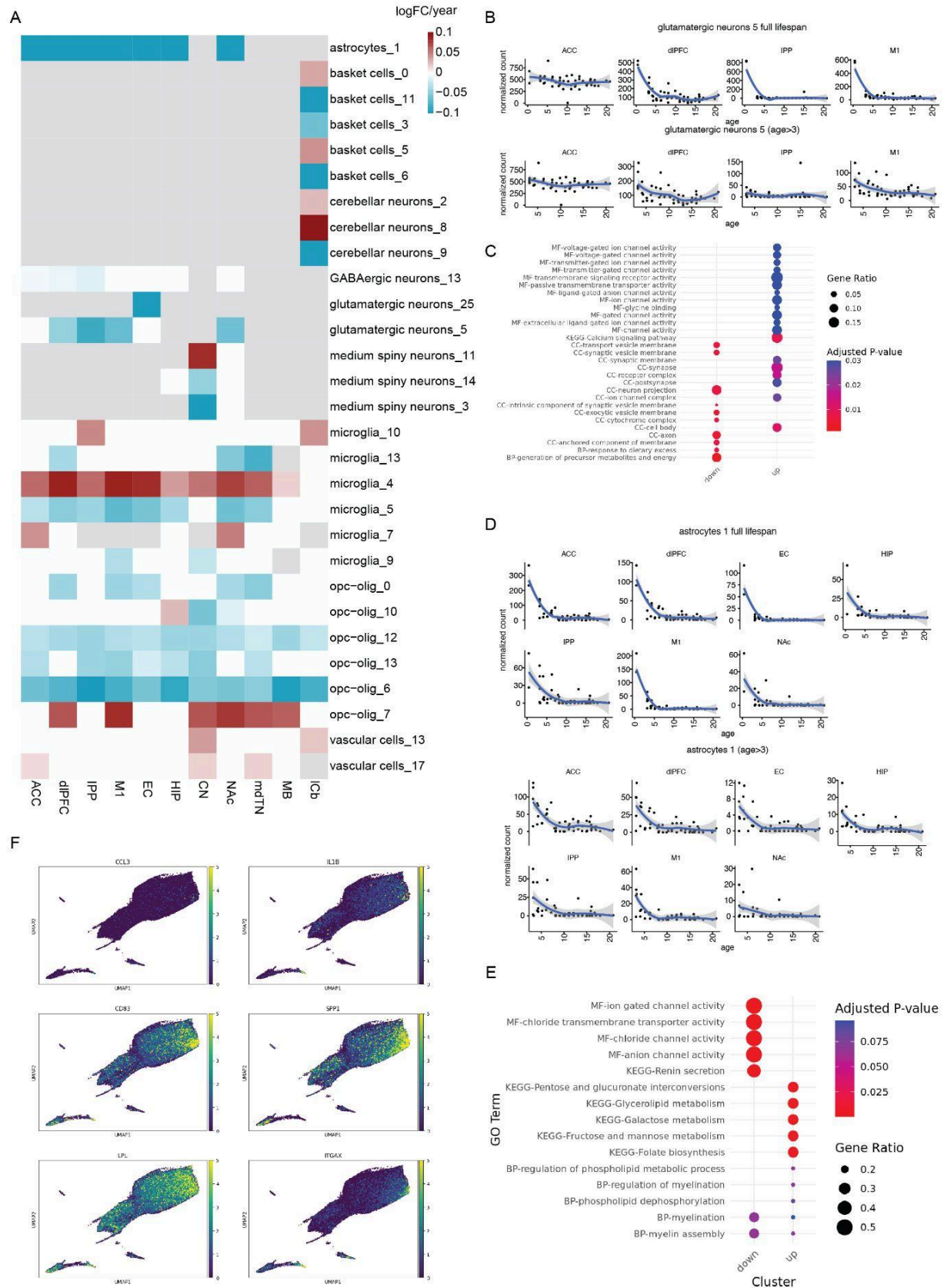


Figure S6.

A) Heatmap showing cell abundance change across different cell subtypes and regions. Value represents log-fold changes per year in the latent abundance from the poisson log-normal model implemented by Hooke²⁰¹. **B)** Loess fit of the latent abundance of glutamatergic neurons 5 (an L2/3 IT neuron subcluster) across macaques of all ages (upper), and of age > 3 (lower). **C)** GO term enrichment of up and down regulated genes of glutamatergic neurons 5 against all other L2/3 IT neuron subclusters. **D)** Loess fit of the latent abundance of astrocytes 1 across macaques of all ages (upper), and of age > 3 (lower). **E)** GO term enrichment of up and down regulated genes of opc-olig 7. **F.** UMAP showing expression of proinflammatory cytokine response genes.

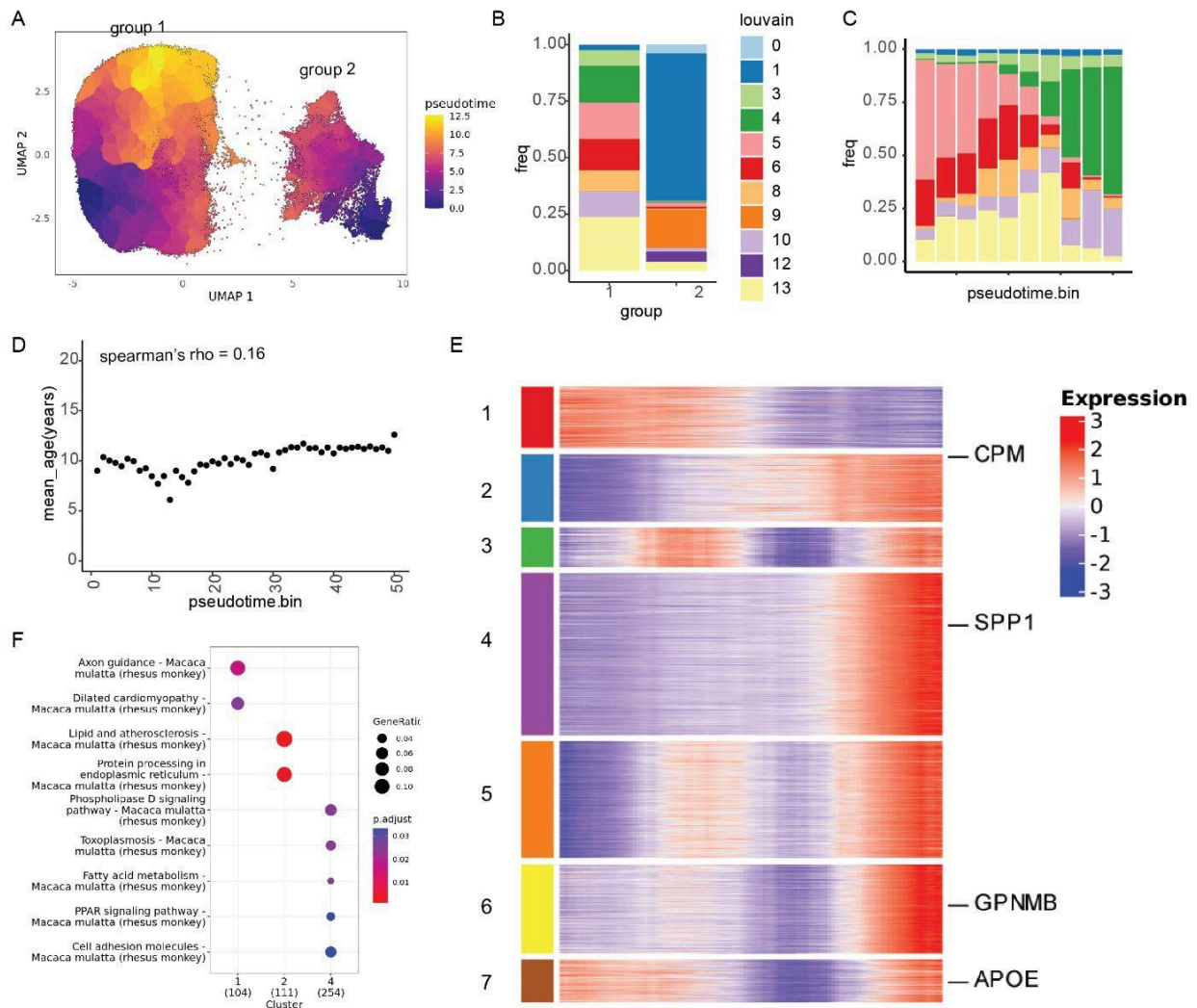


Figure S7

A) UMAP colored by pseudotime output from Monocle3. **B)** Proportion of cells from each subcluster in the graph partitions identified by Monocle 3. **C)** Proportion of cells from each subcluster in each pseudotime bin (10 bins). **D)** Dotplot showing correlation of pseudotime bins (50 bins) and mean age in each pseudotime bin for partition 1. **E)** Gene expression heatmap across pseudotime. Expression values are averaged within defined pseudotime bins (50 bins). Expression patterns are clustered into 7 clusters by k-means clustering. **F)** GO term enrichment of genes in each expression trajectory clusters.

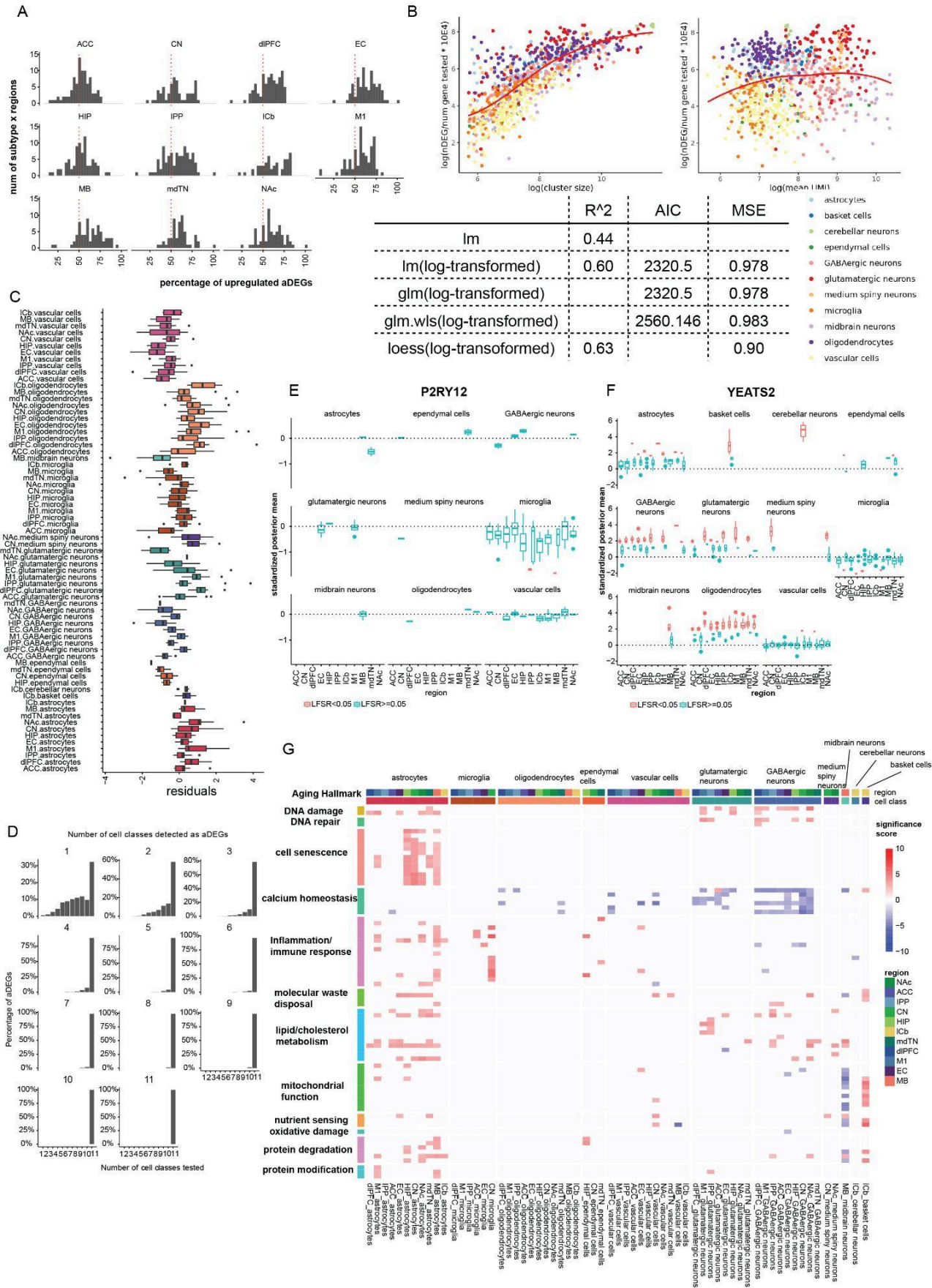


Figure S8.

A) Percentage of upregulated aDEGs in each combination of subtype and region. **B)** Loess fit of normalized nDEGs (total number of DEG/number of tests) and cluster size (left) and number of UMI (right). Goodness-of-fit metrics for different models. **C)** Residuals from loess model for each subtype and region combinations. **D)** Barplots showing the percentage of genes tested across 1 to 11 regions per cell class, stratified by the number of regions in which they were detected as significant aDEGs. Most genes were tested in all 11 regions. **E,F)** Boxplot showing standardized effect size for YEATS2 (E) and P2RY12 (F) showing significant association with age (LFSR<0.05) and coefficients of all other combinations (LFSR>=0.05). **G)** GSEA enrichment of GO pathways among combinations of subtypes and regions (significance score = -LFC sign x p-value) on brain aging hallmark sets(61).

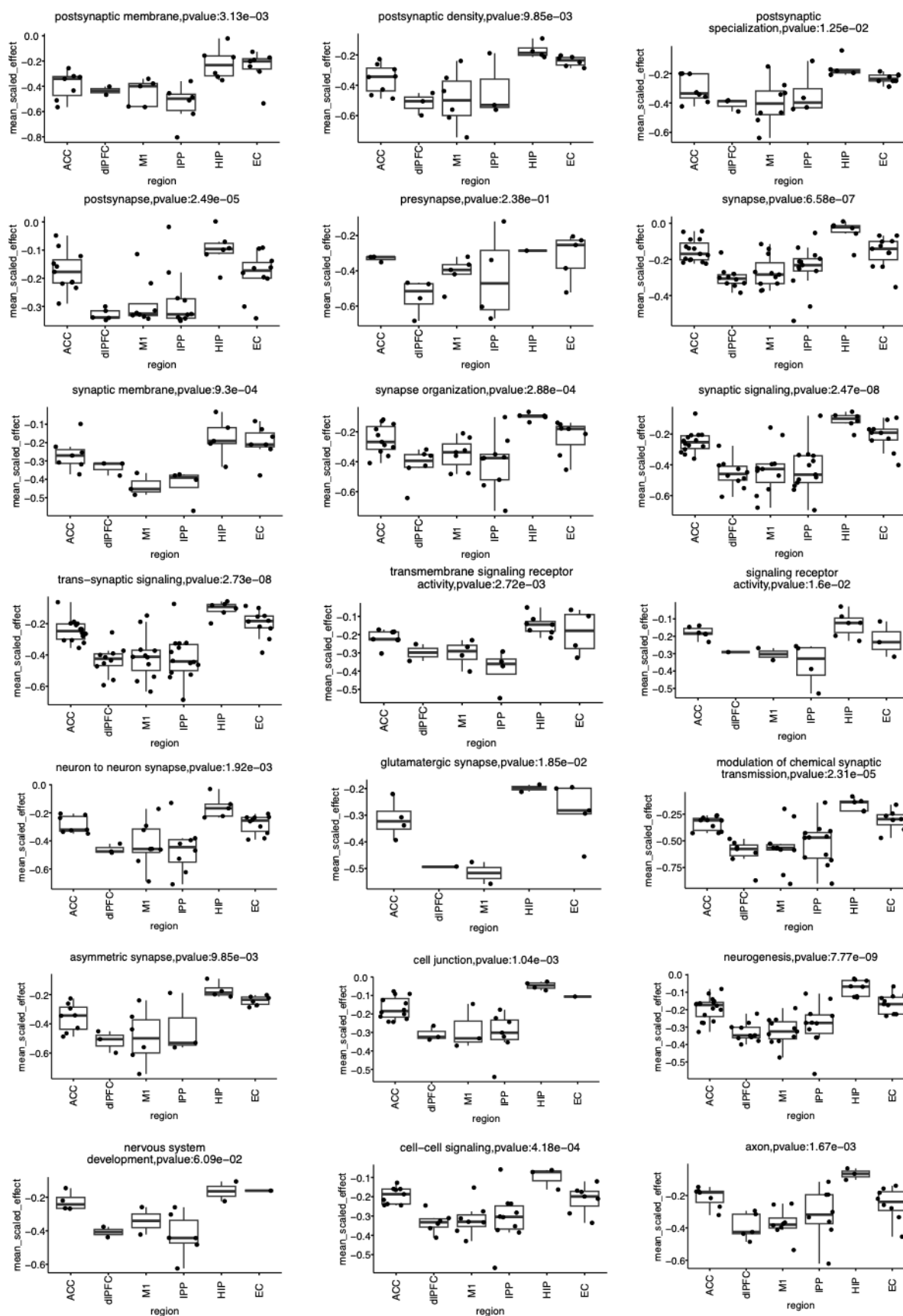


Fig S9.

Dotplots showing scaled effects (posterior mean/posterior standard deviation) averaged across genes in a given pathway. Each dot represents a subcluster x region combination with enrichment in cortical regions and the hippocampal formation. For each pathway, an anova model was fit to model the effects of origin of region and external subtype annotation (scaled effect $\sim$ region + subtype). We show the p-value without correction for region effect for each pathway.

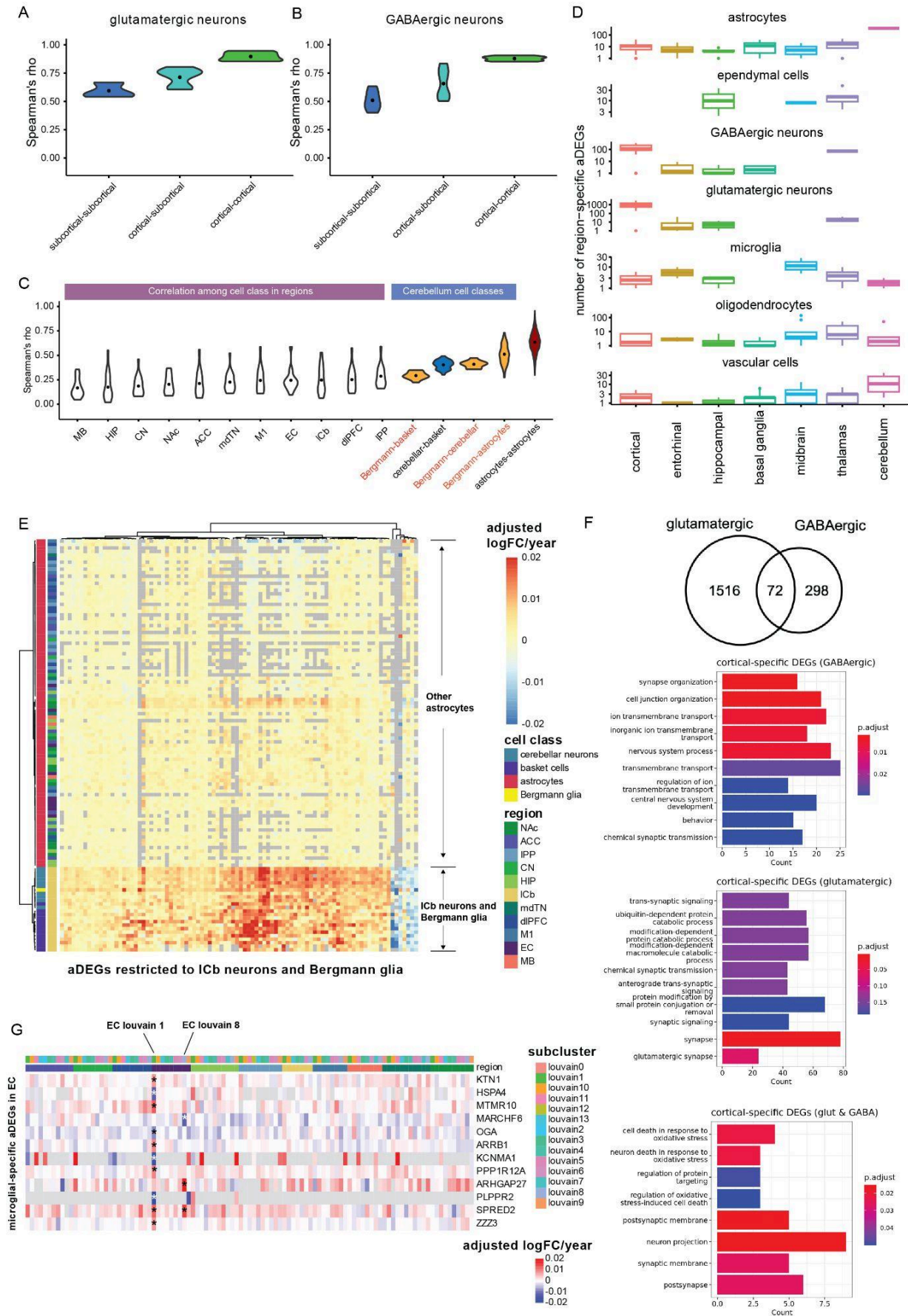


Fig S10.

A-B) Violin plots of spearman's rho of age coefficients of all genes from the linear mixed model after MASHR correction with correlation across these three categories (1) subcortical vs subcortical, (2) subcortical vs cortical, and (3) cortical vs cortical in glutamatergic neurons (A) and GABAergic neurons (B). **C)** Violin plots illustrate spearman's rho across (1) different cell classes within the same brain region, (2) different pairs of cell classes within cerebellum, and (3) different regions within astrocytes. **D)** Number of region-specific aDEGs (LFSR<0.05), defined as those specifically detected in all regions within each anatomical structure and present in at least 3 out of 4 the cortical structure. Cortical: ACC, dIPFC, M1, IPP; entorhinal: microglia; hippocampal: HIP; basal ganglia: CN, NAc; midbrain: MB; thalamus: mdTN; cerebellum: ICb. **E)** Heatmap showing adjusted logFC (posterior mean after MASHR) per year for aDEGs specific to and shared by cerebellum neurons and Bergmann glia across combinations of subcluster and region. **F)** GO pathways enriched in cortical specific aDEGs in glutamatergic neurons (upper left) or GABAergic neurons (upper right) or both (lower right). Venn plot showing intersection of cortical specific aDEGs in glutamatergic neurons and GABAergic neurons (lower right). **G)** Heatmap showing adjusted logFC (posterior mean after MASHR) per year for microglial aDEGs specific to the entorhinal cortex across combinations of subcluster and region.

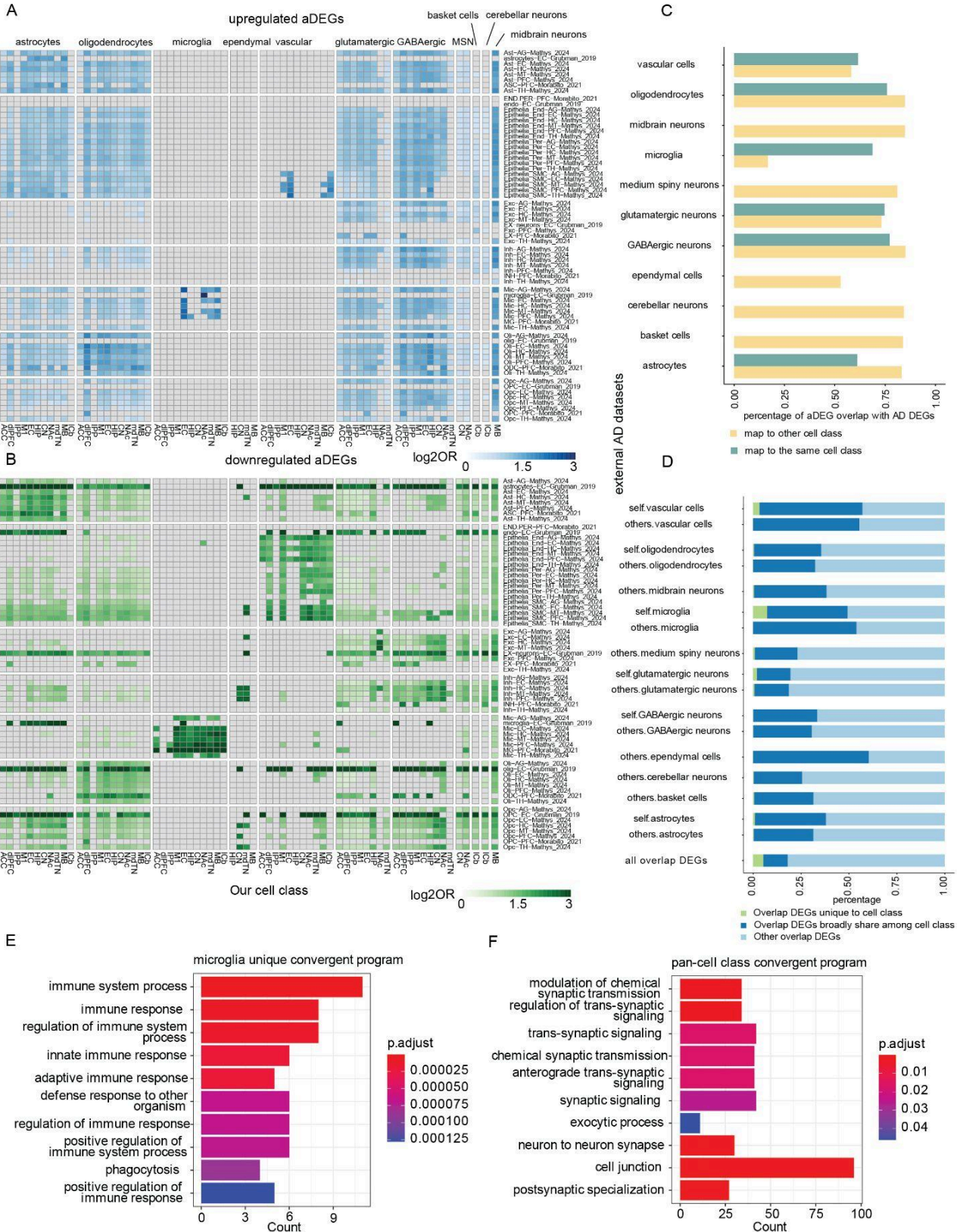


Fig S11.

Heatmap of log2OR of enrichment performed by a fisher's exact test of overlaps between **(A)** upregulated aDEGs or **(B)** downregulated aDEGs and AD DEGs (p adjusted < 0.001). **C)** Proportion of aDEGs overlapping with the same cell class(blue) or other cell class(yellow) within aDEGs of each cell class. **D)** Proportion of aDEGs that are uniquely detected (green) within one cell class, or broadly detected (dark blue) in >50% of the cell classes in both the our dataset and external AD datasets. Others include the rest of the overlap genes not fitting the previous two categories. Proportions are calculated separately for gene overlap within the same cell class or other cell class. **E)** GO term enrichment of genes uniquely detected in microglia and overlap between aDEGs and AD DEGs. **F)** GO term enrichment of genes broadly detected in >50% of the cell classes in both our dataset and external AD datasets.

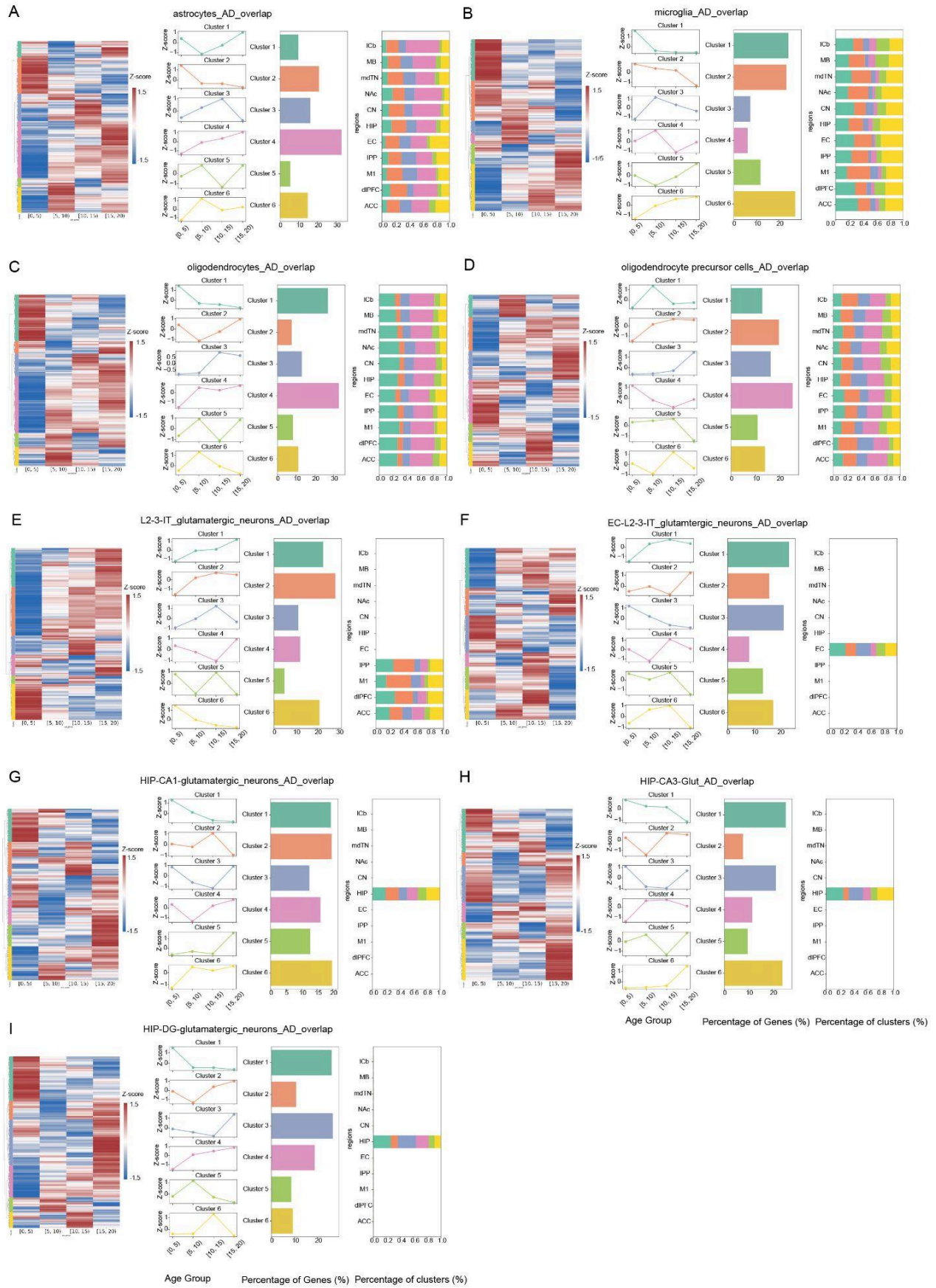


Fig S12.

Nonlinear temporal trajectory of convergent DEGs (genes overlap between aDEGs and AD DEGs) in each glial cell class or neuronal subtypes. In each panel, from left to right, (1) we showed a heatmap of average expression across individual animal of region and convergent DEGs combinations within four age bins: early life (0–5 years), adolescence (5–10 years), middle life (10–15 years), and later life (15–20 years) (Method) Z-transformation was applied for each gene across the age bins. K-means cluster was applied to sort convergent DEGs by their temporal patterns into 6 groups; (2) a lineplot showing average z-score values within each k-cluster and across age bins; (3) a barplot showing proportion of genes bellowing to each k-cluster; 4) a barplot showing proportion of gene in each k-cluster coming from each region. The panel orders are A) astrocytes, B) microglia, C) oligodendrocytes, D) oligodendrocyte precursor cells, E) neocortical L2/3 IT glutamatergic neurons, F) EC L2/3 IT glutamatergic neurons, G) HIP CA1 glutamatergic neurons, H) HIP CA3 glutamatergic neurons, I) HIP DG glutamatergic neurons.

3.12 Supplementary Materials

snRNA-seq data generation

Sample prep and snRNA-seq library construction were performed in 11 waves, one for each brain region.

All nuclei isolation and fixation were performed following the optimized sci-RNA-seq3 protocol¹⁷⁶, with minor modifications. Briefly, tissue samples were manually pulverized on dry ice, transferred to pre-chilled tubes, and stored at -80°C . Pulverized tissue was suspended in PBS-hypotonic lysis buffer A (0.3 M sucrose, 0.1% Triton X-100, 3 mM magnesium chloride, 0.025% IGEPAL CA-630, and 1% DEPC) for 10 minutes. Lysate was passed over a 40-micron cell strainer, pelleted, and resuspended in a buffer containing 0.3 M sucrose, 1X isotonic phosphate buffered solution, 0.1% TritonX-100, 3 mM magnesium chloride (0.3 M SPBSTM) and DEPC; the nuclei-containing suspension was fixed with a mixture of 123 mM Dithiobis(succinimidyl Propionate) (DSP) dissolved in dimethyl sulfoxide (DMSO) further diluted to 1.5 mM DSP in methanol and for 10 minutes. Nuclei were rehydrated with 0.3 M SPBSTM, pelleted, and resuspended in 0.3 M SPBSTM. To create a sucrose gradient, the resuspended nuclei were added to a 10:1 volume of 1.4 M SPBSTM and centrifuged at 3,000xg for 20 minutes at 4°C . The pellet was resuspended in 0.3 M SPBSTM before being passed through a 20-micron filter and counted. Nuclei suspensions were aliquoted and pelleted, then resuspended in a solution of 90% 0.3 M SPBSTM and 10% anhydrous DMSO. Nuclei were stored at -80°C until use.

Nuclei aliquots were shipped in waves on dry ice to the Brotman Baty Institute Advanced Technology Lab (BAT-Lab) at the University of Washington for single-cell combinatorial indexing RNA-seq (sci-RNA-seq3)^{13,176}. One library per region was construction followed the optimized sci-RNA-seq3 protocol [Martin et al. 2023] with the following modifications: samples were counted with the ImageExpress Pico System (Molecular Devices), 15-20K nuclei were loaded per reverse transcription well, and tagmentation with N7 adaptor-loaded Tn5 transposases (Diagenode cat. no. C01070010-20) was performed at 6uL Tn5 per 550 uL of 2X TD with 5uL per well across PCR plates for all experimental runs. Libraries were cleaned twice with 0.8X AMPure XP beads to remove lower molecular weight bands followed by a 0.55X AMPure XP supernatant step to remove higher MW bands and a Qiagen GeneRead Size Selection purification column for the final library purification in Qiagen elution buffer. All libraries were sequenced on Illumina NovaSeq S4 flow cells at the University of Washington Northwest Genomics Center core sequencing facility with the following cycles: read 1, 34 cycles; read 2, 100 cycles; index 1, 10 cycles; index 2, 10 cycles.

snRNA-seq preprocessing

snRNA sequencing reads were processed into a gene-by-nucleus matrix of UMI counts following the pipeline developed by the sci-team of Brotman Baty institute (<https://github.com/bbi-lab/bbi-sci/tree/master>). Briefly the pipeline include the following steps: (i) converts base calls to fastq files with bcl2fastq/ v.2.20 (RRID:SCR_015058) (Illumina), (ii) removes polyA tails using Trim Galore/v.0.6.7 (RRID:SCR_011847) (99), (iii) aligns trimmed reads to a reference genome with STAR/v.2.7.6 (RRID:SCR_004463)²³², (iv) extracts mapped reads, (v) removes duplicates, and (vi) generates UMI counts for exonic and intronic regions of each gene, tabulated according to the unique three-level barcode design in sci-RNA-seq3. We used the rhesus macaque reference genome (Mmul_10)²³³ and annotation, obtained from Ensembl (version 101) (RRID:SCR_002344). We extended the 30 untranslated region annotations of genes and transcripts by 500 bp to avoid misclassifying genic reads as intergenic. After generating the count matrix, we removed nuclei of low quality or ambient RNA with the following standard: (i) UMI counts < 100, (ii) number of gene detected ≤ 100 , (iii) percentage of mitochondria $\geq 10\%$, (iv) percentage of reads mapping to intronic region $\geq 20\%$.

For each sample, we then imported the filtered gene-by-nucleus count matrices into the AnnData/v.0.8.0 (RRID:SCR_018209)²³⁴ framework and then ran Scrublet/v.0.2.3

(RRID:SCR_018098)^{234,235}(expected_doublet_rate = 0.05) to calculate doublet scores. We marked nuclei as Scrublet-inferred doublets if they had Scrublet doublet scores > 0.20 .

To identify doublets with the scrublet output, we used an iterative clustering strategy using Scanpy/v.1.9.1 (RRID:SCR_018139)²³⁶ implemented by bioalpha(Vuong et al. 2023). First, we combined the count matrices of all samples from the same brain region into a single AnnData object. Second, we normalized the data to the total UMI per nucleus, logarithmized the data, and subsetted the data to the 10,000 most variable genes. For each cell, we regressed out total UMI counts per nucleus and then mean-centered and scaled the data. The dimensionality of the data was then reduced by principal components analysis (PCA) (50 components). We then constructed a neighborhood graph using 30 nearest neighbors (n_neighbors=30) and 30 principal components (n_pcs=30), and performed Louvain clustering at a high resolution (resolution = 6) to partition the nuclei into small clusters. Finally, we calculated the proportion of Scrublet-inferred doublets in each cluster and observed a bimodal distribution across all clusters, with a clear separation of the two peaks at 40%. Therefore, we filtered all nuclei in clusters with $\geq 40\%$ of Scrublet-inferred doublets.

To further filter out nuclei of low quality or doublets that are remaining, we implemented a distribution based approach. For putative non-neuronal and neuronal nuclei, we modeled their UMI count distributions separately and computed the mean and standard deviation (SD) for each group. Nuclei with UMI counts $\leq \text{mean} + 1 \text{ SD}$ and $\geq \text{mean} + 2 \text{ SD}$ were excluded from further analysis.

After the filtering steps as described above, we repeated the normalization and dimensionality reduction. We then visualized the filtered dataset by UMAP (min_dist=0.25, spread=1.0, n_components=2). We confirmed adequate removal of doublets by observing the clean separation of distinct cell types and the absence of clusters expressing obviously ambiguous marker gene profiles.

snRNA-seq cell class and cell subcluster annotation

Cell class

For each region, we annotate the cell class by a combination of label transfer and majority ruling. First, we annotate each cell to its putative cell class by an optimal transport based label transfer method implemented by TACCO²³⁷. We performed label transfer with all cell classes present in the corresponding region in BICCN¹⁹¹ as the reference. Due to substantial increase in UMI counts of UMI counts in the current dataset compared to BICCN, we normalize our data to conform to the reference (normalize_to='reference'). After annotation with TACCO, we applied a majority ruling

approach on the Louvain clusters (resolution = 6) generated above. We annotate a cluster to a particular cell class if 70% of the nuclei in the cluster belong to the same class, or the proportion of the top annotated cell class are 3x higher than the second annotated one. Clusters that are not annotated with the majority ruling will be subjected to manual annotation based on marker gene expression and subcluster annotation described below (1-15% of nuclei in each region). Unknown clusters with high mean doublet scores (> 0.15) and inconsistent annotation between cell class and subcluster are marked as doublet and excluded (0-8% of nuclei in each region). We also identified two cell classes that are not expected in a certain region (i.e. basket cells in midbrain, medium spiny neurons in hippocampus), likely coming from dissection errors. They are kept for cell abundance analysis at the cell class level to account for unidentified cells from dissection errors in these regions, but excluded in other analyses.

Cell subclusters

After finalizing the annotation of the cell class in each region, we combine nuclei of the same cell class among regions to generate subclusters under each cell class. We performed normalization and dimensionality reduction as above except subsetting to 3,000 most variable genes. We then constructed a neighborhood graph as above and performed Louvain clustering (resolution = 1) for each cell class. For each cell class, we then annotated the cell subclusters by label transfer implemented by TACCO as described above. We used BICCN [191](#) and human brain datasets [192,193](#) as reference. We also compared the Allen mouse brain atlas by MapMyCell [238](#). For microglia and astrocytes subclusters, we have performed additional functional annotation by comparing their transcriptomes to external datasets [78](#) and Gene Ontology with msigDB [239,240](#).

After generation and annotation of cell subclusters, we combine all nuclei into a global AnnData object (~5.3M nuclei). We then inspected the localization of subclusters annotated to the same cell class. For subclusters that are not localizing closer to their annotated cell class, we reassign their cell class and subcluster labels based on marker gene expression (1% of all subclusters).

TF Regulon analysis

To identify TF regulons that are enriched in cell subclusters, we have performed single cell regulatory network inference and clustering (SCENIC) analysis with pySCENIC [241](#). We filtered macaque genes in our dataset to retain only those with one-to-one human orthologs, and then mapped the macaque genes to their corresponding human orthologs using orthology annotations

from Ensembl BioMart (release 113). To accelerate the analysis, we filtered out genes expressed in <100 cells and subset cell class objects to 100k cells. We followed the pipeline of SCENIC, which in briefly: i) generate co-expression modules based on positive correlation between transcription factors (annotated in hg38 human genome) and candidate target genes with GRNBoost2; ii) each co-expressed module is analyzed with cisTarget using v10 human motif databases and annotations to identify enriched motifs: modules and targets for which the motif of TF is enriched retained; iii) the activity of each regulon in each cell is evaluated using AUCell, which calculates the Area Under the recovery Curve. We evaluated the activity of a particular regulon in a subcluster by averaging the regulon activities across all cells.

Cell composition analysis

Poisson Lognormal models (Hooke)

To perform differential analysis of cell state abundances, we modeled cell abundance change with Poisson Lognormal (PLN) models implemented in Hooke²⁰¹. We retained only animals (age>3 years) for modeling linear abundance change. For each region, we first converted our single cell dataset into a matrix containing the number of cells of each cell class/subcluster observed in each sample, along with metadata for each sample. Then Hooke estimates per-sample “size factors” that will control for sample-to-sample variation in cell capture levels. Next, it initializes a PLN model that estimates proportions of each cell state as a function of age and sex by `new_cell_count_model(vhat_method = 'bootstrap', num_bootstraps = 100)`. We extracted the coefficients and standard errors from the full model and calculated two-sided p-values under the null hypothesis that each coefficient equals zero, using the Student’s t-distribution. We then performed false discovery rate (FDR) correction on the resulting p-values and considered genes significant at an adjusted p-value < 0.05. To maintain sufficient statistical power, we excluded region–cell class combinations with fewer than 100 cells in differential analysis within cell class. To visualize non-linear abundance change, we input cell count in all animals into the PLN models and extract the ‘normalized counts’ after normalization with size factor.

Multivariate adaptive shrinkage (MASH)

To further boost sensitivity, especially in regions with low cell counts, we applied multivariate adaptive shrinkage (MASH) through its R package `mashR` ^{201,205}. We imported the coefficients output and standard errors from Hooke and applied the data-driven approach: (i) select strong

signals (local false discovery rate, $\text{lfsr} < 0.05$) from running a condition-by-condition analysis on all the data; (ii) Perform PCA and compute initial covariance matrices based on the top 5 PCs of the strong signals; (iii) apply the Extreme Deconvolution algorithm from the initialized matrices to the strong signals; (iv) fit mash to all tests.

Pseudotime analysis

The pseudotime analysis was performed with Monocle3 [13](#). We followed the instruction to (i) perform normalization and dimensionality reduction of the full dataset with default parameters; (ii) cluster cells into partitions using a statistical test [242](#); (iii) For each partition, fit a principal graph and set the root node to the subclusters with abundance decrease over age; (iv) calculate pseudotime values for each cell using `order_cells`.

Differential expression analysis

Linear mixed model (NEBULA)

To assess differential gene expression by age and sex across combinations of cell subclusters and brain regions, we applied the NEBULA framework, which fits a negative binomial gamma mixed model (NBGM) using a large-sample approximation. Following NEBULA guidelines, we excluded subcluster–region combinations with fewer than 30 animals. Within each combination, we filtered out lowly expressed genes using a threshold of counts per cell ($\text{cpc} < 0.05$ (i.e., expressed in fewer than 5% of cells)). The model included age, sex, and UMI counts as fixed effects, and animal ID as a random effect. We performed false discovery rate (FDR) correction on the resulting p-values and considered genes significant at an adjusted p-value < 0.05 . To further improve statistical robustness, we applied a post-hoc heuristic filter, excluding combinations with fewer than 300 cells, resulting in 820 unique combinations of region and cell subclusters. This threshold was based on the minimum cell count at which a linear relationship between cell number and the number of detected differentially expressed genes became evident.

Multivariate adaptive shrinkage (MASH)

To further boost sensitivity, especially in combinations of cell subclusters and regions with low cell counts, we applied multivariate adaptive shrinkage (MASH) as above among all combinations of cell subclusters and regions within each cell class.

Gene set enrichment

All age-associated differentially expressed genes (aDEGs) identified by NEBULA were ranked in ascending order based on their local false discovery rate (lfsr) values obtained from MASH. This ranked gene list was then used for gene set enrichment analysis (GSEA) using the GSEA function from the clusterProfiler package²⁴³. Enrichment analysis was conducted using gene sets from msigDB ^{239,240}, KEGG ^{244,245} and synGO ²⁴⁴.

Supplementary Note 1

We first used unsupervised Louvain clustering and label transfer from two reference atlases [\(16, 17\)](#) to identify 12 molecularly distinct cell classes across the 11 regions (Fig 1B). Based on known cell-type-specific marker genes gene markers and expected regional representation, these cell classes were annotated as either (i) neuronal cells, including glutamatergic neurons (*SLC17A7*, *SLC17A6*), GABAergic neurons (*GAD1*, *GAD2*), medium spiny neurons (*DRD1*, *DRD2*), midbrain neurons (neurons residing in midbrain), cerebellar basket cells (*SORCS3*, *KIT*) and other cerebellar neurons (granule cells and Purkinje cells, *GRM4*); or (ii) non-neuronal cells, including oligodendrocyte precursor cells (OPCs; *PCDH15*), oligodendrocytes (*MBP*, *MOG*), astrocytes (*SLC1A2*, *GFAP*), microglia (*DOCK2*, *P2RY12*), vascular cells (*CFH*), and ependymal cells (*SPAG17*) (Method, Fig. 1E).

What is the coverage of these major cell classes in our dataset? The major cell classes were captured over the course of aging with no dropouts (Fig. 1C). As expected, the distribution of major cell classes differed extensively across regions, reflecting the cellular makeup underlying known region-specific functions (Fig. 1D,S1D) [\(17\)](#). Most of the major cell classes recovered are as expected, except for basket cells in midbrain and medium spiny neurons in hippocampus. The presence of these unexpected cell classes likely indicates minor dissection errors into nearby regions: basket cells from cerebellum and medium spiny neurons from the basal ganglia. To account for the proportion of cells from dissection errors, we retain these unexpected cell classes for differential abundance study at the cell class level. Within a region, the expected major cell classes were present in >80% individuals, except for ependymal cells (Table S2). Ependymal cells are a rare cell class (0.4% of the full dataset), so their recovery might be more variable in each individual.

Supplementary Note 2

We first aimed to characterize how different covariates (i.e. region, age, sex) pattern transcriptional variance. To do so, we divided the macaque lifespan to different age groups and created pseudobulk transcriptomes among individuals in each age group for a given region (Fig S1E). The pseudobulk transcriptomes were then projected to a principal component analysis (PCA) space. As visualized in the PCA space, transcriptomes were largely separated by regions rather than by age. Therefore, it is important to dissect age-associated change within individual regions. We did observe progressive changes of the transcriptome in each region, with a considerable gap between infancy (age >3 years) and the other age groups (ages 3 - 21). By analyzing the genes most highly correlated with age, we found that the infant samples were transcriptionally distinct from the rest of the donors in the major cell classes (Fig S1F). Indeed, the brain tissue volumes of macaques continues to grow throughout infancy, only stabilizing or decelerating at the onset of adolescence, which occurs around 3 or 4 years of age in macaques [\(21, 22\)](#).

We further looked at variance explained by different covariates (i.e. age and sex) within each region. Age explained a small proportion of variance in gene expression (median = 1.1-2.6%), which is consistent with previous studies using bulk RNA-seq (median = 0.6-6.4%) and human brain transcriptomic data from the Genotype-Tissue Expression (GTEx) Consortium V8 dataset (median = 0.3-4.2%) [\(14\)](#). Among regions, age explained the most changes in cerebellum, followed by the primary motor cortex and caudate nucleus (Fig. S2A). Sex explained a much smaller proportion of variance, explaining variance of 15-21% of all genes detected (median = 1.4-2.2% among those explained by sex) (Fig S2B, Table S3).

Supplementary Note 3

To examine the heterogeneity within each cell class, we then performed unsupervised sub-clustering analysis for each cell class (oligodendrocyte precursor and oligodendrocytes were analyzed together to capture transition states). Overall, we identified a total of 226 subclusters, with 193-195,742 cells in each subcluster (Fig. 1E). Hierarchical clustering of all cell subclusters reflected ontogenic relationships, with two clades of neuronal classes and non-neuronal classes (except OPCs were clustering with neurons potentially due to expression of similar receptors such

as GRID2). The subclusters show consistent marker gene expression as their corresponding cell class and are defined by a distinct set of marker genes (e.g. GABAergic neurons subclusters are marked by GAD1 and GAD2, Fig. 1E, fig. S2C,S3).

The majority of the GABAergic neuron subclusters were observed in cortical and subcortical regions (19 out of 26 subclusters), while some subclusters have regional bias (Fig. 2B). Most of these specializations were reported in a human study (8). We identified the four major neocortical types of GABAergic neurons in our dataset (SST^+ , $PVALB^+$, VIP^+ , or $LAMP5^+$), including the $LAMP5^+LHX6^+$ subtype specific to the primate cortex (24) (Fig. S5B). A subpopulation of SST^+ neurons were enriched in the entorhinal cortex and hippocampus, including an NPY^+ subtype reported. A unique GABAergic neuron subtype ($OTX2^+ GATA3^+$) was specific to thalamus, which show corresponding markers ($MEIS2$, $FOXP2$) with the counterpart of humans, also enriched in genes involved in neurite outgrowth ($SEMA3C$, $SPON1$), and receptors for serotonin ($HTR2A$), acetylcholine ($CHRM2$, $CHRNA3$), and glutamate ($GRM3$). We also discovered multiple subclusters that are specific to basal ganglia, including LHX6 ($TACR3^+ CRABP1^+$), LHX8 ($DLK1^+ PKHDIL1^+$), SST($NPY^+ CHODL^+$) and cholinergic ($CHAT^+ NGFR^+$) neurons.

We have identified 14-25 subclusters for each non-neuronal cell class and further characterized them by distinct expression profiles, marker genes, and enriched pathways. The oligodendrocyte precursors and oligodendrocytes were partitioned into 14 subclusters. All oligodendrocytes subclusters are found in all regions, showing minimal regional specialization. These subclusters mapped to 4 distinct states along oligodendrocyte differentiation and maturation with differential activities of transcription factor regulons (25, 26): oligodendrocyte precursor cells (olig-opc 12 and 13; regulons of $PDGFRA$, $PCDH15$), pre-myelinating oligodendrocytes (olig-opc 4 and 8, regulons of $ASCL1$, $SOX5$ |6|10), differentiating oligodendrocytes (olig-opc 6, regulons of $REST$) and myelinating oligodendrocytes (olig-opc 0, 1, 2, 3, 5, 7, 9, 11, regulons of $MYRF$) (Fig. S4A, S5C).

We have observed both microglia and peripheral immune subclusters in our dataset, including border associated macrophage (microglia 11; $DAB2^+ LYVE1^+ F13A1^+$), neutrophils (microglia 2; $FCGR3^+$), T lymphocytes (microglia 7; $CD3D^+ SLAMF^+$). Similar to oligodendrocytes, the microglial and immune cells show minimal regional specificity. The microglia subclusters show

differential enrichment for signature genes of different microglial states: homeostatic, surveilling, stress response, lipid metabolism, neuroinflammation, etc (Fig. S4B, S5D).

The majority of astrocytes subclusters are distributed across the brain while a subset of them (5 out of 16) exhibited region specificity, including ICb-specific Bergmann glia cells (astrocytes 9; *GABRA2*⁺), a subcluster that is specific to the basal ganglia (astrocytes 3, *PENK*⁺ *SYNDIGIL*⁺), and 3 subclusters (astrocytes 1, 12, 13) enriched in the thalamus. The astrocyte subclusters show differential enrichment for signature genes of homeostatic, reactive, mitophagic, and stress responsive astrocyte states (Fig. S4C, S5E).

For rarer cell classes, vascular cells and ependymal cells, we have captured their distinct subtypes in our dataset. Vascular cells subclusters show exclusive enrichment of markers of endothelial cells, pericytes, vascular leptomeningeal cells (VLMC), and vascular smooth muscle cells (VSMCs), most of which distribute broadly across the brain except subcluster 10 (*HTR2C*⁺ *LMX1A*⁺) was specific to midbrain and cerebellum (Fig. S4D). Ependymal subclusters show more regional variations, with bias for hippocampus, basal ganglia, midbrain and thalamus, respectively. This potentially reflects distinct molecular signatures of different ventricle structures present in different regions (Fig. S4E).

Chapter 4 Discussion and Future directions

In the first project, we used single cell RNA-seq to delineate the transition of cellular states in an *in vitro* model called gastruloids. We identified a retinoic acid signaling axis which restores the bipotency of neuromesodermal progenitors, resulting in formation of embryonic structures, further development of the neuronal lineage, and development of intermediate mesoderm lineage (human RA gastruloids). We successfully developed a computational framework to align *in vitro* stem-cell models and *in vivo* embryos to systematically stage human RA gastruloids. We further validated that human RA gastruloids to be a robust model for large-scale perturbation experiments.

Although human RA gastruloids model specific aspects of *in vivo* human early embryogenesis, there are still limitations:

- It is developing on a much faster time scale as the *in vivo* counterpart: developing from hESCs equivalent to embryonic day 4-7 to a stage equivalent to day 23-26 in just 6 days. The acceleration was potentially caused by addition of Wnt agonist and retinoic acid. While essential processes such as somitogenesis remain in the same time scale (each somite pair established every 5 hours), it is unclear how this acceleration of time scale influences the establishment of cell identities.
- We did not observe a heart structure as those seen in embryoid bodies but rather an unorganized heart structure in the anterior part of human RA gastruloids, which could be due to the addition of the high concentration of Wnt agonist to induce mesoderm.

- Human RA gastruloids did not form the embryonic disc and primitive streak as in their *in vivo* counterparts, suggesting cell identities can be established without these structures. How these structures and geometric constraints in the uterus shape *in vivo* embryonic development still remain elusive.
- Human RA gastruloids only model the posterior part of their *in vivo* counterparts, lacking the anterior brain structures. Indeed, we observed a more disorganized anterior part in human RA gastruloids than its posterior parts. This is potentially due to the lack of signaling from the anterior parts.
- Endoderm lineage is underrepresented in human RA gastruloids, potentially due to the high concentration of Wnt agonist during pretreatment.

As for the staging method, it establishes a computational axis to do comparative staging across species and *in vivo* vs *in vitro*. However, there are certain caveat and limitations:

- Since it is based on pseudobulk expression, it might be influenced by the representation of different lineages in the original model. We observed less accurate staging of the renal lineage which was less represented and PGC which was not present in gastruloids.
- The axis was established partially on human gastruloids, which only models the posterior part of the embryo, which might show different temporal gene expression as compared to the anterior embryos, especially the pluripotency marker of SOX2 in the posterior embryo are marker genes of many cell types in the anterior part. The anterior lineages at E8.5 (e.g. placodal area, forebrain/midbrain) were staged slightly earlier than E8.5.

Despite the above limitations, the human RA gastruloids still offer a valuable model to study gene regulatory programs in development of the lineages represented. By perturbing different signaling axes and developmental relevant genes, one could investigate the cell-autonomous and cell-nonautonomous factors that drive the establishment of cellular identities. It will also be a reliable model to investigate genetic programs responsible for formation of neural tube and somites, and how genetic variation contributes to congenital defects in these structures.

By manipulating the retinoic acid axis and the number of cells, we have successfully developed embryonic structures and new lineages as *in vivo* counterparts. Future studies could explore if a combination of signaling regimes with the appropriate cell number could further develop the under-represented lineages (e.g. endoderm lineage). Lately, microfluidics devices²⁴⁶ were developed to control spatial exposure to different morphogens, while it is possible to combine stem cell population undergoing exposure to different morphogens separately, one could imagine creating the anterior part which might require a different signaling regimes than the posterior part.

On the other hand, we demonstrated that there are differences in the development of human and mouse stembryo models that human stembryo models require an additional retinoic acid pulse to form embryonic structures. Whether that's due to species differences or cell lines differences requires more investigation and might inform improvement of other stembryo models which have a more developed mouse counterpart than human counterparts^{43,44}.

The current *in silico* staging method provides a framework to systematically align posterior embryonic structures with the *in vivo* development timeline. However, this approach is limited to posterior structure and might be impacted by the representation of lineages within a stem cell based model. Therefore, an improved method at the single cell level would be ideal to overcome these challenges. Methods based on variational autoencoder^{246,247} and support vector machine²⁴⁸ are developed to align embryonic time across modality. It could potentially be applied to align developmental time across species at the single cell level.

Overall, we develop a human stembryo model which provides a platform to understand genetic programs in development and genetic disease, while creating a framework to align stembryo models to *in vivo* embryos on developmental stage.

In the second project, we generated a comprehensive single-cell map of cell type- and region-specific aging in the brain of non-human primates (i.e., rhesus macaques). This dataset enabled us to quantify both inter-regional and intra-cellular heterogeneity in the aging process. We observed substantial variability in the aging trajectories across brain regions and cell types, and identified selectively vulnerable cell populations that change in abundance with age. Importantly, by comparing these aging trajectories to transcriptomic data from human neurodegenerative diseases, we discovered convergent molecular programs between normal aging in non-human primates and pathological processes in the human brain.

While this study provides novel insights into the cellular trajectories and gene regulatory networks associated with brain aging in non-human primates, several limitations remain.

- Dissection errors across certain brain regions may introduce confounding effects in downstream analyses. These errors are difficult to detect or correct, especially for widely distributed cell types such as glia, where no region-specific marker is available to flag contamination.
- Although we implemented statistical frameworks to account for imbalance in sample sizes across regions, residual differences in statistical power could still lead to false negatives or exaggerated heterogeneity.

- Because non-human primates are exposed to diverse natural environmental conditions, their transcriptomes exhibit substantial inter-individual variability—especially in adulthood—potentially masking subtle age-associated signals. Future studies may benefit from a denser sampling design, where more individuals are profiled per age bracket, even at the expense of fewer timepoints, to better capture non-linear aging trajectories.
- Our dataset spans the macaque lifespan up to 21 years, which is below the median lifespan of ~27 years for rhesus macaques in both wild and captive settings²⁴⁹. Therefore, we may be observing only the onset of age-related decline, missing more advanced stages of neurodegeneration. Extending sampling into late-life stages could reveal additional features of neuronal dysfunction and cell-type-specific pathology.

Despite these caveats, our study represents the first large-scale, multi-region, lifespan-spanning single-cell transcriptomic atlas of brain aging in a non-human primate. It reveals major shifts in cell abundance and gene expression with age, providing a critical resource for dissecting molecular and cellular features of primate brain aging. Still, several open questions remain. For example, while we identified selectively vulnerable neuronal subtypes, the molecular factors underlying their vulnerability are not yet clear. Perturbation studies targeting genes within their age-associated programs may help pinpoint mechanisms that render these neurons particularly susceptible to age-related decline.

A key limitation of scRNA-seq is the loss of spatial context due to tissue dissociation. Consequently, we lack information about the *in vivo* cellular environments and intercellular signaling cues. Although computational tools exist to infer spatial origin²⁵⁰, they often rely on human reference datasets, which may not fully reflect primate-specific spatial organization. Integrating our data with macaque spatial transcriptomic datasets will offer valuable insights into the non-autonomous influences shaping the aging process in different brain cell types.

The majority of genetic variants associated with neurodegenerative disease risk reside in non-coding regions of the genome. By integrating chromatin accessibility data (e.g., from scATAC-seq), we can identify regulatory elements that control age-associated gene programs, revealing how genetic variation may contribute to pathological aging. Since age-associated genes are typically defined by their expression correlation with age or co-expression with other genes, adding regulatory information will help elucidate causal gene-regulatory relationships and potential mechanisms linking genetic risk to functional decline.

Notably, non-human primates do not naturally develop full-blown neurodegenerative disorders such as Alzheimer's disease. While aged macaques may exhibit amyloid- β (A β) deposition and occasional tau hyperphosphorylation, neurofibrillary tangles and extensive neuronal loss are rarely observed. Yet, our transcriptomic analyses show that aging macaques activate gene programs that closely resemble those found in human Alzheimer's disease. This convergence opens a unique window to investigate protective mechanisms—genetic or environmental—that may buffer macaques from the more severe neuro-pathological outcomes seen in humans.

In summary, our work establishes a foundational framework for studying brain aging in a non-human primate model. By constructing regional and cell types-resolved aging trajectories across the lifespan, we have set the stage for deeper mechanistic investigations into the key drivers of brain aging and resilience to neurodegeneration.

Domcke et al. (2022)²⁵¹ proposed a novel framework for defining cell types based on developmental lineage trees, capturing cell states through their molecular profiles and ancestry. Building on this concept, our work offers the opportunity to extend these developmental trees beyond embryogenesis, tracing the ordered progression of cell states into aging. By doing so, we can systematically examine whether aging represents a reversal of developmental programs, a reactivation of early-life pathways, or a distinct continuation of cellular transitions. Recent studies, such as Ling et al. (2024)⁷⁹, have shown that inter-individual variation in synaptic gene programs contributes to the selective vulnerability of schizophrenia to certain human populations. Mapping the continuum from development to aging may help reveal how such variation arises over a lifetime and provide insights into the molecular origins of resilience or susceptibility. Ultimately, a unified view of development and aging could uncover new opportunities to intervene in age-related decline by identifying points of divergence along an individual's cellular trajectory.

Bibliography

1. Kreider, J. J., Pen, I. & Kramer, B. H. Antagonistic pleiotropy and the evolution of extraordinary lifespans in eusocial organisms. *Evol Lett* **5**, 178–186 (2021).
2. Blagosklonny, M. V. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. *Cell Cycle* **5**, 2087–2102 (2006).
3. Dilman, V. M. Age-associated elevation of hypothalamic, threshold to feedback control, and its role in development, ageing, and disease. *Lancet* **1**, 1211–1219 (1971).
4. Langley-Evans, S. C. Developmental programming of health and disease. *Proc Nutr Soc* **65**, 97–105 (2006).
5. Silva-García, C. G. Devo-Aging: Intersections Between Development and Aging. *Geroscience* **45**, 2145–2159 (2023).
6. McKenna, A. *et al.* Whole-organism lineage tracing by combinatorial and cumulative genome editing. *Science* **353**, aaf7907 (2016).
7. McDole, K. *et al.* In Toto Imaging and Reconstruction of Post-Implantation Mouse Development at the Single-Cell Level. *Cell* **175**, 859–876.e33 (2018).
8. Trapnell, C. *et al.* The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol* **32**, 381–386 (2014).

9. Farrell, J. A. *et al.* Single-cell reconstruction of developmental trajectories during zebrafish embryogenesis. *Science* **360**, (2018).
10. Wagner, D. E. *et al.* Single-cell mapping of gene expression landscapes and lineage in the zebrafish embryo. *Science* **360**, 981–987 (2018).
11. Packer, J. S. *et al.* A lineage-resolved molecular atlas of embryogenesis at single-cell resolution. *Science* **365**, (2019).
12. Pijuan-Sala, B. *et al.* A single-cell molecular map of mouse gastrulation and early organogenesis. *Nature* **566**, 490–495 (2019).
13. Cao, J. *et al.* The single-cell transcriptional landscape of mammalian organogenesis. *Nature* **566**, 496–502 (2019).
14. Qiu, C. *et al.* Systematic reconstruction of cellular trajectories across mouse embryogenesis. *Nat Genet* **54**, 328–341 (2022).
15. Qiu, C. *et al.* A single-cell time-lapse of mouse prenatal development from gastrula to birth. *Nature* **626**, 1084–1093 (2024).
16. Tyser, R. C. V. *et al.* Single-cell transcriptomic characterization of a gastrulating human embryo. *Nature* **600**, 285–289 (2021).
17. Cao, J. *et al.* A human cell atlas of fetal gene expression. *Science* **370**, (2020).
18. Domcke, S. *et al.* A human cell atlas of fetal chromatin accessibility. *Science* (2020)
doi:10.1126/science.aba7612.
19. Xu, Y. *et al.* A single-cell transcriptome atlas profiles early organogenesis in human embryos. *Nat Cell Biol* **25**, 604–615 (2023).
20. Turner, D. A., Rué, P., Mackenzie, J. P., Davies, E. & Martinez Arias, A. Brachyury cooperates with Wnt/ β -catenin signalling to elicit primitive-streak-like behaviour in differentiating mouse embryonic stem cells. *BMC Biology* **12**, 1–19 (2014).
21. Turner, D. A., Trott, J., Hayward, P., Rué, P. & Martinez Arias, A. An interplay between extracellular signalling and the dynamics of the exit from pluripotency drives cell fate decisions in mouse ES cells. *Biol Open* **3**, 614–626 (2014).
22. Martin, G. R. & Evans, M. J. Differentiation of clonal lines of teratocarcinoma cells: formation of

embryoid bodies in vitro. *Proc Natl Acad Sci U S A* **72**, 1441–1445 (1975).

23. Brickman, J. M. & Serup, P. Properties of embryoid bodies. *Wiley Interdisciplinary Reviews: Developmental Biology* **6**, e259 (2017).
24. ten Berge, D. *et al.* Wnt signaling mediates self-organization and axis formation in embryoid bodies. *Cell Stem Cell* **3**, 508–518 (2008).
25. Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262–265 (2009).
26. Eiraku, M. *et al.* Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* **472**, 51–56 (2011).
27. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013).
28. Rivron, N. C. *et al.* Blastocyst-like structures generated solely from stem cells. *Nature* **557**, 106–111 (2018).
29. Amadei, G. *et al.* Inducible Stem-Cell-Derived Embryos Capture Mouse Morphogenetic Events In Vitro. *Dev Cell* **56**, 366–382.e9 (2021).
30. Sozen, B. *et al.* Self-assembly of embryonic and two extra-embryonic stem cell types into gastrulating embryo-like structures. *Nat Cell Biol* **20**, 979–989 (2018).
31. Marikawa, Y., Tamashiro, D. A. A., Fujita, T. C. & Alarcón, V. B. Aggregated P19 mouse embryonal carcinoma cells as a simple in vitro model to study the molecular regulations of mesoderm formation and axial elongation morphogenesis. *Genesis* **47**, 93–106 (2009).
32. Turner, D. A. *et al.* Anteroposterior polarity and elongation in the absence of extra-embryonic tissues and of spatially localised signalling in gastruloids: mammalian embryonic organoids. *Development* **144**, 3894–3906 (2017).
33. van den Brink, S. C. *et al.* Symmetry breaking, germ layer specification and axial organisation in aggregates of mouse embryonic stem cells. *Development* **141**, 4231–4242 (2014).
34. Girgin, M. U. *et al.* Bioengineered embryoids mimic post-implantation development in vitro. *Nat Commun* **12**, 5140 (2021).
35. Beccari, L. *et al.* Multi-axial self-organization properties of mouse embryonic stem cells into

- gastruloids. *Nature* **562**, 272–276 (2018).
36. Rossi, G. *et al.* Capturing Cardiogenesis in Gastruloids. *Cell Stem Cell* **28**, 230–240.e6 (2021).
 37. Veenvliet, J. V. *et al.* Mouse embryonic stem cells self-organize into trunk-like structures with neural tube and somites. *Science* **370**, (2020).
 38. Moris, N. *et al.* An in vitro model of early anteroposterior organization during human development. *Nature* **582**, 410–415 (2020).
 39. Sanaki-Matsumiya, M. *et al.* Periodic formation of epithelial somites from human pluripotent stem cells. *Nature Communications* **13**, 1–14 (2022).
 40. Yamanaka, Y. *et al.* Reconstituting human somitogenesis in vitro. *Nature* **614**, 509–520 (2023).
 41. Miao, Y. *et al.* Reconstruction and deconstruction of human somitogenesis in vitro. *Nature* **614**, 500–508 (2023).
 42. Olmsted, Z. T. & Paluh, J. L. Co-development of central and peripheral neurons with trunk mesendoderm in human elongating multi-lineage organized gastruloids. *Nature Communications* **12**, 1–19 (2021).
 43. Tarazi, S. *et al.* Post-gastrulation synthetic embryos generated ex utero from mouse naive ESCs. *Cell* **185**, 3290–3306.e25 (2022).
 44. Oldak, B. *et al.* Complete human day 14 post-implantation embryo models from naive ES cells. *Nature* **622**, 562–573 (2023).
 45. van den Brink, S. C. *et al.* Single-cell and spatial transcriptomics reveal somitogenesis in gastruloids. *Nature* **582**, 405–409 (2020).
 46. Alldritt, S. *et al.* Brain Charts for the Rhesus Macaque Lifespan. *bioRxiv* (2024)
doi:10.1101/2024.08.28.610193.
 47. Dash, S. *et al.* Brain volumetrics across the lifespan of the rhesus macaque. *Neurobiol Aging* **126**, 34–43 (2023).
 48. Kramer, J. H. *et al.* Longitudinal MRI and cognitive change in healthy elderly. *Neuropsychology* **21**, 412–418 (2007).
 49. Crivello, F., Tzourio-Mazoyer, N., Tzourio, C. & Mazoyer, B. Longitudinal assessment of global and regional rate of grey matter atrophy in 1,172 healthy older adults: modulation by sex and age. *PLoS One*

- 9**, e114478 (2014).
50. Salat, D. H. *et al.* Thinning of the cerebral cortex in aging. *Cereb Cortex* **14**, 721–730 (2004).
 51. Cardenas, V. A. *et al.* Brain atrophy associated with baseline and longitudinal measures of cognition. *Neurobiol Aging* **32**, 572–580 (2011).
 52. Mungas, D. *et al.* Longitudinal volumetric MRI change and rate of cognitive decline. *Neurology* **65**, 565–571 (2005).
 53. Shamy, J. L. T. *et al.* Hippocampal volume is preserved and fails to predict recognition memory impairment in aged rhesus monkeys (*Macaca mulatta*). *Neurobiol Aging* **27**, 1405–1415 (2006).
 54. Nobis, L. *et al.* Hippocampal volume across age: Nomograms derived from over 19,700 people in UK Biobank. *Neuroimage Clin* **23**, 101904 (2019).
 55. Dickstein, D. L. *et al.* Changes in the structural complexity of the aged brain. *Aging Cell* **6**, 275–284 (2007).
 56. Pannese, E. Quantitative, structural and molecular changes in neuroglia of aging mammals: A review. *Eur J Histochem* **65**, (2021).
 57. Haney, M. S. *et al.* APOE4/4 is linked to damaging lipid droplets in Alzheimer’s disease microglia. *Nature* **628**, 154–161 (2024).
 58. Kunkle, B. W. *et al.* Genetic meta-analysis of diagnosed Alzheimer’s disease identifies new risk loci and implicates A β , tau, immunity and lipid processing. *Nat Genet* **51**, 414–430 (2019).
 59. Huang, Y.-L. *et al.* Toll-like receptor agonists promote prolonged triglyceride storage in macrophages. *J Biol Chem* **289**, 3001–3012 (2014).
 60. Pal, S. & Tyler, J. K. Epigenetics and aging. *Sci Adv* **2**, e1600584 (2016).
 61. Wang, K. *et al.* Epigenetic regulation of aging: implications for interventions of aging and diseases. *Signal Transduction and Targeted Therapy* **7**, 1–22 (2022).
 62. Berdyshev, G. D., Korotaev, G. K., Boiarskikh, G. V. & Vaniushin, B. F. [Nucleotide composition of DNA and RNA from somatic tissues of humpback and its changes during spawning]. *Biokhimiia* **32**, 988–993 (1967).
 63. Ishimi, Y. *et al.* Changes in chromatin structure during aging of human skin fibroblasts. *Exp Cell Res* **169**, 458–467 (1987).

64. Greer, E. L. *et al.* Members of the H3K4 trimethylation complex regulate lifespan in a germline-dependent manner in *C. elegans*. *Nature* **466**, 383–387 (2010).
65. Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biology* **14**, 1–20 (2013).
66. Sen, P. *et al.* Histone Acetyltransferase p300 Induces De Novo Super-Enhancers to Drive Cellular Senescence. *Mol Cell* **73**, 684–698.e8 (2019).
67. Singh, P. & Thakur, M. K. Histone Deacetylase 2 Inhibition Attenuates Downregulation of Hippocampal Plasticity Gene Expression during Aging. *Mol Neurobiol* **55**, 2432–2442 (2018).
68. Marzi, S. J. *et al.* A histone acetylome-wide association study of Alzheimer's disease identifies disease-associated H3K27ac differences in the entorhinal cortex. *Nat Neurosci* **21**, 1618–1627 (2018).
69. Klein, H.-U. *et al.* Epigenome-wide study uncovers large-scale changes in histone acetylation driven by tau pathology in aging and Alzheimer's human brains. *Nat Neurosci* **22**, 37–46 (2019).
70. Ham, S. & Lee, S.-J. V. Advances in transcriptome analysis of human brain aging. *Experimental & Molecular Medicine* **52**, 1787–1797 (2020).
71. Lee, J. & Kim, H.-J. Normal Aging Induces Changes in the Brain and Neurodegeneration Progress: Review of the Structural, Biochemical, Metabolic, Cellular, and Molecular Changes. *Front. Aging Neurosci.* **14**, 931536 (2022).
72. Chiou, K. L. *et al.* Multiregion transcriptomic profiling of the primate brain reveals signatures of aging and the social environment. *Nat Neurosci* **25**, 1714–1723 (2022).
73. Li, M.-L. *et al.* 547 transcriptomes from 44 brain areas reveal features of the aging brain in non-human primates. *Genome Biol* **20**, 258 (2019).
74. Greene, A. N. *et al.* In silico gene expression and pathway analysis of DEK in the human brain across the lifespan. *Eur J Neurosci* **56**, 4720–4743 (2022).
75. Chien, J.-F. *et al.* Cell-type-specific effects of age and sex on human cortical neurons. *Neuron* **112**, 2524–2539.e5 (2024).
76. Jin, K. *et al.* Brain-wide cell-type-specific transcriptomic signatures of healthy ageing in mice. *Nature* **638**, 182–196 (2025).
77. Ximerakis, M. *et al.* Single-cell transcriptomic profiling of the aging mouse brain. *Nat Neurosci* **22**, 1696–1708 (2019).

78. Green, G. S. *et al.* Cellular communities reveal trajectories of brain ageing and Alzheimer's disease. *Nature* **633**, 634–645 (2024).
79. Ling, E. *et al.* A concerted neuron-astrocyte program declines in ageing and schizophrenia. *Nature* **627**, 604–611 (2024).
80. Hammond, T. R. *et al.* Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity* **50**, 253–271.e6 (2019).
81. Odawara, A., Katoh, H., Matsuda, N. & Suzuki, I. Physiological maturation and drug responses of human induced pluripotent stem cell-derived cortical neuronal networks in long-term culture. *Sci Rep* **6**, 26181 (2016).
82. Vera, E., Bosco, N. & Studer, L. Generating Late-Onset Human iPSC-Based Disease Models by Inducing Neuronal Age-Related Phenotypes through Telomerase Manipulation. *Cell Rep* **17**, 1184–1192 (2016).
83. Miller, J. D. *et al.* Human iPSC-based modeling of late-onset disease via progerin-induced aging. *Cell Stem Cell* **13**, 691–705 (2013).
84. Pitrez, P. R. *et al.* Cellular reprogramming as a tool to model human aging in a dish. *Nature Communications* **15**, 1–11 (2024).
85. Molecular and spatial signatures of mouse brain aging at single-cell resolution. *Cell* **186**, 194–208.e18 (2023).
86. Prater, K. E. *et al.* Human microglia show unique transcriptional changes in Alzheimer's disease. *Nature Aging* **3**, 894–907 (2023).
87. Roth, G. S. *et al.* Aging in rhesus monkeys: relevance to human health interventions. *Science* **305**, 1423–1426 (2004).
88. Kessler, M. J. & Rawlins, R. G. A 75-year pictorial history of the Cayo Santiago rhesus monkey colony. *American Journal of Primatology* **78**, 6–43 (2016).
89. Chiou, K. L. *et al.* Rhesus macaques as a tractable physiological model of human ageing. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **375**, (2020).
90. Van Dam, D. & De Deyn, P. P. Non human primate models for Alzheimer's disease-related research and drug discovery. *Expert Opin Drug Discov* **12**, 187–200 (2017).

91. Paspalas, C. D. *et al.* The aged rhesus macaque manifests Braak stage III/IV Alzheimer's-like pathology. *Alzheimers Dement* **14**, 680–691 (2018).
92. Peters, A. & Kemper, T. A review of the structural alterations in the cerebral hemispheres of the aging rhesus monkey. *Neurobiol Aging* **33**, 2357–2372 (2012).
93. Zhang, Z. *et al.* A panoramic view of cell population dynamics in mammalian aging. *Science* **387**, eadn3949 (2025).
94. Read, D. F. *et al.* Single-cell analysis of chromatin and expression reveals age- and sex-associated alterations in the human heart. *bioRxiv* (2022) doi:10.1101/2022.07.12.496461.
95. He, L. *et al.* NEBULA is a fast negative binomial mixed model for differential or co-expression analysis of large-scale multi-subject single-cell data. *Communications Biology* **4**, 1–17 (2021).
96. Deglincerti, A. *et al.* Self-organization of the in vitro attached human embryo. *Nature* **533**, 251–254 (2016).
97. Shahbazi, M. N. *et al.* Self-organization of the human embryo in the absence of maternal tissues. *Nat. Cell Biol.* **18**, 700–708 (2016).
98. Xiang, L. *et al.* A developmental landscape of 3D-cultured human pre-gastrulation embryos. *Nature* **577**, 537–542 (2019).
99. Yu, L. *et al.* Blastocyst-like structures generated from human pluripotent stem cells. *Nature* **591**, 620–626 (2021).
100. Zheng, Y. *et al.* Controlled modelling of human epiblast and amnion development using stem cells. *Nature* **573**, 421–425 (2019).
101. Weatherbee, B. A. T., Cui, T. & Zernicka-Goetz, M. Modeling human embryo development with embryonic and extra-embryonic stem cells. *Dev. Biol.* **474**, 91–99 (2021).
102. Shao, Y. *et al.* A pluripotent stem cell-based model for post-implantation human amniotic sac development. *Nat. Commun.* **8**, 1–15 (2017).
103. Xue, X. *et al.* Mechanics-guided embryonic patterning of neuroectoderm tissue from human pluripotent stem cells. *Nat. Mater.* **17**, 633–641 (2018).
104. Gribaudo, S. *et al.* Self-organizing models of human trunk organogenesis recapitulate spinal cord and spine co-morphogenesis. *Nat. Biotechnol.* (2023) doi:10.1038/s41587-023-01956-9.

105. Yaman, Y. I. & Ramanathan, S. Controlling human organoid symmetry breaking reveals signaling gradients drive segmentation clock waves. *Cell* **186**, 513–527.e19 (2023).
106. Faustino Martins, J.-M. *et al.* Self-Organizing 3D Human Trunk Neuromuscular Organoids. *Cell Stem Cell* **26**, 172–186.e6 (2020).
107. Junker, J. P. *et al.* Genome-wide RNA Tomography in the zebrafish embryo. *Cell* **159**, 662–675 (2014).
108. Wilson, V., Olivera-Martinez, I. & Storey, K. G. Stem cells, signals and vertebrate body axis extension. *Development* **136**, 1591–1604 (2009).
109. Kondoh, H. & Takemoto, T. Axial stem cells deriving both posterior neural and mesodermal tissues during gastrulation. *Curr. Opin. Genet. Dev.* **22**, 374–380 (2012).
110. Henrique, D., Abranches, E., Verrier, L. & Storey, K. G. Neuromesodermal progenitors and the making of the spinal cord. *Development* **142**, 2864–2875 (2015).
111. Kimelman, D. Tales of tails (and trunks): Forming the posterior body in vertebrate embryos. *Curr. Top. Dev. Biol.* **116**, 517–536 (2016).
112. Sambasivan, R. & Steventon, B. Neuromesodermal Progenitors: A Basis for Robust Axial Patterning in Development and Evolution. *Front Cell Dev Biol* **8**, 607516 (2020).
113. Blumberg, B. *et al.* An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124**, 373–379 (1997).
114. Janesick, A., Wu, S. C. & Blumberg, B. Retinoic acid signaling and neuronal differentiation. *Cell. Mol. Life Sci.* **72**, 1559–1576 (2015).
115. Durston, A. J. *et al.* Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340**, 140–144 (1989).
116. Jones-Villeneuve, E. M., McBurney, M. W., Rogers, K. A. & Kalnins, V. I. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94**, 253–262 (1982).
117. Okada, Y., Shimazaki, T., Sobue, G. & Okano, H. Retinoic-acid-concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells. *Dev. Biol.* **275**, 124–142 (2004).
118. Shibata, M. *et al.* Regulation of prefrontal patterning and connectivity by retinoic acid. *Nature* **598**, 483–488 (2021).

119. Gouti, M. *et al.* A Gene Regulatory Network Balances Neural and Mesoderm Specification during Vertebrate Trunk Development. *Dev. Cell* **41**, 243–261.e7 (2017).
120. Schubert, F. R. *et al.* Early mesodermal phenotypes in splotch suggest a role for Pax3 in the formation of epithelial somites. *Dev. Dyn.* **222**, 506–521 (2001).
121. Sadler, T. W., Greenberg, D., Coughlin, P. & Lessard, J. L. Actin distribution patterns in the mouse neural tube during neurulation. *Science* **215**, 172–174 (1982).
122. Escuin, S. *et al.* Rho-kinase-dependent actin turnover and actomyosin disassembly are necessary for mouse spinal neural tube closure. *J. Cell Sci.* **128**, 2468–2481 (2015).
123. Nelemans, B. K. A., Schmitz, M., Tahir, H., Merks, R. M. H. & Smit, T. H. Somite Division and New Boundary Formation by Mechanical Strain. *iScience* **23**, 100976 (2020).
124. Müller, F. & O’Rahilly, R. Somitic-vertebral correlation and vertebral levels in the human embryo. *Am. J. Anat.* **177**, 3–19 (1986).
125. Hubaud, A. & Pourquié, O. Signalling dynamics in vertebrate segmentation. *Nat. Rev. Mol. Cell Biol.* **15**, 709–721 (2014).
126. Martyn, I., Kanno, T. Y., Ruzo, A., Siggia, E. D. & Brivanlou, A. H. Self-organization of a human organizer by combined Wnt and Nodal signalling. *Nature* **558**, 132–135 (2018).
127. Libby, A. R. G. *et al.* Axial elongation of caudalized human organoids mimics aspects of neural tube development. *Development* **148**, (2021).
128. Achilleos, A. & Trainor, P. A. Neural crest stem cells: discovery, properties and potential for therapy. *Cell Res.* **22**, 288–304 (2012).
129. Zhai, J. *et al.* Primate gastrulation and early organogenesis at single-cell resolution. *Nature* **612**, 732–738 (2022).
130. Karzbrun, E. *et al.* Human neural tube morphogenesis in vitro by geometric constraints. *Nature* **599**, 268–272 (2021).
131. Haremake, T. *et al.* Self-organizing neuruloids model developmental aspects of Huntington’s disease in the ectodermal compartment. *Nat. Biotechnol.* **37**, 1198–1208 (2019).
132. De Santis, R., Etoc, F., Rosado-Olivieri, E. A. & Brivanlou, A. H. Self-organization of human dorsal-ventral forebrain structures by light induced SHH. *Nat. Commun.* **12**, 6768 (2021).

133. Sasai, Y. & De Robertis, E. M. Ectodermal patterning in vertebrate embryos. *Dev. Biol.* **182**, 5–20 (1997).
134. Chal, J. & Pourquié, O. Making muscle: skeletal myogenesis in vivo and in vitro. *Development* **144**, 2104–2122 (2017).
135. Murtaugh, L. C., Chyung, J. H. & Lassar, A. B. Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev.* **13**, 225–237 (1999).
136. Maroto, M., Bone, R. A. & Dale, J. K. Somitogenesis. *Development* **139**, 2453–2456 (2012).
137. Kojima, Y. *et al.* The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. *Cell Stem Cell* **14**, 107–120 (2014).
138. Stirparo, G. G. *et al.* Integrated analysis of single-cell embryo data yields a unified transcriptome signature for the human pre-implantation epiblast. *Development* **145**, (2018).
139. Srivatsan, S. R. *et al.* Massively multiplex chemical transcriptomics at single-cell resolution. *Science* **367**, 45–51 (2020).
140. Rosen, L. U. *et al.* Inter-gastruloid heterogeneity revealed by single cell transcriptomics time course: implications for organoid based perturbation studies. *bioRxiv* 2022.09.27.509783 (2022)
doi:10.1101/2022.09.27.509783.
141. Aulehla, A. & Pourquié, O. Signaling gradients during paraxial mesoderm development. *Cold Spring Harb. Perspect. Biol.* **2**, a000869 (2010).
142. Wahl, M. B., Deng, C., Lewandoski, M. & Pourquié, O. FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. *Development* **134**, 4033–4041 (2007).
143. Gridley, T. The long and short of it: somite formation in mice. *Dev. Dyn.* **235**, 2330–2336 (2006).
144. Takemoto, T., Uchikawa, M., Kamachi, Y. & Kondoh, H. Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1. *Development* **133**, 297–306 (2006).
145. Bond, A. M., Bhalala, O. G. & Kessler, J. A. The Dynamic Role of Bone Morphogenetic Proteins in Neural Stem Cell Fate and Maturation. *Dev. Neurobiol.* **72**, 1068 (2012).
146. Yamaguchi, T. P., Bradley, A., McMahon, A. P. & Jones, S. A Wnt5a pathway underlies outgrowth of

- multiple structures in the vertebrate embryo. *Development* **126**, 1211–1223 (1999).
147. Boylan, M., Anderson, M. J., Ornitz, D. M. & Lewandoski, M. The Fgf8 subfamily (Fgf8, Fgf17 and Fgf18) is required for closure of the embryonic ventral body wall. *Development* **147**, (2020).
 148. Nguyen, P. D. *et al.* Haematopoietic stem cell induction by somite-derived endothelial cells controlled by meox1. *Nature* **512**, 314–318 (2014).
 149. Kobayashi, I. *et al.* Jam1a-Jam2a interactions regulate haematopoietic stem cell fate through Notch signalling. *Nature* **512**, 319–323 (2014).
 150. Tani, S., Chung, U.-I., Ohba, S. & Hojo, H. Understanding paraxial mesoderm development and sclerotome specification for skeletal repair. *Exp. Mol. Med.* **52**, 1166–1177 (2020).
 151. Koushik, S. V., Chen, H., Wang, J. & Conway, S. J. Generation of a conditional loxP allele of the Pax3 transcription factor that enables selective deletion of the homeodomain. *Genesis* **32**, 114–117 (2002).
 152. Chapman, D. L. & Papaioannou, V. E. Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. *Nature* **391**, 695–697 (1998).
 153. Takemoto, T. *et al.* Tbx6-dependent Sox2 regulation determines neural or mesodermal fate in axial stem cells. *Nature* **470**, 394–398 (2011).
 154. Choi, J. *et al.* A time-resolved, multi-symbol molecular recorder via sequential genome editing. *Nature* **608**, 98–107 (2022).
 155. Tremblay, P., Kessel, M. & Gruss, P. A transgenic neuroanatomical marker identifies cranial neural crest deficiencies associated with the Pax3 mutant Splotch. *Dev. Biol.* **171**, 317–329 (1995).
 156. Relaix, F., Rocancourt, D., Mansouri, A. & Buckingham, M. Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev.* **18**, 1088–1105 (2004).
 157. Delile, J. *et al.* Single cell transcriptomics reveals spatial and temporal dynamics of gene expression in the developing mouse spinal cord. *Development* **146**, (2019).
 158. Misra, K. & Matise, M. P. A critical role for sFRP proteins in maintaining caudal neural tube closure in mice via inhibition of BMP signaling. *Dev. Biol.* **337**, 74–83 (2010).
 159. Suppinger, S. *et al.* Multimodal characterization of murine gastruloid development. *Cell Stem Cell* **30**, 867–884.e11 (2023).
 160. Loh, K. M. *et al.* Efficient endoderm induction from human pluripotent stem cells by logically directing

signals controlling lineage bifurcations. *Cell Stem Cell* **14**, 237–252 (2014).

161. Prummel, K. D. *et al.* A conserved regulatory program initiates lateral plate mesoderm emergence across chordates. *Nat. Commun.* **10**, 1–15 (2019).
162. Fleming, B. M., Yelin, R., James, R. G. & Schultheiss, T. M. A role for Vg1/Nodal signaling in specification of the intermediate mesoderm. *Development* **140**, 1819–1829 (2013).
163. Stuhlmiller, T. J. & García-Castro, M. I. Current perspectives of the signaling pathways directing neural crest induction. *Cell. Mol. Life Sci.* **69**, 3715–3737 (2012).
164. Wang, R. N. *et al.* Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes Dis* **1**, 87–105 (2014).
165. Veenvliet, J. V., Lenne, P.-F., Turner, D. A., Nachman, I. & Trivedi, V. Sculpting with stem cells: how models of embryo development take shape. *Development* **148**, (2021).
166. Lovell-Badge, R. *et al.* ISSCR Guidelines for Stem Cell Research and Clinical Translation: The 2021 update. *Stem Cell Reports* **16**, 1398–1408 (2021).
167. Watanabe, K. *et al.* A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat. Biotechnol.* **25**, 681–686 (2007).
168. Moris, N. *et al.* Generating human gastruloids from human Embryonic Stem Cells. *Research Square* (2020) doi:10.21203/rs.3.pex-812/v1.
169. Choi, H. M. T. *et al.* Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* **145**, (2018).
170. Zheng, G. X. Y. *et al.* Massively parallel digital transcriptional profiling of single cells. *Nat. Commun.* **8**, 14049 (2017).
171. Hao, Y. *et al.* Integrated analysis of multimodal single-cell data. *Cell* **184**, 3573–3587.e29 (2021).
172. Tirosh, I. *et al.* Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* **352**, 189–196 (2016).
173. Stuart, T. *et al.* Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888–1902.e21 (2019).
174. Pijuan-Sala, B. *et al.* Single-cell chromatin accessibility maps reveal regulatory programs driving early mouse organogenesis. *Nat. Cell Biol.* **22**, 487–497 (2020).
175. Santos, A., Wernersson, R. & Jensen, L. J. Cyclebase 3.0: a multi-organism database on cell-cycle

regulation and phenotypes. *Nucleic Acids Research* vol. 43 D1140–D1144 Preprint at <https://doi.org/10.1093/nar/gku1092> (2015).

176. Martin, B. K. *et al.* Optimized single-nucleus transcriptional profiling by combinatorial indexing. *Nat. Protoc.* **18**, 188–207 (2023).
177. Yang, J., Qu, J. & Ma, H. Recent developments in understanding brain aging: sex differences, mechanisms, and implications in diseases. *OAE Publishing Inc.* **2**, (2022).
178. Bianco, A., Antonacci, Y. & Liguori, M. Sex and Gender Differences in Neurodegenerative Diseases: Challenges for Therapeutic Opportunities. *International Journal of Molecular Sciences* **24**, 6354 (2023).
179. Jagust, W. & D’Esposito, M. *Imaging the Aging Brain*. (2009).
180. Schulz, M. *et al.* Association of Age and Structural Brain Changes With Functional Connectivity and Executive Function in a Middle-Aged to Older Population-Based Cohort. *Front. Aging Neurosci.* **14**, 782738 (2022).
181. Smith, S. M. *et al.* Brain aging comprises many modes of structural and functional change with distinct genetic and biophysical associations. (2020) doi:10.7554/eLife.52677.
182. Lu, T. *et al.* Gene regulation and DNA damage in the ageing human brain. *Nature* **429**, 883–891 (2004).
183. Mathys, H. *et al.* Single-cell multiregion dissection of Alzheimer’s disease. *Nature* **632**, 858–868 (2024).
184. Fröhlich, A. S. *et al.* Single-nucleus transcriptomic profiling of human orbitofrontal cortex reveals convergent effects of aging and psychiatric disease. *Nat. Neurosci.* **27**, 2021–2032 (2024).
185. Pandya, V. A. & Patani, R. Region-specific vulnerability in neurodegeneration: lessons from normal ageing. *Ageing Res. Rev.* **67**, 101311 (2021).
186. Fu, H., Hardy, J. & Duff, K. E. Selective vulnerability in neurodegenerative diseases. *Nat. Neurosci.* **21**, 1350–1358 (2018).
187. Ferretti, M. T., Dimech, A. S. & Chadha, A. S. *Sex and Gender Differences in Alzheimer’s Disease*. (Academic Press, 2021).
188. Dumitriu, D. *et al.* Selective changes in thin spine density and morphology in monkey prefrontal cortex correlate with aging-related cognitive impairment. *J. Neurosci.* **30**, 7507–7515 (2010).
189. Hernandez-Pacheco, R. *et al.* Managing the Cayo Santiago rhesus macaque population: The role of

- density. *Am. J. Primatol.* **78**, 167–181 (2016).
190. Thierry, B., Singh, M. & Kaumanns, W. *Macaque Societies: A Model for the Study of Social Organization*. (Cambridge University Press, 2004).
 191. Chiou, K. L. *et al.* A single-cell multi-omic atlas spanning the adult rhesus macaque brain. *Science Advances* (2023) doi:10.1126/sciadv.adh1914.
 192. Siletti, K. *et al.* Transcriptomic diversity of cell types across the adult human brain. *Science* **382**, eadd7046 (2023).
 193. Gabitto, M. I. *et al.* Integrated multimodal cell atlas of Alzheimer’s disease. *Nat Neurosci* (2024) doi:10.1038/s41593-024-01774-5.
 194. Yao, Z. *et al.* A high-resolution transcriptomic and spatial atlas of cell types in the whole mouse brain. *Nature* **624**, 317–332 (2023).
 195. GTEx Consortium. The GTEx Consortium atlas of genetic regulatory effects across human tissues. *Science* **369**, 1318–1330 (2020).
 196. MapMyCells. <https://portal.brain-map.org/atlasses-and-data/bkp/mapmycells>.
 197. Single-cell spatial transcriptome reveals cell-type organization in the macaque cortex. *Cell* **186**, 3726–3743.e24 (2023).
 198. Hao, S. *et al.* Cross-species single-cell spatial transcriptomic atlases of the cerebellar cortex. *Science* **385**, eado3927 (2024).
 199. Website.
 200. Nascimento, M. A. *et al.* Protracted neuronal recruitment in the temporal lobes of young children. *Nature* **626**, 1056–1065 (2024).
 201. Duran, M. *et al.* A statistical framework for inferring genetic requirements from embryo-scale single-cell sequencing experiments. *bioRxiv* 2025.04.03.646654 (2025) doi:10.1101/2025.04.03.646654.
 202. The effects of aging in the hippocampus and cognitive decline. *Neuroscience & Biobehavioral Reviews* **79**, 66–86 (2017).
 203. Sams, E. C. Oligodendrocytes in the aging brain. *Neuronal Signaling* **5**, (2021).
 204. Luebke, J., Barbas, H. & Peters, A. Effects of normal aging on prefrontal area 46 in the rhesus monkey. *Brain Res. Rev.* **62**, 212–232 (2010).

205. Urbut, S. M., Wang, G., Carbonetto, P. & Stephens, M. Flexible statistical methods for estimating and testing effects in genomic studies with multiple conditions. *Nat Genet* **51**, 187–195 (2019).
206. Urbut, S. M. *et al.* Bayesian multivariate genetic analysis improves translational insights. *iScience* **26**, 107854 (2023).
207. Sun, S. *et al.* Translational profiling identifies a cascade of damage initiated in motor neurons and spreading to glia in mutant SOD1-mediated ALS. *Proceedings of the National Academy of Sciences* **112**, E6993–E7002 (2015).
208. Mancuso, R. *et al.* Xenografted human microglia display diverse transcriptomic states in response to Alzheimer's disease-related amyloid- β pathology. *Nature Neuroscience* **27**, 886–900 (2024).
209. Mastenbroek, L. J. M., Kooistra, S. M., Eggen, B. J. L. & Prins, J. R. The role of microglia in early neurodevelopment and the effects of maternal immune activation. *Semin Immunopathol* **46**, 1 (2024).
210. Walker, L. C. & Jucker, M. The Exceptional Vulnerability of Humans to Alzheimer's Disease. *Trends Mol Med* **23**, 534–545 (2017).
211. Morelli, L. *et al.* Cerebrovascular amyloidosis in squirrel monkeys and rhesus monkeys: apolipoprotein E genotype. *FEBS Lett* **379**, 132–134 (1996).
212. Muzio, L., Viotti, A. & Martino, G. Microglia in Neuroinflammation and Neurodegeneration: From Understanding to Therapy. *Front. Neurosci.* **15**, 742065 (2021).
213. Yeewa, R. *et al.* The histone acylation reader ENL/AF9 regulates aging in *Drosophila melanogaster*. *Neurobiol Aging* **144**, 153–162 (2024).
214. Wood, S. H. *et al.* Transcriptome analysis in calorie-restricted rats implicates epigenetic and post-translational mechanisms in neuroprotection and aging. *Genome Biol* **16**, 285 (2015).
215. Zhang, H. *et al.* Single-nucleus transcriptomic landscape of primate hippocampal aging. *Protein Cell* **12**, 695–716 (2021).
216. Graham, L. C. *et al.* Regional Molecular Mapping of Primate Synapses during Normal Healthy Aging. *Cell Rep* **27**, 1018–1026.e4 (2019).
217. Dillman, A. A. *et al.* Transcriptomic profiling of the human brain reveals that altered synaptic gene expression is associated with chronological aging. *Sci Rep* **7**, 16890 (2017).
218. Peters, A., Sethares, C. & Luebke, J. I. SYNAPSES ARE LOST DURING AGING IN THE PRIMATE

PREFRONTAL CORTEX. *Neuroscience* **152**, 970 (2007).

219. Young, M. E., Ohm, D. T., Dumitriu, D., Rapp, P. R. & Morrison, J. H. Differential effects of aging on dendritic spines in visual cortex and prefrontal cortex of the rhesus monkey. *Neuroscience* **274**, 33 (2014).
220. Morrison, J. H. & Baxter, M. G. The Aging Cortical Synapse: Hallmarks and Implications for Cognitive Decline. *Nature reviews. Neuroscience* **13**, 240 (2012).
221. Knox, E. G., Aburto, M. R., Clarke, G., Cryan, J. F. & O'Driscoll, C. M. The blood-brain barrier in aging and neurodegeneration. *Molecular Psychiatry* **27**, 2659–2673 (2022).
222. Jaye, S., Sandau, U. S. & Saugstad, J. A. Clathrin mediated endocytosis in Alzheimer's disease: cell type specific involvement in amyloid beta pathology. *Front. Aging Neurosci.* **16**, 1378576 (2024).
223. Mattson, M. P. & Arumugam, T. V. Hallmarks of Brain Aging: Adaptive and Pathological Modification by Metabolic States. *Cell metabolism* **27**, 1176 (2018).
224. Eroglu, C. Astrocytes, hidden puppet masters of the brain. *Science* (2025).
225. Lefton, K. B. *et al.* Norepinephrine signals through astrocytes to modulate synapses. *Science* (2025).
226. Guttenplan, K. A. *et al.* GPCR signaling gates astrocyte responsiveness to neurotransmitters and control of neuronal activity. *Science* **388**, 763–768 (2025).
227. Baker, D. J. *et al.* Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* **530**, 184–189 (2016).
228. Xu, M. *et al.* Senolytics improve physical function and increase lifespan in old age. *Nat Med* **24**, 1246–1256 (2018).
229. Suda, M. *et al.* Senolytic vaccination improves normal and pathological age-related phenotypes and increases lifespan in progeroid mice. *Nat Aging* **1**, 1117–1126 (2021).
230. Grubman, A. *et al.* A single-cell atlas of entorhinal cortex from individuals with Alzheimer's disease reveals cell-type-specific gene expression regulation. *Nat Neurosci* **22**, 2087–2097 (2019).
231. Morabito, S. *et al.* Single-nucleus chromatin accessibility and transcriptomic characterization of Alzheimer's disease. *Nat Genet* **53**, 1143–1155 (2021).
232. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).
233. Warren, W. C. *et al.* Sequence diversity analyses of an improved rhesus macaque genome enhance its

- biomedical utility. *Science* **370**, (2020).
234. Virshup, I., Rybakov, S., Theis, F. J., Angerer, P. & Alexander Wolf, F. anndata: Annotated data. *bioRxiv* 2021.12.16.473007 (2021) doi:10.1101/2021.12.16.473007.
 235. Wolock, S. L., Lopez, R. & Klein, A. M. Scrublet: Computational Identification of Cell Doublets in Single-Cell Transcriptomic Data. *Cell Syst* **8**, 281–291.e9 (2019).
 236. Wolf, F. A., Angerer, P. & Theis, F. J. SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol* **19**, 15 (2018).
 237. Mages, S. *et al.* TACCO unifies annotation transfer and decomposition of cell identities for single-cell and spatial omics. *Nature Biotechnology* **41**, 1465–1473 (2023).
 238. Website. MapMyCells. <https://portal.brain-map.org/atlas-and-data/bkp/mapmycells>.
 239. Subramanian, A. *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences* **102**, 15545–15550 (2005).
 240. Liberzon, A. *et al.* The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell systems* **1**, 417 (2015).
 241. Van de Sande, B. *et al.* A scalable SCENIC workflow for single-cell gene regulatory network analysis. *Nature Protocols* **15**, 2247–2276 (2020).
 242. Wolf, F. A. *et al.* PAGA: graph abstraction reconciles clustering with trajectory inference through a topology preserving map of single cells. *Genome Biology* **20**, 1–9 (2019).
 243. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. (2012) doi:10.1089/omi.2011.0118.
 244. SynGO: An Evidence-Based, Expert-Curated Knowledge Base for the Synapse. *Neuron* **103**, 217–234.e4 (2019).
 245. Kanehisa, M., Furumichi, M., Sato, Y., Matsuura, Y. & Ishiguro-Watanabe, M. KEGG: biological systems database as a model of the real world. *Nucleic Acids Res* **53**, D672–D677 (2024).
 246. Regier, M. C. *et al.* User-defined morphogen patterning for directing human cell fate stratification. *Scientific Reports* **9**, 1–12 (2019).
 247. Zhang, R. *et al.* Cross-species imputation and comparison of single-cell transcriptomic profiles. *Genome*

Biology **26**, 1–22 (2025).

248. Li, G. *et al.* Pseudotime analysis for time-series single-cell sequencing and imaging data. *bioRxiv* 2023.11.03.565575 (2023) doi:10.1101/2023.11.03.565575.

249. Simmons, H. A. Age-Associated Pathology in Rhesus Macaques (*Macaca mulatta*). *Veterinary pathology* **53**, 399 (2016).

250. Jin, S. *et al.* Inference and analysis of cell-cell communication using CellChat. *Nature Communications* **12**, 1–20 (2021).

251. A reference cell tree will serve science better than a reference cell atlas. *Cell* **186**, 1103–1114 (2023).