Anteroposterior polarity and elongation in the absence of extra-embryonic tissues and of spatially localised signalling in gastruloids: mammalian embryonic organoids

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ABSTRACT

The establishment of the anteroposterior (AP) axis is a crucial step during animal embryo development. In mammals, genetic studies have shown that this process relies on signals spatiotemporally deployed in the extra-embryonic tissues that locate the position of the head and the onset of gastrulation, marked by T/Brachyury (T/Bra) at the posterior of the embryo. Here, we use gastruloids, mESC-based organoids, as a model system with which to study this process. We find that gastruloids localise T/Bra expression to one end and undergo elongation similar to the posterior region of the embryo, suggesting that they develop an AP axis. This process relies on precisely timed interactions between Wnt/β-catenin and Nodal signalling, whereas BMP signalling is dispensable. Additionally, polarised T/Bra expression occurs in the absence of extra-embryonic tissues or localised sources of signals. We suggest that the role of extra-embryonic tissues in the mammalian embryo might not be to induce the axes but to bias an intrinsic ability of the embryo to initially break symmetry. Furthermore, we suggest that Wnt signalling has a separable activity involved in the elongation of the axis.

KEY WORDS: Gastruloids, Axial organisation, Organoids, Symmetry-breaking

INTRODUCTION

The establishment of the anteroposterior (AP) and dorsoventral (DV) axes during the early stages of animal development is a fundamental patterning event that guides the spatial organisation of tissues and organs. Although this process differs from one organism to another, in all cases it involves a break in an initial molecular or cellular symmetry, resulting in the precise positioning of signalling centres that will drive subsequent patterning events (Meinhardt, 2006). Dipteran and avian embryos provide extreme examples of the strategies associated with these processes. For example, in Drosophila, the symmetry is broken before fertilisation within a single cell, the oocyte, that acquires information for both the AP and DV axes. This occurs through interactions with surrounding support cells that control processes of RNA and protein localisation, which then serve as references for the rapid patterning of the embryo as the zygote turns into a multicellular system (Riechmann and Ephrussi, 2001; Roth and Lynch, 2009). On the other hand, in chickens the processes take place in the developing embryo, within a homogeneous multicellular system that lacks external references (Bertocchini and Stern, 2002; Stern, 2006). In mammalian embryos, the axes are also established within a homogeneous cellular system, the epiblast, but in this case they are under the influence of an initial symmetry-breaking event that takes place within the extra-embryonic tissues, which is then transferred to the developing embryo (Rivera-Pérez and Hadjantonakis, 2015; Rossant and Tam, 2009; Stern, 2006; Takaoka and Hamada, 2012).

Efforts to understand the molecular mechanisms that pattern early embryos have relied on genetic approaches such as perturbation through genetic mutations and a correlation between specific processes and molecular events, as highlighted by the activity of specific genes (Anderson, 2000; St Johnston, 2002). Although successful, these approaches have limitations, as they often conflate correlation and causation, and, importantly, cannot probe the role of mechanical forces that have been shown to play a role in the early events (Hamada, 2015; Hiramatsu et al., 2013). This suggests a need for a complementary experimental system in which, for example, rather than removing components, we attempt to build tissues and organs from cells and learn what the minimal conditions are that allow this (Sasai et al., 2012). We have recently established a non-adherent culture system for mouse embryonic stem cells (ESCs) in which small aggregates of defined numbers of cells undergo symmetry breaking, polarisation of gene expression and axial development in a reproducible manner that mirrors events in embryos (Turner et al., 2014a, 2016b preprint; van den Brink et al., 2014). We call these polarised aggregates gastruloids and believe that they provide a versatile and useful system with which to analyse the mechanisms that mediate cell fate assignments and pattern formation in mammalian embryos (Simunovic and Brivanlou, 2017).

Here, we show that gastruloids become polarised along two axes that resemble the AP and DV axes of the mouse embryo in the absence of extra-embryonic tissues. We focus on the AP polarity and find that, unlike the embryo, in gastruloids this process does not require BMP signalling but relies on interactions between Nodal and...
Gastruloids exhibit anteroposterior and dorsoventral organisation in the elongating domain

Our previous studies using gastruloids revealed a longitudinal polarisation with the expression of T/Bra located towards one end that will lead an elongation process (Bailie-Johnson et al., 2015; Turner et al., 2014a; van den Brink et al., 2014). This generates an axis reminiscent of the AP axis of early mammalian embryos. To follow these observations and determine whether other markers of the embryonic axis are present in the emerging structures, we cultured gastruloids for 120 h and mapped the expression domain of reporters for three major signalling pathways involved in axial organisation in the embryo (Wnt/β-catenin, Nodal and BMP) as well as of Cdx2, which identifies the posterior of the embryo (Fig. 1, Fig. S1 and Materials and Methods). At 120 h after aggregation (AA), gastruloids that have been exposed to the Wnt signalling agonist CHIR99021 (Chi) between 48 and 72 h AA, are polarised, with localised expression of T/Bra (Fig. 1A,C, Fig. S1A,C) and Cdx2 (Fig. 1A, Fig. S1B) at one end of the protruding tip; they also exhibit a shallow gradient of Wnt signalling away from the T/Bra-expressing region (Fig. 1C, Fig. S1C). In most replicate experiments there is no detectable BMP signalling activity at 120 h (Fig. 1D, Fig. S1C), although on one occasion we detected expression of the BMP reporter in the anterior region (Fig. S1C). This arrangement suggests that the elongating domain of the gastruloid is similar to the tail bud of an embryo (Beddington et al., 1992; Herrmann, 1991; Wilkinson et al., 1990), supporting our previous observations that gastruloids have AP axial organisation.

The extension of the gastruloids is characterised by the expression of neural progenitor markers (Turner et al., 2014a; van den Brink et al., 2014). When we correlate the expression of T/Bra, Cdx2, Sox2 and a Sox1::GFP reporter (Ying et al., 2003) (Fig. 1A,B, Fig. S1A), we observe an organisation perpendicular to that of the AP axis, in which high levels of expression of the neural markers Sox1, Sox2, as well as Cdx2 extend away from the T/Bra-expressing tip on one side of the gastruloid, with a weak Cdx2 expression domain directly opposite and just anterior to the T/Bra-expressing cells (Fig. 1A,B, Fig. S1A). This organisation of gene expression is reminiscent of the DV organisation of the embryonic caudal lateral epiblast (CLE) at around E8.5 (see Kanai-Azuma et al., 2002; Zhao et al., 2014). Furthermore, at this stage in the embryo, some ventral endodermal cells express Sox17 (see Choi et al., 2012; Saund et al., 2012) and we observe such a domain here (Fig. 1B, Fig. S3).

Taken together, these results suggest that by 120 h AA, Chi-treated gastruloids have an organisation reminiscent of that of the post-occipital region of the embryo. The lack of anterior Sox1 expression suggests that gastruloids lack brain and head structures (van den Brink et al., 2014); in this sense, they are very similar to gain-of-function β-catenin mutants (Fossat et al., 2011, 2012; Tam and Loebel, 2007), consistent with their being exposed to high levels of Wnt signalling during their early development.

Wnt/β-catenin signalling provides robustness to the polarisation of T/Bra expression

To understand the emergence of the AP polarisation in gastruloids, we monitored the temporal expression of a T/Bra::GFP reporter line (Fehling et al., 2003) from the moment of their aggregation, as well as the patterns of Wnt, Nodal expression (using the Nodal::YFP reporter mentioned above) and activity [using an AR8::mCherry line to report on Nodal signalling transduction (Serup et al., 2012)], and BMP signalling [IBRE::Cerulean (Serup et al., 2012)].

We also assessed the transition from pluripotency towards differentiation using the miR-290-mCherry/mir-302-eGFP (Fig. 2A,A′), which marks distinct stages of pluripotency based on the expression of the...
**Fig. 2. Gastruloids progress through stages similar to the early embryonic to late epiblast.** (A) mir-290-mCherry/mir-302-eGFP gastruloids imaged by wide-field microscopy for 102 h \((n=6\) for 24-48 h and \(8\) for 72-120 h). The colour changes schematic is shown in A’ (see Parchem et al., 2014 and Turner et al., 2016b). (B-F) Gastruloids made from the (B) TNGA \((n=21)\), (C) TLC2 \((24\) and 48 h \(n=84\); 72 h \(n=42\)) and (D) Nodal::YFP (Nodal expression; 24 and 48 h \(n=84\); 72 h \(n=42\)) and (E) AR8::mCherry (Nodal signalling; \(n=14\)) and (F) IBRE4::Cerulean (BMP reporter; 24 h \(n=70\); 48 and 72 h \(n=14\)) cell lines and treated with a pulse of Chi between 48 and 72 h AA (B-E), or pre-treated with a pulse of BMP4 (24-48 h) followed by a pulse of Chi (48-72 h; F). Schematic for the stimulation regime shown in the top-right corner. Scale bars: 100 μm.

Addition of Chi or Wnt3a to the medium between 48 and 72 h resulted in enhanced levels of T/Bra::GFP expression by 72 h AA compared with the vehicle controls (Fig. 3A,B), which is maintained in all gastruloids at the posterior tip at higher levels than the control (Fig. 3A). Similarly, Nodal expression is greatly enhanced following the Chi pulse and is expressed across the whole gastruloid (Fig. 2D), although the Nodal signalling reporter is not activated as strongly (Fig. 2E). This is consistent with the role of Wnt signalling in controlling Nodal expression in the post-implantation epiblast. Gastruloids also alter the expression of the miRNA reporters, downregulating mir-290 and greatly upregulating mir-302 (Fig. 2A,A’).

To garner an understanding of the heterogeneities in T/Bra::GFP expression over time, we quantified the fluorescence levels of the reporter in a posterior-to-anterior direction along the spine of the gastruloids (Fig. 3B-D, Fig. S2A,B; see Materials and Methods) (Bailie-Johnson et al., 2015). We notice that the changes in shape and patterns of gene expression are highly reproducible and have used this feature to extract quantitative information about gene expression and morphogenesis at single time-points or at regular intervals over time. Exposure of gastruloids to Chi 48 and 72 h AA results in a tighter distribution of all the measured variables and a higher level of sustained fluorescence than when they are exposed to DMSO (Fig. 3B-D, Fig. S2A; \(P<0.001\) at 72 h and \(P<0.01\) at 120 h). Stimulation with Wnt3a is able to substitute for Chi and results in similar fluorescence expression profiles over time with a similar rate of acquisition of an elongated morphology (Fig. 3B-D, Fig. S2B; \(P>0.05\)).

Live imaging of the T/Bra::GFP reporter throughout the process confirms that Chi enhances its intrinsically polarised expression but also reveals a global transient response to the Chi pulse throughout the gastruloid that relaxes to the original position after the pulse (Fig. 3E, Movies 1 and 2). Using a Sox17::GFP line (Niakan et al., 2010), which reveals endodermal progenitors, we observe the initial expression in the anterior pole of the aggregate followed by a complex migration of some of the expressing cells towards the posterior region. At 120 h, Sox17::GFP-expressing cells localise anterior to the T/Bra expression domain following the Chi pulse (Fig. S3). The final patterning of the reporter showed some heterogeneity, examples of which are shown in Fig. S1C. Taken together, these results suggest that during the first 48 h AA, gastruloids undergo an intrinsic symmetry-breaking process that is reflected in an AP axis made robust and stable by Wnt/β-catenin signalling.

**Extra-embryonic tissues are not required for axial organisation in gastruloids**

In the embryo, the spatial restriction of T/Bra is concomitant with the establishment of the AP axis and the onset of gastrulation at the posterior end of the embryo (Rivera-Pérez and Hadjantonakis, 2015; Tam and Gad, 2004). Genetic analysis has shown that this pattern arises from interactions between signalling systems asymmetrically deployed in the extra-embryonic tissues (Rossant and Tam, 2009).

To determine the mechanism whereby gastruloids are patterned along the AP axis and to compare the process with that taking place in embryos, we first analysed the expression of several genes involved in the AP patterning at 48 h AA, when we first observe signs of polarisation in gene expression (Fig. 4). At this stage, gastruloids expressed Fgf4, Fgf5, Axin2, Wnt3, Nodal and cripto (Cfc1) all of which are expressed in the epiblast in the embryo (Fig. 4). We also detect low levels of Lefty1 (Fig. 4), which in the
Embryo is expressed mainly in the extra-embryonic tissues but also in the epiblast as gastrulation begins. On the other hand, we do not detect significant expression of genes associated with extra-embryonic tissues e.g. Bmp4, Dkk, Furin, Lrp2 and Dab2 (disabled homolog 2) with very low levels of cerberus (Cer1) (Fig. 4). By 72 h AA in N2B27, we observed increases in expression of Nodal, Lefty1 and Fgf5, decreases in Fgf4 and the emergence, at low levels, of Wnt3a (Fig. 4). Some of these patterns are Wnt/β-catenin signalling-dependent, as exposure to Chi from 48 to 72 h AA leads to a clear increase in Nodal, Lefty1 and Wnt3a,
expression analysis.

E7.0 Precise timing will require more-detailed and extensive gastrulation in the E6.0 embryo and 72 h AA is an approximation of landmarks for correlating the development of gastruloids with that of the cell behaviours associated with gastrulation expression domain.

96 h AA in a domain of cells at the opposite end of the T/Bra visceral endoderm and, in the gastruloids, it is first expressed around implantation in the early embryo, Gata6 is associated with the embryonic composition of the gastruloids (Fig. S4). Before lack of taking place as previously described (Fig. 2F, right). Additionally, the during the first 48 h AA, when the polarisation of T/Bra expression is into this process, we exposed gastruloids to agonists and antagonists relies on Nodal and Wnt signalling (Figs 3 and 4). To gain insights associated with the epiblast, extra-embryonic tissues or those expressed in embryonic tissues and progressively activate posterior markers. The patterns of gene expression at different times AA, together with the timing of the cell behaviours associated with gastrulation that we have described before (Baillie-Johnson et al., 2015; Turner et al., 2014a, 2016b preprint; van den Brink et al., 2014), provide landmarks for correlating the development of gastruloids with that of embryos. They suggest that 48 h AA corresponds to the onset of gastrulation in the E6.0 embryo and 72 h AA is an approximation of E7.0. Precise timing will require more-detailed and extensive expression analysis.

**Nodal signalling promotes T/Bra expression**

The expression of signalling reporters suggests that, by 48 h AA, gastruloids are being patterned through an intrinsic mechanism that relies on Nodal and Wnt signalling (Figs 3 and 4). To gain insights into this process, we exposed gastruloids to agonists and antagonists of both signalling pathways before or at the time of exposure to Chi. Treatment with the Nodal ALK4 receptor inhibitor SB431542 (SB43) (Inman et al., 2002) between 48-72 h AA in the absence of Chi abolished both the expression of T/Bra::GFP and the elongation, with gastruloids remaining essentially spherical (Fig. 5, Fig. S5). Co-treatment with Chi and SB43 (48-72 h) severely reduced the levels of fluorescence and greatly impacted the ability of the gastruloids to elongate in a typical manner, with a large degree of variation between experimental replicates (Fig. 5, Fig. S5; P<0.001 from 72-120 h). These results indicate an absolute requirement for Nodal signalling in the expression of T/Bra. To identify a temporal element to this requirement, we pre-treated gastruloids with SB43 between 24 and 48 h before pulsing them with Chi (48-72 h). These gastruloids are delayed in expressing T/Bra::GFP and the levels, generally low, exhibit a high degree of variation in the location and expression of T/Bra between individuals (Fig. 5, Fig. S6; P<0.01 for 72-120 h); however, their ability to elongate is not affected and is occasionally enhanced relative to the Chi control (Fig. 5, Fig. S6). These results confirm a requirement for Nodal in the expression of T/Bra and suggest that it is possible to separate the axial elongation from T/Bra expression.

Addition of Nodal, alone or together with Chi from 48 and 72 h AA results in an increase in T/Bra expression similar to that observed with Chi alone (Fig. 5, Figs S5, S6; P>0.05 at all time-points except Nodal+Chi at 96 h, where P<0.05). However, the elongation is severely reduced with respect to Chi alone, with gastruloids tending to remain spheroid or ovoid (Fig. 5, Figs S5,S6). This suggests a synergy between the two signalling events. To test this further, we tried to rescue the effects of Nodal inhibition between 24 and 48 h on T/Bra expression. The maximum average expression of T/Bra::GFP in gastruloids treated with SB43 between 24-48 h AA, followed by Chi and Nodal co-stimulation between 48-72 h AA was not as high as that produced by Chi and Nodal co-stimulation at 48 and 72 h AA. Although the levels of expression at 96 h were enhanced compared with Chi and Nodal co-stimulation with less variation (Fig. 5, Fig. S6); P<0.01), the gastruloids were less polarised and peak expression was shifted anteriorly; however, the expression was maintained at higher levels at 120 h (Fig. 5, Fig. S6). Additionally, the increased elongation that was observed with SB43 (24-48 h) and Chi (48-72 h) treatment is suppressed in this condition, and gastruloids tended to stay more spherical, indicating that increased Nodal signalling at this period negatively impacts the elongation, similar to Nodal stimulation alone (48-72 h; Fig. 5, Fig. S6).

These results demonstrate an absolute requirement for Nodal signalling in the expression of T/Bra and its requirement for precise modulation of its levels at specific phases for the elongation. Furthermore, they suggest a negative impact of Nodal signalling on axial elongation.

**Wnt signalling promotes T/Bra expression and axial elongation in gastruloids**

To test the role of Wnt signalling on the patterning process, gastruloids were treated in different regimes with either recombinant Wnt3a or its antagonist Dkk1, as well as with small-molecule inhibitors of Wnt signalling (IWP2, which inhibits secretion of all Wnt proteins (Chen et al., 2009); and XAV939, which increases β-catenin degradation through tankyrase inhibition (Huang et al., 2009) (Fig. 6, Fig. S7). As demonstrated above, Wnt3a is able to substitute for Chi during the 48-72 h AA period with no significant difference in the normalised fluorescence traces at any time-point (Figs 3B,C, 6A,B; P>0.05). Pre-treatment with Wnt3a prior to a pulse of Chi enhanced the expression of T/Bra::GFP (P<0.05 at 48 h and 120 h), reduced expression heterogeneity at later time-points (shown in Fig. S8, lower panel, by the normalised root square distance) and generated an elongated phenotype more rapidly than in controls (Fig. 6, Fig. S7). By contrast, pre-treatment with Dkk1, XAV939 or IWP2 before Chi exposure results in a significantly delayed and variable expression of T/Bra (Fig. 6, Figs S7, S8; see...
significance matrix in Figs S7, S8); however, we observe differences in the response to Dkk1 and IWP2, which target Wnt expression and receptor binding, compared with XAV939, which targets active β-catenin (Fig. 6, Figs S7, S8). This suggests a requirement for non-canonical Wnt signalling in T/Bra::GFP maintenance, as reductions in Wnt expression (IWP2) or receptor interaction (Dkk1) have a more dramatic effect than reductions in β-catenin activity (XAV939) (Fig. 6, Figs S7, S8). These results reveal that Wnt signalling is essential and the primary signal required for the elongation of gastruloids, but that it cooperates with Nodal in the control of T/Bra expression and polarisation.

A synergy between Nodal and Wnt signalling during axial organisation has been reported in other organisms (Crease et al., 1998; Skromne and Stern, 2001; Steinbeisser et al., 1993) and is supported by our results, which, in addition, suggest different roles for each signalling system. Whereas Nodal is essential for the onset of T/Bra expression, Wnt/β-catenin signalling provides amplification and robustness to the response, promotes Nodal expression by positive feedback, and mediates axial elongation.

**Wnt/β-catenin can generate multiple axes in a Nodal-dependent manner**

To further delimit the requirements for Wnt/β-catenin signalling, we exposed aggregates to Chi for 24 h at different periods between 24 and 72 h AA, and analysed elongation and T/Bra expression (Fig. 7, Fig. S9; D.A.T. and A.M.A., unpublished). The experiments reveal that the 48-72 h period is crucial for both the elongation and correct patterning of the gastruloids. Although in all cases there is localised T/Bra::GFP expression and tissue elongation, exposure to Chi during the 48-72 h period elicits this behaviour most effectively (Fig. 7A, B, Fig. S9). In the course of these experiments, we observed that long exposures to Wnt signalling between 24-72 h...
AA, led to gastruloids with more than one focus of elongation and T/Bra::GFP expression that was significantly different from the 48-72 h control (Fig. 7A,B, Fig. S9; P < 0.05). In contrast, exposure between 48 and 96 h AA tends to abolish the focussed polarisation of T/Bra::GFP expression and the gastruloids are wider, resulting in a less slender elongation phenotype; the fluorescence traces along the spine of the gastruloids, however, are similar to the control 48-72 h Chi pulse (Fig. 7A,B, Fig. S9; P > 0.05).

These results reveal two overlapping events in the patterning of the gastruloids centred around the 48 h AA time that we have...
mapped to \( \sim E6.0 \) in the embryo. Between 24 and 48 h AA there is autonomous axial organisation from within the gastruloid that is stabilised through Wnt/\( \beta \)-catenin signalling but is critically dependent on Nodal signalling. Following this period (after 48 h), it is essential that Nodal signalling is tightly regulated, as it negatively impacts the elongation potential of the gastruloid and long exposures abolish elongation without altering the localisation of T/Bra::GFP expression. This highlights the period between 24 and 48 h as being crucial for axial establishment, which is then consolidated in the period after 48 h AA.

**BMP promotes T/Bra expression but not axial elongation**

In the embryo, the expression of Nodal and Wnt3 is thought to be modulated by BMP signalling (Rossant and Tam, 2009; Stern, 2006; Takaoka and Hamada, 2012) and it has been suggested that this is also the case in vitro (Harrison et al., 2017). As described above, a reporter for BMP signalling does not exhibit any detectable expression in the early stages of patterning (Figs 2E, 8A,B, Fig. S10). Consistent with this, exposure of the gastruloids to dorsomorphin H1 (DMH1) (Neely et al., 2012), a small molecule inhibitor of BMP signalling, prior to the Chi pulse did not significantly alter the expression pattern of T/Bra::GFP or the morphology (length and roundness) of the gastruloids up to 96 h AA (Fig. 8A,B, Fig. S10; \( P>0.05 \)). Addition of BMP between 24-48 h AA followed by a Chi pulse resulted in a more-focused expression of T/Bra::GFP at 120 h and, although the length of the gastruloids was broadly similar to that of the control there was a clear effect on the elongation process (Fig. 8A,B, Fig S10). On the other hand, when BMP is applied instead of Chi between 48 and 72 h AA, although the majority of gastruloids express the BMP reporter (\( \sim 88\%) \) albeit at a much lower level than in the Chi-treated control, only half of these exhibit polarisation. Additionally, the frequency of elongation is greatly reduced when compared with Chi (\( \sim 31\% \) elongated; Fig. 8C). This suggests that, in our in vitro system, BMP cannot substitute for Chi. Application of BMP between 24 and 48 h AA leads to a weak focus of expression that is not consistently placed at the elongating tip, and no elongation is observed (Fig. 8B, Fig. S10). Altogether, these results suggest that

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**Fig. 8.** BMP signalling is dispensable for early gastruloid patterning. (A) T/Bra::GFP gastruloids stimulated with Chi (48-72 h) AA following a 24 h pulse of either vehicle (top), BMP4 (middle) or DMH1 (bottom; \( n=12, 13 \) and 13 at 120 h, respectively), an inhibitor of BMP signalling between 24 and 48 h AA. Normalised fluorescence traces shown per condition (A) with corresponding fluorescence and shape descriptor quantification (B). Inhibition of BMP signalling by DMH1 or activation by BMP4 (24-48 h AA) does not alter the initial patterning of gastruloids; BMP treatment at this time has minimal effect on the subsequent patterning. (C) Gastruloids imaged at 120 h by wide-field microscopy following 24-48 h of vehicle or BMP4 stimulation (pink horizontal box) followed by either vehicle, Chi or BMP4 as indicated (blue horizontal box) between 48 and 72 h AA (\( n=16 \) per condition). (D) Stimulation schematic. BMP4 is unable to substitute for Chi in terms of the elongation and patterning of T/Bra, and its sustained expression over time (refer to Fig. S10 for further details and statistical analysis). Scale bar: 200 µm.
Nodal mutant gastruloids treated with Chi were unable to upregulate vehicle to DMSO controls. Immunofluorescence revealed that reduced, but not eliminated, compared with the vehicle to Chi and phenotype (0 to 50%), and the number of protrusions was greatly proportion of gastruloids displaying an elongated non-protrusion signalising regimes. Addition of Nodal (24-48 h AA) drastically increased the but not statistically significantly (Fig. 9B, Fig. S11). Application of the size of the protrusions was also increased relative to the control, similar to controls, with some showing four or more protrusions; (Fig. 9A). However, the average number of protrusions was ∼50%, supporting a role for Wnt signalling in elongation (Fig. 9B, Fig. S11). We then attempted to rescue these gastruloids using various appropriate vehicle controls, standard conditions and grown in N2B27 supplemented with the appropriate vehicle controls, Nodal mutant gastruloids remain spherical or ovoid, exhibit a number of protrusions and, by 120 h AA, a large proportion (~90%) have developed small bulbous structures at varying locations (Fig. 9A,B, Fig. S11). These data confirm the absolute requirement for Nodal in symmetry breaking. We then attempted to rescue these gastruloids using various signalling regimes. Addition of Nodal (24-48 h AA) reduces the frequency of protrusions but the number is not significantly different from the control (Fig. 9B). Treatment with Chi (48-72 h AA) leads to an increase in the proportion of elongated gastruloids (~50%), supporting a role for Wnt signalling in elongation (Fig. 9A). However, the average number of protrusions was similar to controls, with some showing four or more protrusions; the size of the protrusions was also increased relative to the control, but not statistically significantly (Fig. 9B, Fig. S11). Application of Nodal (24-48 h) followed by Chi (48-72 h) drastically increased the proportion of gastruloids displaying an elongated non-protrusion phenotype (0 to 50%), and the number of protrusions was greatly reduced, but not eliminated, compared with the vehicle to Chi and vehicle to DMSO controls. Immunofluorescence revealed that Nodal mutant gastruloids treated with Chi were unable to upregulate the posterior markers T/Bra and Cdx2 compared with previous observations (Fig. 1). However, addition of Nodal prior to the Chi pulse rescued the patterning and location of the reporters (Fig. 9C).

To assess whether the timing and duration of Nodal addition are important for the rescue of the Nodal mutant phenotype, Nodal was applied at 48-72, 72-96 and 24-72 h AA, in addition to Chi between 48 and 72 h AA, and gastruloid morphology was assessed at 120 h AA (Fig. 9B, Fig. S11). Although there was some variation between experimental replicates, applying Nodal at later time points reduced the ‘no protrusion-elongated’ phenotype while increasing the ‘no protrusions, no elongation’ morphology (Fig. 9B, Fig. S11) compared with the 24-48 h Nodal to 48-72 h Chi condition. A longer duration of Nodal signalling did not result in effects that were different from those obtained for 72-96 h Nodal. These data reveal the absolute requirement for Nodal signalling for the symmetry-breaking event, and that tight control of Nodal signalling is necessary for proper gastruloid elongation.

**DISCUSSION**

We find that gastruloids, mammalian embryonic organoids, develop an embryo-like AP organisation that is characteristic of the tail domain of the embryo in the absence of external patterned influences. Significantly, they organise an AP axis in the absence of extra-embryonic tissues, which have been shown to drive axial organisation during embryogenesis (Rossant and Tam, 2009; Stern, 2006; Takaoka and Hamada, 2012). This observation extends our previous finding (van den Brink et al., 2014) and leads us to suggest that, in vivo, the role of the extra-embryonic tissues might not be to induce axial organisation but rather to bias an intrinsically driven symmetry-breaking event similar to the one we report here that occurs in the embryo (Turner et al., 2016b, 2017 preprint). The deployment of signalling centres around the embryo thus provides a
robust source of spatial information that positions the onset of gastrulation in a defined and reproducible location. If the symmetry breaking were stochastic, it would be difficult to link gastrulation to the interactions of the emerging mesoderm cells with extra-embryonic tissues in a reproducible manner. Our suggestion is supported by the observation that, in the absence of extra-embryonic signals, the embryo still exhibits a degree of patterning and axial organisation, although this is somewhat variable (Perea-Gómez et al., 2002; Yamamoto et al., 2004). In addition, a recent report demonstrates that trophoderm stem cells appear to impose polarisation on T/Bra expression in aggregates of ESCs (Harrison et al., 2017) that, as in our case (van den Brink et al., 2014), lack visceral endoderm. However, this report from Harrison et al. (2017) suggests a strict requirement for extra-embryonic tissues, specifically trophoderm, for the expression of T/Bra, which is at odds with our observations that the expression and localisation of T/Bra occurs in over 90% of the extra-embryonic-free aggregates (Baillie-Johnson et al., 2015; Turner et al., 2016b, 2017 preprint; van den Brink et al., 2014) and with previous reports that also showed T/Bra polarisation in embryoid bodies (ten Berge et al., 2008; Marikawa et al., 2009). There are a number of explanations for this discrepancy. It may be that the interaction between extra-embryonic and embryonic tissues raises the threshold of the patterning events and creates interdependencies for relative spatial biases (see also Turner et al., 2016a). Alternatively, the spatial confinement of the TSCs, and/or receptor-ligand interactions of ESCs with Matrigel components, could create conditions that affect the rate and the frequency of the symmetry-breaking events that we observe in our experiments. The resolution of these discrepancies will require further experiments in both systems. In our case, we have shown that the transition from the pluripotent to the primed state follows a pattern similar to that of the embryo and it will be interesting to see if this is also the case when the ESCs are confined in Matrigel.

A most important consequence of the symmetry breaking event in the embryo is the polarised onset of T/Bra expression (Rivera-Pérez and Magnuson, 2005; Yoon et al., 2015). A connection between the expression of T/Bra and Wnt signalling had been reported in assorted EBs (ten Berge et al., 2008), but the reproducibility and precision of this process in gastruloids allows us to investigate its origin. In gastruloids, the joint action of Nodal and Wnt signalling promotes the expression and localisation of T/Bra expression between 24 and 48 h AA, but the stabilisation of this pattern requires a burst of Wnt signalling between 48 and 72 h AA. An interpretation of our results is that Nodal provides the initial input on the expression of T/Bra and the organisation of an AP axis, but that these effects are enhanced and consolidated by Wnt/β-catenin signalling. This possibility is supported by the observation that, in the embryo, T/Bra expression is initiated and localised in the absence of Wnt signalling, though this pattern is not robust (Tortelote et al., 2013). Similar interactions between Nodal and Wnt/β-catenin signalling have been described in chick and frog embryos (Crease et al., 1998; Skromme and Stern, 2001; Steinbeisser et al., 1993) and we have also shown that they occur in an adherent culture system of primitive streak formation (Turner et al., 2014b). It is therefore likely that they also occur in the mammalian embryo. At the molecular level, this synergy is supported by reports of molecular interactions between Smad2, Smad3 and β-catenin in the regulatory regions of genes expressed in the primitive streak and specifically of Nodal and T/Bra (Dahle et al., 2010; Estarás et al., 2015; Funa et al., 2015).

Mechanisms to explain how Nodal leads to symmetry breaking during AP axis formation often invoke reaction-diffusion mechanisms (Juan and Hamada, 2001; Marcon et al., 2016; Müller et al., 2012). Accordingly, interactions between Nodal and its inhibitor and downstream target Lefty1 lead to the asymmetric localisation of both proteins and to the asymmetric expression of target genes, e.g. T/Bra. Surprisingly, we observe that ubiquitous exposure of gastruloids to Nodal leads to polarisation of T/Bra expression and, moreover, that this will occur when high ubiquitous concentrations of Nodal are provided to a Nodal mutant gastruloid. This observation challenges many of our current notions about the patterning driven by Nodal and demonstrates that Nodal needs not be localised to generate an axis. One possible explanation for this observation that is consistent with our results is that Nodal signalling initiates the expression of T/Bra but that it is not involved in its refinement and maintenance, which depend on a positive feedback between Wnt/β-catenin signalling and T/Bra (Turner et al., 2014b). Indeed, several Wnt genes are known to be downstream targets of T/Bra (Evans et al., 2012), which, in turn, is a target of Wnt/β-catenin signalling, thus providing the elements for a positive-feedback loop that could be involved in the patterning and localisation of T/Bra and its downstream targets. In agreement with this, we observe a spatial correlation between the pattern of Wnt signalling and of T/Bra expression (Fig. 1C,D).

Our results also highlight that, in addition to, and independently of, its role in T/Bra expression and of its interactions with Nodal/Smad2/Smad3 signalling, Wnt/β-catenin signalling is central to axial elongation. This provides independent proof of this well-established phylogenetic relationship (Petersen and Reddien, 2009). Timing of exposure suggests two different phases to this involvement. Long exposures to Wnt signalling early (24-72 h AA; E5.0-E7.0 in the embryo) can lead to multiple axes, only some of which express T/Bra; this mirrors situations with gain of function of Wnt signalling (Merrill et al., 2004; Pöpperl et al., 1997). Increased activity later on (48-96 h AA; E6.0-E8.0) results in abolition of the polarity and ubiquitous expression of T/Bra. These observations highlight two temporally separate activities of Wnt: a first one in the establishment and enhancement of the AP axis, probably together with Nodal signalling; followed by a second phase of stabilisation of T/Bra expression and axial elongation. As in the case of Nodal, but in a more manifest manner, the observation that a localised source of Wnt/β-catenin activity is not necessary for the polarisation of T/Bra expression and the elongation of the gastruloid, questions the widespread notion for a role of Wnt signalling gradients in pattern formation and supports views in which the function of Wnt signalling is to control the signal-to-noise ratio of events induced by other means (Martínez Arias and Hayward, 2006; Mateus et al., 2009).

A remarkable feature of gastruloids is the degree to which their spatial organisation resembles the posterior region of an E8.5 embryo. However, this structure, though coherent, is partial, e.g. gastruloids lack the most anterior structures (van den Brink et al., 2014). In this regard, they resemble Dkk (Fossat et al., 2011) or some Smad2/Smad3 (Dunn et al., 2004) mutants and show that it is possible to orientate an axis without an identifiable head or brain. A likely cause for this deficiency is a combination of the exposure to high levels of Wnt signalling between 48 and 72 h AA, which will suppress anterior development (Arkell et al., 2013; Pöpperl et al., 1997), and the lack of a prechordal plate and anterior mesendoderm, which are essential for anterior neural induction (Andoniadou and Martínez-Barbera, 2013). Thus, although signalling from the extra-embryonic tissues might not be strictly necessary for the
establishment of an AP axis, it might be essential not only for the reliable positioning of the initiation of gastrulation, but also for the location of the brain at the opposite pole.

Over the past few years a number of experimental systems have emerged in which ESCs become spatially patterned and each of them can contribute to a construction of our understanding of the connection between cell fate assignments and the polarisation of the embryo (Bauwens et al., 2008; Desbaillets et al., 2000; Etoe et al., 2016; Harrison et al., 2017; Warmflash et al., 2014). The system that we have developed has some advantages, in particular its 3D self-organisation, reproducibility and robustness allow it to be used in long-term studies and screens. However, despite the resemblance to early embryos, the current generation of gastruloids exhibit differences in detail that create the challenge of what it takes to make the similarities more obvious. In this process, engineering will play an important role and help the rational design of tissues and organs. Importantly, we feel that our findings suggest that gastruloids could be a useful substitute for embryos in the study of early development.

**MATERIALS AND METHODS**

**Cell lines and routine cell culture**

AR8::mCherry [Nodal signalling reporter (Serup et al., 2012)], T/Bra::GFP (Fehling et al., 2003), GATA6::H2B-Venus (Freyer et al., 2015), IBRE4::Cerulean (Serup et al., 2012), miR-290-mCherry/mir-302-eGFP (Parchem et al., 2014), Nodal::YFP reporter (Papanayotou et al., 2014), Nodal−/− (Camus et al., 2006), Sox17::GFP (Niakan et al., 2010) and TCF/LEF::mCherry (TLC2) (Faunes et al., 2013; Ferrer-Vaquer et al., 2010) were cultured in GMEM supplemented with LIF, foetal bovine serum, non-essential amino acids, glutamax, sodium pyruvate and β-mercaptoethanol (ESL medium) on gelatinised tissue-culture flasks and passaged every second day as previously described (Faunes et al., 2013; Kalmar et al., 2009; Turner et al., 2014a,b,c). If cells were not being passaged, half the medium in the tissue culture flask was replaced with ESL. All cell lines were routinely tested and confirmed to be free from mycoplasma. See Table S3 for the cell lines used and Table S4 for the small molecules and recombiant proteins used in this study.

**Gastruloid culture and application of specific signals**

Aggregates of mouse ESCs were generated using an optimised version of the previously published protocol (Baillie-Johnson et al., 2015; van den Brink et al., 2014) (for further details, see supplementary Materials and Methods). Table S3 details the number of cells required to generate gastruloids for the cell lines used in this study.

**Immunofluorescence, microscopy and data analysis**

Gastruloids were fixed, stained with the required antibodies (Table S2) and imaged by confocal microscopy according to the protocol previously described (Baillie-Johnson et al., 2015). Wide-field, single-time-point and time-lapse images of gastruloids were acquired using a Zeiss AxioObserver. Z1 in a humidified CO2 incubator (5% CO2, 37°C) with Illumination provided by an LED white-light system (Laser2000) and emitted light recorded using a back-illuminated iXon888 Ultra EMCCD (Andor Technology). Images were analysed using FIJI (Schindelin et al., 2012) and plug-ins therein as previously described (Baillie-Johnson et al., 2015; Preibisch et al., 2009; Sorollo et al., 2014). The were data analysed and plotted as described in the supplementary Materials and Methods.

**Statistical analysis**

Statistical analysis of the normalised fluorescence traces of the gastruloids was performed in Matlab (Mathworks) and is described in the supplementary Materials and Methods.

**Quantitative RT-PCR**

Gastruloids (n=64 per time-point) from T/Bra::GFP mouse ESCs, subjected to a Chi or DMSO pulse (between 48 and 72 h AA), harvested at 48 or 72 h AA, trypsinised, pelleted and RNA extracted using the RNaseasy Mini kit (Qiagen, 74104) according to the manufacturer’s instruction as previously described (Turner et al., 2014c). Samples were normalised to the housekeeping gene *Ppia*. The sequences for the primers are described in Table S5.

**Orientation of gastruloids**

To define the AP orientation of gastruloids, we have assigned the point of T/Bra::GFP expression as the ‘posterior’, because the primitive streak, which forms in the posterior of embryo, is the site of T/Bra expression in the embryo (Beddington et al., 1992; Herrmann, 1991; Wilkinson et al., 1990). At least two biological replicates were performed for each condition.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.150391.supplemental

**References**


Supplemental Materials and Methods

**Immunofluorescence, Microscopy and data analysis:** Gastruloids were fixed and stained for as required according to the protocol previously described (Baillie-Johnson et al., 2015). Hoechst3342 was used to mark the nuclei (see **table S2** for the antibodies used and their dilutions). Confocal z-stacks of Gastruloids were generated using an LSM700 (Zeiss) on a Zeiss Axiovert 200 M using a 40× EC Plan-NeoFluar 1.3 NA DIC oil-immersion objective. Hoechst3342, Alexa-488, -568 and -633 were sequentially excited with 405, 488, 555 and 639 nm diode lasers respectively as previously described (Turner et al., 2014). Data capture was carried out using Zen2010 v6 (Carl Zeiss Microscopy Ltd, Cambridge UK). The z-stacks were acquired for at least 4 Gastruloids per condition with a z-interval of 0.5µm. Images were analysed using the ImageJ image processing package FIJI (Schindelin et al., 2012).
Widefield, single-time point images of *Gastruloids* were acquired using a Zeiss AxioObserver.Z1 (Carl Zeiss, UK) in a humidified CO₂ incubator (5% CO₂, 37°C) with a 20x LD Plan-Neofluar 0.4 NA Ph2 objective with the correction collar set to image through plastic. Illumination was provided by an LED white-light system (Laser2000, Kettering, UK) in combination with filter cubes GFP-1828A-ZHE (Semrock, NY, USA), YFP-2427B-ZHE (Semrock, NY, USA) and Filter Set 45 (Carl Zeiss Microscopy Ltd. Cambridge, UK) used for GFP, YFP and RFP respectively, and emitted light recorded using a back-illuminated iXon888 Ultra EMCCD (Andor, UK). Images were analysed using FIJI (Schindelin et al., 2012) and plugins therein as previously described (Baillie-Johnson et al., 2015) and when required, images were stitched using the ‘Pairwise Stitching’ plugin in FIJI (Preibisch et al., 2009). Briefly, the fluorescence intensity was measured by a line of interest (LOI) drawn from the posterior to anterior region of the *Gastruloid* with the LOI width set to half the diameter of a typical *Gastruloid* at 48h (100px with the 20x objective). The background for each position was measured and subtracted from the fluorescence for each *Gastruloid*. Shape-descriptors were generated by converting brightfield images of Gastruloids to binary images and measuring them by particle detection in FIJI.

Fluorescence levels were normalised to the maximum obtained in following Chi stimulation, and the maximum length of each *Gastruloid* was rescaled 1 unit. Average fluorescence traces of *Gastruloids* ±S.D. are shown in the main figures, and the raw data and individual traces in the supplemental data. For live imaging experiments, each well of a 96-well plate containing individual *Gastruloids* were imaged as described above using both the 20x (24-72h) and the 10x (72-96h) objectives, and images captured every 20 min for a maximum of 96h (120h AA). All images were analysed in FIJI (Schindelin et al., 2012) using the LOI interpolator (Soroldoni et al., 2014) with the LOI set as described above.

Data processing and graph plotting was performed in the Jupyter IPython notebook environment (Kluyver et al., 2016; Perez and Granger, 2007) using the following principle modules: Matplotlib (Hunter, 2007; McDougall et al., 2016), NumPy & SciPy (Oliphant, 2007; Terrel et al., 2015a; Terrel et al., 2015b), tifffile (Silvester, 2015), Statsmodels (Fulton et al., 2014) and Pandas (Van den Bossche et al., 2015). All code is freely available upon request.

**Statistical Analysis:** Statistical analysis of the normalised fluorescence traces was performed in Matlab (Mathworks, 2016a). Let \( f_{c,i}(x) \) denote the signal intensity profile for T/Bra expression over the normalized length of the \( i^{th} \) *Gastruloid* in condition \( c \); \( x \) denotes the coordinate along the normalized length of the \( i^{th} \) *Gastruloid* where \( x = 0 \) denotes the posterior end and \( x = 1 \) denotes
the anterior end. $\mu(x)$ and $\sigma(x)$ denote the mean and standard deviation, respectively, of the signal intensity profile for T/Bra expression over the normalised length of the control Gastruloids. We define a measure of assessing differences between intensity profiles, of a Gastruloid in a given condition and the control Gastruloids, similar to the root-mean-square deviation used to measure differences between values of an estimator and the values observed. We call this measure the Normalised Root Square Distance ($\eta$) and for the $i^{th}$ Gastruloid in condition $c$ it is defined as follows:

$$\eta_{c,i} = \sqrt{\frac{1}{N} \sum_{j=1}^{N} \left( f_{c,i}(x_j) - \mu_0(x_j) \right)^2 / \sigma_0^2(x_j)}$$

where $N$ denotes the maximum number of points (typically 100) defining the normalised length of the Gastruloid. As a physical interpretation of this measure, it can be seen that $\eta_{c,i} = 0$ means that the signal intensity profile for the $i^{th}$ Gastruloid in condition $c$ is identical to the mean intensity profile of the control Gastruloids. $\eta_{c,i} \leq 1$ means that the signal intensity profile for the $i^{th}$ Gastruloid in condition $c$ is within the standard deviation around the mean intensity profile of the control Gastruloids, thereby implying that the Gastruloid in condition $c$ is similar to the control. Significance between treatments within each time-point was determined using a non-paired Student’s t-test.

**Gastruloid culture and application of specific signals:** Aggregates of mouse ESCs were generated using an optimised version of the previously described protocol (Baillie-Johnson et al., 2015; van den Brink et al., 2014). Mouse ESCs harvested from tissue-culture flasks were centrifuged and washed twice in warm PBS. After the final wash, the pellet was resuspended in 3ml warm N2B27 and cell concentration determined using a Moxi™ Z automated cell counter with curve-fitting (Orflo Technologies). The number of cells required to generate Gastruloids of ~150µm in diameter by 48h (optimised for each cell line, ~300 cells; table S3) was then plated in 40µl droplets of N2B27 in round-bottomed low-adhesion 96-well plates. Counting cells after washing in PBS in this way instead of prior to the washes (as described previously (Baillie-Johnson et al., 2015; van den Brink et al., 2014)) results in the number of cells required for Gastruloid formation being ~100 fewer than previously described as fewer are lost during washing. See table S3 for the number of cells required for each cell line.
In experiments which required the addition of specific factors to Gastruloids on the second day of aggregation (24-48h), 20µl medium was carefully removed with a multichannel pipette, and 20µl of N2B27 containing twice the concentration of the required factors was added. This method was preferable to the addition of smaller volumes containing higher concentrations of agonist/antagonists, as the data from these experiments showed more variation between Gastruloids (DAT, PB-J, AMA unpublished). Control experiments showed that replacement of half the medium at this stage did not significantly alter the ability of Gastruloids to respond to signals on the third day (DAT, PB-J, AMA unpublished). The next day, 150µl fresh N2B27 was added to each of the wells with a multichannel pipette and left for no more than 30 min to wash the Gastruloids; a time delay ensured that sample loss was prevented. Following washing, 150µl N2B27 containing the required factors was then applied. The small molecules used in this study and their concentrations are described in table S4.
Supplemental Movies

**Movie 1. T/Bra::GFP expression in Gastruloids following DMSO treatment (48-72h AA).** Gastruloids made from T/Bra::GFP mESCs stimulated with a mock pulse of DMSO and imaged by wide-field microscopy from 24h to 120h AA every 20 min. The 20x objective was used between 24 and 72h, followed by the 10x objective from 72h to the end of the experiment. Quantification of both the length and fluorescence as a function of time can be seen in Fig. 3D (top).

**Movie 2. T/Bra::GFP expression in Gastruloids following Chi treatment (48-72h AA).** Gastruloids made from T/Bra::GFP mESCs stimulated with a pulse of Chi and imaged by wide-field microscopy from 24h to 120h AA every 20 min. The 20x objective was used between 24 and 72h, followed by the 10x objective from 72h to the end of the experiment. Quantification of both the length and fluorescence as a function of time can be seen in Fig. 3D (bottom).
Supplemental Tables

Tables S1-S5

**Table S1.** Expression phenotype of T/Bra::GFP mESCs. The proportion of T/Bra::GFP Gastruloids not expressing the reporter (No Expression) or displaying either Polarised or Ubiquitous expression at 24, 48 and 72h AA followed by a pulse of DMSO or Chi (72h). The standard deviation is shown in brackets and the number of Gastruloids analysed are shown.

<table>
<thead>
<tr>
<th>Condition</th>
<th>No Expression</th>
<th>Polarised</th>
<th>Ubiquitous</th>
<th>Spherical</th>
<th>Ovoid</th>
<th>Elongated</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2B27</td>
<td>26.8 (21.5)</td>
<td>62.5 (16.1)</td>
<td>10.7 (15.2)</td>
<td>100.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>23.7 (13.2)</td>
<td>74.1 (11.8)</td>
<td>2.2 (3.4)</td>
<td>67.0 (9.4)</td>
<td>33.0 (9.4)</td>
<td>0.0 (0.0)</td>
<td>140</td>
</tr>
<tr>
<td>48h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>3.6 (-)</td>
<td>89.3 (-)</td>
<td>7.1 (-)</td>
<td>10.7 (-)</td>
<td>85.7 (-)</td>
<td>3.6 (-)</td>
<td>28</td>
</tr>
<tr>
<td>Chi</td>
<td>0.0 (-)</td>
<td>91.2 (11.7)</td>
<td>8.8 (11.7)</td>
<td>23.3 (18.2)</td>
<td>52.9 (18.1)</td>
<td>23.8 (26.3)</td>
<td>28</td>
</tr>
</tbody>
</table>

**Table S2.** Antibodies and their concentrations used for Gastruloid immunofluorescence with the associated supplier details.

<table>
<thead>
<tr>
<th></th>
<th>Species</th>
<th>Dilution</th>
<th>Cat. Number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brachyury</td>
<td>Goat</td>
<td>1:200</td>
<td>sc-17743</td>
<td>Santa Cruz Biotechnologies</td>
</tr>
<tr>
<td>CDX2</td>
<td>Rabbit</td>
<td>1:200</td>
<td>MA5-14494</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>GFP</td>
<td>Chicken</td>
<td>1:2000</td>
<td>A11122</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Nanog</td>
<td>Mouse</td>
<td>1:300</td>
<td>14-5761-80</td>
<td>e-Biosciences</td>
</tr>
<tr>
<td>Sox2</td>
<td>Rabbit</td>
<td>1:200</td>
<td>AB5603</td>
<td>Millipore</td>
</tr>
<tr>
<td>Sox17</td>
<td>Goat</td>
<td>1:200</td>
<td>AF1924</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat-A633</td>
<td>Donkey</td>
<td>1:500</td>
<td>A21082</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Mouse-A568</td>
<td>Donkey</td>
<td>1:500</td>
<td>A10037</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Rabbit-A488</td>
<td>Donkey</td>
<td>1:500</td>
<td>A21206</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Hoechst3342</td>
<td>n/a</td>
<td>1:1000</td>
<td>H3570</td>
<td>Invitrogen (ThermoFisher)</td>
</tr>
</tbody>
</table>
Table S3. Cell lines used and numbers of cells required for Gastruloid culture. The average diameter of the Gastruloids at 48h AA is indicated with the standard deviation and the number of Gastruloids measured. ND: not determined.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Reference</th>
<th>Cells/40µl</th>
<th>48h diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR8::mCherry</td>
<td>(Serup et al., 2012)</td>
<td>450</td>
<td>182.7 ±17.3 (n = 83)</td>
</tr>
<tr>
<td>T/Bra::GFP</td>
<td>(Fehling et al., 2003)</td>
<td>300</td>
<td>161.0 ±26.2 (n = 222)</td>
</tr>
<tr>
<td>miR-290-mCherry/mir-302-eGFP (DRC)</td>
<td>(Parchem et al., 2014)</td>
<td>300-400</td>
<td>N.D.</td>
</tr>
<tr>
<td>GATA6::H2B-Venus</td>
<td>(Freyer et al., 2015)</td>
<td>300</td>
<td>154.2 ± (n = 10)</td>
</tr>
<tr>
<td>IBRE4-TA-Cerulean</td>
<td>(Serup et al., 2012)</td>
<td>300</td>
<td>152.6 ±12.2 (n = 39)</td>
</tr>
<tr>
<td>Nodal::YFP</td>
<td>(Papanayotou et al., 2014)</td>
<td>400</td>
<td>138.7 ±16.1 (n = 124)</td>
</tr>
<tr>
<td>Nodal(\d) (FC-15)</td>
<td>(Camus et al., 2006)</td>
<td>300</td>
<td>181.6 ±23.7 (n = 251)</td>
</tr>
<tr>
<td>Sox17::GFP</td>
<td>(Niakan et al., 2010)</td>
<td>400</td>
<td>N.D.</td>
</tr>
<tr>
<td>TCF/LEF::mCherry (TLC2)</td>
<td>(Faunes et al., 2013; Ferrer-Vaquer et al., 2010)</td>
<td>200-300</td>
<td>194.9 ±20.7 (n = 56)</td>
</tr>
</tbody>
</table>

Table S4. Concentrations of Small molecules and recombinant proteins used in this study, and the associated supplier details.

<table>
<thead>
<tr>
<th>Reference</th>
<th>[Working]</th>
<th>[Stock]</th>
<th>Cat. Number</th>
<th>Supplier</th>
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<tr>
<td>CHIR99201</td>
<td>(Ring et al., 2003)</td>
<td>3µM</td>
<td>10mM</td>
<td>4423</td>
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<tr>
<td>DMH1</td>
<td>(Neely et al., 2012)</td>
<td>500nM</td>
<td>5mM</td>
<td>HY-12273</td>
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<tr>
<td>IWP2</td>
<td>(Chen et al., 2009)</td>
<td>1µM</td>
<td>5mM</td>
<td>04-0034</td>
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<tr>
<td>SB431542</td>
<td>(Inman et al., 2002)</td>
<td>10µM</td>
<td>100mM</td>
<td>1614</td>
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<tr>
<td>XAV939</td>
<td>(Huang et al., 2009)</td>
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<td>10mM</td>
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<tr>
<td>BMP4</td>
<td>-</td>
<td>1ng/ml</td>
<td>100µg/ml</td>
<td>314-BP</td>
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<tr>
<td>DKK</td>
<td>-</td>
<td>200ng/ml</td>
<td>100µg/ml</td>
<td>5897-DK</td>
</tr>
<tr>
<td>Nodal</td>
<td>-</td>
<td>1µg/ml</td>
<td>50µg/ml</td>
<td>1315-ND-025</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>-</td>
<td>100ng/ml</td>
<td>40µg/ml</td>
<td>1324-WN-002</td>
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Table S5. Primer Sequences used for qRT-PCR.

<table>
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<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
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<td>Axin2</td>
<td>CTAGACTACGCGCATCAGGAA</td>
<td>GCTGGCAGACAGGACATA</td>
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<td>Bmp4</td>
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<td>ATGCTTGGGACTACGTGTTG</td>
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<td>CDX2</td>
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<td>CCCTTCCTGATTTGTGGAGA</td>
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<tr>
<td>Cer1</td>
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<td>AGGGTCAGAATTGACCTTTG</td>
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<tr>
<td>Chordin</td>
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<td>Dab2</td>
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<td>CAACTTGGCAGGAACAAA</td>
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<td>Dkk1</td>
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<td>CAATCCCTCCCCCTCAATAA</td>
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<td>Furin</td>
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<td>ppiA</td>
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<td>Wnt3a</td>
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</tbody>
</table>

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**Fig. S1. Expression of axial markers in Gastruloids.** (A,B) Stereo images of Gastruloids from Nodal::YFP (A) and Sox1::GFP (B) mESCs stained for anti YFP (green) and either CDX2 (A) or T/Bra (B) (red) at 120h AA. (C) Further examples of Gastruloids from the indicated cell lines at 120h AA (see Fig. 1C-F). Asterisks represent Gastruloids from a different replicate experiment.
Supplementary Figure S2

**Fig. S2. Quantification of T/Bra::GFP Gastruloid Fluorescence.** (A,B) Expression of the T/Bra::GFP reporter at the indicated time-points (DMSO or Chi (A) and Chi or Wnt3a (B) stimulation) prior to length normalisation (top) and following normalisation of the length to from 0 to 1 (middle). The bottom panel in each shows the length and roundness of the *Gastruloids* in the indicated conditions.
Supplementary Figure S3

**Fig. S3.** *Sox17::GFP is expressed anterior to the elongating region of the Gastruloids at 120h AA.*

*Gastruloids* made from *Sox17::GFP* mESCs were grown in standard conditions (see materials and methods), pulsed with Chi between 48 and 72h AA and imaged by widefield microscopy continuously for 96h with a time-interval of 20 min. Top row displays still images from the time-lapse experiment using the 20x objective (24h, 48h, 69h) and the 10x objective (72, 96, 120h; \(n = 21\)). Quantification of the length and fluorescence along the ‘mid-line’ of the *Gastruloid* every 20 min (bottom row; see materials and methods in main text and supplemental for explanation of quantification). Colour map represents the fluorescence and the time of Chi addition indicated. Gaps in the quantification are due to the *Gastruloid* leaving the field of view, an example of which is indicated at the 69h time-point (top row) with the hashed line representing the edge of the field of view. The posterior of the *Gastruloid* is orientated towards the base of the figures, as time-lapse imaging revealed the *Sox17::GFP* negative region was absent from the elongating, posterior region. Scale bar indicates 100µm in all images.
Fig. S4. Expression of GATA6::H2B-Venus in Gastruloids over time. Gastruloids made from GATA6::H2B-Venus mESCs were grown in standard conditions and imaged by widefield microscopy continuously for 115 h with a time-interval of 20 min (n = 9). GATA6 expression is apparent at approximately 88 h AA and becomes restricted to the anterior region of the Gastruloid (as judged by morphology).
Fig. S5. Quantifying the Effect of modulating Nodal signalling in Gastruloids (#1). (A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression at the indicated time-points prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). (E) Statistical analysis of the normalised fluorescence traces showing (upper panel) the normalised root square distance as a measure of the heterogeneity within each condition of the Gastruloids in the indicated conditions (see supplemental materials and methods), and (lower panel) the significance matrix showing the pairwise p values between individual treatments per time-point. Significance determined by non-paired Student’s t-test; p-values highlighted in red indicate p < 0.05. Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100 µm.
Fig. S6. Quantifying the Effect of modulating Nodal signalling in Gastruloids (#2). (A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). (E) Statistical analysis of the normalised fluorescence traces showing (upper panel) the normalised root square distance as a measure of the heterogeneity within each condition of the Gastruloids in the indicated conditions (see supplemental materials and methods), and (lower panel) the significance matrix showing the pairwise p values between individual treatments per time-point. Significance determined by non-paired Student’s t-test; p-values highlighted in red indicate p < 0.05. Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100 µm.
Supplementary Figure S7
Fig. S7. **Quantifying the Effect of modulating Wnt/β-Catenin signalling in Gastruloids (#1).** (A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). (E) Statistical analysis of the normalised fluorescence traces showing (upper panel) the normalised root square distance as a measure of the heterogeneity within each condition of the Gastruloids in the indicated conditions (see supplemental materials and methods), and (lower panel) the significance matrix showing the pairwise p values between individual treatments per time-point. Significance determined by non-paired Student’s t-test; p-values highlighted in red indicate p < 0.05. Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100 µm.
Supplementary Figure S8
Fig. S8. Quantifying the Effect of modulating Wnt/β-Catenin signalling in Gastruloids (#2). (A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). (E) Statistical analysis of the normalised fluorescence traces showing (upper panel) the normalised root square distance as a measure of the heterogeneity within each condition of the Gastruloids in the indicated conditions (see supplemental materials and methods), and (lower panel) the significance matrix showing the pairwise p values between individual treatments per time-point. Significance determined by non-paired Student’s t-test; p-values highlighted in red indicate p < 0.05. Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100 µm.
**Supplementary Figure S9**

Fig. S9. Quantifying the Effect of modulating the time of Wnt/β-Catenin signalling in Gastruloids. (A,B) Quantification of the reporter expression prior to length normalisation (A) and following normalisation of the length from 0 and 1 (B). The length and roundness of the Gastruloids in the indicated conditions (C). (D) Statistical analysis of the normalised fluorescence traces showing (upper panel) the normalised root square distance as a measure of the heterogeneity within each condition of the Gastruloids in the indicated conditions (see supplemental materials and methods), and (lower panel) the significance matrix showing the pairwise $p$ values between individual treatments per time-point. Significance determined by non-paired Student’s t-test; $p$-values highlighted in red indicate $p < 0.05$. Vertical line and coordinates in B correspond to the location and position of the peak maximum.
Supplementary Figure S10
Fig. S10. Quantifying the Effect of modulating BMP signalling in Gastruloids. (A) examples of T/Bra::GFP reporter expression in Gastruloids treated as indicated. (B, C, D) Quantification of the reporter expression prior to length normalisation (B) and following normalisation of the length from 0 and 1 (C). The length and roundness of the Gastruloids in the indicated conditions (D). (E) Statistical analysis of the normalised fluorescence traces showing (upper panel) the normalised root square distance as a measure of the heterogeneity within each condition of the Gastruloids in the indicated conditions (see supplemental materials and methods), and (lower panel) the significance matrix showing pairwise p values between individual treatments per time-point. Significance determined by non-paired Student’s t-test; p-values highlighted in red indicate p < 0.05. Vertical line and coordinates in C correspond to the location and position of the peak maximum. Scale bar indicates 100 µm.
Supplementary Figure S11

Fig. S11. Modulation of Nodal signalling in Nodal mutants. (A) Examples of Gastruloids treated with Chi between 48 and 72h with a 24h pulse of either vehicle or Nodal at the indicated time-points (24-48h, 48-72h, 72-96h and 24-72h AA). Pie charts indicated the proportion which do not show protrusions (‘no’), show protrusions (‘yes’), show protrusions with a defined AP axis (‘yes+APaxis’) or don’t show protrusions but still have a defined AP axis (‘no+APaxis’). The schematic for the time-course is indicated on the right of the panel. (B) Quantification of the area of the protrusions in the indicated experimental conditions. Significance determined following Mann-Whitney U test followed by Bonferroni adjustment, comparing selected columns. Asterisk indicates p < 0.05.
References from Supplemental Material


McDougall, D., Firing, E., Perez, F., Thomas, I., Ivanov, P., Nielsen, J. H., Seppänen, J. K., Kniazev,


Terrel, A. R., Harris, C., Cournapeau, D., Laxalde, D., Burovski, E., Moore, E., Pedregosa, F.,

