Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids

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The emergence of multiple axes is an essential element in the establishment of the mammalian body plan. This process takes place shortly after implantation of the embryo within the uterus and relies on the activity of gene regulatory networks that coordinate transcription in space and time. Whereas genetic approaches have revealed important aspects of these processes1, a mechanistic understanding is hampered by the poor experimental accessibility of early post-implantation stages. Here we show that small aggregates of mouse embryonic stem cells (ESCs), when stimulated to undergo gastrulation-like events and elongation in vitro, can organize a 3D spatial and temporal gene expression. The establishment of the three major body axes in these ‘gastruloids’2,3, suggests that the mechanisms involved are interdependent. Specifically, gastruloids display the hallmarks of axial gene regulatory systems as exemplified by the implementation of collinear Hox transcriptional patterns along an extending antero-posterior axis. These results reveal an unprecedented self-organizing capacity of aggregated ESCs and suggest that gastruloids could be used as a complementary system to study early developmental events in the mammalian embryo.

Recent work on organoids derived from stem cells has revealed a surprising autonomy in the development of particular tissues and organs4,5. When around 250 ESCs are aggregated, given a pulse of the Wnt agonist CHIR99021 (Chi) between 48 and 72 h after the start of culture, and returned to N2B27 medium (Fig. 1a), a pole of Bra (brachyury, also known as T) expression emerges reproducibly6 (Fig. 1b, Extended Data Fig. 1), resembling the elongating embryonic tail bud. The aggregates continue to elongate up to 120 h after aggregation (AA), when they display a ‘rostral’ cell-dense region and a polar extension towards a ‘caudal’ extremity, reaching up to 500 μm in size (Fig. 1b). Shaking the culture enables aggregates to grow to 850–1,000 μm in length at 168 h AA (Fig. 1c, d). At 120 h AA, a Gata6-positive domain is visible opposite to a Bra and Cdx2-expressing region, probably corresponding to the cardiac crescent, which delimits the embryonic post-occipital region7 (Fig. 1b–d, Extended Data Fig. 1, Supplementary Videos 1, 2). By contrast, at 120–168 h AA Sox1/Sox2-positive cells are localized centrally, with the exception of those at the rostral extremity (Fig. 1c, d).

To characterize the transcriptional programmes of these gastruloids, we carried out RNA-sequencing (RNA-seq) analysis on duplicate pools of gastruloids and compared their profiles with those of developing mouse embryos from E6.5 to E9.5. Because gastruloids display

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hallmarks of post-occipital embryos (Fig. 1b–d) we excluded the anterior portion of E7.5–E9.5 embryos (Fig. 1e, left). Principal component analysis (PCA) demonstrated reproducibility between samples and a clear clustering along principal component 1 (PC1) corresponding to their temporal order (Fig. 1e), whereas embryo samples segregated from gastruloids along PC2 only. The main (top 100) clustering determinants of gastruloid samples included several pluripotency-related genes, epiblast markers and genes involved in gastrulation. They also comprised different Hox genes and other transcription factors usually expressed in post-occipital structures of the developing mouse embryo such as \textit{Cdx1}, \textit{Cdx2}, \textit{Meis1}, \textit{Meis2}, \textit{Meox1}, \textit{Bra} and \textit{Gata4} (Fig. 2a).

Twenty-five of these 100 PCA determinants were identified independently in both gastruloid and embryonic temporal series (Fig. 2a, genes in red), supporting the idea that gastruloids and embryos elaborate by implementing similar transcriptional programs. The analysis of specific genes associated with particular developmental landmarks confirmed this point (Fig. 2b, Extended Data Fig. 2a, b). For instance, genes associated with gastrulation, such as \textit{Mix1}, \textit{Eomes}, \textit{Gsc} (gosecod) or \textit{Chrd} (chordin) were transiently and orderly transcribed at around 48 h AA (Fig. 2b, Extended Data Fig. 2b), suggesting that at this stage the gastruloid transcriptome resembles that of mouse epiblast at the onset of gastrulation. By 72 h AA, we observed an increase in the complexity of gene-expression profiles, with the appearance of markers for different embryonic lineages, including mesendoderm and neuroectoderm, and the transcription of \textit{Hox} gene clusters (Fig. 2a, b, Extended Data Fig. 2a, b; see below). Genes associated with either extra-embryonic structures or anterior neural plate derivatives were not (or were poorly) expressed in gastruloids (Fig. 2b, Supplementary Information datasets 1, 2).

PCA analysis using single gastruloids revealed robust clustering at all assessed developmental stages and a correspondence with the pooled RNA-seq datasets (Extended Data Fig. 2c), showing that the population of gastruloids was comparatively homogenous at the time points analysed, and hence that the pooled RNA-seq datasets reflected the transcriptional status of single gastruloids. Transcriptome analyses of gastruloids revealed mRNAs that are usually associated with neural, endodermal and mesodermal derivatives, including paraxial, cardiac, intermediate and haematopoietic progenitors as well as neural...
We observed clumps of cells positive for either SOX1 and OLG2, SOX1 and PAX3, or SOX1 and PAX7, indicative of dorsal and ventral neural tube progenitors\textsuperscript{11} (Extended Data Fig. 4d); however, they lacked a clear segregation along the dorso-ventral extension of the SOX1 domain. Similarly, Trp515-expressing cells did not condense into somites (Extended Data Fig. 3b).

Analysis of different endodermal markers revealed temporal dynamics that were also reminiscent of those in the embryo\textsuperscript{12} (Extended Data Figs. 3e, f, 4e, f). Transcripts of Gsc and Cdx2, markers of definitive endoderm\textsuperscript{13,14}, were upregulated soon after Chi induction (72 h AA), followed by upregulation of Cer1 (cerberus) (96–120 h AA) and subsequently Sorcs2, Pax9 or Shh (120–144 h AA). All assayed endoderm-expressed genes were active in the ventral-like domain of gastruloids (Extended Data Figs. 3f, 4e, f), resembling expression in the embryo. In a majority of cases, gut-endoderm progenitors appeared as a continuous tubular structure (Extended Data Fig. 4a, e, f, red arrowheads), often spanning the entire antero-posterior extension, reminiscent of an embryonic digestive tract.

We next investigated this unanticipated level of organization and capacity to self-organize an integrated axial system by assessing the expression of genes associated with the developing embryonic axes (Fig. 3). Transcripts of Wnt3a and Cyp26a1 were detected at the caudal extremity of gastruloids, similar to those of Bra and Cdx2 (compare Fig. 3a and Extended Data Figs. 5a with Fig. 1c, d and Extended Data Figs. 3b, 4f), and complementary to the localization of Raldh2 (also known as Aldh1a2) mRNAs (Fig. 3a)—further supporting the existence of an antero-posterior axis. This was also supported by the spatial segregation of the presumptive mesoderm-like domain (marked by Cyp26a1) and the Meox1 somitic mesoderm (Fig. 3a (right), Extended Data Fig. 5a). On the other hand, Lfng, Sox1 and Sox2 were transcribed in a central and dorsal domain at 144 h AA (Fig. 3b, Extended Data Figs. 3d, 4a), in a complementary fashion to the ventrally located intestinal tract markers (Fig. 3b, Extended Data Figs. 3d, 4e, f, 5b). Additional signs of multi-axial organization were provided by the expression of mesoderm-specific genes Ors1, Pecam1, Meox1 and Pax2 in a medio-lateral symmetry flattening the centrally located Sox2-positive domain (Fig. 3c, Extended Data Fig. 3b). Double staining of Sox2 and Meox1 (Fig. 3c (right), Extended Data Fig. 5c) and cross-sections (Extended Data Fig. 4b) confirmed the non-overlapping medio-lateral and dorso-ventral distribution of neural and mesodermal progenitors.

Nodal expression was confined to a small and compact region on the ventral most posterior aspect at 120 h AA (Extended Data Figs. 6, 7). These Nodal-expressing cells displayed high levels of Cadh1 (also known as E-cadherin) and dense phalloidin staining (Extended Data Fig. 6a, b), suggestive of a node-like identity\textsuperscript{15,16}, a hypothesis supported by the presence of Nodal mRNA in a domain comparable to that of Gsc, Bra and Chrd at 96 h AA (Extended Data Fig. 6c, d). Nodal mRNA in these cells rapidly decreased and was almost undetectable at 144 h AA. However, despite these signs of a node-like structure, we did not observe any notochord derivatives, which usually originate from the node. The downregulation of Nodal in the presumptive node-like cells at 120 h AA coincided with the appearance of patches of Nodal-expressing cells along the posterior half of extending gastruloids, which were often distributed in an asymmetric manner (Fig. 3d, Extended Data Figs. 6d, e, 7, Supplementary Videos 3, 4) at 120 h and 144 h AA. Cer1 also displayed a left–right asymmetric expression, particularly evident at 144 h AA (Extended Data Fig. 6f). This pattern was not observed with Meox1, which was predominantly expressed on both sides (Fig. 3d, e, Supplementary Information dataset 3). Together, these data suggest that besides the establishment of a medio-lateral axis, gastruloids may also implement the beginning of left–right asymmetry.

The formation and patterning of post-ocipital embryonic territories is associated with the sequential activation of clustered Hox genes. Because these genes appeared to be differentially regulated in the RNA-seq time course (Fig. 2a, Extended Data Fig. 2a, b), we assessed whether their sequential activation in time and space\textsuperscript{16} was also recapitulated. A pooled PCA analysis using only Hox transcripts revealed robust...
clustering along the time axis (81% variance) and a close correspondence with the dynamic activation of these genes in embryos (Fig. 4a, Extended Data Fig. 8a–c). The variability in Hox mRNA content among gastruloids was evaluated using ten individual specimens from three different stages (Extended Data Fig. 9a). Gastruloids at identical time points clustered tightly together based solely on their Hox transcripts. Transcript profiles over Hox clusters revealed signs of collinear activation, the hallmark of this gene family in E6.5 embryos, some Hoxa and Hoxd genes are expressed before gastrulation in extra-embryonic tissues (Extended Data Fig. 8a). Between E7 and E9.5, Hox genes start to be transcribed in an order that reflects their 3' to 5' position within each cluster (Extended Data Fig. 8a, b). RNA-seq profiling revealed an activation dynamic comparable to that observed in embryos (Fig. 4a, Extended Data Fig. 8c). For instance, whereas Hoxa1 RNA was not detected until 48 h AA, Hoxa1 to Hoxa3 expression was robust at 72 h, and was followed by sustained transcription of Hoxa5, Hoxa7 and Hoxa9 at 96–120 h. Hoxa10 and Hoxa11 RNA appeared at 144 h AA, at the same time that Hoxa1, Hoxa2 and Hoxa3 transcripts started to disappear (Fig. 4b, Extended Data Fig. 8c). Similar dynamics were observed for Hoxd genes (Extended Data Fig. 8c–e). The early transcription of 5' Hoxa/Hoxd genes (Extended Data Fig. 8a, b) was not observed in gastruloids (Extended Data Figs. 4b, 8c, d), consistent with the absence of extra-embryonic derivatives.

Comparative expression profiles were observed when single organoids were examined (Extended Data Fig. 9a, b), again revealing the reproducibility of this activation process. In the embryo, this temporal activation is paralleled by a collinear distribution of transcripts in space (Extended Data Fig. 8a–c). Likewise, Hoxa4/Hoxd4 displayed an antero-posterior boundary near the anterior aspect of the gastruloid, whereas Hoxa9/Hoxd9, Hoxa11/Hoxd11 and Hoxd13 exhibited successively posterior boundaries (Extended Data Fig. 8a–c). Notably, Hoxd13 transcripts appeared in cells that were located centrally at the posterior extremity, resembling Hoxd13 expression in the embryonic cloacal area (Extended Data Fig. 8c). Hoxa13 expression was also detected at 168 h AA in the posterior aspect, yet rarely (one in 20 gastruloids examined, consistent with the low transcript levels detected in the pooled RNA-seq analysis (Extended Data Fig. 9c). Double staining for Hoxd4 and either Sox2 or Meox1 (marking the neural and mesodermal precursors, respectively) showed that Hoxd4 expression colocalized with both markers, suggesting that gastruloids implement both neural and mesodermal Hox gene expression. The expression patterns are representative of four independent experiments. Scale bar, 200 µm.
circuits, particularly during early post-implantation development and the emergence of body axes.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0578-0.

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METHODS

Culture of gastruloids. E14Tg2a (ref. 28) (E14), Bra-GFP (ref. 27), Gaeta6H2B-Venus (ref. 28), NodalGFP (ref. 28) and Sox2xGFP,Bra-Cherry double reporter (SBR30) mouse embryonic stem (ES) cells were cultured on gelatinized tissue-culture flasks in a humidified incubator (5% CO2, 37 °C) as previously described3,6,7. The E14Tg2a ESC line was obtained from the group of A. Smith. The Bra-GFP ESC line was obtained from the Keller laboratory. The Gaeta6H2B-Venus ESC line was generated by the laboratories of A. Martinez Arias and C. Schröter. The NodalGFP cell line was obtained from the Collignon laboratory. The SBR30 ESC line was obtained through mutual transfer agreement from the laboratory of D. Suter.

The different cell lines used in this study were validated as follows: for the Bra-GFP ESC line, stimulation with Activin/Chi resulted in an increase of the GFP reporter which overlapped with the signal of the anti-BRA antibody; for the NodalGFP ESC line, we tested whether expression of the reporter was upregulated after Activin stimulation, while its expression was blocked by Nodal inhibitor SB43; the Gaeta6H2B-Venus and SBR30 ESC lines were validated by genotyping and co-staining with GATA6 and BRA antibodies, respectively. All cell lines were routinely tested and confirmed to be free of mycoplasma via the MYCOPLASMACHECk service of GATC Biotech.

The E14, Bra-GFP, Gaeta6H2B-Venus and NodalGFP mouse ESC lines were cultured in GMEM supplemented with 10% fetal bovine serum (FBS), non-essential amino acids (NEAA), sodium pyruvate, GlutaMax, beta-mercaptoethanol (β-ME) and LIF (ESL). The SBR30 cell line was cultured in DMEM supplemented with 10% FBS, NEAA, sodium pyruvate, β-ME, 3 μM Chil2, 2 μM PdO25901 (PD03) and LIF. If cells were not being passaged, half the medium was replaced with fresh medium. Gastruloids were generated as previously described with modifications (a detailed protocol describing gastruloid culture has been deposited in the Protocol Exchange repository34). Mouse ES or iPSCs were collected from tissue-culture flasks, centrifuged and washed twice with warm PBS (containing Ca2+ and Mg2+). After the final wash, cells were resuspended in 3–4 ml fresh and warm N2B27 (NDiff) and the cell concentration was determined.

For ESCs, the number of cells required to form gastruloids with a diameter of ~150 μm at 48 h AA (~300 cells) was determined and seeded in each well of a round-bottomed, low-adherence 96-well plate as 40 μl droplets of N2B27 (Supplementary Information File 1). For iPSCs, we performed a titration of the initial number of cells required to form gastruloids capable of elongating until at least 144 h AA. Amongst the different conditions tested (200, 400, 600, 800 and 1,200 cells per well) the best results were obtained with a starting number of 800 cells per well. In all cases, a 24 h pulse of 150 μl 3 μM Chii was added at 48 h AA. Medium (150 μl) was replaced with the same volume of fresh N2B27 daily. To extend the culture period, gastruloids were transferred onto low-attachment 24-well plates in 700 μl fresh N2B27 at 120 h and cultured in an incubator-compatible shaker for 48 h at 40 r.p.m. Four hundred microlitres medium was replenished at 144 h, and gastruloids fixed at 168 h.

Gastruloids for the different time-points analysed in this study were allocated in 1.5 ml 3-ml tubes and pelleted by centrifuging at 1,000 r.p.m. for 5 min. After washing with 80°C until RNA extraction. The number of ES cells was estimated by qPCR analysis. Purified RNA from iPSC-derived gastruloids was reverse transcribed using the Promega GoScript Reverse Transcription Kit. QRT-PCR analysis of mRNA levels for different HoxD genes, Bra and the housekeeping gene Hsmd was performed using the SYBR Select Master Mix for CFX (Thermo Fisher) kit according to the manufacturer’s instructions with specific primers3,6,7. The Biorage CFX96 thermocycler was used. At least two technical (PCR) replicates and two biological replicates were analysed per time point after aggregation.

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RNA-seq data processing. No biological replicate was excluded from the RNA-seq analysis. Mitochondrial and non-autosomal genes were excluded as they were not relevant for the biological question addressed in this study. These exclusion criteria were established before the analysis of the data. RNA-seq reads were aligned on the mouse mm10 genome assembly (https://www.ncbi.nlm.nih.gov/assembly/ GCF_000001045.2) using TopHat 2.0.9, implemented in Galaxy45. TopHat output files were processed with SAMTools38 and BedTools39. RNA-seq coverages were normalized to the millions of reads mapped for each sample. For the replicates of pooled gastruloids RNA-seq samples, average coverage files were calculated from the normalized coverages of each replicate. We used HTSeq46 implemented in the Galaxy server to count the number of uniquely mapped reads attributable to each gene (based on genomic annotations from Ensembl release 8247). We used DESeq2 to perform differential expression analyses. Specifically, we contrasted a generalized linear model that explains the variation in read counts for each gene, as a function of organoid stage, to a null model that assumes no effect of the treatment time. We ran the Wald test and the P values were corrected for multiple testing with the Benjamini–Hochberg approach. We computed reads per kilobase of exon per million mapped reads gene-expression levels using Cufflinks48.

Fragments per kilobase of transcript per million mapped reads (FPKM) levels were log2-transformed after adding an offset of 1 to each value. The log2-transformed values were centred across samples before PCA; no variance scaling was performed. For single-gastruloid PCA, only the 1,000 most highly expressed genes were used. For this, an average FPKM expression level of all the replicates of the 1,000 most expressed genes (the gene with the greatest expression was chosen) was calculated and the gene order was determined accordingly.

For cluster analysis of the most variably expressed genes, the top 250 most variable genes were determined by row variance using the genefilter::rownVars function. All heat map clustering, as identified by accompanying dendrogram, was performed using Euclidean distances and complete hierarchical clustering. For each gene cluster, enrichment of gene ontology (GO) terms was performed using GORilla43, by comparing the unranked list of gene of each cluster versus the totality of GO-term-annotated genes and by using a P value threshold of P < 10−4 and a false discovery rate (FDR) < 0.05. When more than 10 GO-term categories satisfying these criteria were identified, we used the REVIGO tool42 to summarize them, using an allowed similarity threshold of 0.7.

Probe cloning, in vitro transcription and in situ hybridization. Specific primers (Supplementary Information File 1) were used to amplify fragments of approximately 400–700 bp of the genes analysed using Topqaq DNA polymerase. The PCR fragments were gel-purified using the Qiagen Gel Extraction Kit and cloned in the pGEM-T Easy Vector System (Promega). Positive clones were verified by standard Sanger sequencing. For antisense RNA probe synthesis, the plasmids were digested with specific enzymes (Supplementary Information File 1) and purified with the Qiagen PCR purification kit. A total of 2 μg of the digested plasmid was used for in vitro transcription using either T7, T3 or Sp6 polymerase (Promega) and the Sigma DIG labelling mix or fluorescein labelling mix (Supplementary Information). The probes were purified using Qia-gen RNAeasy mini kit.

Fluorescent and non-fluorescent whole mount in situ hybridization. Gastruloids at different stages AA and E8.5–E9.5 wild-type mouse embryos were collected in 5-ml tubes, fixed overnight at 4 °C in 4% paraformaldehyde (PFA) and stored in methanol at −20 °C. In situ hybridization on whole-mount gastruloids was performed as previously described47, with some modifications. For non-fluorescent in situ hybridization, gastruloids were transferred into 12-Well Costar Netwell permeable inserts (ref. 3477) and rehydrated through a series of decreasing methanol concentrations. After washing in TBST (20 mM Tris 137 mM NaCl, 2.7 mM KCl, 0.1% Tween, pH = 7.4), gastruloids were digested in proteinase K...
solution and post-fixed in 4% PFA. The duration and concentration of the proteinase K treatment depended on the developmental stage of the embryo or the gastruloid time point after aggregation. E8 and E9 mouse embryos were incubated for 5 and 7 min in a 5 μg/ml proteinase K solution. Gastruloids at 72–120 h AA and 144–168 h AA were incubated for 1 or 2 min, respectively, in a 1.6 μg/ml proteinase K solution. Proteinase K treatment was stopped by rinsing embryos or gastruloids 3 times in a 2 mg/ml glycine-TBST solution. After post-fixation, gastruloids were prehybridized at 68 °C for 4 h to block non-specific RNA-probe interactions and incubated overnight at 68 °C with specific probes at approximately 200 ng/ml. The next day, probe washes were performed at 68 °C and the gastruloids were transferred to blocking solution at room temperature to impair nonspecific antibody recognition. Subsequently, digoxigenin (DIG)-labelled RNA probes were detected using anti-DIG antibody coupled to alkaline phosphatase (Sigma) at 1:3,000 dilution for 4 h at room temperature. Non-specific antibody background was removed by washing overnight in MABT (0.1 M Tris pH 7.5, 150 mM NaCl, 0.1% Tween) and stained with BM purple solution (Sigma).

For fluorescent in situ hybridization, gastruloids were processed as described above up to the antibody incubation step, but a higher probe concentration was used (500–700 ng/ml). In this protocol, fluorescein-labelled and DIG-labelled probes targeting each of the two genes to be detected were incubated simultaneously. After probe washing (see above), gastruloids were incubated in blocking solution containing anti-fluorescein antibody coupled to horseradish peroxidase (HRP) 1:100 (Perkin Elmer) for 3–4 h at room temperature. Gastruloids were subsequently washed 3 times in MABT and 3 times in TNT solution (0.1 M Tris pH 7.5, 150 mM NaCl, 0.05% Tween). The fluorescein-labelled probe was then developed using the TSA PLUS Fluorescin system (Perkin Elmer) for 10–12 min, following the manufacturer's instructions. To stop the first developing reaction, the anti-fluorescein-coated HRP was inactivated by washing the gastruloid 2 times (5 min each) in PBS-Triton 0.3% followed by one hour incubation with PBS-Triton 0.3% + 1% H2O2, and post-fixing the gastruloid for 35 min in 4% PFA solution. After 3 washes in TBST and 2 washes in MABT (5 min each), gastruloids were again incubated in blocking solution with anti-DIG antibody coupled to HRP (1:200; Perkin Elmer) for 4 h at room temperature. Subsequently, gastruloids were washed overnight in MABT. The next day, gastruloids were washed 3 times for 5 min in TBST and 3 times in TNT solution. The DIG-labelled probe was then developed using the TSA PLUS Cyanine 3.5 system (Perkin Elmer) for 10 min, following the manufacturer's instructions. The developing reaction was stopped as described above.

**Histology.** Histology was performed on the EPFL platform (Lausanne). For cryo-sectioning after WISH, gastruloids were placed in Histogel (Thermo Fisher) and oriented under a binocular microscope. Solidified gels were placed in a plastic mould filled with Cryomatrix (Thermo Fisher) and frozen with isopentane.

**Immunostaining and confocal microscopy.** Gastruloids were fixed and either Hoechst 33342 or DAPI was used to mark the nuclei. The primary and secondary antibodies used are listed in Supplementary Information File 1. Confocal images of gastruloids were generated using an LSM700 (Zeiss) on a Zeiss Axiovert 200M microscope equipped with a Plan-Apochromat 100×/1.40 oil-immersion objective. Solidified gastruloids were placed in a plastic mould filled with Cryomatrix (Thermo Fisher) and frozen with isopentane. Histology was performed on the EPFL platform (Lausanne). For cryo-sectioning, sections were used following standard procedures.

**Left-right asymmetry quantification methods.** Gastruloids formed from nodalGFP ES cells were fixed at 120 h AA, stained for GFP indicating nodal expression, Bra and Sox2, and 2 stacks were acquired from two opposite directions, 0° and 180°. The stacks from both sides of the gastruloids were then aligned and registered in FIJI. Gastruloids were scored as having a node-like structure if a region of nodal expression was found on the ventral surface directly opposite the dorsal expression of Sox2, and near the posterior Bra-expressing region. The ‘left’ or ‘right’ sides of the gastruloids were then inferred from the expression of Sox2 (dorsal-ventral axis) and Bra (antero-posterior axis), and the frequency of those displaying asymmetric Nodal expression on the bilateral axis was quantified. To test the significance of this asymmetry, the occurrence of asymmetry in a control gene, Mox1 (probed by WISH), which is usually expressed on both sides, was quantified and a binomial test of expected versus observed was performed. The fraction of gastruloids displaying symmetric or asymmetric Nodal and Cer1 expression after WISH detection was visually inferred under a stereoscope (Leica MZ2005). In these cases, as for Mox1, no reference gene was used to determine the left and right side of the gastruloids. In all cases, the frequency of gastruloids displaying symmetric versus asymmetric gene expression was contrasted with the expected frequency based on the expression of these genes in wild-type embryos (Mox1: 100% symmetric; Nodal and cerberus: 100% asymmetric). The observed proportions of Nodal and Cer1 expression pattern in gastruloids were then compared to those of Mox1, using the latter as expected frequency for laterally symmetric expression. The Wilson/Brown hybrid test was used to determine the confidence interval.

**Data availability**

All RNA-seq datasets produced in this study are publicly available in the Gene Expression Omnibus (GEO) database under accession code GSE106227 (for gastruloids) and GSE113885 (for embryos). All the scripts used for the analyses of the RNA-seq data are freely available upon request.

Extended Data Fig. 1 | z stacks used for 3D rendering of gastruloids.

a–d, Gastruloids produced using Gata6\textsuperscript{H2B-Venus} ESCs treated with a pulse of the GSK3 inhibitor Chi between 48 h and 72 h AA and fixed at 48 h (a), 72 h (b), 96 h (c) or 120 h AA (d) and imaged by confocal microscopy. BRA and SOX2 proteins are stained in red and white, respectively. VENUS signal (green) reports Gata6 expression and Hoechst 33342 (blue) marks the nuclei. Gastruloids correspond to the 3D renderings shown in Fig. 1a.

Each fluorescent channel is displayed to the right of the merged image. Gata6 (a) or Gata6 and SOX2 (b) signals were undetectable, and are therefore not shown. Three z sections are shown for each gastruloid. The bright-field outline of each gastruloid is indicated by the dashed lines. Each panel is representative of an experiment performed in parallel in seven independent biological replicates showing the same expression pattern. Scale bars are as indicated.

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Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Transcriptional profiling of mouse embryos and gastruloids. **a.** Heat map showing the temporal evolution of 97 out of the 250 most variable genes throughout embryonic development from E6.5 to E9.5 (left) and their corresponding expression over the gastruloid time course, from 24 h to 168 h AA (right). Expression levels are indicated by colour scale from blue to red (bottom left). Genes were clustered according to their expression behaviour in the embryo. Enriched GO term categories were identified for each cluster using the Gorilla and REVIGO tools (see Supplementary Information dataset 1). Finally, a functional classification of each cluster was established based on the identified GO term categories and literature-based evidence. **b,** Expression of markers for different embryonic tissues through the gastruloid time course. The two replicates of each time point are represented by a triangle and a circle, respectively. The black dotted line in each plot represents the average behaviour of the genes displayed in the plot. For gastruloids, \( n = 2 \) independent biological replicates per time point; for E6.5 and E7.8 embryos, \( n = 3 \) independent biological replicates; for E8.5 and E9.5 embryos, \( n = 2 \) independent biological replicates. **c,** PCA analysis of RNA-seq datasets from either pooled or individual gastruloids using the top 1,000 most highly expressed genes. Despite different strategies used for RNA-seq of pooled versus individual gastruloids (accounting for the sample segregation across PC1), their clustering illustrates both the homogeneity of gastruloid cultures and the representativeness of pooled samples to single gastruloid samples. For individual gastruloid RNA-seq: \( n = 10 \) independent biological replicates per time point.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Gastruloids display spatio-temporal organization in the expression profiles of neural, mesodermal and endodermal marker genes. a–f, The expression profiles of several genes usually expressed in the embryonic neural, mesodermal and endodermal domains were analysed by plotting RNA-seq data from the pooled gastruloids in heat maps of scaled gene expression (2 independent biological replicates per time point) (a, c, e) and/or by WISH (b, d, f). a, b, Genes usually expressed in different types of mesoderm precursors in the embryo (for example, Tcf15 in paraxial somatic mesoderm, Osr1 in intermediate mesoderm, Bra in tail bud, notochord and presomitic mesoderm, and Pecam1 in lateral plate mesoderm) were expressed in reproducible and spatially restricted domains within the gastruloids. c, d, Expression of different neural markers was detected in our RNA-seq (c). Transcripts of genes such as Lfng or Irx3 formed continuous and homogenous domains located in the central and dorsal portion of the gastruloids, reminiscent of their expression domains in the embryo (d, top panels). Genes involved in Notch signalling in neural progenitors (Hes5, Dll1) and in the terminal differentiation of neural precursors (Phox2a, Mnx1) displayed a salt-and-pepper expression pattern, consistent with the lack of an organized neural-tube structure (see also Extended Data Figs. 4a, c, 5). However, the latter mRNAs also displayed a graded distribution along the anterior-to-posterior extension of the gastruloid axis and were absent from its posterior half (empty red arrowheads). e, f, Endoderm-specific genes were also expressed in gastruloids. In particular, genes expressed in the embryonic digestive tract were consistently found on the ventral side of gastruloids. For each gene, the proportion of gastruloids displaying the reported expression pattern is shown in the upper right corner of the image, expressed as a fraction of the total number. Experimental statistics are provided in Supplementary Information dataset 3. Scale bar, 100 µm.
Extended Data Fig. 4 | Tissue organization in gastruloids. **a**, Gastruloids formed from Sox1\(^{GFP}\);Br\(^{mCherry}\) (SBR) line and stained for Sox2 expression (Sox1\(^{GFP}\) and SOX2 signals are displayed in green and magenta, respectively). White arrowheads indicate tubular SOX2/Sox1-positive neural structures. Red arrowheads point to the presumptive digestive tube. **b**, WISH on 8-\(\mu\)m transverse cryosections of gastruloids at 144 h AA using Sox2 and Meox1 antisense probes, counter-stained with Nuclear Fast Red. Sox2-positive cells localized predominantly in a compact dorsal domain, whereas Meox1 signals were found in two bilateral domains. The domain of expression of each gene is outlined with white dashed lines. **c**, Haematoxylin and eosin staining of transverse paraffin sections of different gastruloids at 120 h AA, showing the diversity of cell types and several levels of tissue organization. **d**, Gastruloids formed from Sox1\(^{GFP}\);Br\(^{mCherry}\) ESCs were fixed and stained at 168 h AA for OLIG2 (top, white), PAX3 (middle, red) and PAX7 (bottom, red). Scale bars as indicated. **c**, **d**, Gastruloids formed from Sox1\(^{GFP}\);Br\(^{mCherry}\) ESCs collected at 168 h AA and stained for SOX17 (magenta, **c**) or CDX2 (magenta, **d**). Scale bars as indicated. All immunostaining experiments were repeated twice, with three biological replicates per experiment, with similar results.
Extended Data Fig. 5 | Double-FISH staining shows organized gene expression across the three main gastruloid axes. a–e, Double-FISH staining of gastruloids at 144 h AA with Meox1 and Cyp26a1 (a), Sox2 and Shh (b), Sox2 and Meox1 (c), Meox1 and Hoxd4 (d) or Sox2 and Hoxd4 (e). a–e, Experiments were repeated twice in three biological replicates with similar results. Scale bar, 200 µm.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | A node-like structure and left–right asymmetry in gastruloids. **a, b,** Gastruloids formed from Nodal^{YFP} ESCs were fixed at 120 h AA. They were stained for CDX2, YFP (Nodal^{YFP}) and E-cadherin (a, top panel), CDX2, YFP (Nodal^{YFP}, green) and phalloidin (a, bottom panel) or for CDX2, YFP and E-cadherin (both with an Alexa-488 secondary antibody), and SOX2 (b). Maximum intensity projection of a representative gastruloid in b, with the node-like structure highlighted. Hoechst 33342 labels the nuclei (greyscale in a, blue in b). Data are representative of one experiment performed in three independent biological replicates. **c, d,** In situ hybridization showing expression of the indicated genes in gastruloids at different time-points AA. *, presumptive node-like cells. White arrowheads point towards Nodal-expressing cells distributed asymmetrically, on the lateral side of the gastruloid. Whereas Nodal was expressed in the presumptive node region from 96 h AA, no clear asymmetry in transcript distribution was observed at that stage. **e,** Three-dimensional renderings of confocal stacks of 120 h gastruloids containing a Nodal^{YFP} reporter gene (green) and stained for SOX2 (white) and BRA (red) proteins. SOX2 signal identifies dorsal cells. Left and right panels show the same gastruloid, imaged from two different polar directions that is, top (dorsal) and bottom (ventral) or ‘left’ and ‘right’, depending on the orientation of the gastruloid. Insets in specific panels show a cross-section through the gastruloid at the indicated z plane. White arrowheads indicate the region of biased Nodal expression. Empty white arrowheads point to the node-like cells marked by the Nodal^{YFP} reporter gene (see also Fig. 4d). These results are consistent with the asymmetric distribution of Nodal transcripts at 120–144 h AA. **f,** In situ hybridization showing expression of Cer1 in 120 h AA (left) and 144 h AA (right) gastruloids. The gastruloid midline is marked by a dashed white line. At this stage, Cer1 is expressed in the presumptive embryonic somitic territory⁴⁷ and the pattern in gastruloids may reflect this specificity. In c, d and f, the proportion of gastruloids displaying the reported expression pattern is shown at the bottom left corner of each image, expressed as a fraction of the total number of specimens analysed (see Supplementary Information dataset 3 for a complete statistical report).
Extended Data Fig. 7 | Z stacks used for 3D rendering of gastruloids. 

**a**, Dorsal (a) and ventral (b) sections of the same representative gastruloid shown in the 3D renderings in Fig. 3d, fixed and stained at 120 h for Nodal<sup>YFP</sup> (green), BRA (red) and SOX2 (white). Hoechst 33342 was used to label nuclei. Data are representative of two independent experiments with n = 13 biological replicates in total (see Supplementary Information dataset 3 for a detailed statistical report). Scale bar, 100 µm.
Extended Data Fig. 8 | Hox expression profiles in mouse embryos and gastruloids. a, Heat map of unscaled gene expression in E6.5–E9.5 mouse embryos, showing levels of Hox gene transcripts over time. Between 2 and 3 independent biological replicates were used for each time point (indicated below each graph). b, RNA-seq mapping showing Hoxa and Hoxd gene expression in these embryos. After a first wave of transcription of 5′ Hoxa and Hoxd genes, which is likely to reflect their activation in extra-embryonic tissues, the HoxA and HoxD clusters were progressively transcribed between E7.8 and E9.5, when expression of Hox13 paralogues was detected. Each profile was averaged from independent biological replicates indicated in a. c, Heat map of unscaled gene expression in pooled gastruloids, showing Hox gene transcript levels over time. Two independent biological replicates were used per time point. d, RNA-seq mapping showing Hoxd gene expression in pooled gastruloids at different time points. Sub-groups of Hoxd genes are progressively activated between 72 h and 168 h AA, when expression of Hoxd13 starts to be detected (e). This resembles the temporal activation described in vivo (a, b). Each profile represents the average of two independent biological replicates. e, WISH of gastruloids collected at different time points, showing the detectable initiation of expression of different Hoxd genes. Each panel shows the earliest stage at which the indicated gene was detected (black arrowhead). Expression of Hoxd4 was already strong at 96 h AA, indicating that its transcripts are rapidly upregulated compared to Hoxd9, which is expressed at low levels at this stage. Scale bar, 100 μm. The fraction of gastruloids displaying the reported expression pattern is indicated in the upper right corner of each image. Experimental statistics are provided in Supplementary Information dataset 3.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Homogeneity in Hox transcript profiles for individual gastruloids. a, PCA based on Hox-transcript datasets only, extracted from individually sequenced gastruloids across time points (10 individual organoids per time point, representing independent biological replicates). The analysis was carried out using the log₂-transformed FPKM+1 value of all 39 Hox genes. Replicate batches of organoids primarily cluster according to their age at collection. The clustering revealed the low sample-to-sample variation. however, replicates were clearly separated by the temporal parameter, representing 93.6% of total sample variation. b, Comparison of Hoxa (top) and Hoxd (bottom) gene-expression profiles among individual gastruloids confirmed the low inter-sample variation among time points, illustrated with the 120 h AA condition. c, WISH of 168 h AA gastruloids showing the expression of different Hoxa paralogues. The proportion of gastruloids displaying the reported expression pattern is shown in the upper right corner of the image, expressed as a fraction of the total number. Experimental statistics are provided in Supplementary Information dataset 3. Scale bar, 100 µm.
Extended Data Fig. 10 | Axial extension and spatio-temporal Hox expression patterns in iPSC-derived gastruloids. a, Dot plot representing the progression in the measured longitudinal extension of gastruloids produced either from ESCs or from iPSCs. In each case, 10 different gastruloids were measured at the different time points indicated. The median (round points) and the interquartile range (vertical bars) are reported. b, Light microscopy images showing representative examples of gastruloids at the different time points analysed in a. Zoom: 10×. Note that iPSC-derived gastruloids exhibit delay in their longitudinal extension rate and at 120 h AA they are markedly smaller than their ESC-derived counterparts. For this analysis, gastruloids were produced starting from the same number of cells (800 cells per well). c, Dot plots representing the Bra mRNA levels, showing comparable dynamics of this gene in both types of gastruloids. Circles represent individual data points and the short horizontal line represents the mean. The number of biological independent replicates (n) per condition is indicated. d, Confocal images showing the expression of Oct4, SOX2 and BRA (top) or of Oct4, SOX1 and CDX2 (bottom) in 120 h AA gastruloids derived from the iPSC line Oct4::Gfp (IpSL40N). iPSC-derived gastruloids were fixed and stained for SOX2 and BRA (top) and CDX2 and SOX1 (bottom). Oct4::GFP signal is shown in grey. Scale bar, 200 µm. In each case, data are representative of one experiment with three independent biological replicates. e, Dot plots representing the Hoxd mRNA levels in ESC- or iPSC-derived gastruloids collected at different time points AA. Each circle represents an independent biological replicate, the horizontal bars represent the mean value of the replicates. Both sets of gastruloids sequentially activated Hoxd gene expression. However, their temporal activation seemed to be delayed in iPSC gastruloids (especially that of the most 3′ Hoxd paralogues). f, WISH of 144 h AA gastruloids showing the expression of different Hoxd paralogues. Even though iPSC-derived gastruloids reproduced the antero-posterior Hoxd collinear expression, the Hoxd9 expression domain often extended more anteriorly in comparison to that in ESC-derived gastruloids (see Fig. 4c), occupying roughly the same domain as Hoxd4. Patches of Hoxd-negative cells were often observed within the Hoxd4/Hoxd9 expression domain (white). The fraction of gastruloids displaying the reported expression pattern is indicated in the upper right corner of each image. Experimental statistics are provided in Supplementary Information dataset 3. Scale bar, 100 µm.
Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

☐ ❌ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ ❌ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐ ❌ The statistical test(s) used AND whether they are one- or two-sided
   ✓ Only common tests should be described solely by name; describe more complex techniques in the Methods section.
☐ ❌ A description of all covariates tested
☐ ❌ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐ ❌ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐ ❌ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
   ✓ Give P values as exact values whenever suitable.
☐ ❌ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐ ❌ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐ ❌ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
☐ ❌ Clearly defined error bars
   State explicitly what error bars represent (e.g. SD, SE, CI)

*Our web collection on statistics for biologists may be useful.*

Software and code

Policy information about availability of computer code

Data collection

Confocal image acquisition was performed with Zeiss Zen2010 v6; ImageJ/Fiji and Imaris were used for confocal data processing.

Data analysis

R studio Version 1.0.153 and 1.0.143, implementing the R software version 3.2.4 (2016-04-14) or 3.3.3 (2017-03-06), was used for RNA-seq data processing and statistical analysis. Libraries Ade4, ggplot2_2.2.1, RColorBrewer_1.1-2, pheatmap_1.0.8, genefilter_1.56.0, ape_4.1, cluster_2.0.6, phytools_0.6-30, factoextra_1.0.4, FactoMineR_1.36, reshape2_1.4.2 and DESeq2_1.14.1 were used alongside base packages for the statistical analysis and visualisation of RNA-seq data.

Graphpad prism 6 was used for the plotting and statistical analysis of the qPCR results, gastruloid size mesurement and Nodal/assymetry statistics and graphics.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Provide your data availability statement here.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/Reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No statistical methods were used to predetermine sample sizes. For gene expression pattern analysis by in situ hybridization or immunohistochemistry a number of gastruloids was used to test the reproducibility of the results across biological replicates and is indicated on their corresponding panels of the figures. For pooled organoids or embryonic transcriptomic studies we used at least two biological replicates. This number was enough to identify significantly variant genes across time-points, with little variance between replicates. For individual organoids RNAseq analysis we used 10 independent biological replicates in order to have an estimation of the variability in gene expression levels across replicates.

For qPCR, gene expression analysis in ES and iPS cells at least two technical (PCR) replicates and at least 2 biological replicates were performed.

Data exclusions

No biological replicate was excluded from the RNAseq analysis. Mitochondrial and non-autosomal genes were excluded as they were not relevant for the biological question addressed in this study. This exclusion criteria was established before the analysis of the data.

Replication

All the data presented in this study were reliably reproduced with the exception of the reported Hoxa13 expression pattern in 168AA gastruloids (Figure 4e) which was scored only in one gastruloid out of the 20 analyzed. The frequency of the reported expression pattern is stated in the description of the results. For the other gene expression pattern described in this study the frequency of the pattern displayed in each panel is reported.

Randomization

Gastruloids for the different time-points analyzed in this study were allocated randomly. Only gastruloids showing clear signs of apoptosis were removed from the experimental group before processing/analysis of the samples.

Blinding

Blinding was not relevant to the present study since any specific assumption or hypothesis was postulated a priori, nor was any specific comparison between different treatments or genotypes performed.

Reporting for specific materials, systems and methods

Materials & experimental systems

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<td>Antibodies</td>
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Methods

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<td>MRI-based neuroimaging</td>
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Unique biological materials

Obtaining unique materials

Gata6::H2B-Venus ES cell line is available upon request to Alfonso Martinez Arias /Christian Schröter’s laboratories. Bra::GFP ES cell line is not publicly available and was obtained through mutual transfer agreement with the Keller’s lab. Nodal::YFP cell line is not publicly available and was obtained through mutual transfer agreement with the Collignon’s lab. SBR ES cell line is freely available from David Suter’s group upon request. E14Tg2A ES cell line is freely available from Austin Smith’s group upon request.

Antibodies

Antibodies used

Antibodies used in this study were: anti-Brachyury (Santa Cruz Biotechnologies, sc-17749), anti-CDX2 (Thermo Fisher, MA5-14494), anti-E-Cadherin (Takara, M108), anti-GFP (Molecular Probes, A11122), anti-Olig2 (Merck Millipore, AB9610), anti-Pax3 (DSHB), anti-Pax7 (DSHB), anti-Sox17 (R&D Systems, AF1924), anti-Sox2 (Millipore, AB5603), anti-Chicken-A488 (Molecular Probes, A11039), anti-Goat-A568 (Molecular Probes, A11057), anti-Rabbit-A647 (Molecular Probes, A31573), anti-Digoxigenin-AP (Roche, 11093274910), anti-Digoxigenin-HRP (Perkin Elmer, NEF832001EA), anti-Fluorescein-HRP (Perkin Elmer (NFF710001FA)).

Validation

Antibodies were previously validated in Turner DA et al 2017.

Eukaryotic cell lines

Cell line source(s)

Gata6::H2B-Venus ES cell line was generated by the Alfonso Martinez Arias /Christian Schröter’s laboratories. Bra::GFP ES cell line was obtained from Keller’s Lab. Nodal::YFP cell line was obtained from the Collignon’s Lab. SBR ES cell line was obtained through mutual transfer agreement with David Suter Lab. E14Tg2A ES cell line was obtained from Austin Smith’s group.

All cell lines used in this study have the 129 genetic background.

Authentication

For the Bra::GFP ES cell line: Stimulation with Activin/ChR showed an increase of the GFP reporter which overlapped with the signal of anti-T/Bra antibody. Nodal::YFP, expression of the reporter was up-regulated after Activin stimulation, while its expression was blocked by Nodal inhibitor SB433. Gata6::H2B-Venus and SBR ES cell lines were validated by genotyping and via co-staining with Gata6 and Bra antibodies respectively.

Mycoplasma contamination

All cell lines were tested regularly and confirmed free of mycoplasma. We scored a low percentage of reads (0.11% max of the total number of reads) mapping into different mycoplasma genomes. We attribute this percentage to low levels of contamination during gastruloid collection/RNA extraction.

Commonly misidentified lines

No cell lines used in this study are in the data-base of commonly misidentified cell lines.

Palaeontology

Specimen provenance

Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).

Specimen deposition

Indicate where the specimens have been deposited to permit free access by other researchers.

Dating methods

If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance or state that no new dates are provided.

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research.

Laboratory animals

Mouse embryos for RNAseq and WISH experiments were obtained from CD1 wild type animals grown crossed in house. Adult animals of 3-5 month old were used for the crosses. Embryos were collected at E6.5, E7.8, E8.5 and E9.5 dpc. All experiments were performed in agreement with the Swiss law on animal protection (LPA) under license number GE 81/14 (to D. Duboule).

Wild animals

Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals...
Wild animals

were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method, if released, say where and when) OR state that the study did not involve wild animals.

Field-collected samples

For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state the study did not involve samples collected from the field.

Human research participants

Policy information about studies involving human research participants

Population characteristics

This study did not involve human research participants

Recruitment

Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

For "initial submission" or "revised version" documents, provide reviewer access links. For your "final submission" document, provide a link to the deposited data.

Files in database submission

Provide a list of all files available in the database submission.

Genome browser session

Provide a link to an anonymized genome browser session for "initial submission" and "revised version" documents only, to enable peer review. Write "no longer applicable" for "final submission" documents.

Methodology

Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth

Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the CHIP, control and index files used.

Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5 fold enrichment.

Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage [with statistics] is provided.

Methodology

Sample preparation

Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.

Instrument

Identify the instrument used for data collection, specifying make and model number.

Software

Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.
Cell population abundance: Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.

Gating strategy: Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

## Magnetic resonance imaging

### Experimental design

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<th>Indicate task or resting state; event-related or block design.</th>
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<td>Behavioral performance measures</td>
<td>State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).</td>
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### Acquisition

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<td>Area of acquisition</td>
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### Preprocessing

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<td>Normalization</td>
<td>If data were normalized/standardized, describe the approach(es); specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.</td>
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<td>Normalization template</td>
<td>Describe the template used for normalization/ transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.</td>
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<td>Noise and artifact removal</td>
<td>Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).</td>
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<td>Volume censoring</td>
<td>Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.</td>
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### Statistical modeling & inference

| Model type and settings | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation). |
| Effect(s) tested        | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |
| Specify type of analysis | Whole brain | ROI-based | Both |
| Statistic type for inference (See Eklund et al., 2016) | Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. |
| Correction | Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). |

### Models & analysis

<table>
<thead>
<tr>
<th>n/a</th>
<th>Involved in the study</th>
<th>Functional and/or effective connectivity</th>
<th>Graph analysis</th>
<th>Multivariate modeling or predictive analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional and/or effective connectivity</td>
<td>Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).</td>
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<tr>
<td>Graph analysis</td>
<td>Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).</td>
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<tr>
<td>Multivariate modeling and predictive analysis</td>
<td>Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.</td>
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</tr>
</tbody>
</table>