1 Single-cell and spatial transcriptomics reveal somitogenesis in gastruloids
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18 **Gastruloids are three-dimensional aggregates of embryonic stem cells (ESCs) that display key**
19 **features of mammalian post-implantation development, including germ layer specification and**
20 **axial organization[1–3]. So far, the expression pattern of only a small number of genes in gastruloids**
21 **has been explored with microscopy, but it is still unclear to what extent genome-wide expression**
22 **patterns mimic those in embryos. Here, we compared mouse gastruloids with mouse embryos**
23 **using single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics (tomo-seq). We**
24 **identify various embryonic cell types that were not known to be present in gastruloids, and show**
25 **that key regulators of somitogenesis are expressed similarly between embryos and gastruloids.**
26 **Using live-imaging we then show that the somitogenesis clock is active in gastruloids with**
27 **dynamics resembling those *in vivo*. Since gastruloids can be grown in large quantities, we**
28 **perform a small screen that revealed how reduced FGF signalling induces a short-tail phenotype**
29 **in embryos. Finally, we demonstrate that Matrigel-embedding induces gastruloids to generate**
30 **somites with correct rostral-caudal patterning, which appear sequentially in anterior to posterior**
31 **direction over time. This study thus shows the power of gastruloids as a model system to explore**
32 **development and somitogenesis *in vitro* in a high-throughput manner.**

33   It has previously been shown that transcriptomes of entire gastruloids at 120 hours after
34 aggregation (120 h) resembles that of E8.5 mouse embryos[3]. To extend this characterization to the
35 single-cell level, we applied scRNA-seq to more than 25,000 cells obtained from 100 gastruloids (120 h)
36 that were generated using either E14-IB10 or LfngT2AVenus mouse ESCs (Extended Data Fig. 1a-b,
37 Methods), and clustered cells based on highly variable genes (Fig. 1a, Extended Data Fig. 1c-f,
38 Supplementary Tables 1-2). To annotate the 13 resulting clusters, we compared their transcriptomes to a
39 recently published scRNA-seq dataset from E8.5 mouse embryos[4] (Fig. 1b, Methods, Supplementary
40 Table 3). We confirmed the absence of anterior neuronal cell types and the presence of ectodermal cells

41   resembling embryonic spinal cord[1,3] (cluster 8; Extended Data Fig. 1g-h and 2). Additionally, we for the

42   first time identified endothelial and haematoendothelial cells (cluster 10), and found a cluster with

43   signatures of primordial germ cells and extra-embryonic ectoderm (cluster 12). Cluster 13 correlates with

44   the visceral endoderm (VE); however, we suggest that this represents definitive endoderm (DE) since

45   previous studies showed that VE has been incorporated into DE in E8.5 mouse embryos[5,6]. We find the

46   olfactory receptor genes *Olfr959* and *Olfr129* upregulated in cluster 9, suggesting the presence of

47   sensory neuron precursors. This cluster also expresses markers linked to head mesenchyme, pharyngeal

48   pouches, branchial arches and neural crest and correlates with mesenchyme in embryos. Cluster 11

49   might represent allantoic cells, as it expresses *Tbx4*, which in E8.5 embryos is expressed exclusively in

50   the allantois[4,7]. A comparison between both mouse ESC lines revealed that some cell types are more

51   prevalent in one of the two lines (Extended Data Fig. 1e, Supplementary Tables 1,4), indicating that

52   genetic background can skew the composition of gastruloids.

53   Many of the cells in gastruloids correspond to mesodermal subtypes, including neuro-

54   mesodermal progenitors (NMPs), caudal, paraxial, somatic, pharyngeal and cardiac mesoderm (clusters

55   1-7; Fig. 1b). After careful examination, we concluded that the cells in clusters 1-8 are ordered along

56   neural and mesodermal differentiation trajectories. To further explore this, we linearized the part of the

57   UMAP containing clusters 1-8 (Methods) and plotted the expression of genes linked to neural and

58   mesodermal differentiation processes along this linearized UMAP (Fig. 1c). First, we observed an NMP to

59   neural differentiation trajectory from cluster 7 to 8 that starts with the expression of the tail bud genes *T*

60   (*Brachyury*), *Nkx1-2*, *Cyp26a1* and that is followed by the expression of neural differentiation markers

61   such as *Sox2*, *Hes3*, *Sox1* and *Pax6*[8]. Second, we observed a mesodermal differentiation trajectory from

62   cluster 6 to 2. In good agreement with what happens in embryos, the expression levels of tail bud and

63   Wnt/FGF signalling genes (*Fgf8*, *Fgf17* and *Wnt3a*) gradually decline in cells that differentiate towards a

64   pre-somitic fate (characterized by the expression of *Tbx6* and *Hes7*[9]), with expression levels being lower

65   in the somite differentiation front (which expresses *Ripply2*). Upon somitic differentiation, cells first

66   express *Uncx4.1* and *Tbx18*, and later express markers more differentiated somites, such as *Meox2* and

67   *Pax3*[9]. Finally, cluster 1 expresses heart markers (*Gata6* and *Hand2*[10]).

68   In embryos, neural and mesodermal differentiation trajectories have a strong spatial component,

69   with NMPs being located within the tail bud and differentiated tissues being located more anteriorly[8]. To

70   determine whether the differentiation trajectories detected in gastruloids also have a spatial anterior-

71   posterior (AP) component, we performed tomo-seq[11] on 120 hours E14-IB10 and LfngT2AVenus

72   gastruloids (Methods, Extended Data Fig. 3-5). For each cell line, we selected reproducible genes

73   between replicates, and clustered these according to their AP expression pattern (Methods,

74   Supplementary Tables 5-6). The overall gene expression patterns between gastruloids generated from

75   the two ESC lines are similar (Fig. 1d, Extended Data Fig. 6, Supplementary Tables 6-8). To annotate the

76   various expression domains, we projected the mean expression of the genes in each tomo-seq cluster

77   onto the UMAP (Fig. 1e). This revealed that NMPs (cluster 7 in Fig. 1a and cluster II in Fig. 1e) are

78 located in the most posterior tip of gastruloids. More differentiated neural cells are found slightly more

79 anterior (Extended Data Fig. 3e). Furthermore, mesodermal clusters in the UMAP are sequentially

80 ordered along the AP axis of gastruloids, with 6 being the most posterior and 2 the most anterior (cluster

81 V-VIII in Fig. 1d-e; see also Extended Data Fig. 3e). This revealed that the neural and mesodermal

82 differentiation trajectories in gastruloids are linked to their AP axis, which agrees with what occurs in

83 embryos[8,9]. Additionally, we found that the anterior domain in gastruloids (clusters VI-VIII) contains

84 cardiac, endothelial and head mesenchymal cells (Fig. 3d-e, Extended Data Fig. 3e). This is consistent

85 with the locations of these tissues in embryos.

86 To further investigate to what extent AP gene expression patterns in gastruloids recapitulate

87 those in embryos, we applied tomo-seq to E8.5 embryos (Fig. 1f, Extended Data Figs. 3-6,

88 Supplementary Tables 5-8 and Methods). This revealed that mesoderm genes and genes that regulate

89 somitogenesis, are expressed very similarly between embryos and gastruloids. We detected cardiac and

90 brain domains in embryos (cluster VII and I in Extended Data Figure 5b, respectively) that are not clearly

91 defined and absent, respectively, in gastruloids. We found additional differences and similarities between

92 embryos and gastruloids that are presented in detail in the supplement (Extended Data Fig. 5 and

93 Supplementary Tables 7-8; for visualization, see https://avolab.shinyapps.io/962095337353856/). We

94 also compared our gastruloid tomo-seq dataset to a previously published microarray dataset where the

95 posterior mesoderm (from the tail bud to the newly formed somite) of E9.5 mouse embryos was

96 dissected[12] (Fig. 1g, Extended Data Figs. 4-5 and Supplementary Tables 5-8). This comparison reveals a

97 striking similarity between gastruloids and the mesoderm of embryos.

98 In embryos, the organization of the mesoderm is established by dynamic gene regulatory

99 networks that are tightly linked to the process of somitogenesis[9]. During somitogenesis, AP retinoic acid

100 and opposing Wnt/FGF signalling gradients determine the position of the differentiation front, which

101 induces the differentiation of the mesoderm into epithelial blocks called somites (Fig. 2a). These somites

102 have defined rostral and caudal halves, and appear sequentially in AP direction. During this process, the

103 tail bud of the embryo grows, and consequently, the signalling gradients and differentiation front move

104 posteriorly over time. A second component of somitogenesis entails oscillations of Wnt, Notch and FGF

105 signalling, where signalling waves travel from the tail bud towards the differentiation front every ~2 hours

106 in mice[9,13]. This cyclic component of the somitogenesis process is known as the "segmentation clock" and

107 is thought to regulate the timing of somite formation[9,14]. To investigate whether the segmentation clock is

108 active in gastruloids, we monitored Notch signalling activity by performing fluorescence time-lapse

109 imaging on gastruloids generated from LnfgT2AVenus mouse ESCs[15] (Methods). Similar to what has

110 been seen in embryos[15], we observed a dynamic differentiation front, which expresses high levels of *Lfng*

111 and regresses posteriorly as the gastruloids extend (Fig. 2b, Extended Data Fig. 7-8, Supplementary

112 Video 1). Additionally, we observed oscillating waves with low expression of *Lfng* and a period of about 2

113 hours that travel from the tip of the tailbud towards the differentiation front, where they stall (Fig. 2c-e).

114 The expression of *Lfng* disappears in the presence of the Notch inhibitor DAPT (Extended Data Fig. 7,

115   Supplementary Video 2), confirming that the reporter expression is dependent on Notch signalling in

116   gastruloids, as it is in embryos[16]. These experiments indicate that the segmentation clock is active in

117   gastruloids with dynamics that are very similar to the *in vivo* situation.

118   Gastruloids can be easily generated in large numbers, opening the possibility to perform screens.

119   To exemplify this, we performed a small compound screen on LfngT2AVenus gastruloids and

120   investigated the effect of inhibitors and agonists of FGF, Wnt, and BMP signalling pathways on the speed

121   of the differentiation front (Supplementary Videos 3, Extended Data Figs. 7 and 8e-f). This revealed that

122   the application of the MEK/ERK pathway inhibitor PD03, which inhibits FGF signalling, speeds up the

123   differentiation front in a dose-dependent manner without altering the speed by which gastruloids grow

124   posteriorly (Fig. 2f, Extended Data Fig. 9a, Supplementary Video 4). This imbalance between the speed

125   of the differentiation front and gastruloid growth results in a progressive decrease in the length of the

126   presomitic mesoderm, and in gastruloids that stop growing prematurely (Fig. 2g). Similar results were

127   obtained with the FGF receptor inhibitors PD17 and BGJ398 (Extended Data Figs. 7 and 8f,

128   Supplementary Video 5). Our observations provide an explanation for the observed short-tail phenotype

129   of FGF-mutant mouse embryos[17] and posteriorly shifted differentiation fronts after FGF inhibition[18,19].

130   Even though our experiments reveal that key regulators of somitogenesis are expressed in the

131   correct location and that the segmentation clock is active in gastruloids, gastruloids that are generated

132   with previously published protocols do not form somites[1,3,20]. Remarkably, during our real-time imaging

133   experiments, we occasionally observed small "indentations" that appeared anteriorly to the differentiation

134   front (Supplementary Video 4). These segments were only visible in gastruloids mounted in Matrigel at 96

135   h, which was done prior to the real-time imaging experiments to stabilize them (Methods). We then

136   performed *in situ* hybridization (ISH) stainings for *Uncx4.1* (a marker for the caudal halves of somites[9];

137   Fig. 2a) and found that *Uncx4.1* was expressed in a stripy pattern in 4% (4 out of 100) of the 120 h

138   gastruloids that were embedded in 100% Matrigel at 96 h (Fig. 3a). Such a pattern was never detected in

139   120 h gastruloids cultured without Matrigel. To explore the effect of the concentration of Matrigel, we

140   performed a titration experiment. We found that embedding 96 h gastruloids in 10-25% Matrigel resulted

141   in the formation of clear segments of which the posterior half is marked by *Uncx4.1* expression in up to

142   50% of the gastruloids (ISH and hybridization chain reaction (HCR[21]) stainings; Fig. 3a-b, Extended Data

143   Fig. 9b). Time-lapse imaging movies on these gastruloids revealed that the segments appear sequentially

144   in AP direction, anteriorly to the *Lfng* expression domain (Fig. 3c, Supplementary Video 6 and Extended

145   Data Fig. 9c). Lastly, double stainings for *Uncx4.1* and *Ripply2* (which is expressed in the newly forming

146   somite) and for *Uncx4.1* and *Tbx18* (a marker of rostral somites[9]) revealed that *Uncx4.1* and *Tbx18* are

147   expressed in an alternating pattern (Fig. 3d), and it is indeed the caudal half of the segments that

148   expresses *Uncx4.1* (Extended Data Fig. 10). At 120 h of culture (after 24 h in 10% Matrigel), gastruloids

149   have ~10-11 somites (Fig. 3d, Extended Data Fig. 10), whose size decreases in the AP direction, from on

150   average 183 to 43.4 µm (Extended Data Fig. 10c-e). In embryos, the size of these somites decreases

151   from 120 to 80 µm (Methods). Our experiments thus reveal that embedding gastruloids in low-percentage

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152 Matrigel induces the formation of somites, which have correct rostral-caudal patterning and appear
153 sequentially along the AP direction over time. We have so far not observed gastruloids with two
154 neighbouring rows of somites, and it will be interesting to explore why this is the case in future studies.

155   Using single-cell and spatial transcriptomics we demonstrate that gene expression in murine
156 gastruloids is very similar to embryos. Gastruloids can therefore be used as a model system for
157 embryology, and have some key advantages over embryos: they can be grown in large quantities
158 allowing screens, are easier to genetically modify as they can be grown directly from ESCs, and can be
159 used to study human development (see accompanying manuscript[22]). We utilized several of these
160 advantages to study somitogenesis *in vitro*. Recent pioneering studies have explored *ex vivo* and *in vitro*
161 models for somitogenesis, such as monolayer-PSM cultures[23,16] and cultures of embryoid body-like
162 aggregates of mouse ESCs that display travelling somitogenesis waves *in vitro*[24]. However, such cultures
163 do not form proper somites, lack a correctly defined AP axis and do not elongate in posterior direction.
164 Here, we have shown that gastruloids overcome these limitations, and thus provide a powerful tool to
165 study somitogenesis *in vitro.* In general, *in vitro* mimics of development, such as gastruloids, are
166 promising systems with which we are starting to obtain new insights that could not readily be obtained
167 with embryos. We therefore anticipate many applications of this system, which will aid to unravel the
168 complex processes that regulate embryogenesis.
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171 **Extended data**

172 Ten Extended Data Figures, nine Supplementary Tables and six Supplementary Videos are available for

173 this publication.

174

Author contributions

S.C.v.d.B. and A.v.O. conceived and designed the project. S.C.v.d.B. and V.v.B. generated gastruloids, and S.C.v.d.B., M.B. and J.V. performed scRNA-seq experiments. Embedding of mouse gastruloids for tomo-seq was done by S.C.v.d.B.; N.M. and P.B.J. embedded mouse embryos for tomo-seq with help from J.N.. S.C.v.d.B. cryosectioned gastruloids and embryos and performed tomo-seq experiments, and J.V. developed the robotized tomo-seq protocol. A.A. performed the mapping and analysis, including comparisons with embryonic datasets, of the scRNA-seq and tomo-seq data. A.v.O. performed the linearized UMAP analysis. S.C.v.d.B., M.B., A.A., N.M. and A.M.A. interpreted the sequencing datasets. P.B.J. performed the first Matrigel-embedding pilot experiments. V.v.B. performed time-lapse imaging experiments, ISH and HCR stainings, with help from S.C.v.d.B. and K.F.S.. V.v.B. analysed the microscopy data, with support from K.F.S., and V.v.B., S.c.v.d.B., A.v.O. and K.F.S. interpreted the imaging results. S.C.v.d.B., A.A., V.v.B. and A.v.O. wrote the manuscript with support from K.F.S. and A.M.A., and A.M.A. and A.v.O. guided the project.

212 **Author information**

213 Susanne C. van den Brink, Anna Alemany and Vincent van Batenburg contributed equally to this work.

214

215 **Data availability**

216 All RNA-seq datasets produced in this study are deposited in the Gene Expression Omnibus (GEO)

217 under accession code GSE123187. All the scripts used to analyse the data are freely available upon

218 request. All scRNA-seq and tomo-seq data can be explored at

219 https://avolab.shinyapps.io/962095337353856/.

220

221 **Competing interests**

222 There are no competing interests for this work.

223

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227

228 **Figure legends**

229 **Fig. 1 | scRNA-seq and tomo-seq on mouse gastruloids and comparison to embryos. a,** Uniform

230 manifold approximation and projection (UMAP) plot showing cells isolated from 120 h gastruloids (26 and

231 74 gastruloids grown using E14-IB10 and LfngT2AVenus[15] ESC lines, respectively) cultured in

232 standard[1,20] conditions. Cells are coloured and numbered by their cluster annotation. **b,** Dot plot showing

233 overlapping genes between significantly upregulated genes for each gastruloid cluster and each E8.5

234 mouse embryonic cell type[4]. Dot colour indicates the probability of finding such a number of overlapping

235 genes between the two sets by random chance (*P*-value). Dot size represents the number of overlapping

236 genes. **c,** Linearized UMAP of clusters 1-8 (top) and expression profiles of genes related to neural and

237 mesodermal differentiation[8,9] (bottom). Green and grey shades indicate location of cardiac cells and

238 NMPs, respectively. The position of each cell along the x-axis relates to its differentiated state towards a

239 neural or mesodermal fate. **d,** Heatmap showing the average AP expression pattern of 514 genes

240 detected by tomo-seq[11] in 120 h gastruloids generated from E14-IB10 and LfngT2AVenus[15] mouse ESCs

241 using standard[1,20] culture protocols. Only genes reproducible between all replicates of E14-IB10 (n = 5)

242 and LfngT2AVenus (n = 3) gastruloids are shown. Genes are clustered based on AP expression pattern

243 (Supplementary Tables 5-6); Roman-numbered bars represent tomo-seq clusters. **e,** Mean log expression

244 of genes present in each tomo-seq cluster plotted on the UMAP. **f, g,** As in d, but showing 222 genes (f)

245 or 239 genes (g) found reproducible between replicates of E14-IB10 and LfngT2AVenus gastruloids, and

246 (f) E8.5 mouse embryos (n = 3); or (g) posterior mesoderm of E9.5 mouse embryos[12] MD, mesoderm;

247 ExE, extra-embryonic; EcD, ectoderm; EnD, endoderm; PGC, primordial germ cells; prog, progenitors;

248 Haemato, haemato-endothelial; NMP, neuro-mesodermal progenitors; PSM, presomitic mesoderm; E14,

249 E14-IB10; Lfng, LfngT2AVenus.

250

251 **Fig. 2 | Real-time imaging and perturbation of the segmentation clock in mouse gastruloids. a,**

252 Illustration of somitogenesis in mouse embryos. Dark blue, retinoic acid (RA) gradient; red area and

253 arrows, dynamic expression of *Lfng*; green, FGF/Wnt signalling gradient in PSM (presomitic mesoderm);

254 magenta/cyan blocks, somites; blocks with dotted lines, newly forming somites; posterior dotted line,

255 posterior elongation of the PSM. **b**, Real-time imaging of a LfngT2AVenus[15] gastruloid embedded in

256 100% Matrigel at 96 h and subsequently imaged for 17 hours (Supplementary Video 1). Blue arrowheads

257 show the AP displacement of the differentiation front (*Lfng* expressing; red). **c,** Kymograph along the AP

258 axis of a LfngT2AVenus gastruloid embedded in 100% Matrigel at 96 h and subsequently imaged for 30

259 h. Highest intensity signal reflects the posteriorly moving differentiation front (blue arrowhead in b); white

260 arrowheads indicate periodic oscillations in the PSM. **d,** Detrended LfngT2AVenus intensity along the

261 dashed white line in d. A.U., arbitrary units. **e,** Periodogram of the *Lfng* oscillations detected in 13

262 LfngT2AVenus gastruloids, as determined by Lomb-Scargle decomposition (Methods). **f,** Speed of

263 elongation and differentiation front in LfngT2AVenus gastruloids treated with PD03. Box plots: center line,

264 median; box limits, 1st and 3rd quartiles; whiskers, range. Each point is one replicate. **g,** Illustration

265 explaining the effect of FGF inhibition, which increases the speed of the differentiation front (red arrows,
266 V_{Diff}) without altering the elongation rate (blue arrows, V_{PSM}) of gastruloids. Three timepoints (t_1, t_2, t_3) are
267 depicted. White tissue, non-differentiated tissue (PSM), grey tissue, differentiated tissue; A, anterior; P,
268 posterior; scale bar, 200 µm.

269

270 **Fig. 3 | Stainings and real-time imaging of somite formation in gastruloids embedded in low**
271 **percentages of Matrigel. a,** ISH staining for *Uncx4.1* on 120 h LfngT2AVenus gastruloids that were not
272 embedded in Matrigel (0%; standard, previously published protocol[1,20]) or that were embedded in 25% or
273 100% Matrigel at 96 h. Numbers below panels indicate number of gastruloids where stripy *Uncx4.1*
274 expression patterns were observed. **b,** Somites in a LfngT2AVenus gastruloid (zoomed in; Extended Data
275 Fig. 9b) embedded in 10% Matrigel at 96 h and stained for *Uncx4.1* using HCR[21] at 120 h. Magenta
276 arrowheads, segment boundaries. **c,** Real-time imaging (Supplementary Video 6) of LfngT2AVenus
277 gastruloids embedded in 10% Matrigel at 96 h. Blue arrowheads, differentiation front (*Lfng* expressing,
278 red); magenta arrowheads, appearing segment boundaries. **d,** HCR[21] double staining for *Uncx4.1* (cyan)
279 and *Tbx18* (magenta) (Fig. 2a)[9], on a 120 h LfngT2AVenus gastruloid embedded in 10% Matrigel at 96 h
280 and to which 1.3 µM of PD03 was added at 96.5 h. White asterisks mark *Uncx4.1* expression stripes. A,
281 Anterior; P, Posterior; scale bar in panels a and d: 200 µm; scale bar in panels b and c, 100 µm.

282

283 **Methods**
284 **Mouse gastruloid culture, with and without Matrigel.** E14-IB10 (subclone of 129/Ola-derived E14 ES
285 cells from The Netherlands Cancer Institute), LfngT2AVenus[15] (Notch-signalling reporter; contains a
286 single copy of Venus that was inserted in the endogenous *Lfng* locus[15]; the selection cassette was
287 removed), *Brachyury*[GFP(25)], Wnt/β-catenin transcriptional reporter *TCF/LEF*[mCherry(26,27)] and *Nodal*[YFP(28)]
288 mouse ESCs were maintained in standard conditions in serum + LIF (ESLIF medium) on gelatinized 6-
289 well plates and in a humidified incubator (5% CO_2, 37 °C) as described before[20,26,29–32]. Gastruloids for
290 scRNA-seq and tomo-seq experiments were generated as described previously[1,20], with the following
291 minor modifications: after neutralization of trypsin with ESLIF, cells were washed with PBS (containing
292 Ca2+ and Mg2+) twice. Next, cells were resuspended in N2B27 medium (NDiff 227 medium, Takara,
293 Y40002), and the cell concentration was determined only after resuspension in N2B27 medium. Cells
294 were then diluted in N2B27 to a concentration of 7.5 cells/µl, and 40 µl (with ~300 cells) of this
295 suspension was transferred to each well of a U-bottomed 96-well plate (Greiner Bio-One, 650185).
296 N2B27 aliquots were stored at -20 °C and thawed by rocking them at 4 °C for several hours, after which
297 aliquots were transferred to a cell culture flask in a CO_2-controlled 37 °C incubator for pH-equilibration
298 one day before gastruloid formation. Aggregates that did not elongate and that did not form gastruloids
299 were excluded from this study, and curved gastruloids were excluded from tomo-seq experiments. For the
300 scRNA-seq and tomo-seq experiments, 120 h gastruloids generated with the original gastruloids
301 protocol[1,20] were used, as these gastruloids were in our hands more reproducible (significantly less

302   variation in morphology between wells) than more recent versions of the protocol, that allow culture up to
303   168 h[3]. For ISH and HCR staining and real-time imaging experiments, gastruloids were cultured as
304   described above, but then embedded in Matrigel at 96 h. To embed gastruloids in 50-100% Matrigel
305   (Corning, 356231, lot number 6137007, protein concentration 9.8 mg/mL), Matrigel was thawed on ice,
306   mixed with the required amount of cold N2B27 medium, and 60 µl was added to each well of a multi-well
307   imaging chamber (Sigma, EP0030741021 or M9312) on ice. 96 h gastruloids were then transferred to the
308   Matrigel using a 20 µl pipet and allowed to settle for approximately 5 min before the chamber was
309   incubated at 37 °C for 10 min, allowing the Matrigel to solidify. After this, 500 µL N2B27 medium was
310   added to each well. Embedding gastruloids in diluted 10-25% Matrigel was done by first pooling the
311   gastruloids in a 5 mL low binding Eppendorf tube on ice, replacing the N2B27 medium with fresh cold
312   medium and then adding the correct volume of Matrigel. The gastruloids were then transferred to a 24
313   wells plate (Sigma, EP0030741021 or M9312) using a p1000 pipet with the tip cut off, at a concentration
314   of ~8 gastruloids per mL, 500 µL per well.

315   **Dissociation and FACS of gastruloids prior to scRNA-seq.** To dissociate gastruloids for scRNA-seq,
316   gastruloids were washed with PBS 2x, incubated in Trypsin-EDTA at 37 °C for 5 min and titrated with a
317   p200 pipette, after which ESLIF (see above) was added to neutralize the Trypsin. After centrifugation
318   (170g, 3 min), cells were resuspended in PBS with 10% serum and filtered through a 35 µm filter (Falcon,
319   352235). Prior to FACS, DAPI (Thermo Fisher) was added to assess cell viability. For SORT-seq,
320   individual live cells were sorted into the wells of a 384-well plate as described previously[33] using a BD
321   FACSJazz[TM] Cell Sorter (BD Biosciences) that was equipped with BD FACS software (version 1.2.0.124).
322   For 10x Genomics scRNA-seq, washes were done using PBS0 (PBS without calcium and magnesium),
323   and 100,000 live cells were sorted into 1.5 ml DNA lowbind tubes (Eppendorf, 022431021) that were pre-
324   filled with 50 µl PBS0, after which cells were centrifuged for 3 min at 200g, resuspended in 80 µl PBS0
325   containing 5-10% serum, and filtered through a 35 µm filter (Falcon, 352235). After resuspension and
326   filtering, the cell concentration was determined using a counting chamber (Bürker-Türk, Marienfeld).

327   **scRNA-seq (SORT-seq and 10x Genomics).** For scRNA-seq, cells extracted from 120 h gastruloids
328   (120 h; generated with a previously published, non-Matrigel based protocol[1,20]) were processed using
329   either SORT-seq (CEL-seq2 based scRNA-seq on cells that were sorted into 384-well plates[33]) or using
330   the 10x Genomics Chromium Single Cell 3' (v3 Chemistry) gene expression kit, according to
331   manufacturer's instructions.

332   **Animal experimentation.** Mouse embryos (n = 3) used for tomo-seq were derived from crosses between
333   CD-1 females and CD-1 stud males. Experiments were performed in accordance with EU guidelines,
334   under the authority of appropriate UK governmental legislation. Use of animals for this project was
335   approved by the Animal Welfare and Ethical Review Body for the University of Cambridge. Relevant
336   Home Office licenses are in place.

337   **Tomo-seq.** Tomo-seq was performed using a robotized (SORT-seq[33] based) version of a previously
338   published tomo-seq protocol[11]. Briefly, 120 h gastruloids (n = 3 E14-IB10 gastruloids sectioned using 20

339   µm sections; n = 2 E14-IB10 gastruloids sectioned using 8 µm sections; n = 3 LfngT2AVenus gastruloids
340   sectioned using 20 µm sections, generated with previously published, non-Matrigel based gastruloid
341   protocols[1,20]) or E8.5 mouse embryos (n = 3 sectioned using 20 µm sections) were embedded in
342   cryosolution (Leica, 14020108926), snap-frozen on dry-ice, stored at -80 °C and sectioned using a
343   cryotome. Sections were collected in the wells of a Hard-Shell PCR Low-profile, semi-skirted 96-well plate
344   (Bio-rad, HSL9601) that was already prefilled with mineral oil (Sigma, M8410-1L) and CEL-seq2 primers.
345   For each well, a unique, barcoded CEL-seq2 primer was used, which allowed us to pool the content of
346   the wells after second strand synthesis. To sequence the mRNA content of the wells, SORT-seq
347   (robotized CEL-seq2 based scRNA-seq[33]) was performed using a Nanodrop II liquid handling platform
348   (GC biotech).

349   **Sequencing.** Sequencing was performed on the Illumina Next-seq sequencing platform. For SORT-seq
350   and tomo-seq, paired end (75 bp) sequencing was performed; for 10x Genomics, sequencing was
351   performed according to 10x Genomics manufacturer's instructions (Read1, 28 cycles; Index i7, 8 cycles;
352   Read2, 91 cycles).

353   **Mapping sequencing data.** For SORT-seq and tomo-seq, the first 6 bases of read 1 contain the unique
354   molecular identifier (UMI) and the next 7 bases contain the cell or section barcode. For 10x Genomics,
355   the first 16 bases of read 1 contain the cell barcode, and the next 12 contain the UMI. For all sequencing
356   experiments, read 2 contains the biological information. Reads 2 with a valid cell/section barcode were
357   selected, trimmed using TrimGalore-0.4.3 with default parameters, and mapped using STAR-2.5.3a with
358   default parameters to the mouse mm10 genome (Ensembl 93). Only reads mapping to gene bodies
359   (exons or introns) were used for downstream analysis. Reads mapping simultaneously to an exon and to
360   an intron were assigned to the exon. For each cell or section, the number of transcripts was obtained as
361   previously described[34]. We refer to transcripts as unique molecules based on UMI correction.
362   Mappabilities for both scRNA-seq and tomo-seq experiments range from 35% to 60%. Spike-ins,
363   ribosomal, and mitochondrial genes were removed from downstream analysis, together with *Kcnq1ot1*,
364   *Mir5109*, *Lars2*, *Malat1*, *Rn45s*, because these genes seem to be linked to mapping errors and have
365   been shown to be erroneous in earlier studies[34].

366   **Processing single-cell data.** scRNA-seq analysis was performed using the Scanpy package[35] (v1.4.3).
367   In each experiment, cell barcodes with more than 1,000 transcripts and fewer than 6,000 genes were
368   selected. Genes detected in fewer than 3 cells were excluded. Expression levels for each cell were size-
369   normalized to 10,000 transcripts. Highly variable genes were defined as those with a mean expression
370   value between 0.0125 and 5, and with a minimum dispersion, and used to generate the UMAPs shown in
371   Fig. 1 and Extended Data Figs. 1, 2, 4, 5. Next, cells from the three independent experiments were
372   analysed together. Here, we kept cells with more than 700 and fewer than 8,000 genes, and more than
373   1,000 and fewer than 40,000 transcripts. Selection of highly variable genes and cell normalization were
374   performed as described above. To remove batch effects, we used the combat function from Scanpy (a
375   Python implementation (https://github.com/brentp/combat.py) of the R-package Bioconductor[36,37]). Cells

376   were clustered using a combination of k-medoids and Leiden algorithms[38] (Supplementary Table 1).

377   Differentially expressed genes in each cluster were determined using the t-test (Supplementary Table 2).

378   **Comparison between gastruloid cell types and mouse embryonic cell types.** Common genes

379   between marker genes detected in the gastruloid cell clusters (Supplementary Table 1, *P*-value < 0.01

380   and log2(fold-change) > 1.01) and markers genes found for the different embryonic cell types defined in a

381   previously published mouse embryo scRNA-seq dataset[4] were found. *P*-value for significance was

382   assigned using a binomial test, where the probability of sharing a number of common marker genes

383   between a gastruloid cell type and an embryonic cell type was determined by randomizing the list of

384   marker genes for the embryonic cell type from the full list of marker genes in the embryonic cell types (n =

385   200). Fig. 1 only shows comparison to embryonic cell types found at E8.5. Extended Data Fig. 1h shows

386   the comparison to all embryonic cell types detected from E7.0 until E8.5. Only embryonic cell types with

387   at least one cluster comparison with a *P*-value below 0.2 are shown. Using different P-value thresholds to

388   define up-regulated genes does not have a significant impact on the results of the comparison between

389   gastruloid cell populations and embryonic cell types.

390   **Linearization of the UMAP.** Cells in clusters 1-8 were projected on the symmetry axis along the clusters

391   1-8 in the UMAP (Extended Data Fig. 1d). The position of each cell along this symmetry axis defines the

392   x-position in Fig. 1c. To plot gene expression along the linearized UMAP, 1,000 evenly spaced bins were

393   defined along the x-axis for which the expression average of all cells per respective bin was scaled and

394   plotted. For visualization, a LOESS smoother was used with span set to 0.2.

395   **Processing tomo-seq data.** 20 μm sectioned slides with fewer than 3,200 genes and 8 μm sectioned

396   slices with fewer than 6,000 genes were filtered out (Extended Data Fig. 3). In each tomo-seq sample,

397   data was normalized to the median number of unique transcripts per slide. Sequencing libraries contain a

398   maximum of 96 slices. In samples with more than 96 sections, several libraries were generated. For these

399   samples, we corrected batch effects between sequenced libraries by imposing the continuity of

400   expression profiles along the AP axis for each gene separately.

401   **Gene reproducibility analysis between replicates.** The Pearson correlation coefficient between the AP

402   expression pattern (in z-score units) of two different samples is computed for all possible pairs of

403   replicates. Linearly interpolated gene expression profiles are used when the number of sections is

404   different between replicates. To assess for significant correlations, we randomly generate 10,000

405   expression profiles with the same number of sections as in the pair of replicates and determine a

406   threshold for the correlation value at which less than n random profiles have larger correlation values (n =

407   100 for *P*-value < 0.01; n = 500 for *P*-value < 0.05, etc; Supplementary Table 5). Only genes that are

408   significantly correlated (*P*-value < 0.01) in at least five possible pairs of replicates are considered as

409   reproducible between replicates (Supplementary Tables 6 and 9). Custom made code was used for this

410   analysis.

411   **Clustering genes based on AP expression patterns.** Genes were first clustered based on z-score AP

412   expression pattern using self-organizing maps with an initial number of clusters set to ~5√N, where N is

413 the total number of genes. Average z-score expression patterns for each cluster were then hierarchically

414 clustered using Euclidean distances and the Wart.D method.

415 **Comparison between tomo-seq data of mouse embryos and mouse gastruloids.** Gene

416 reproducibility analysis between the individual replicates of the systems that are being compared are

417 performed independently, as described above (Supplementary Tables 5-9). For heatmaps in Fig. 1d, f-g,

418 only genes present in the two separate lists of significantly correlated genes are used for downstream

419 analysis (Supplementary Tables 7 and 9). For heatmaps in Extended Data Fig. 5, genes that were

420 present in only one of the two separate lists were included as well (Supplementary Tables 8-9). Genes

421 were clustered based on their AP expression pattern in the systems that are being compared

422 simultaneously, as described above. The Pearson correlation coefficient for each gene is calculated

423 between the AP expression pattern of two different samples (in z-score units). To assess for significantly

424 correlated genes, we randomly generate 10,000 expression profiles with the same number of sections as

425 in the pair of replicates and determine the correlation value at which less than 500 random profiles have

426 larger correlation values (P-value < 0.05).

427 **Comparison between genes in tomo-seq clusters and mouse embryonic cell types.** As above, but

428 then calculating the number of overlapping genes, and the P-value of this overlap, by comparing the

429 genes in each tomo-seq cluster with the list of genes upregulated in the cell types of a previously

430 published E8.5 mouse embryo scRNA-seq dataset[4] (Supplementary Tables 5-9).

431 **Wide field microscopy.** Widefield images of gastruloids made from *Brachyury*[GFP 25], *Nodal*[YFP 28] and

432 *TCF/LEF*[mCherry] (TLC2[26,27]) mouse ESCs were acquired at 120 h using a Zeiss AxioObserver Z1 in a

433 humidified CO_2 incubator (5% CO_2, 37 °C) and a 20x LD Plan-Neofluar 0.4 NA Ph2 objective with the

434 correction collar set to image through plastic, as previously described[2]. Illumination was provided by an

435 LED white-light system (Laser2000, Kettering, UK) in combination with filter cubes GFP-1828A-ZHE

436 (Semrock, NY, USA), YFP-2427B-ZHE (Semrock, NY, USA) and Filter Set 45 (Carl Zeiss Microscopy Ltd.

437 Cambridge, UK) used for GFP, YFP and RFP respectively. Emitted light was recorded using a back-

438 illuminated iXon888 Ultra EMCCD (Andor, UK) and images were processed using FIJI[39].

439 **Multi-photon time-lapse imaging of gastruloids.** Gastruloids were embedded in 10-100% Matrigel in

440 24-well plates (Sigma, EP0030741021 or M9312) at 96 h as described above, and imaged immediately

441 following embedding at 37 °C, 5% CO_2 with humidified air influx on a Leica SP8 multi-photon microscope

442 system using an HC PL APO 20x/0.75 air CS2 objective, a Coherent Chameleon Vision-S multi-photon

443 laser tuned to 960 nm and the pinhole maximally opened. The brightfield channel was recorded using a

444 488 nm laser set at low intensity in combination with a transmission PMT. A z-stack of around 4 images

445 with a z-interval of 15 μm was taken every 15 min (10 images per stack and at 12 min interval (Fig. 3c))

446 for each individual gastruloid (frame accumulation 2 times, pixel dwell time 2.425 μs). Photons with a

447 wavelength between 505-555 nm, and 555-680 nm were collected with two separate hybrid detectors and

448 assigned to a 16-bit pixel range. Alternatively, in Extended data Fig. 7d, a 514 nm solid state laser was

449 used during which photons were collected with a wavelength between 524-575 nm, and 600-700 nm with

450 two separate hybrid detectors and assigned to a 16-bit pixel range. In this case the brightfield channel
451 was recorded simultaneously with the other channels using a transmission PMT.

452 **Treatment of Matrigel-embedded gastruloids with inhibitors.** Gastruloids were embedded in 10-100%
453 Matrigel at 96 as described above, and real-time imaging was started immediately after embedding. After
454 recording at least 2 timepoints and at most 4 timepoints for each replicate (~30-60 min in total) the
455 microscope was paused and inhibitors were added without removing the culturing plate from the stage.
456 DAPT (Sigma, D5942; stock 10 mM in DMSO; used at 27 µM); PD0325901 (Sigma, PZ0162; stock 10
457 mM in DMSO); BGJ398 (Selleckchem, S2183; stock 1 mM in DMSO; used at 0.2 µM); PD173074
458 (Peprotech, 2191178; stock 10mM in DMSO; used at 0.5 µM); FGF1 (Peprotech, 100-17A; stock 10
459 µg/mL in H_2O; used at 0.02 µg/mL); FGF10 (Peprotech, 100-26; stock 100 µg/mL in H_2O; used at 0.2
460 µg/mL); Chiron (CHI99021; Sigma, SML1046; stock 10 mM in DMSO; used at 10 µM); IWP-2 (Sigma,
461 I0536; stock 2 mM in DMSO; used at 2 µM); IWR-1 (Sigma, I0161; stock 10 mM in DMSO; used at 10
462 µM); LDN193189 (Sigma, SML0559; stock 0.1 mM in H_2O; used at 0.2 µM).

463 **Analysis of multi-photon time-lapse imaging data.** Image analysis was done similar to previously
464 described image-analysis methods[15,23]. Time-lapse imaging data was analysed using the ImageJ data
465 processing package FIJI[39]. To filter out autofluorescence, the first channel (555-680 nm) was multiplied
466 by 0.3 and subtracted from the second channel (505-555 nm). Then, a sum projection of all z-slices was
467 generated for all timepoints. The resulting image was convolved using a gaussian filter with a sigma value
468 of 1 µm. Kymographs were generated using the plug-in KymoResliceWide
469 (https://github.com/ekatrukha/KymoResliceWide) by tracing the path of the differentiation front as it
470 moves along the AP axis with a segmented line (60 pixels wide) and then blurred using a gaussian filter
471 with a sigma value of 1 pixels. The intensity profile of the oscillations was measured at a constant
472 distance from the differentiation front (dashed white line Fig. 2c) on the kymograph. The intensity profile
473 of the oscillations was decomposed into a trend- and a cycle-component using Hodrick-Prescott filtering
474 with an l of 800. Trend and cycle component for all replicates are shown in Extended Data Fig. 8. To
475 make an estimation of the period of the *Lfng* oscillations, Lomb-Scargle analysis was performed with the
476 maximum scanned frequency at half the temporal resolution and over-sampling set to 3[40]. The speed of
477 the differentiation front and the elongation speed of the gastruloid were measured by first drawing a line
478 along the differentiation front or posterior tip of the gastruloid on the kymograph, respectively, and then
479 measuring the angle, as explained in Extended Data Fig. 9a.

480 **Sample fixation for stainings.** For gastruloids grown in 100-50 % Matrigel, the medium was removed
481 and the samples were washed twice for 5 min in PBS before fixation in 4% PFA/PBS overnight at 4 °C.
482 For gastruloids grown in 25-10% Matrigel, the medium/Matrigel was not removed in the first washing step
483 with PBS. After fixation, all samples were washed 3 times for 5 min in PBS-Tween (0.1% Tween-20 (v/v))
484 and washed 3 times for 3 min in TBS-Tween (0.1% Tween-20 (v/v)) before digesting for 4 min with 25
485 µg/mL Proteinase-K in TBS-Tween. The samples were then rinsed briefly 3 times with 2 mg/mL Glycine in

486 TBS-Tween20, washed with TBS-Tween once, refixed for 30 min in 4% PFA and 0.05% GA in PBS at
487 room temperature and washed 3 times in TBS-Tween.

488 ***In situ* hybridization.** ISH was performed as described before[3,15]. Briefly, samples were incubated for 4-5
489 hours in hybridization mix (5 mg/ml torula RNA (Sigma, R6625), 50% deionized formamide (Sigma,
490 AM9342) (v/v), 1.33x SSC, 0.1% BSA (w/v), 125 µg/ml Heparin (Sigma, H3393), 10 mM EDTA 0.5 pH =
491 8.0, 0.1% Tween 20 (v/v)) at 68 °C followed by incubation overnight in 150 ng/mL DIG-labelled probe in
492 hybridization mix at 68 °C. Carryover Matrigel that was still present degraded during this incubation step
493 in most instances. The hybridization mix with the probe was pre-incubated for 10 min at 80 °C. Samples
494 were then washed twice for 30 min in pre-heated hybridization mix at 68 °C, 4 times for 20 min in pre-
495 heated 2x SSC-Tween (0.1% Tween-20 (v/v)) at 68 °C, allowed to cool down and washed twice for 5 min
496 in MAB-Tween (0.1% Tween-20 (v/v)) at room temperature. The samples were blocked for 1.5 hours in
497 blocking buffer (10% heat inactivated sheep serum (Sigma, S3772) (v/v) and 1% BSA (w/v) in MAB-
498 Tween) at room temperature, incubated for 4-5 hours in blocking buffer containing 1:2,000 anti-DIG-AP
499 antibody (Sigma, 11093274910) at room temperature and washed 5 times for 10 min followed by washing
500 overnight in MAB-Tween. Finally, the samples were washed 3 times in TBS-Tween, washed 3 times for
501 10 min in AP-buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween-20), stained for
502 several hours in 1 mL BM purple **(**Sigma, 11442074001), washed 3 times for 5 min in TBS-Tween and
503 refixed in 4% PFA/PBS for 20 min at room temperature.

504 **Imaging of gastruloids stained with *in situ* hybridization.** *In situ* samples were imaged on a Leica
505 M165FC stereo microscope with DMC5400 digital camera (Fig. 3a, right panel) or using a Nikon
506 SMZ800N microscope (Fig. 3a left two panels) in TBS-Tween.

507 **Hybridization chain reaction of 10% Matrigel-embedded gastruloids.** *In situ* whole mount HCR V3
508 was performed as described previously[21] using reagents from Molecular Instruments. Briefly, each
509 condition (up to 100 gastruloids) was incubated in 200-500 µL of probe hybridization buffer for 5 min at
510 room temperature and 30 min at 37 °C before incubation with 4 pM of each probe stock in 200-500 µL
511 probe hybridization buffer for 12-16 hours at 37 °C. Next, samples were washed 4x with 500 µL probe
512 wash buffer for 15 min at 37 °C, 2x with 1 mL 5x SSC-Tween for 10 min at room temperature and 1x with
513 200-500 µL amplification buffer for 5 min at room temperature. The hairpin mixture was prepared by
514 separately heating both h1 and h2 of each hairpin to 95 °C for 90 seconds and incubating these at room
515 temperature for 30 min in the dark. All the hairpin mixtures were then added to 200-500 µL amplification
516 buffer at a concentration of 48 pM, which was then added to the samples and incubated for 12-16 hours
517 at room temperature in the dark. Samples were then washed at least 2x with 1 mL SSC-Tween for 30 min
518 before imaging. HCR probe design: *Uncx4.1* (Accession NM_013702.3, hairpin B1); *Tbx18* (Accession
519 NM_023814.4, hairpin B3); *Ripply2* (Accession NM_001037907, hairpin B2); hairpin B1 was labelled with
520 Alexa 594 and B2 and B3 with Alexa 488.

521 **Multi-photon microscopy of HCR-stained gastruloids.** HCR stained samples were imaged in TBS-T
522 on a Leica SP8 multi-photon microscope system using an HC PL APO 20x/0.75 air CS2 objective, a

523   Coherent Chameleon Vision-S multi-photon laser tuned to 810 nm for the Alexa-594 dye, a 488 nm OPS-
524   laser for the Alexa-488 dye and the pinhole maximally opened. A z-stack of around 30 images with a z-
525   interval of 5 µm was taken with frame accumulation set to 4. Photons with a wavelength between 505-555
526   nm, and 555-680 nm were collected with two separate hybrid detectors and assigned to a 16-bit pixel
527   range for the Alexa-594 channel; photons with a wavelength between 498-550 nm were collected with a
528   hybrid detector and assigned to a 16-bit pixel range for the Alexa-488 channel. The brightfield channel
529   was recorded simultaneously with the Alexa-488 channel using a transmission PMT detector.

530   **HCR data analysis.** HCR imaging data was analysed using the ImageJ data processing package FIJI[39].
531   First, all the images in a single stack were aligned using the ImageJ plug-in Correlescence
532   (https://github.com/ekatrukha/Correlescence), after which a maximum projection was generated for the
533   fluorescence channels. The posterior region of gastruloids was identified visually (the anterior end of
534   gastruloids is darker than the posterior end), and confirmed with *Ripply2* stainings. To plot the intensity
535   profile along the AP axis, a segmented line with a width of 100 pixels was drawn, and the intensity was
536   measured along this line. To measure the peak-to-peak distances in the *Uncx4.1* intensity profiles, a
537   LOWESS smoother (0.002 span) was applied, after which the maximal values corresponding to the peaks
538   were selected in R.

539   **Somite-size measurements in embryos.** Somite-sizes were measured in 10 somite-stage paraffin-
540   embedded mouse embryos that were sectioned with 6 µm sections, stained using a standard
541   haematoxylin and eosin staining and imaged with a Leica dm 4000 b led microscope with Leica DFC450
542   camera that was size-calibrated using a microscope calibration slide (Pyser-SGI). Somite-sizes were next
543   measured using Fiji. Measurements were validated by comparing results to somite-sizes in the EMAP
544   eMouse Atlas Project (http://www.emouseatlas.org)[41].

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545   **Extended Data Figure legends**

546 **Extended Data Fig. 1 | scRNA-seq on 120 h mouse gastruloids and comparison to embryos. a,**
547 Fluorescence-activated cell sorting (FACS) gating strategy prior to scRNA-seq. Live cells were selected
548 based on DAPI staining. Four sequential gates (P1-P4) were used; cells from gate P4 were used for
549 scRNA- seq. SSC, side scatter; FSC, forward scatter; H, height; W, width; A, area. **b,** Box plot showing
550 the median number of transcripts (left) and genes (right) detected per cell for SORT-seq experiments on
551 E14-IB10 (E14-S) and LfngT2AVenus gastruloids (Lfng-S), and for 10x Genomics experiments on
552 LfngT2AVenus gastruloids (Lfng-10x). The box extends from the lower to the upper quartile. Whiskers,
553 1.5x interquartile range; flier points are those past the end of the whiskers. **c,** Uniform manifold
554 approximation and projection (UMAP) plot for each experiment separately. Colour of each cell is the
555 same as the colour of that particular cell in Fig. 1a. **d,** UMAP obtained by analysing all the cells from the
556 different experiments together, where cells are coloured according to their batch (Methods,
557 Supplementary Table 1). The black line indicates the symmetry line in clusters 1-8 used to generate the
558 linearized UMAP in Fig. 1c (Methods). **e,** Fraction of E14-IB10 and LfngT2AVenus cells in each scRNA-
559 seq cluster from Fig. 1a. Blue, green and black numbers, number of E14-IB10, LfngT2AVenus and total
560 cells in each cluster (Supplementary Tables 1, 4). **f,** Fraction of cells for each cell type in each plate in
561 SORT-seq experiments (Lfng-S, E14-S), and in each experimental batch in 10x Genomics experiments
562 (Lfng-10x). Box plots: center line, median; box limits, 1st and 3rd quartiles; whiskers, range. **g,** Fraction of
563 cells detected in the E8.5 mouse embryo scRNA-seq dataset[4] used to compare our gastruloid scRNA-seq
564 data with. Exact numbers in each cluster are indicated. **h,** Dot plot showing overlapping genes between
565 significantly upregulated genes for each gastruloid scRNA-seq cluster (Supplementary Table 2), and
566 upregulated genes for each E7.0-E8.5 mouse embryonic cell type[4]. Dot colour indicates the probability of
567 finding such a number of overlapping genes between the two sets by random chance (P-value, Methods),
568 and dot size represents the number of overlapping genes. Blue colouring, embryonic stage. E14, E14-
569 IB10; Lfng, LfngT2AVenus; S, SORT-seq[33]; 10x, 10x Genomics; MD, mesoderm; EcD, ectoderm; NMP,
570 neuro-mesodermal progenitors; ExE, extra-embryonic; EnD, endoderm; Haemato, haemato-endothelial;
571 prog, progenitors; PGC, primordial germ cells; Ant, anterior; PSM, presomitic mesoderm.

572

573 **Extended Data Fig. 2 | Expression of relevant markers in gastruloid scRNA-seq dataset. a,** Mean
574 log expression of relevant markers of outlier populations (clusters 9-13) plotted on the UMAP from Fig.
575 1a. *Olfr129* and *Onecut1*, head mesenchyme (cluster 9); *Etv2*, haemato-endothelial progenitors (bottom
576 part of cluster 10); *Kdr*, haemato-endothelial progenitors and endothelium (cluster 10); *Cdh5* and *Tie1*,
577 endothelium (top part of cluster 10); *Tbx4*, *Hoxa11*, *Ass1* and *Bmp7*, allantois (cluster 11); *Ephx2*, *Mt1*,
578 *Utf1* and *Pou5f1*, primordial germ cell like or extra-embryonic ectoderm (cluster 12); *Col4a1*, *Epcam* and
579 *Sox17*, endoderm (cluster 13). **b,** Mean log normalized expression of relevant markers of clusters 1-8
580 plotted on the UMAP from Fig. 1a. *Hand2* and *Gata6*, heart (cluster 1); *Meox2* and *Pax3*, differentiated
581 somite (cluster 3); *Aldh1a2* and *Uncx4.1*, somite (cluster 4); *Lfng*, *Mesp2*, *Ripply2* and *Dll1*, differentiation
582 front (cluster 5); *Hes7* and *Tbx6*, presomitic mesoderm (cluster 6); *Wnt3a*, *Fgf17*, *Fgf8*, *Cyp26a1*, *Nkx1-2*

583 and *T*, tail bud containing neuro-mesodermal progenitors (cluster 7); *Pax6*, *Sox1*, *Hes3* and *Sox2*,
584 differentiated neural cells (spinal cord; cluster 8). Expression was first count-normalized to 10,000 for
585 each cell (Methods), and then log-transformed. Additional markers of all clusters are provided in
586 Supplementary Table 2.

587

588 **Extended Data Fig. 3 | Number of genes and reads in gastruloid and embryo tomo-seq datasets,**
589 **and comparison to microscopy data. a-c,** Number of unique transcripts and genes detected in 3 E14-
590 IB10 120 h mouse gastruloids that were sectioned using 20 µm sections and 2 E14-Ib10 120 h mouse
591 gastruloids that were sectioned using 8 µm sections (a); in 3 LfngT2AVenus 120 h mouse gastruloids that
592 were sectioned using 20 µm sections (b); and in 3 E8.5 mouse embryos that were sectioned using 20 µm
593 sections (c). Due to their length, embryo sections were collected in two sequential 96-well plates. **d,**
594 Validation of tomo-seq data with microscopy. Top panels, *Brachyury^GFP*, *Wnt* signalling activity (as
595 reported using a *TCF/LEF^mCherry* mouse ESC line) and *Nodal^YFP* expression in 120h mouse gastruloids as
596 measured by microscopy (Methods). Barplots showing the normalized expression levels of *Brachyury*,
597 *Wnt3a* and *Nodal* in 120 h E14-IB10 gastruloids, 120 h LfngT2AVenus gastruloids and E8.5 mouse
598 embryos as determined by tomo-seq (Methods), and in the posterior mesoderm of E9.5 mouse embryos
599 as determined by microarray[12]. **e,** Scaled average z-score of significantly upregulated genes detected in
600 each single cell cluster from Fig. 1a (Supplementary Table 2) as measured in the averaged
601 LfngT2AVenus tomo-seq gastruloid. Scale bar, 100 µm; A, anterior; P, posterior.

602

603 **Extended Data Fig. 4 | Individual replicates of gastruloids, E8.5 embryo tomo-seq and E9.5**
604 **posterior mesoderm datasets, and comparison to gastruloid and E8.5 embryonic scRNA-seq**
605 **datasets. a,** Heatmaps showing the AP expression patterns of 1,199 genes as detected by tomo-seq[11] in
606 individual replicates of 120 h E14-IB10 gastruloids (n = 3 gastruloids, 20 µm sections and n = 2
607 gastruloids, 8 µm sections) that were cultured in standard[1,20] (non-Matrigel based) conditions; average
608 heatmap of the 5 replicates; average expression of genes found in each tomo-seq domain in the E14-
609 IB10 tomo-seq dataset, projected in the UMAP from Fig. 1a; dot plot showing overlapping genes between
610 genes detected in each tomo-seq domain in the E14-IB10 tomo-seq dataset, and upregulated genes for
611 each E8.5 mouse embryonic cell type[4]. Dot colour represents the probability of finding such a number of
612 overlapping genes between the two sets by random chance (Methods), and dot size represents the
613 number of overlapping genes. Only genes that were reproducible between all replicates are shown
614 (Methods). Genes are clustered based on their AP expression pattern (Methods); Roman-numbered bars
615 represent tomo-seq clusters. **b,** Similar to panel a, but for 1,456 genes in 120 h LfngT2AVenus[15] (n = 3
616 gastruloids; 20 µm sections) gastruloids that were cultured in standard[1,20] (non-Matrigel based)
617 conditions. **c,** Similar to panel a, but for 1,553 genes in E8.5 embryos (n = 3 embryos, 20 µm sections). **d,**
618 Similar to panel a, but for 1,989 genes in an E9.5 mouse embryo posterior mesoderm dataset (tail bud to
619 newly formed somite; n = 3 embryos; previously published microarray data; ~100 µm sections[12]. All genes

620   are in Supplementary Table 6. E14, E14-IB10; Lfng, LfngT2AVenus; AP, anterior-posterior; MD,
621   mesoderm; NMP, neuro-mesodermal progenitors; EcD, ectoderm; Def, definitive; EnD, endoderm;
622   Haemato, haemato-endothelial; prog, progenitors; ExE, extra-embryonic; FMH, fore- mid - hindbrain.

623

624   **Extended Data Fig. 5 | Comparisons between mouse gastruloid and mouse embryo datasets,**
625   **including genes that are reproducible in at least one system. a,** Heatmap showing the average AP
626   expression pattern of 2,065 genes as detected by tomo-seq[11] in 120 h mouse gastruloids that were
627   generated from E14-IB10 and LfngT2AVenus[15] mouse ESCs and that were cultured in standard[1,20] (non-
628   Matrigel based) conditions; average expression of genes found in each tomo-seq domain in the E14-
629   IB10- LfngT2AVenus comparison heatmap, projected in the UMAP from Fig. 1a; dot plot showing
630   overlapping genes between genes detected in each tomo-seq domain in panel a, and upregulated genes
631   for E8.5 mouse embryonic cell types[4]. Dot colour represents the probability of finding such a number of
632   overlapping genes by random chance (Methods), and dot size represents the number of overlapping
633   genes. In contrast to the heatmaps in Fig. 1, this heatmap contains genes that were reproducible in either
634   E14-IB10 (n = 3 gastruloids, 20 µm sections and n = 2 gastruloids, 8 µm sections) or LfngT2AVenus (n =
635   3 gastruloids; 20 µm sections) gastruloids (Methods, Supplementary Tables 5-6, Extended Data Fig. 4).
636   This means that genes that are reproducible in E14-IB10 replicates but not in LfngT2AVenus replicates,
637   and vice versa, are included. Genes are clustered based on their AP expression pattern (Methods);
638   Roman-numbered bars represent tomo-seq clusters, which are also indicated with the gray-black barplot.
639   The red-to-white barplots indicate the *P*-value of reproducibility of each gene in each heatmap. The order
640   of these barplots correspond to the order of the heatmaps. **b,** Similar to panel a, but for 2,804 genes in
641   that were reproducible in E14-IB10 (n = 3 gastruloids, 20 µm sections and n = 2 gastruloids, 8 µm
642   sections) or LfngT2AVenus (n = 3 gastruloids; 20 µm sections) or E8.5 mouse embryos (n = 3 embryos;
643   20 µm sections). **c,** Similar to panel a, but for 3,086 genes in that were reproducible in E14-IB10 (n = 3
644   gastruloids, 20 µm sections and n = 2 gastruloids, 8 µm sections) or LfngT2AVenus (n = 3 gastruloids; 20
645   µm sections) or the E9.5 mouse embryo posterior mesoderm dataset (tail bud to newly formed somite; n
646   = 3 embryos; previously published microarray data; ~100 µm sections[12]. Here, only the first 15 tomo-seq
647   clusters are projected onto the UMAPs. Gene lists are provided in Supplementary Table 8. E14, E14-
648   IB10; Lfng, LfngT2AVenus; AP, anterior-posterior; MD, mesoderm; NMP, neuro-mesodermal progenitors;
649   EnD, endoderm; Haemato, haemato-endothelial; prog, progenitors; PGC, primordial germ cells; EcD,
650   ectoderm; Def, definitive; ExE, extra-embryonic.

651

652   **Extended Data Fig. 6 | Gene expression profiles in gastruloid and embryo tomo-seq datasets.**
653   Lineplots for the normalized AP expression of genes emphasized in Fig. 1d, f and g for the E14-IB10 and
654   LfngT2AVenus gastruloids, and for the E8.5 mouse embryo, as measured by tomo-seq[11]. Each color is a
655   different replicate.

656

657   **Extended Data Fig. 7 | Kymographs of time-lapse experiments performed on LfngT2AVenus**

658   **gastruloids that were embedded in 100% Matrigel at 96 h. a-d,** Kymographs (space-time plots) of

659   brightfield channel and LfngT2AVenus signal along the AP axis of all replicates from all time-lapse

660   experiments (Experiments 1-4) that are presented in Fig. 2f and in Extended Data Fig. 8e,f. These

661   gastruloids were embedded in 100% Matrigel (Methods) to stabilize them during imaging, and

662   subsequently imaged for at least 17 hours (Supplementary Video 1-2, 4-5). Inhibitors were added at the

663   start of the time-lapse (Methods) and are indicated above the kymographs, together with their

664   concentration. Asterisks refer to gastruloids used to generate Fig. 3e and Extended Data Fig. 8b. **e,** Real-

665   time imaging of a LfngT2AVenus gastruloid that was embedded in 100% Matrigel at 96 h and to which the

666   Notch-inhibitor DAPT was added at 96.5 h (Supplementary Video 2; *Lfng* signal disappears ~6 hours after

667   DAPT addition). Corresponding kymographs in panel a. A, Anterior; P, Posterior.

668

669   **Extended Data Fig. 8 | Detrending procedure and Lomb-Scargle analysis of replicates from Fig. 2,**

670   **and measurements of elongation and differentiation front speed in small panel screening and**

671   **upon BGJ389 and PD17 treatment. a,** Black line, measured intensity of the *Lfng* signal along the white-

672   dashed line in Fig. 2c; blue line, trend (Methods) of this signal, and periodogram of the *Lfng* oscillations in

673   Fig. 2d, as determined by Lomb-Scargle decomposition. **b,** As in a, but then for the 13 DMSO-control

674   LfngT2AVenus gastruloid replicates shown in Extended Data Fig. 7c-d. **c,** cyclical component of the

675   scaled intensity of the LfngT2AVenus oscillations relative to the trendline shown in b. A.U., arbitrary units.

676   **d,** Periodogram of the *Lfng* oscillations in c, as determined by Lomb-Scargle decomposition (Methods).

677   Gastruloids used for this experiment were embedded in 100% Matrigel at 96 h, and subsequently imaged

678   for at least 17 hours (Supplementary Video 6). **e**-**f,** Speed of posterior gastruloid elongation (V_{PSM}) and

679   speed of posteriorly moving differentiation front (V_{DIFF}; see explanation in Extended Data Fig. 9a) in

680   LfngT2AVenus gastruloids treated with DMSO (control), or with various inhibitors (Supplementary Videos

681   3, 5). Points refer to replicates; kymographs of replicates in Extended Data Fig. 7. Box plots: center line,

682   median; box limits, 1st and 3rd quartiles; whiskers, range.

683

684   **Extended Data Fig. 9 | Explanation on how elongation and differentiation front speed were**

685   **measured, and HCR stainings and live-imaging kymographs of gastruloids embedded in 10%**

686   **Matrigel. a,** Kymographs (space-time plots) of brightfield channel and LfngT2AVenus signal along the AP

687   axis of a DMSO-treated (control) and a PD03-treated (MEK/ERK inhibitor) LfngT2AVenus gastruloid.

688   Gastruloids were embedded in 100% Matrigel at 96 h; DMSO or PD03 (66.7 µM) was added at 96.5 h.

689   Kymographs were used to measure the elongation speed of the gastruloid (angle of blue dashed line;

690   V_{PSM}; Methods) and the speed of the differentiation front (angle of red dashed line; V_{DIFF}). **b,**

691   LfngT2AVenus gastruloids that were embedded in 10% Matrigel (Methods) at 96 h and stained for

692   *Uncx4.1* using HCR[21] at 120 h. Zoom in of the left gastruloid is shown in Fig. 3b. **c,** Kymographs of

693   LfngT2AVenus signal and brightfield channel along the AP axis of gastruloids that were embedded in

694   10% Matrigel at 96 h, and subsequently imaged for 20 hours (Supplementary Video 6). Top kymograph

695   belongs to the gastruloid that is shown in Fig. 3c. A, Anterior; P, Posterior; Scale bar, 200 µm.

696   **Extended Data Fig. 10 | *Uncx4.1*/*Tbx18*/*Ripply2* stainings and somite size measurements. a,** HCR[21]

697   double staining for *Uncx4.1* (cyan) and *Tbx18* (magenta) on a 120 h LfngT2AVenus gastruloids

698   embedded in 10% Matrigel at 96 h. To replicate 4, 1.3 µM of PD03 was added at 96.5 h. **b,** Similar to

699   panel a, but now for *Uncx4.1* (cyan) and *Ripply2* (yellow). **c,** Intensity of *Uncx4.1* and *Tbx18* signal along

700   the AP axis of the gastruloids in panel a. Peaks (circles) are called on the smoothened *Uncx4.1* profile

701   (dark blue; Methods). **d,** Similar to panel c, but now for the *Uncx4.1* and *Ripply2* stained gastruloids from

702   panel b. **e,** Distance between *Uncx4.1* peaks in the 120 h LfngT2AVenus gastruloids (n = 7) from

703   replicates 1-6 in panels a-d and in replicate 7 (which is shown in Fig. 3d). Replicate 8 was excluded from

704   quantification and both replicate 4 and 7 were incubated in 1.3 µM PD03 from 96 - 120 h. A, Anterior; P,

705   Posterior; Scale bar, 200 µm.