Boundary constraints can determine pattern emergence

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SUMMARY

The robust patterning of cell fates during embryonic development requires precise coordination of signalling gradients within defined spatial constraints. Using a geometrically confined *in vitro* system derived from human embryonic stem cells, we demonstrate that patterning of neuromesodermal progenitors (NMPs) during axial elongation is driven by boundary-dependent mechanisms. A minimal reaction-diffusion model coupled with a simplified gene regulatory network accurately predicts spatial patterns across diverse geometries. We identify Wnt signalling as a key component of the activator signal. Inhibition of Wnt secretion preserved initiation of patterning but disrupted subsequent morphogenesis, indicating distinct mechanisms govern pattern establishment versus maintenance. Our findings reveal how geometry encodes positional information that directs molecular patterning, providing insight into how spatial constraints and signalling dynamics guide robust tissue self-organisation during development.

KEYWORDS

embryonic stem cells, organoid, reaction-diffusion, patterning, phenomenological model

INTRODUCTION

Embryonic development relies on robust collective self-organisation to sculpt complex tissue architectures. Cells display a broad range of behaviours, including differentiation, proliferation and migration[1]. Such processes typically occur within spatially constrained environments, either in the form of surrounding tissues, an external shell, or ECM compartmentalisation. Physical boundaries shape key aspects of development, including morphogen gradient distribution, cell positioning, and force generation[2–6].

Such factors can impact early development. For example, spatial confinement of blastomeres governs their interactions and signalling environment[7]; physical constraints imposed by the shell are essential for consistent and robust blastomere positioning in *C. elegans*[8]. Boundaries continue to influence development beyond early stages, guiding large-scale tissue organisation and body axis formation. In vertebrate embryos, the body axis progressively extends through a process of axial elongation, during which neural and mesodermal tissues are laid down in precise

proportions and spatial domains along the anterior-posterior axis[9–12]. Volumetric growth during elongation is driven by multipotent axial progenitors in the tailbud, which continuously differentiate and exit the progenitor zone. The posterior presomitic mesoderm (pPSM) exhibits intrinsic motility, believed to generate the forces required for elongation; however, directional tissue extension only arises in the presence of physical boundaries[13,14]. When cultured *ex vivo*, pPSM tissue elongates uniaxially only under confinement, demonstrating that spatial constraints are essential for guiding morphogenesis[15]. These boundary conditions may also influence the progenitor zone itself, suggesting a feedback loop in which confinement helps shape progenitor behaviour and cell fate transitions in response to mechanical cues[16].

Due to the difficulty of isolating cellular behaviours, tissue mechanics, and confinement effects *in vivo*, the role of boundaries in vertebrate patterning and axial elongation remains poorly understood. To address this challenge, several *in vitro* 2D organoid models derived from pluripotent stem cells (PSCs) have been developed, using substrate micropatterning to impose spatial constraints. These provide a tractable, reproducible system to study developmental mechanisms (reviewed in[17]). Systems such as 2D gastruloids generate robust, concentric patterns of cell fates - an organisation not seen under unconstrained conditions[12,18,19]. In neuruloids - a micropatterned model of human trunk formation - cycles of differentiation generate spatially organised cell fates. Mesodermal populations typically form near colony edges and neural cells towards the centre - a behaviour reminiscent of posterior tissue arrangement *in vivo*[20]. Transcriptomic analysis confirmed that day-3 neuruloids had posterior identity characteristic of mid- to late-stage axial elongation, comprising cell types such as pPSM, neural progenitors, and axial progenitors. Under confinement, the first symmetry-breaking event in neuruloids is the emergence of a ring of cells at the colony edge expressing the primitive streak and mesoderm-associated transcription factor Brachyury (TBXT). Later, neuruloids displayed a remarkable transition from a 2D monolayer into a complex 3D structure three days after induction. The initial patterning of cell fates appears to be influenced by boundary cues, which may act as key upstream signals that guide and enable the later morphogenetic transition.

Alongside improvements in experimental models of tissue patterning, there have recently been significant advances in theoretical approaches to understand cell fate determination[21] and robust patterning[22,23]. Reaction-diffusion theory, originally proposed by Alan Turing[24], remains one of the most powerful and widely used frameworks for modelling spontaneous symmetry breaking and pattern formation in initially homogeneous systems. Alongside Wolpert's concept of positional information[25,26], such models have successfully captured key features of diverse biological processes[22,27,28]. Notably, these models are particularly well-suited for explaining phenomena where patterns emerge without pre-existing spatial cues. Despite differences in biological context and specific mechanisms of each system, reaction-diffusion models have been applied to study the formation of biological patterns across a range of spatial scales, from cellular[29], to tissue[30–32], and up to population dynamics in ecological systems scales[33]. Importantly, this approach can make quantitative predictions about the system behaviour without requiring precise molecular or mechanistic details[22,23,31,34]. However, it remains unclear how well a minimal reaction-diffusion model can account for spatially constrained patterning in systems where symmetry is broken within defined geometric boundaries.

In this study, we show that the width of the region expressing TBXT remains constant regardless of the sizes of the micropatterns for posterior neuruloids grown within circular domains. This suggests that cell fate patterning is induced by a signal (*e.g.*, morphogen) at the boundary with a fixed length scale[25,35]. Further, morphogen-driven patterning precedes cell differentiation and migration[36,37]. These observations motivated a phenomenological model to describe how boundary constraints shape the concentration profile of signalling molecules. Combined with a minimal gene regulatory network (GRN), the model was able to predict how boundaries affect the balance between competing cell fates, marked by the neuromesodermal expressed genes

SOX2 and TBXT. Spurred by these predictions, we show that a gradient of Wnt activation forms prior to TBXT pattern emergence. Interestingly, blocking Wnt ligand secretion did not prevent the initiation of TBXT patterning, but did impair later morphogenetic processes, suggesting that Wnt pathway activation alone is sufficient for early fate patterning but ligand secretion is essential for subsequent tissue organisation.

Overall our findings demonstrate that boundary constraints can provide positional information through reaction-diffusion principles, offering insight into the architecture of the gene regulatory network that underlies spatial pattern emergence.

RESULTS

A phenomenological reaction-diffusion model robustly captures neuruloid patterning

To investigate initial pattern emergence during vertebrate trunk formation, we used *neuruloids*, an organoid model of posterior trunk development[20]. Human embryonic stem cells were seeded onto circular, laminin-coated micropatterns to impose spatial confinement. Starting from a homogeneous population, neuruloids self-organised over the course of three days[18,20,38]. Specifically, the initially uniform layer of pluripotent SOX2-expressing cells differentiated following induction with Wnt/Fgf agonists and BMP/NODAL inhibitors (Fig. 1A), leading to the emergence of axial progenitors — neuromesodermal progenitors (NMPs) – precursors to posterior neural and trunk mesodermal lineages[20]. Over its development, the monolayer underwent morphological changes as a second layer of cells gradually emerged at the neuruloid periphery, transitioning the structure into 3D (Fig. 1B, lower panel). Prior to this morphogenetic transition, neuruloids exhibited an initial symmetry-breaking event: TBXT expression became restricted to a ring at the colony edge, marking the onset of mesodermal differentiation (Fig. 1A-B.). *In vivo*, the presomitic mesoderm drives mechanical elongation[13]. Hence, this early pattern of mesodermal induction, which appears to be boundary-localised, may contribute to initiating the morphogenetic changes that follow.

To test the hypothesis that TBXT expression is induced by proximity of the cells to the boundary, we reasoned that the width of this region should be invariant to changes in colony size. We examined this by varying the diameter of the micropatterns and analysing the cell fate patterning in different colony sizes. We found that despite substantial tissue size differences, a similar radial pattern consistently emerged 24 hours post-induction at the neuruloid periphery (Fig. 1C and S1A). By quantifying the width of the TBXT-positive ring in 24 to 48-hour neuruloids, we found that the spatial extent of TBXT expression pattern was invariant to changes in system size (Fig. 1C and S1B-C); the width of the TBXT ring remained consistent, ranging from 40–70μm with no significant differences in width between colony sizes (Fig. S1B). These observations suggest that TBXT patterning occurs within a characteristic distance from the boundary, consistent with a morphogen gradient of fixed range driving pattern emergence. Therefore, we hypothesised that the sensitivity of cells to signalling molecules to the boundary, either as a source or sink, determined the region of TBXT expression.

To gain theoretical insight into how such a mechanism would pattern colonies, we hypothesised a reaction-diffusion mechanism to explain patterning (Fig. 1D). This consisted of two coupled partial differential equations (PDEs), which capture the interaction between a diffusible activator (of TBXT) and its endogenous inhibitor (Fig. S1D). A key question is which boundary conditions best encode the observed effects. The ability of cells to respond to colony edges has been demonstrated previously in micropatterned colonies, with the main edge-sensing mech-

anism proposed to be receptor accessibility to the activating ligand[18,19,39] (Fig. S1D). However in our system, a key activator is Chiron, a Wnt agonist that acts independently of cell surface receptors. Despite this, neuruloids clearly exhibit edge-sensing. To model edge detection, we applied a mixed boundary condition[40]: a fixed activator concentration to impose a uniform state at the boundary, representing constant cellular sensitivity to signalling, and a no-flux condition for the inhibitor to reflect its endogenous secretion (Fig. S1D). Fixing the activator concentration at the boundary isolates pattern formation to the interior of the domain. By enforcing the role of a consistent boundary, this reduces sensitivity to initial perturbations, thus promotes robust and biologically meaningful patterns[40]. The equations were solved numerically on a two-dimensional domain using a finite element method, yielding the predicted steady-state activator concentration profile across the domain (Supplementary Material, S2 for details).

We performed a sensitivity analysis of the model, to explore the patterns that can be obtained from changing the domain size and strength of the fixed activator concentration at the boundary relative to concentrations at equilibrium (Fig. 1E and S1E). We focused on two regions, highlighted by dashed boxes in Fig. 1E. In the first region (black box), we identified parameter sets that produced radially symmetric patterns resembling the TBXT expression observed in neuruloids. This pattern is inverted when boundary input is lower than the concentration in equilibrium, concentrating high activator levels at the centre rather than the boundary. As the domain size is increased, the activator concentration profile on a normalised spatial domain steepened (Fig. 1F, right). This aligns with the earlier observation that TBXT-positive regions maintain a consistent width, regardless of the overall size of the micropattern. As a result, in larger micropatterns, TBXT expression appears more restricted relative to the total area (Fig. 1C and S1B). Further increasing the domain size in the model led to multiple foci of high activator concentration (Fig. 1E, red dashed box). Other parameter sets led to different patterns, including stripes and concentric rings (Supplementary Material, S2 for details).

We experimentally tested the prediction that larger domains would lead to TBXT clusters in the cell population by seeding the cells at the same density on a much larger substrate (18mm diameter). In agreement with the simulation predictions, we observed the formation of discrete clusters of cells expressing high TBXT (Fig. S2A). Quantifying the distribution of the cluster sizes, we found that most clusters had diameters of 50-100μm, consistent with the range of TBXT expression width observed on circular micropatterns (Fig. 1B and S2B). When the domain size was constrained, no clusters formed within the micropattern, and only cells near the edge up-regulated TBXT, consistent with a reaction-diffusion mechanism in which larger domains can accommodate more pattern repetitions. In summary, our phenomenological reaction-diffusion model effectively captures the TBXT spatial patterning observed in neuruloids with a length scale set by the system boundary.

Predicted activator profiles align with geometry-dependent patterning

If boundary constraints are determining the domain of TBXT expression then altering colony geometry should impact the TBXT pattern. We first parameterised our model using the circular domains as calibration data. To do this, we quantified the TBXT signal across multiple circular domains, accounting for variations in cell density by normalising using DAPI signal (Fig. S2C-E). Since the reaction-diffusion system here is a phenomenological model, we fixed parameters such as activator concentration at boundary that qualitatively replicated the observed TBXT radial patterns through sensitivity analysis (Fig. S1E). This reduced the model to a single-free parameter. We fitted the simulated activator profile to the TBXT concentration (Fig. 2A) to find the relation between the dimensionless length scale of the model, γ to the change in micropattern domain size. More details on the dimensionless parameters of the model and fitting are in

the Supplementary Material, Section S2. We kept all the parameters and the relation between the length scale, γ and area of micropattern constant in our analysis below.

To investigate the influence of geometric confinement on pattern formation, we experimentally determined the TBXT profiles of neuruloids cultured on micropatterns of various shapes: annuli, triangles, and squares (Fig. 2B and S3A). With the exception of annuli, all shapes were matched in area to the circular domains which were previously used. Annulus-shaped micropatterns featured both inner and outer boundaries. Notably, TBXT expression emerged at both boundaries, providing further evidence that edge-associated cues can drive self-organisation (Fig. 2B top row). Conversely, triangle and square geometries allowed us to assess whether the presence of straight edges and sharp vertex angles influence the positioning of cell fate boundaries. In general, pattern formation on these shapes displayed TBXT profiles and band widths comparable to those on circular micropatterns (Fig. 2B and S3B-C). We observed an expansion of the TBXT signal near corners. This effect was more pronounced on triangular domains than on squares, suggesting that sharper angles enhanced TBXT induction (Fig. S3B). This is consistent with the effects at acute angles, where there are overlapping signals from adjacent boundaries. This increases the amplitude near these sharp corners and hence the range of signalling due to the diffusion process. This further suggests that rather than a pure mechanical effect of edge sensing, a diffusive molecular signal emanating from the boundaries may underlie the enhanced width of TBXT patterning at sharp corners[41].

When we compared experimental TBXT expression with predictions from the reaction-diffusion model, we found good agreement in the overall trend between their spatial profiles (Fig. 2B right). The greatest mismatch between experiment and predictions occurred on the smallest square geometries. This may arise because of the differences in the 3D morphology acquisition of these neuruloids.

A minimal gene regulatory network predicts curvature-dependent SOX2 and TBXT patterning

We have so far assumed that the boundary signal activates TBXT expression. However, the underlying interactions between downstream transcriptional regulators are typically more complicated[21,42,43]. To investigate how activator concentration regulates the expression of these genes, we developed a minimal model with ordinary differential equations (ODEs) to capture the gene regulatory network (GRN) of SOX2 and TBXT. As we are principally interested in the initial symmetry breaking event, we focused only on the early interactions of genes of interest in the system and excluded contributions from other downstream genes.

The balance between SOX2 and TBXT expression is crucial for maintaining the progenitor state of NMPs. However, during lineage commitment, these transcription factors act antagonistically[44,45]. This mutual inhibition, together with local Wnt/FGF signalling levels, plays a key role in directing cell fate toward mesodermal (high TBXT) or neural (high SOX2) lineages[46]. Accordingly, in our ODE model, SOX2 and TBXT mutually repress one another (Fig. 3A). We hypothesised that the activator stimulates TBXT expression. Thus, in this GRN, the activator concentration from the reaction-diffusion model (step 1) is a direct input to the ODE model (step 2), providing spatial information (Fig. 3A, Methods and Supplementary sec S4). Given that SOX2 is highly-expressed in self-renewing stem cells, we incorporated self-activation of SOX2 into the model to reflect its role in maintaining pluripotency and sustained expression in the absence of differentiation cues[47].

In this two-step model, we assumed that chemical reactions and diffusion occurred on faster timescales than the gene regulatory interactions. With this separation of time scales, we solved the reaction-diffusion model to steady-state to obtain the stable spatially-varying activator con-

centration. This was then input into the ODE model governing SOX2 and TBXT dynamics. We explored how the activator concentration varied the balance of SOX2 and TBXT expression levels. With varying activator concentration, the model exhibited a bifurcation behaviour, capturing bistable states at intermediate levels of activator concentrations (Fig. 3B). There existed fixed points with co-expression of SOX2 and TBXT, representing the NMP state. At higher activator concentrations, the trajectories consistently transitioned towards a mesodermal fate, characterised by high TBXT expression with no SOX2 expression (Fig. 3B).

We fitted our two-step model, using Markov chain Monte Carlo (MCMC) methods, to TBXT intensity profiles from 24-48 hour neuruloids, where spatial patterning was prominent prior to the onset of 3D morphogenesis (Fig. 3C and S4A-B and Methods). Extracting the transcription and degradation rates parameters for SOX2 and TBXT from MCMC, our two-step model resulted in a better fit to the experimental TBXT intensity data (Fig. 3C) compared with considering the activator alone, as expected. This fitting procedure also yielded an estimate of the activator concentration profile required to obtain the observed TBXT expression (Fig 3C orange dashed lines).

To further investigate the role of boundary geometry in pattern formation, neuruloids were generated on micropatterns with more complex boundary properties (Fig. 3D-E). We used “sharp star” and “curved star” domains, which include both convex and concave edges in the micropattern. These geometries allowed us to compare similar shapes with identical surface areas, isolating the effects of vertex sharpness and curvature type (positive vs. negative). The two domains have different degrees of curvature. Under the assumption that signalling is primarily boundary-driven, we hypothesised that regions near sharper boundaries would show increased sensitivity to the activator, as they received input from two adjacent boundary segments. In contrast, regions near concave boundaries would show reduced sensitivity, as cells are positioned further from the nearest boundary source.

We examined SOX2 and TBXT patterns at 24 and 48 hours post-induction in these domains. At these time points, neuruloids patterned on ”sharp star” and ”curved star” geometries exhibited striking changes in expression (Fig. 3D-E). On both geometries, we observed TBXT up-regulation within the ’limbs’ of the shape over time (indicated by magenta arrows in Fig. 3E). At 48 hours, this effect was more pronounced at sharp vertices, consistent with previous observations on the triangle domain (Fig. 2B and S4C-D). SOX2 expression was notably down-regulated along these same regions. Consistent with our prediction, concave edges appeared to have a weaker influence on SOX2 and TBXT patterning compared to convex ones. A similar, though subtler, effect was seen in the annulus domains, where TBXT intensity was lower along the concave inner boundary than the convex outer edge (Fig. S3C).

These spatiotemporal patterns were successfully recapitulated by the two-step model (Fig. 3D-E lower panels) simulated on the corresponding 2D domains with identical parameters to those fitted from Fig. 3C. In particular, the model-predicted SOX2 and TBXT profiles were up-regulated and down-regulated in similar spatial regions as experimentally generated neuruloids. Given the predictive ability of our model parameterised on only a subset of the data (circular domains) to a range of different challenges with no further fitting, we were confident that the close agreement was not due to over-fitting.

To conclude, a minimal and mutually repressive gene regulatory network, downstream of a boundary-driven activation was sufficient to capture the emergence of SOX2 and TBXT patterns arising from a variety of geometric perturbations. We note that neuruloids are a complex self-organising system, and thus there are likely other factors contributing to patterning, yet our minimal model predicted global patterns under different boundary conditions.

Wnt secretion inhibition preserves initial patterning but disrupts subsequent morphological development

Our focus so far has been to develop a phenomenological model of observed cell fate patterns within neuruloids, assuming the existence of a general activator molecule. Wnt signalling has been shown to play a critical and concentration-dependent role in regulating TBXT expression[48]. Additionally, high local Wnt concentrations drive NMPs toward mesodermal fates by promoting the Wnt/FGF signalling cascade and expression of TBXT, whereas low levels of Wnt facilitate commitment to neural fates during vertebrate axial elongation[44,46,49]. Wnt and its inhibitor Dkk are well-established components of an activator-inhibitor system in the canonical Wnt/β-catenin signalling pathway[50], which has been previously modelled in the context of hair follicle spatial patterning[51]. Thus, we explored the possibility that the Wnt pathway may form at least part of the activator signal.

We first searched for Wnt signalling pathway components within transcriptomic data from day-3 neuruloids[20]. Ligand-receptor interactions involving Wnt3 and Dkk ligands[52] predominantly originated from mesoderm populations within the neuruloid system (Fig. S5A). Furthermore, a population of cells with high co-expression of Wnt, DKK and TBX6 (Fig. S5B), a transcription factor marking presomitic mesoderm cell fates, was present at later development times. Given that TBXT directly up-regulates TBX6, which then represses SOX2 to drive commitment of NMPs to mesodermal fates[44], this suggested that the emergence of TBXT patterning may be mediated by local variations in Wnt-Dkk activity.

To test this hypothesis, we used LEF1 as a readout of Wnt activation. As a downstream effector of the canonical Wnt/β-catenin signalling pathway[50], LEF1 serves as a proxy for the Wnt concentration gradient. The expression of LEF1 behaved similarly to TBXT in 24-hour neuruloids (Fig. 4A). The expression profiles of LEF1 (taken to represent the activator) and TBXT quantified for neuruloids of different micropattern sizes closely resembled the predictions from our two-step model (Fig. 3C and Fig. 4). To confirm that the time scales of gradient formation were much faster than cell differentiation, we checked LEF1 expression at 8 hours post-induction (Fig. 4B-C). Under control conditions, we obtained a clear LEF1 gradient without TBXT expression (Fig. 4C). The emergence of the 'activator' prior to the downstream patterning is consistent with our quasi-static approximation utilised above.

As canonical Wnt signalling is activated uniformly upon induction, we reasoned that spatial patterning might arise from endogenously secreted Wnt ligands[53]. To investigate this, we inhibited Wnt secretion using IWP2, a small molecule that blocks Porcupine-mediated Wnt ligand processing[54]. Initially, we saw a clear difference in LEF1 profiles 8 hours after induction, before the onset of TBXT (Fig. 4B-C). Surprisingly, at 24 hours post-induction, we observed no significant variations in the spatial expression of TBXT or LEF1 between control and IWP-treated conditions (Fig. 4D-E). This suggests that pattern emergence is not immediately disrupted by interference with Wnt secretion. However, by 48 hours, TBXT and LEF1 expression profiles in IWP-treated samples deviated markedly from controls (Fig. 4F-G), indicating a pronounced effect of Wnt inhibition on further pattern maintenance and development. Cross-sectional views at 48 hours post induction revealed striking morphological differences between the control and IWP2 treated conditions (Fig. 4G). In particular, the IWP2 treated condition did not display the characteristic 'doughnut' shape reported previously[20], which is also seen in our 48hr control data in Fig. 4G. This suggests that boundary-driven pattern emergence may initially rely on additional molecular cues, while Wnt signalling is crucial for maintaining proper development, shaping both morphogenesis and downstream spatial patterning.

DISCUSSION

Neuruloid pattern emergence can be quantitatively predicted from boundary-informed reaction-diffusion dynamics

We have quantitatively revealed how boundary constraints influence the spatial patterning of TBXT expression in neuruloids, an *in vitro* model recapitulating NMPs and their differentiation into neural and mesodermal cell types. By varying the size and curvatures of the micropatterned domains, we demonstrated that TBXT up-regulation is dependent on boundary geometry, exhibiting a consistent length scale from the colony edge. However, sharp convex curvatures led to increased TBXT strength and pattern width with a down-regulation of SOX2 compared to concave curvatures. This is consistent with a model in which gene regulation depends on the local boundaries. *In vivo*, neuromesodermal progenitors exhibit location-dependent fate decisions within the progenitor zone: anterior cells predominantly form neural tissue, while medial and posterior cells contribute to mesoderm[55]. Heterogeneous gene expression within confined geometries appears essential for robust axial elongation[10,11], supporting the view that developmental patterning arises from an interplay of signalling gradients, mechanical forces, and spatial constraints rather than biochemical signals alone[56]. Although a role for confinement has been proposed, direct links between boundary conditions and fate specification remain limited. Our findings offer quantitative evidence that physical boundaries help organise gene expression patterns and thus influence fate allocation in NMP populations.

Mechanisms of boundary sensing in organoid systems

Micropatterned organoid models have successfully recapitulated various stages and aspects of embryonic development *in vitro*, from gastruloids that model early germ layer formation by BMP-induced signalling[18,57] to neuruloids that use Chiron (CHIR) activation to study caudal cell fate patterning[20] for embryonic trunk formation. However, these systems have each been shown to sense boundaries through distinct mechanisms. In WNT-treated 2D gastruloids, early TBXT expression initially appeared throughout the colony before becoming edge-restricted, driven by E-Cadherin dynamics linked to radially varying cell density and β-catenin levels. Neuruloids, despite activation of Wnt signalling using CHIR, exhibit TBXT edge restriction. This is in contrast to previous findings where activation through CHIR instead of Wnt3 abolished spatial patterning in micropatterned hESCs[19]. Likewise, IWP2 eliminated TBXT in 2D gastruloids[58], but not in neuruloids, suggesting distinct sensitivities to WNT perturbation across *in vitro* models.

While these systems show distinct sensitivities to pathway perturbations, reaction-diffusion frameworks have proven remarkably versatile in describing pattern emergence across multiple micropatterned systems[59,60]. This convergence suggests that reaction-diffusion mechanisms represent a fundamental principle underlying biological self-organisation[31,61], thus providing a common theoretical framework to explain diverse patterning behaviours. These phenomenological models can generate testable predictions and capture complex spatial self-organisation even without complete molecular characterisation, as evidenced by successful applications in both developmental and skin patterning systems[23,34].

Molecular mechanisms underlying neuruloid self-organisation

Integrating the reaction-diffusion model with a minimal gene regulatory network successfully recapitulated several experimental observations, providing a rationale to investigate the underlying molecular mechanisms. The prediction of activator gradient using this model guided the search

for elements of the signalling pathway underlying the patterning. In probing the molecular identity of the activator in our reaction-diffusion model, our findings suggest that WNT signalling alone does not fully account for pattern emergence. TBXT patterning at early stages remains robust even when WNT secretion is inhibited, though later morphogenetic processes are clearly disrupted. This points to the involvement of additional pathways—such as FGF signalling or YAP-mediated mechanotransduction[20]—in early pattern emergence. This result has emphasised the distinction of the role of Wnt signalling on patterning establishment and maintenance. Since CHIR selectively activates canonical WNT signalling, the observed disruption of morphogenesis under WNT secretion inhibition suggests a role for non-canonical WNT pathways. This is supported by our single-cell RNA-seq data, which show higher expression of WNT5 than WNT3 in the mesodermal compartment, the main driver of morphogenesis in this system and *in vivo*[13].

FGF signalling, which directly interacts with Wnt/β-catenin pathways, is essential for posteriorising tissues, maintaining the bipotent NMP state, and promoting mesodermal differentiation[62,63]. In Xenopus, FGF has been shown to regulate expression of the TBXT homolog Xbra[64]. In neuruloids, FGF/ERK signalling patterns similarly to TBXT[20], and FGF receptors were recently found to localise basally on micropatterned gastruloids, akin to BMP receptors[57,65]. These findings raise the possiblity that FGF ligands contribute to the molecular basis of the reaction-diffusion system underpinning TBXT patterning. Further work will better inform the specific contribution of the FGF/Erk pathway in spatial self-organisation during NMP specification.

Overall, our combined systems approach - bringing together both experimental and theoretical approaches - provides a rigorous and predictive framework for understanding how changes in boundary constraints impact tissue self-organisation.

Limitations of the study

This study combines experimental and theoretical approaches to decipher how boundaries impact cell fate determination, but raises several questions.

First, our results show that Wnt signalling is important but not the sole contributory factor. Here, our focus has been on the role of boundaries, but identifying the other factors involved would increase our understanding of the patterning process. Second, how do cells ‘sense’ the boundary? There could be either (or both) mechanical or biochemical signals. While a role for biochemical cues in fate specification is well-established, recent work has highlighted the important role of mechanical feedback[66]. Yap signalling plays a role in neuruloid fate determination[20] but how this mechanistically (if at all) links to boundary effects is unknown. Finally, we have focused our analysis on a 2D system. Yet, after they have established patterning in 2D, neuruloids develop a complex 3D morphology over 3 days. Cell fate determination continues to evolve over this time period[20]. An extension to this study is to incorporate the GRN and cell fate specification to mechanical and morphological change of the neuruloids. This will then strengthen the relevance of this model to *in vivo* systems.

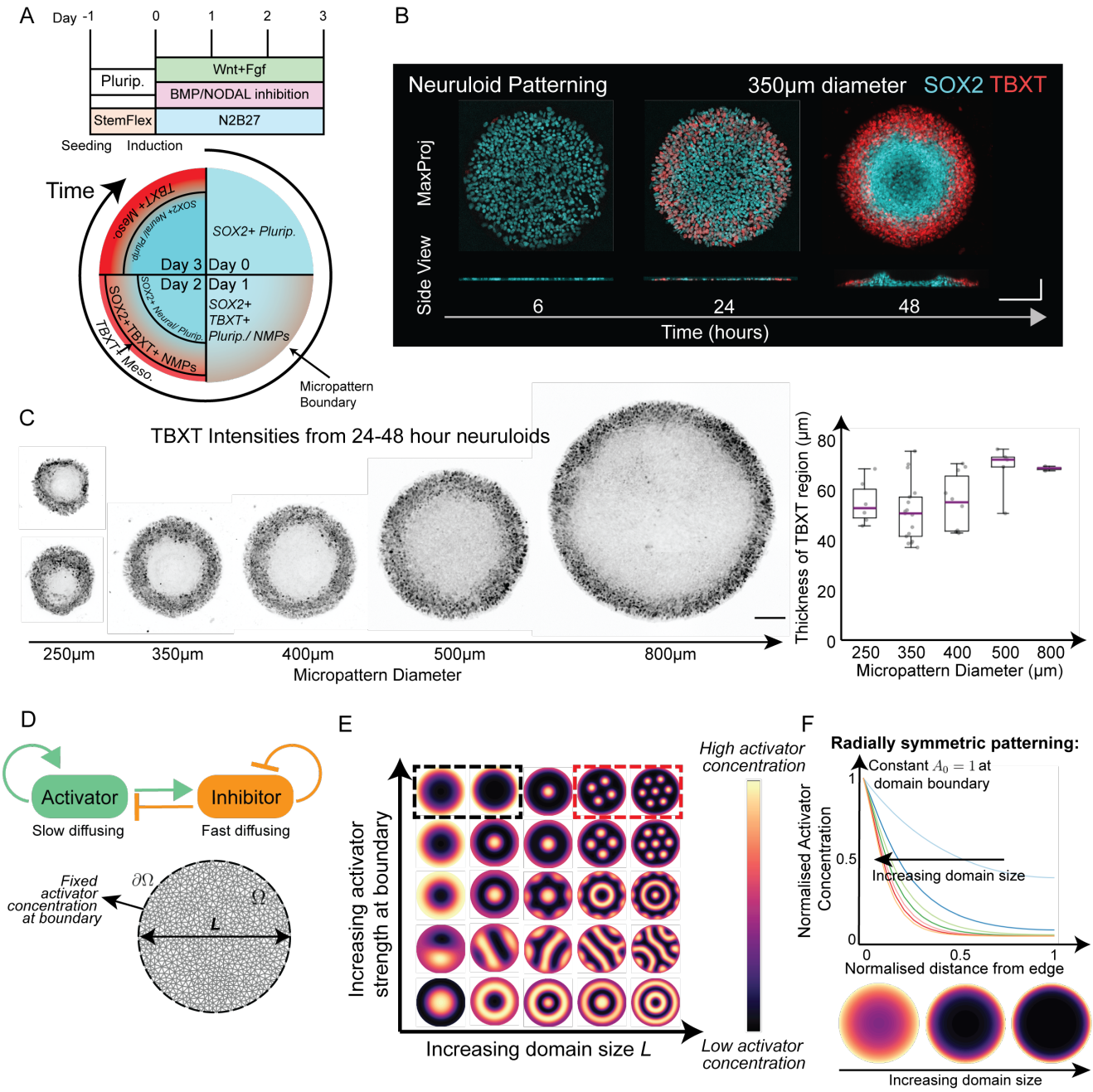

Figure 1: ***Neuruloids reproducibly pattern SOX2 and TBXT positive cells upon induction.*** **A.** Schematics of neuruloid protocol and cell fate patterning during neuruloid development, specifically pluripotent, neuromesodermal progenitors (NMPs), neural and mesoderm cells, upon induction. **B.** Patterning timeline during the first two days of neuruloid development post-induction. Maximum projections shows radial patterning of TBXT expression, which was reproducible across multiple experiments. Cross-sectional views show an initially homogeneous layer of cells developing to a 3D shape at 48 hour post-induction. **C.** (Left) TBXT patterning in neuruloids grown in circular micropatterns of different sizes. (Right) Quantification of the thickness of TBXT ring, where $n = 30$ neuruloids were used for quantification. No statistically significant difference was found between the 5 groups of sizes (Kruskal-Wallis test, H=9.085, p=0.059). **D.** Schematic of activator-inhibitor interaction modelled using a reaction-diffusion model (Supplementary Material, S2 details). **E.** Parameter sensitivity analysis of the reaction-diffusion model. Black and red regions referred to in text. **F.** Relative activator gradients across different domain sizes from the reaction-diffusion model on a normalised spatial axis. Scale bars: All horizontal scale bars are 100μm, vertical scale bar is 50μm.

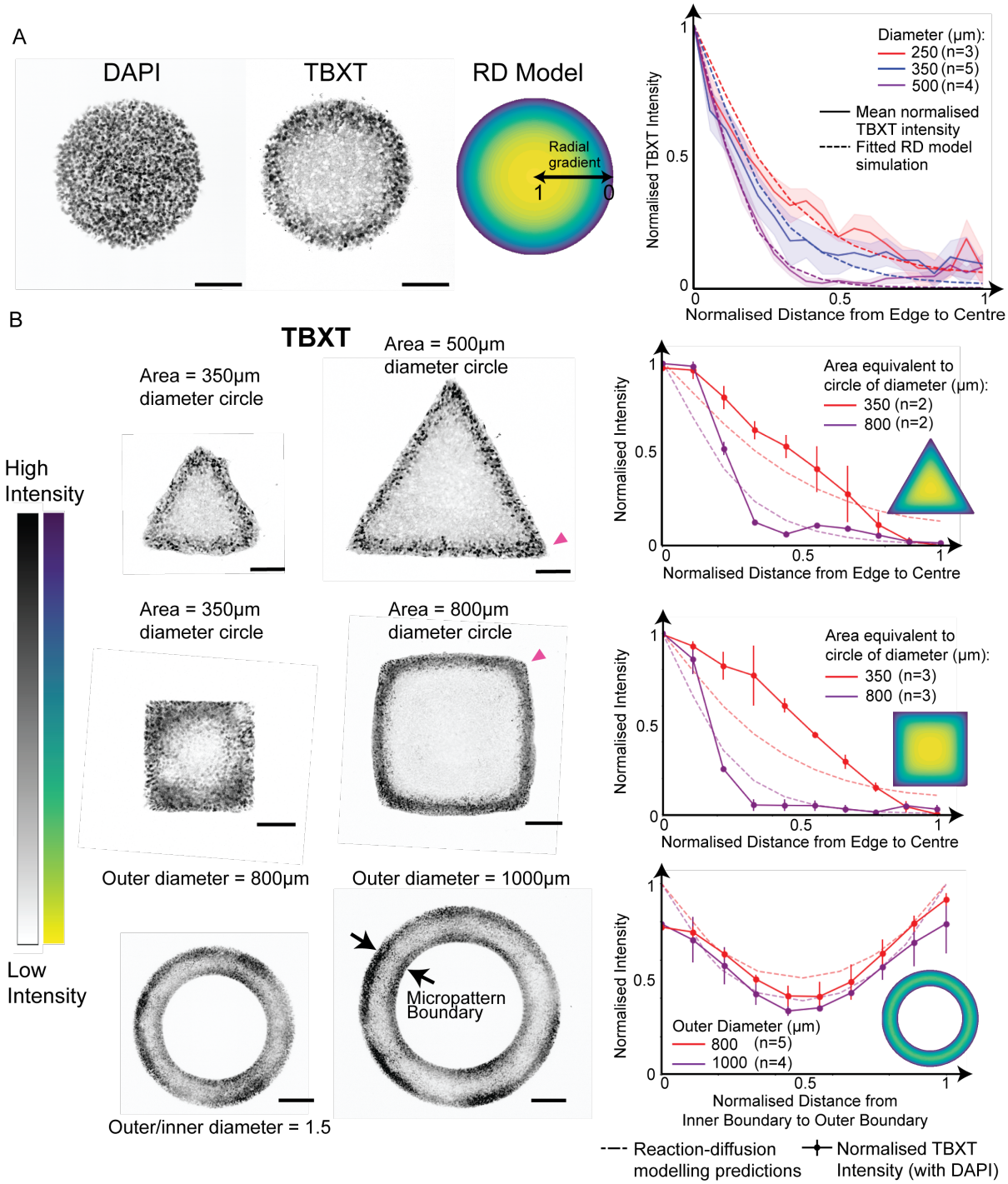

Figure 2: ***TBXT patterning in neuruloids of varying geometry.*** **A.** DAPI and TBXT intensities of circular neuruloids were used to extract normalised TBXT expression. The radial gradient generated by the reaction-diffusion model was fitted to the experimental TBXT expression profile (see also Fig. S3). (Right) Normalised TBXT expression. Solid lines and shaded regions show the mean and standard deviation respectively. At least $n = 3$ for each micropattern diameter. Dashed lines show the best fitted gradient from the model to the data. **B.** (Left) Neuruloids grown on micropatterns of different shapes. Micropattern sizes correspond to the areas of circular micropatterns of specific radii. The heatmap shows TBXT intensity. Magenta arrows indicate outer convex boundary (annulus) and corners (triangle and squares), cyan arrow indicates the inner concave boundary of annulus. (Right) Quantification of TBXT intensity in different geometries. Error bars show s.d. (see also Fig. S3B-C). Dashed lines are predictions from the reaction-diffusion model. Scale bars: All scale bars are 100μm.

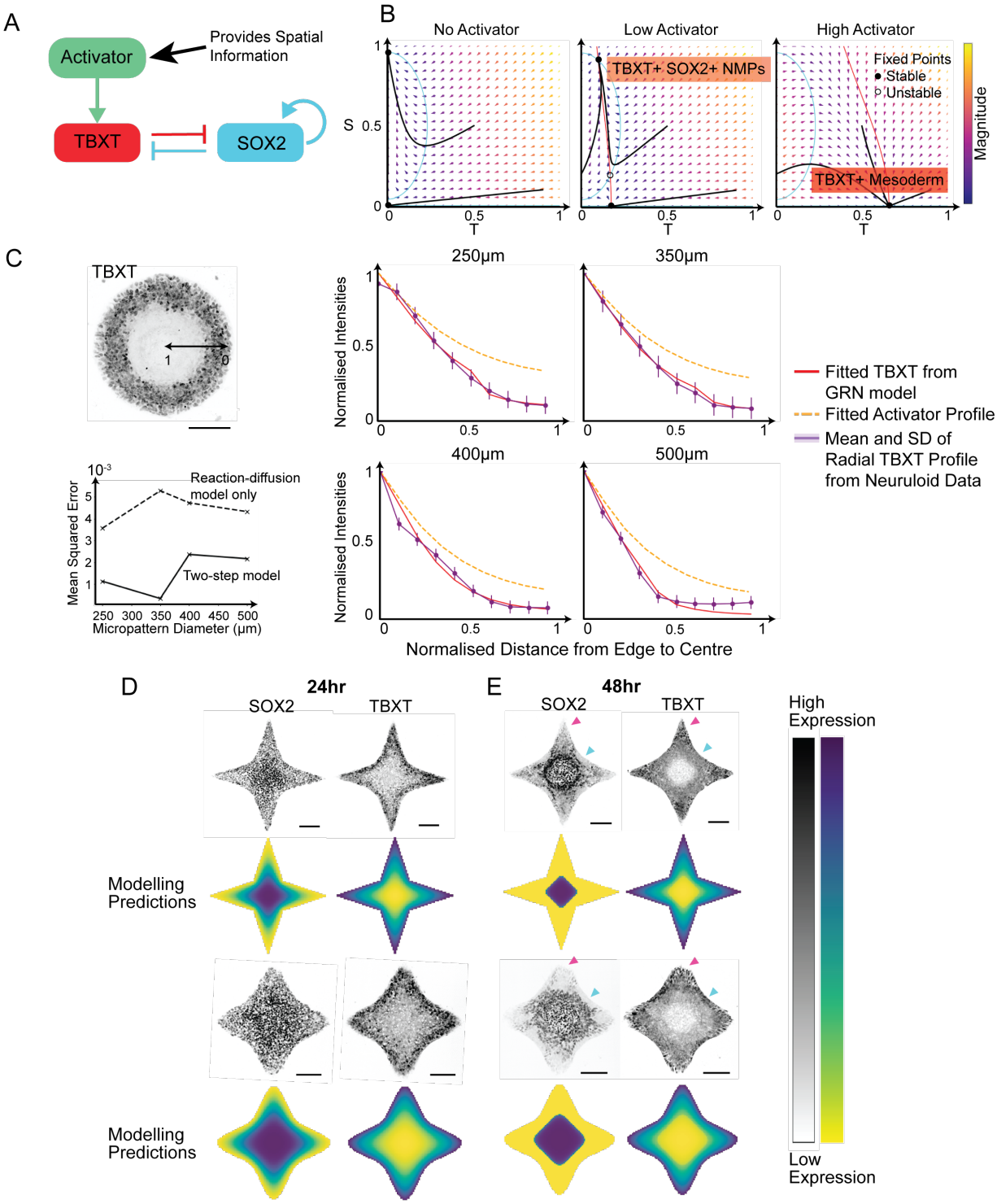

Figure 3: ***Two-step model reproduces SOX2 and TBXT profiles in varying boundary geometries.*** **A.** Schematic of the GRN modelled. The activator concentration is an input from the reaction-diffusion model (Fig. 1). **B.** Phase diagrams with gene expression trajectories for different activator levels. The system is bistable with low activator concentration, but has only one stable fixed point for high activator concentration values. **C.** Two-step model (red solid lines) fitted to TBXT intensity (purple solid lines) from neuruloid data, and corresponding activator profile (orange dashed line) required for increasing micropattern diameters. At least $n = 3$ neuruloids were used for each micropattern diameter. Lower left plot shows mean squared error between model and TBXT data for reaction-diffusion model only (dashed line) and two-step model (solid line). **D-E.** SOX2 and TBXT patterning at 24 (D) and 48 (E) hours post induction on sharp and curved domains with both convex and concave boundaries. Grayscale images show experimental data, with heatmaps at the bottom showing model predictions. Magenta and cyan arrows indicate convex and concave corners respectively. Scale bars: All scale bars are 100μm.

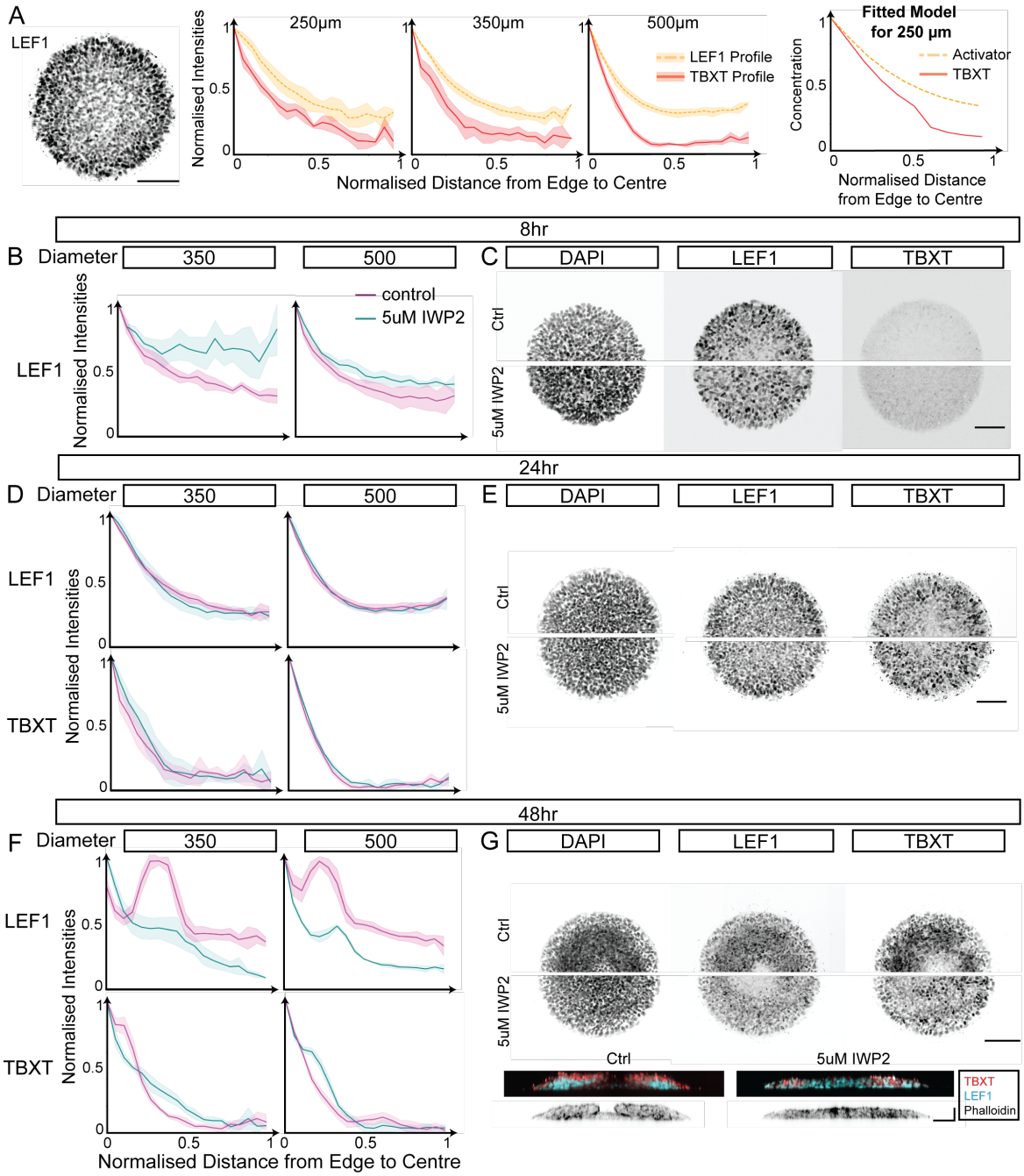

Figure 4: ***LEF1 gradient matches activator gradient from model fitting but pattern emergence was not disrupted when Wnt secretion was inhibited.*** **A.** LEF1 (orange dashed lines) and TBXT (red solid lines) profiles extracted from 24-hour neuruloid data for increasing micropattern diameters. At least $n = 3$ neuruloids were used for each micropattern diameters. **B, D, F.** LEF1 and TBXT intensity profiles for control (magenta) and IWP-treated (teal) conditions for 8-hour (B), 24-hour (D) and 48 hour (F) neuruloids. At least $n = 3$ neuruloids were used for each micropattern diameters and experimental conditions. **C, E, G.** DAPI, LEF1 and TBXT signals, with control (top) and inhibited conditions (lower). **G** Lower panels show cross-sectional view of neuruloids at 48-hours. Neuruloids chosen in C, E and G had similar cell densities as detected using segmentation. Scale bars: All horizontal scale bars are 100μm, vertical scale bar is 50μm.

METHODS

Experimental methods

Human embryonic stem cell culture

H9 human embryonic stem cells (hESCs) were cultured using StemFlex medium (Thermo Fisher Scientific; A3349401) in T25 flasks coated with 0.5 μl/cm^2 laminin-521 (Thermo Fisher Scientific; A29249). When flasks reached 75-80% confluence, cells were passaged at ratios of 1:6 and 1:8 using ReLeSR as per manufacturer's instructions (StemCell Technologies; 05872).

All human embryonic stem cell experiments were conducted at the University of Warwick in accordance with the Guidelines for Stem Cell Research and Clinical Translation from the International Society for Stem Cell Research, as well as the UK Code of Practice for the Use of Human Stem Cell Lines. Our culture system represents a defined stage of development and adheres to section 2.2.1A of the ISSCR Guidelines.

Micropatterning coverslips

Micropatterns were generated following the protocol described in[67]. Briefly, IPA-treated coverslips were first activated in a UVO-cleaner for 10 min, and then incubated with PLL(20)-g[3.5]-PEG(5) for 1.5 hours. Coated coverslips were placed onto a chrome mask that had been UV-ozone activated for 2 minutes and contained the desired pattern geometries. Adherence between the coverslip and mask was aided by adding dH_2O. The assembly was then inverted so the silver side faced upward and exposed to UV light for 8 minutes. Coverslips were subsequently immersed in 70% ethanol for 15 minutes, air-dried, and used within 4 weeks.

Generation of posterior neuruloids

Posterior neuruloids were produced according to[20]. In summary, micropatterned coverslips were thoroughly washed using PBS+/+ (PBS with calcium and magnesium; Thermo Fisher Scientific, 14040-091), and then incubated with rh-Laminin-521 (Thermo Fisher Scientific, A29248) diluted 1:10 in PBS+/+ for 3 hours at 37℃, or overnight at 4℃. hESCs were washed using PBS, dissociated using Accutase, and then manually adjusted to 600,000 cells/ml in StemFlex with 10μM Y-27632. 1.5ml of the single-cell suspension was added to each coated coverslip in a 6-well plate well. After 3 hours, wells containing coverslips were washed once with PBS, and the medium was replaced with fresh StemFlex for overnight incubation. After 18 hours, colonies were induced using 2.5ml of induction media consisting of N2B27 media with CHIR99021 (3M, StemCell Technologies), FGF2 (5ng/mL), SB431542 (10μM) and LDN193189 (0.1μM). Medium was replaced again the following day. Coverslips were fixed using 4% freshly prepared PFA for 30 minutes at room temperature, then washed twice with PBS and stored at 4℃ for subsequent analysis.

Immunostaining

Cells cultured on micropatterns and within 3D colonies were blocked and permeabilised at room temperature for one hour in a solution containing DPBS-/-, 1% Triton-X, 10% DMSO, 10% SDS, and 4% Normal Donkey Serum. After a brief rinse in DPBS-/- (1 minute), samples were incubated overnight at 4℃ with primary antibodies. The following day, samples were washed three times for 5 minutes each in PBS-/-, then incubated overnight with secondary antibodies conjugated to Alexa Fluor dyes (488, 555, 594, or 647; 1:1000 dilution) and DAPI. A final 10-minute

wash in DPBS-/- was performed before mounting with ProLong Glass Antifade Mountant (Invitrogen). Mounted samples were imaged using a spinning disk confocal microscope.

Mathematical Modelling

Reaction-diffusion modelling on 2D domain

The true dynamics of interactions between the hypothesised activator and inhibitor component are not known. Hence, we used a general reaction-diffusion model solved in the domain $\Omega \in \mathbb{R}^2$ describing concentrations of activator and inhibitor, denoted by A and I respectively. This model was first proposed by the Kondo group[68,69] to study patterning on the fish skin. The coupled partial differential equations (PDEs) are

$$\frac{\partial A}{\partial t} = D_A \nabla^2 A - d_A A + F_A(A, I), \tag{1}$$

$$\frac{\partial I}{\partial t} = D_I \nabla^2 I - d_I I + F_I(A, I), \tag{2}$$

where the three terms for both equations model the diffusion, degradation and production, respectively, for both of the activator and inhibitor. D_A and D_I are diffusion coefficients; while d_A and d_I are degradation rates of activator and inhibitor molecules respectively.

We model the production functions with linear terms

$$F_A(A, I) = \max\{0, \min\{A^{\max}, a_A A + b_A I + c_A\}\}, \tag{3}$$

$$F_I(A, I) = \max\{0, \min\{I^{\max}, a_I A + b_I I + c_I\}\}, \tag{4}$$

where nonlinearity is added by truncating the function. The parameters a_A, b_A, c_A, a_I, b_I and c_I are arbitrary parameters to model the interactions between activator and inhibitor. In this model, we ensure that the parameters are chosen so that they are in the diffusion driven instability regime from linear stability analysis and Turing patterns can be formed (Supplementary sec S2). A mixed boundary condition is used for this model, in the form of

$$A = A^{\max}, \mathbf{n} \cdot \nabla I = 0 \text{ for all } \mathbf{x} \in \partial\Omega. \tag{5}$$

Previous analyses of mixed boundary conditions have shown this produces more symmetrical spatial patterning[40] that is robust to fluctuations, making it an ideal framework for capturing differential responses to boundaries in a simplified, phenomenological manner. Hence, we used a Dirichlet boundary condition for the activator concentration to impose the condition that cells at the edge have an increased sensitivity to signalling molecules, as detailed in the results section[19,39,57]. The Neumann boundary condition was used for the inhibitor concentration, indicating no flow of inhibitor out of the micropattern since we hypothesised that the inhibitors are endogeneously and intracellularly produced. The equations were solved using finite element methods and sensitivity analysis is performed to determine a suitable set of parameters to model radially symmetric patterning (see Supplementary sec S2). Finite element method was implemented on Python *FEniCS* library[70]. Meshes for simulations were generated using *mshr* library according to the micropattern domains from experiments. Non-dimensionalisation and analysis of parameters is shown in Supplementary sec S2.

Minimal model of mutually inhibiting SOX2 and TBXT

To study the effects of activator concentration on gene regulation, we construct a minimal ordinary differential equation (ODE) model to obtain SOX2 and TBXT profile predictions. We considered the concentrations of two mutually inhibiting transcription factors SOX2 and TBXT, S and

T, respectively,

$$\frac{dS}{dt} = \alpha_S \frac{\left(\frac{S}{S_S}\right)^2}{\left(\frac{T}{T_S}\right)^2 + \left(\frac{S}{S_S}\right)^2 + 1} - \beta_S S. \tag{6}$$

$$\frac{dT}{dt} = \alpha_T \frac{A(\mathbf{x})^2}{\left(\frac{S}{S_T}\right)^2 + A(\mathbf{x})^2 + 1} - \beta_T T, \tag{7}$$

$A(\mathbf{x})$ is the activator concentration which varies in spatial coordinates, $\mathbf{x} \in \Omega$ (Ω is the micropattern domain) and hypothesised to promote TBXT transcription. The model takes the stable concentration profile of activator from the reaction-diffusion model, $A(\mathbf{x})$ (see previous section of Methods) as input that promotes the expression of TBXT. The activation and inhibition of gene interactions as shown in Fig. 3A are represented using Hill functions, where S_S, T_S and S_T are scaling constants that represent the concentration when production rate is half of the maximum. α_S and α_T are maximum transcription rates; while β_S and β_T are the degradation rates of the transcription factors SOX2 and TBXT respectively. The general form for the production term of the transcription factors,

$$\text{Rate} = \text{Rate}_{\text{max}} \frac{\sum \text{Activators}^n}{\sum \text{Inhibitors}^n + \sum \text{Activators}^n + 1}, \tag{8}$$

is used to model the competition between activators and inhibitors for the regulatory effects in the system.

Initial conditions for the model are $S_0 = 0.2$ and $T_0 = 0$ for initial SOX2 and TBXT signals respectively, since pluripotent stem cells express low levels of SOX2. The activator profile here provides spatial information to the cells, in line with the concept of positional information[25]. The nullclines of the ODE satisfy

$$S = \frac{\alpha_S}{\beta_S} \frac{\left(\frac{S}{S_S}\right)^2}{\left(\frac{T}{T_S}\right)^2 + \left(\frac{S}{S_S}\right)^2 + 1}, \tag{9}$$

$$T = \frac{\alpha_T}{\beta_T} \frac{A(\mathbf{x})^2}{\left(\frac{S}{S_T}\right)^2 + A(\mathbf{x})^2 + 1}, \tag{10}$$

$$\tag{11}$$

where the intersections of these equations are the fixed points of the system. The equations were solved numerically using *scipy odeint* on Python. Details on non-dimensionalisation, stability analysis of the fixed points and the parameter space are in Supplementary sec S4.

Image Analysis

All image analysis were done using a combination of Fiji ImageJ[71] and custom code on Python, including using Cellpose[72,73] for segmentation and libraries including *numpy*, *scikit-image*, and *matplotlib* for visualisation.

Analysis of fluorescence intensity

To quantify gene expression patterns for early neuruloid timepoints (day 1 post-induction), we performed analysis on fluorescence intensity based on nuclear segmentation. First, the best

focal plane was selected from each 3D image stack by computing the Laplacian variance on the DAPI channel. The image slice with the largest value has the sharpest focus. All subsequent analyses were conducted on this selected plane. Nuclear segmentation was performed using a custom-trained Cellpose model[73], fine-tuned to improve accuracy over the default nuclei model.

The segmentation masks were used to generate a binary image, from which colony contours were identified using the *scikit-image.measure.find_contours* function. The colony edge is then detected from the masks of the nuclei segmentation. The centroids of each segmented nucleus was extracted from each mask and used to compute the distance from the colony edge. For each nucleus, gene expression intensity was determined by computing the mean fluorescence intensity value within the nuclear region for the corresponding channel. To account for variability in staining or imaging conditions, fluorescence intensities were normalised to DAPI signal per cell. This is done by dividing transcription factor intensities such as SOX2 and TBXT of the cell by its DAPI signal.

To generate intensity profiles as a function of distance from the colony boundary, the radial distance from the edge to the center (largest nuclei distance from edge) was discretised into N regions. Cell centroids located between distances L_k and L_{k+1}, where $k \in [0, N]$, were grouped and the mean intensity of each group was computed to construct a radial intensity profile. Fig S2 illustrates this analysis pipeline and the resulting quantification. For larger neuruloids where tile scans were required, contrast limited adaptive histogram equalization (CLAHE) method was done on the images before extracting the intensities to correct variation in intensities due to tiling. The same analysis was done for neuruloids cultured on geometrical micropatterns.

For later-stage and larger neuruloids, where lower resolution imaging makes accurate single-cell segmentation unreliable, we analysed fluorescence intensity from maximum intensity projections of the image stacks. To detect the neuruloid boundary in both circular and polygonal micropatterns, we applied a threshold to the DAPI channel to generate a binary image. Using this mask, we extracted the outer contour using scikit-image's contour detection. With the boundary coordinates, we can calculate the centre of area of the contour using the formula

$$x_{\text{centre}} = \frac{1}{6A} \sum_{i=1}^{n_b} (x_i + x_{i+1})(x_i y_{i+1} - x_{i+1} y_i) \tag{12}$$

$$y_{\text{centre}} = \frac{1}{6A} \sum_{i=1}^{n_b} (y_i + y_{i+1})(x_i y_{i+1} - x_{i+1} y_i) \tag{13}$$

where (x_i, y_i) are the coordinates of the ith boundary point and n_b is the total number of points of the contour. A is the area computed from the same coordinate list. For circular neuruloids, the effective radius was estimated by averaging the shortest and longest distances from the centroid to the boundary. Similarly, we can discretise the neuruloid into N regions based on distance to the nearest boundary. Mean fluorescence intensities were calculated for each discretised region. Similarly, the transcription factor intensity profiles can be normalised with DAPI intensity profile. A similar analysis was also applied to the simulated 2D concentration data from the reaction-diffusion model on geometrical domains, enabling direct comparison between experimental and theoretical results.

Parameter fitting

The models are fitted to the normalised signal intensities obtained from neuruloid images. To compare reaction-diffusion model to extracted TBXT intensities, we found parameter values that give the minimum mean absolute error (see Fig. 2A and Fig. S2E).

To fit the full two-stage model to the TBXT data, we utilised Markov Chain Monte Carlo (MCMC) methods to estimate a set of parameters to fit to neuruloid data of different sizes (see Supplementary sec S5 for more details).

Single-cell RNA-seq analysis

Single-cell RNA sequencing data from day 3 neuruloids were obtained from the publicly available dataset published by Rito et al. (2025), accessible via the Gene Expression Omnibus (GEO) under accession number GSE224404[20]. Principal component analysis (PCA) and UMAP were performed using default settings, with the neighbourhood graph constructed using 15 nearest neighbours and 30 principal components. scRNA-seq data were processed using the Python toolkit SCANPY[74], and ligand–receptor interactions were inferred with CellPhoneDB[52].

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, .

Materials availability

This study did not generate new materials.

Data and code availability

Code for image analysis and modelling can be found publicly in the github pages here and here.

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AUTHOR CONTRIBUTIONS

T.E.S, J.B. and G.C. conceived and oversaw the project. Y.T.L performed mathematical modelling and numerical simulations. Y.T.L and J.C. performed image analysis and quantification of data, with support from S.T.. J.C. generated micropatterns, cultured neuruloids, stained and performed experimental perturbations with support from R.H. and T.R.. R.H. trained Cellpose nuclei segmentation model for neuruloids. Y.T.L, J.C. and T.E.S. wrote the original draft. All authors reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION INDEX

Document S1 with Figures S1-S5, their legends and Table 1 and 2 in a PDF.

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