ERK1/2 is an ancestral organising signal in spiral cleavage

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SUMMARY

Embryonic organisers are signalling centres that instruct the establishment of body plans during animal embryogenesis, thus underpinning animal morphological diversity. In spiral cleavage – a stereotypic developmental programme ancestral to 14, nearly half, of the major animal groups (e.g., molluscs, annelids and flatworms), a cell known as the D-quadrant organiser defines cell fates and the body axes. ERK1/2 specifies the embryonic organiser in molluscs, yet how this signalling cascade exerts organising activity and whether this role is conserved in other spiral cleaving groups is unclear. Here, we demonstrate that ERK1/2 promotes the specification and inductive activity of the D-quadrant organiser in *Owenia fusiformis*, an early-branching annelid exhibiting ancestral developmental traits. In this species, active di-phosphorylated ERK1/2 mediated by FGF receptor activity localises to the 4d micromere, establishing the bilateral symmetry and specifying the hindgut and trunk mesodermal progenitor. Accordingly, impairing FGFR and ERK1/2 activity, as well as cell communication results in embryos developing anteroventrally radialised. Differential

26 transcriptomic profiling shows the ParaHox *cdx* and the Notch ligand *delta* as FGFR/ERK1/2
 27 downstream targets in 4d, further revealing that 4d specification instructs the expression of
 28 mesodermal and posterodorsal genes in neighbouring cells, putatively via the Notch pathway.
 29 The instructing role of ERK1/2 in the D-quadrant organiser is thus shared between *O.*
 30 *fusiformis* and molluscs, representing an ancestral trait of spiral cleavage. Altogether, our
 31 study begins to dissect the gene network promoting axial patterning and posterior growth in
 32 spiral cleavage, revealing extensive mechanistic diversification in body plan specification
 33 despite overall conservation of cleavage patterns in Spiralia.

34

35 **Keywords:** spiral cleavage, Spiralia, embryonic organiser, D-quadrant organiser, annelid,
 36 ERK1/2, MAPK pathway, FGF pathway, Notch pathway.

37 **Introduction**

38 Embryonic organisers are cells or groups of cells able to instruct neighbouring tissues towards
 39 certain fates and behaviours during animal embryogenesis [1, 2] (Figure 1A). Embryonic
 40 organisers thus play a critical role in patterning tissues and organs, promoting morphogenetic
 41 processes that establish animal body plans, thereby emerging as potential drivers of
 42 morphological evolution [3-5]. Spiralia is a clade of bilaterally symmetrical animals
 43 (Bilateria) comprising nearly half of the major animal clades, including morphologically
 44 disparate groups such as molluscs, flatworms and annelids [6, 7]. A stereotypic early
 45 developmental programme termed spiral cleavage, characterised by alternating clockwise and
 46 counterclockwise oblique cell divisions from the 4-cell stage onwards [8, 9] is however
 47 widespread, and likely ancestral to Spiralia [8, 10, 11] (Figure 1A). How spiral cleavage
 48 generates the vast diversity of spiralian body plans is still unclear (Figure 1A), albeit this
 49 being fundamental to understand the evolution of animal development and morphology.

50

51 Spiral cleavage involves the formation of an embryonic organiser that instructs cell fates
 52 around four embryonic quadrants, named A to D, which roughly correspond to the left,
 53 ventral, right and dorsal sides of the embryo respectively [8, 9]. It is a cell belonging to the D-
 54 quadrant – e.g., the 3D or 4d blastomeres in molluscs [12] – that acts as embryonic organiser,
 55 and thus this signalling centre is named D-quadrant organiser (Figure 1A). The most common
 56 and probably ancestral mode to specify the D-quadrant and its organising cell is through
 57 inductive signals at around the 32-cell stage [13, 14]. This type of spiral cleavage is termed
 58 conditional; or equal, since early embryos exhibit quadri-radial symmetry during early stages.
 59 However, in some mollusc and annelid species, the specification of the D-quadrant organiser
 60 is largely cell-autonomous, as two asymmetric zygotic divisions segregate maternal mRNAs
 61 to one (the D) blastomere at the 4-cell stage [9, 13, 14]. This autonomous mode of spiral

62 cleavage is also referred to as unequal, because the D founder cell at the 4-cell stage is larger.
63 Regardless of the specification mode, the ERK1/2 signalling pathway, an evolutionarily
64 conserved intracellular cascade of kinases involved in an array of cellular processes [15], is
65 active and necessary in the D-quadrant organiser in molluscs [16-20] (Table S1). However,
66 what activates ERK1/2 and how ERK1/2 orchestrates the instructing role of the D-quadrant
67 organiser in this group is not understood (Figure 1A), and thus how embryonic organisers
68 define the spiralian body plan is still an unresolved question.
69
70 The role of ERK1/2 signalling during spiral cleavage in groups other than Mollusca is in
71 addition ambiguous and only explored in Annelida (Table S1). As in molluscs, the conditional
72 cleaving annelid *Hydroides hexagonus* exhibits active ERK1/2 in the 4d cell [17], which is
73 often called “mesentoblast” and cell lineage analyses identify as the trunk mesodermal
74 precursor in most annelid embryos [21-23]. Yet the role of ERK1/2 in the 4d cell of *H.*
75 *hexagonus* is unknown. In autonomous cleaving annelids, the patterns of ERK1/2 activity
76 differ markedly between species (Table S1), but in general ERK1/2 is not active in 4d and its
77 inhibition does not result in axial polarity defects in any of the studied species [24-27].
78 Indeed, the organiser activity occurs in the 2d blastomere and 2d¹¹ + 4d in the autonomous
79 cleaving annelids *Capitella teleta* and *Tubifex tubifex* respectively [26, 28]; and its inductive
80 role relies on Activin/Nodal activity, and not on ERK1/2 signalling in the autonomous species
81 *C. teleta* and *Chaetopterus pergamentaceus* [24, 29]. Therefore, the cellular and molecular
82 basis underpinning D-quadrant organiser specification and activity varies considerably within
83 Annelida, which ultimately prevents inferring whether ERK1/2 activity in the D-quadrant
84 organiser is a homologous trait to Spiralia or a molluscan innovation that also appeared
85 convergently in some annelid lineages.

86

87 The annelid worm *Owenia fusiformis* belongs to Palaeoannelida – the sister group to all
 88 remaining annelids [30] – and exhibits embryonic traits considered ancestral to Annelida,
 89 including conditional spiral cleavage, formation of a coeloblastula, gastrulation by
 90 invagination and the development of a planktotrophic trochophore-like larva [31] (Figure 1B,
 91 C). While inductive signals appear to control the establishment of the axial polarity in *O.*
 92 *fusiformis* [32], which blastomere acts as D-quadrant organiser and how this signalling centre
 93 becomes specified and controls body patterning is unknown. Therefore, investigating the
 94 nature of the D-quadrant organiser and the role of the ERK1/2 signalling pathway in *O.*
 95 *fusiformis* can, by comparison with other annelid and molluscan lineages, help to infer
 96 ancestral character states to Annelida and Spiralia as a whole, ultimately contributing to our
 97 understanding of the evolution of spiral cleavage and spiralian body plans.

98

99 **Results**

100 ***Di-phosphorylated ERK1/2 is enriched in the 4d cell in O. fusiformis***

101 The annelid *O. fusiformis* has a single ERK1/2 ortholog (Figure S1A), which is expressed at
 102 low levels in active oocytes (Figure S1B). The expression of *erk1/2* is stable during early
 103 cleavage divisions and up to 4 hours post fertilisation (hpf; ~32 cell stage), increasing
 104 thereafter and plateauing at the larval stage (Figure S1B). A cross-reactive antibody against
 105 the active di-phosphorylated form of ERK1/2 (di-P-ERK1/2) [16, 17] shows weak ubiquitous
 106 signal in the zygote and up to 3 hpf (~16 cell stage) (Figure S1C). At 4 hpf, however, the four
 107 most animal/apical micromeres (1q¹¹¹) exhibit enriched di-P-ERK1/2 signal, which disappears
 108 at 5 hpf (coeloblastula) (Figure 1D, Figure S1C). At this stage, a single cell in the vegetal
 109 pole, which positionally corresponds to a fourth quartet micromere (4q), shows strong signal
 110 for di-P-ERK1/2 (Figure 1D, Figure S1C). In *O. fusiformis*, the fourth micromere quartet (4q)
 111 starts to form at 4 hpf, when the 3Q macromeres sharing the vegetal cross furrow divide

(Figure 1E). This creates an asymmetry in the timing and order of appearance of the 4q micromeres that is also observed during cleavage of the 3q micromeres, which divide in a counterclockwise order after all 3Q macromeres have cleaved (Figure 1E). By 5 hpf however, the vegetal pole exhibits again a symmetrical arrangement (Figure 1E). At 6 hpf, with the onset of gastrulation [31], di-P-ERK1/2 signal is enriched in seven vegetal cells forming a bilaterally symmetrical pattern that includes one 4q micromere and the 2q¹² and 2q²² blastomeres of the other three embryonic quadrants (Figure 1D, Figure S1C). At this stage, the 4Q macromeres have cleaved synchronously into the 5Q and 5q cells and all but the 4q micromere enriched in di-P-ERK1/2 have divided, becoming the earliest morphological sign of bilateral symmetry in *O. fusiformis* embryos. This di-P-ERK1/2 positive 4q micromere divides into two large cells only after ingressión during early gastrulation at 7 hpf (Figure 1E), with the later progeny occupying a bilateral position on the posterodorsal side of the archenteron once gastrulation completes at 9 hpf (Figure 1E). The behaviour of this 4q micromere resembles the cellular dynamics described for the 4d micromere in other conditional spiral cleaving annelids [33-36], suggesting that the di-P-ERK1/2 enriched 4q micromere at 5 and 6 hpf is the 4d cell and its two large daughter cells are the MR/ML mesoteloblasts (Figure 1D, E). Therefore, ERK1/2 signalling activates first in the 1q¹¹ animal micromeres and subsequently in the 4d micromere in the annelid *O. fusiformis*, which is similar to the spatiotemporal dynamics of ERK1/2 activity reported in spiral cleaving molluscs (Table S1).

ERK1/2 signalling controls axial polarity in O. fusiformis

To examine the role of the ERK1/2 signalling during *O. fusiformis* development, we treated embryos with brefeldin A (BFA), an inhibitor of intracellular protein trafficking previously used to block the induction of the organiser in other spiral cleaving embryos [32, 37], and

U0126, a selective inhibitor of MEK1/2 and ERK1/2 di-phosphorylation [16, 38] (Figure 2A). For both drugs, treatment from fertilisation (~0.5 hpf) to 5 hpf, when di-P-ERK1/2 is enriched in 4d, effectively blocks activation of ERK1/2 (Figure 2B; Figures S2A; Table S2) and causes the loss of bilateral symmetry, posterior structures (e.g., chaetae and hindgut) and larval muscles in 100% of the embryos at a 10 μ M concentration (Figure 2C; Figure S2B; Table S3, S4). Compared to control samples, 0.5 to 5 hpf treated embryos lack a fully formed apical tuft and apical organ, showing reduced ectodermal expression of the apical organ marker *six3/6* [39] and just a few apical cells positive for the neuronal marker *synaptotagmin-1* (*syt-1*) [31] (Figure 2D). In addition, treated embryos lack expression of hindgut (*cdx*) and trunk mesodermal (*twist*) markers [39], exhibit expanded expression of the oral ectodermal marker gene *gsc* [39] around the single gut opening (Figure 2D; Figure S2C), and retain expression of the midgut endodermal marker *GATA4/5/6b* [39] (Figure S2C). We deem this phenotype as antero-ventrally radialised (or Radial; Figure 2C, E). Therefore, activation of ERK1/2 signalling in the 4d cell at the coeloblastula stage relies on inductive cell-cell communication signals (impaired by BFA treatment) required to specify and develop posterior and dorsal structures during *O. fusiformis* embryogenesis.

To dissect the exact timing of induction and activity of ERK1/2 during *O. fusiformis* cleavage, we treated embryos with 10 μ M BFA/U0126 in overlapping time windows from fertilisation to early gastrulation (Figure 2E; Table S5). Blocking protein secretion with BFA from fertilisation to the 8-cell stage does not affect normal development. However, BFA treatment between the 8-cell stage and 4 hpf results in larvae with all morphological landmarks of a typical mitraria larva and normal expression of tissue-specific markers, but with a compressed morphology (Figure 2F; Figure S2C). Only treatment with BFA from 4 hpf to 6 hpf, and hence spanning the formation of 4d, causes a radial phenotype, with treatments after 5 hpf

being lethal (Figure 2E, Figure S2C). This suggests that the intercellular communication event inducing the activation of ERK1/2 in 4d happens between 4 and 5 hpf, right during 4q micromere formation (Figure 1E). Unexpectedly, preventing ERK1/2 di-phosphorylation with U0126 from the 2-cell stage until 4 hpf, when this induction event might begin, also causes a radial phenotype (Figure 2E), with just slight differences between certain timepoints; embryos treated between 2-cells and 3 hpf show expression of the mesodermal marker *twist* in a few discrete cells, and embryos treated from 4 to 6 hpf are seemingly more elongated along the apical-oral axis (Figure S2C). Therefore, ERK1/2 activity is essential for normal embryonic patterning and posterodorsal development throughout most spiral cleavage in *O. fusiformis*. However, the combination of BFA and U0126 phenotypes suggest that ERK1/2 acts autonomously from the 2-cell stage to 4 hpf, while it requires of inductive cell-to-cell communication signals for its enrichment in 4d.

ERK1/2 signalling activates posterodorsal and mesodermal genes

To investigate the mechanisms through which ERK1/2 controls posterodorsal development in *O. fusiformis*, we hypothesised that genome-wide profiling of gene expression in BFA and U0126 treated embryos would uncover upstream regulators and downstream targets of ERK1/2 activity. We thus treated embryos with either 10 μ M BFA or 10 μ M U0126 from fertilisation to 5 hpf (to cover both the autonomous and conditional phases of ERK1/2 activity) and performed RNA-seq transcriptome profiling in treated and controlled embryos collected right after di-P-ERK1/2 enrichment in the 4d cell (coeloblastula; 5.5 hpf) and at the larval stage (Figure 3A). Principal component analysis (PCA) and sample-to-sample distance matrices clustered transcriptomic profiles according to developmental stage and biological replica (Figure S3A, B). Differential expression analyses revealed 90 and 268 differentially expressed genes (DEGs; Log Fold Change < -1.5 and adjusted p-value < 0.05) in BFA treated

coeloblastula and larva respectively, and 132 (coeloblastula) and 373 (larva) DEGs after U0126 treatment (Figure 3B). When considering all comparisons and removing redundancies, we detected a total of 628 DEGs, 414 (65.92%) of which were functionally annotated and enriched in gene ontology terms related to regulation of transcription, development and cell fate specification (Table S6, S7, S8). Most of these DEGs were downregulated (Figure 3B, C; Figure S3C, D), with 26 genes downregulated under both drug treatments at the coeloblastula stage and 154 genes commonly downregulated at the larval stage. Only three DEGs (*cdx*, *fer3* and *foxH*) appeared systematically downregulated in both drugs and in the two developmental time points, while we did not identify any common upregulated DEG (Figure 3B; Figure S3D). Therefore, our approach revealed a confident and relatively small set of genes whose expression is dependent on ERK1/2 activity and early inductive signals in *O. fusiformis* embryos.

To validate the set of genes affected by 4d misspecification, we selected 22 candidate genes for further gene expression analyses (Figure 3D, Table S9), including a variety of transcription factors recurrently involved in axial embryonic patterning (e.g., *six3/6*, *gsc*, *cdx*, *AP2*, *foxQ2*), mesoderm development (e.g., *twist*, *hand2*, *foxH*) and neurogenesis (e.g., *POU4*, *irxA*), Wnt ligands (*wnt1*, *wntA* and *wnt4*), TGF- β modulators (*noggin* and *BAMBI*), and Notch signalling components (*delta* and *notch-like*) (Table S8). Stage-specific RNA-seq data covering twelve developmental time-points, from the unfertilized oocyte to the mature larva, confirmed that for all candidate genes, their expression upregulates at the time of or just after 4d specification and di-P-ERK1/2 enrichment in this cell (Figure 3D). These genes are expressed either in apical/anterior domains (*noggin*, *BAMBI*, *foxQ2*, *POU4*, *six3/6*, *gsc*), the posterior larval tip and chaetae (*fer3*, *lhx1/5*, *wnt1*, *notch*, *msx2-a*, *irxA*, *AP2*, *wnt4*, *delta*), the hindgut (*cdx*), or mesodermal derivatives (*foxH*, *rhox*, *wntA*, *POU3*, *hand2*, *twist*) (Figure 3D,

Figure S4). Analysis of the expression of these genes in control and treated embryos at the coeloblastula (5.5hpf) and larva (24hpf) stages confirmed the expression domains of these genes disappear after treatment with either BFA or U0126 (Figure 3E, Figure S4), thus validating our RNA-seq approach. Altogether, these results define a set of co-regulated genes that act downstream of ERK1/2 signalling and/or early inductive events, and that are involved in the development of apical, posterodorsal and mesodermal structures in *O. fusiformis*.

ERK1/2 signalling specifies and patterns the D-quadrant

Our RNA-seq study and candidate gene screening revealed nine genes expressed at the vegetal pole at 5.5 hpf and whose expression was affected by either direct inhibition of ERK1/2 di-phosphorylation (*cdx*, *delta*, *foxH*, *wnt1*, *wntA*, *rhox*, *fer3* and *AP2*) or impairing cell-to-cell inductive signals (*gsc*) (Figure 3E, Figure S4). None of these genes are expressed at 5 hpf, at the onset of ERK1/2 activity in the 4d micromere (Figure 4A). Instead, the early endodermal marker *GATA4/5/6a* [39], whose expression is unaffected by BFA and U0126 treatment and thus is cell-autonomous, is symmetrically expressed in the gastral plate (including 4d) at this stage (Figure 4A; Figure S5A). At 5.5 hpf, half an hour after the initial activation of ERK1/2 signalling in 4d, the vegetal pole becomes bilaterally symmetrically patterned, but only two of the ERK1/2 dependent genes (*cdx* and *delta*) are expressed in the 4d micromere (Figure 4A). The ParaHox gene *cdx*, which is detected in the hindgut of *O. fusiformis* at the larval stage [39] (Figure 3E; Figure S5B), becomes expressed in 4d, and later on in two cells of the MR and ML progeny at the gastrula stage (Figure 4B), as expected from the behaviour of 4d at these stages (Figure 1E). Similarly, the Notch ligand *delta* (Figure S5C, D) is expressed in 4d at 5.5 hpf, but also in most of the descendants of 1d at the animal pole, plus 1a-c¹¹²¹ and ectodermal derivatives of the C and D quadrant at the vegetal pole (Figure 4A, C). To investigate how ERK1/2 might control activation of *cdx* and *delta*, we used Assay

for Transposase-Accessible Chromatin using sequencing (ATAC-seq) data at 5 hpf and identified known bound transcription factors in the accessible regions associated with these genes (Figure 4D), which include DNA binding motifs for transcription factors known to be modulated by ERK1/2 phosphorylation, such as ETS, RUNX and GATA factors [15]. Therefore, ERK1/2 di-phosphorylation in 4d seems to delay cell cycle progression in this cell with respect to 4a–c (Figure 1E) and control the activity of transcriptional regulators that induce posterior fates (*cdx*) and cell-cell communication genes (*delta*).

The other six additional ERK1/2-dependent genes pattern the micromeres surrounding 4d at 5.5 hpf, defining mesodermal and posterior ectodermal domains (Figure 4A). The transcription factor *foxH* (Figure S5E), which regulates mesoderm development during gastrulation in vertebrate embryos [40–43], is detected in the 3c²² and 3d²² micromeres adjacent to 4d (Figure 4A), whose progeny contributes to lateral ectomesoderm in the annelid *Urechis caupo* [36]. Later in development, *foxH* is expressed in a posterior V-shaped pattern of putative mesodermal precursors during axial elongation, fading away in larval stages (Figure S5B). The ligands *wnt1* and *wntA* (Figure S5), expressed in the posterior region and D-quadrant during development in the annelid *Platynereis dumerilii* [44], are expressed in two bilaterally symmetrical columns of micromeres (3c²², 3c²¹, 3c¹², 3c¹¹, 3d²², 3d²¹, 3d¹², 3d¹¹) plus the micromeres 2d²²² and 2d¹¹² in *wntA* (Figure 4A). The homeobox *rhox* (Figure S5G), which is expressed in male and female primordial germ cells in vertebrates [45], is detected in the micromeres 2d¹¹² and 2d²¹² (Figure 4A) and thereafter in two small cells inside the blastocoele in the gastrula (Figure S4). The transcription factors *fer3* and *AP2* are expressed in two single micromeres (1c²²² and 1d²²¹) and in a broader posterior ectodermal domain (1c²²², 1d²²¹, 2d¹²², 2d²¹², 2d²¹¹¹, 2d²¹¹², 2d¹¹¹², 2d¹¹¹¹) respectively, becoming restricted to a small expression domain at or near the posterior chaetal sac of the larva (Figure S4; Figure

S5B). The larger size of 4d, which stays as a big posterior cell at 5.5 hpf, allows it to establish almost as double direct cell-cell contacts with its surrounding cells than each of the daughter cells of 4a–c, including most of the cells expressing *foxH*, *wnt1*, *wntA*, *rhox*, and *AP2* (Figure 4E). This data demonstrates the inductive role of the 4d in defining mesodermal and posterodorsal fates, and together with the upregulation of the Notch-ligand *delta* in 4d after ERK1/2 activation, suggests that this role might occur by direct cell-cell communication mediated by the Notch signalling pathway.

The homeobox gene *gsc* is the only candidate expressed outside the D-quadrant at 5.5 hpf, being detected in micromeres $2a^{11}-2c^{11}$, $2a^{12}-2c^{12}$, $2a^{21}-2c^{21}$, and $2a^{22}-2c^{22}$ (Figure 4A). Consistent with its location outside the D-quadrant, *gsc* expression is independent of ERK1/2 activity, but requires of inductive signals, as demonstrated by being downregulated after BFA treatment (Table S3). Accordingly, *gsc* expression disappears in BFA-treated coeloblastulae, while inhibition of ERK1/2 activity with U0126 expands *gsc* domains, which becomes detected in all embryonic quadrants in a radial fashion (Figure 4F). Indeed, in ERK1/2 inhibited embryos all 4q micromeres cleave into $4q^1$ and $4q^2$, as no 4q becomes the 4d cell (Figure 4F). Therefore, all quadrants adopt antero-ventral fates (*gsc*), preventing the expression of posterior marker genes such as *AP2* (Figure 4F). These results demonstrate that the radial phenotype observed after U0126 is a consequence of misspecifying the 4d cell, and not a result of the D-quadrant becoming specified but not developing further. In addition, this data also demonstrates that specification of the 4d cell through ERK1/2 activity represses anterior fates, as shown by limiting *gsc* expression.

FGF signalling regulates ERK1/2 di-phosphorylation in 4d

In *O. fusiformis*, *cdx* and *delta* are expressed in 4d under regulation of ERK1/2, and their expression is associated with hindgut and trunk mesoderm development, as well as posterior growth (Figure 2C). In vertebrate embryos, FGF signalling mediated by ERK1/2 activity regulates the expression of *cdx* and *delta* in the developing hindgut and presomitic mesoderm during posterior elongation [46] and FGF signalling promotes mesoderm development in the spiralian groups Brachiopoda and Phoronida [47]. We thus hypothesised that FGF signalling might be the upstream regulator driving ERK1/2 activity in 4d (Figure 5A). *O. fusiformis* has a single FGF receptor (FGFR; Figure S6A) with high amino acid conservation at key functional residues compared to the human FGFR1 ortholog (Figure 6B). While it is transcriptionally upregulated at the gastrula stage, *FGFR* appears weakly expressed at the gastral plate at the coeloblastula stage in *O. fusiformis* (Figure S6C, D), as observed in brachiopods and phoronids [47]. At this stage, FGF ligands and VEGFR, the other tyrosine kinase receptor inhibited by SU5402, are only weakly expressed (Figure S6C) and not detected by *in situ* hybridisation (Figure S6D). Treatment with 30 μ M SU5402, a selective inhibitor of FGFR phosphorylation and activation [48] (Figure 5A), prevents di-P-ERK1/2 enrichment at 4d cell in 96% of the treated embryos at 5 hpf (Figure 5B, Figure S6E, Table S2). Indeed, treatment with SU5402 during the specification of the 4d micromere (4 to 6 hpf and 3 to 7 hpf; Figure 5C) results in an anteriorly radialised phenotype, with embryos developing into larvae lacking posterior and reduced mesodermal structures and showing radially expended oral ectodermal fates (*gsc*) (Figure 5D, Table S3). Conversely, treatment before di-P-ERK1/2 enrichment in 4d (3 to 5 hpf) causes a slightly compressed phenotype (Figure S6F). Therefore, inhibition of FGFR blocks ERK1/2 activation and phenocopies the effect of U0126 and BFA (Figure 2C), suggesting that FGF signalling activity is upstream of and necessary for 4d specification in *O. fusiformis*.

Discussion

Our study provides compelling evidence that FGFR and ERK1/2 signalling specify and control the organising activity of the 4d micromere in the conditional spiral cleaving annelid *O. fusiformis* (Figure 6A). FGFR/ERK1/2 signalling at around 5 hpf is necessary for instructing one of the recently emerged 4q micromeres into the D fate (Figure 2E, Figure 5C, Figure 6A). However, 4q micromeres form already asymmetrically at 4 hpf in *O. fusiformis* (Figure 1E), yet whether there is an exact correspondence between the order of formation and the fate of each 4q remains elusive. Asynchronies in the cleavage of 3Q have not been described for other conditional cleaving annelids [33-35], but are more common in molluscs, where 4d can be either the first or the last micromere to form [3, 12, 14]. This asynchrony in 3Q cleavage, together with the fact that ERK1/2 signalling affects axial polarity independently of cell communication (i.e., unaffected by BFA) earlier than 4 hpf (Figure 2E, Figure S2C) suggest that cell-autonomous ERK1/2-dependent mechanisms might act before the inductive specification of 4d in *O. fusiformis*. In other systems, MAPK controls unequal protein segregation during mitosis via pericentrosomal material [49], which is the subcellular region where maternal mRNAs condense before asymmetric inheritance during spiral cleavage in the mollusc *Tritia obsoleta* [50], and MAPK controls mRNA stability during early zygotic divisions in the annelid *Helobdella robusta* [51]. Therefore, basal levels of ERK1/2 signalling during early cleavage might control the activity or differential segregation of determinants required to respond adequately to the inductive signals specifying the 4d cell in *O. fusiformis*. Although further work is needed to test this scenario, our findings indicate that both autonomous and conditional mechanisms are essential to specify the D-quadrant organiser in *O. fusiformis*, challenging traditional views that conditional/equal spiral cleaving species rely mostly on inductive signals to establish their body pattern.

The specification of 4d micromere has at least four immediate consequences during *O. fusiformis* embryogenesis (Figure 6A). First, and contrary to its role in promoting cell proliferation [15], ERK1/2 activity correlates with 4d cell cycle arrest, breaking the quadri-radial symmetry of the embryo as the 4a–4c micromeres cleave (Figure 1E; Figure 6A). In some molluscs, activation of ERK1/2 in the 3D blastomere also correlates with cleavage delays [12, 17], and thus a potential role of ERK1/2 signalling in arresting, instead of promoting, cell division appears to be common in the context of the embryonic organiser in spiralian embryos. Second, specification of 4d and the D-quadrant represses anterior fates to the A–C quadrants, as observed by the expression of the BFA-affected oral ectodermal gene *gsc* in all four quadrants in ERK1/2 inhibited embryos (Figure 4F). These findings suggest that two as yet unknown opposing signals – one specifying anterior fates and promoting *gsc* expression, and another driving 4d specification via FGFR/ERK1/2 – might act simultaneously at 4–5 hpf (Figure 6A). Considering that *gsc* expression is eventually recovered in BFA treated larvae (Figure S2C), we hypothesise that this anteriorising signal must come from cell lineages already specified by 4–5 hpf, such as the endoderm/midgut (Figure 4A) or the apical organ (Figure 2D). Third, FGF/ERK1/2 activity upregulates the hindgut gene *cdx* in 4d (Figure 3C; Figure 4A; Figure 6A). Inhibition of both FGF and ERK1/2 activity affects hindgut but not foregut and midgut development (Figure 2C; Figure 5D), indicating that unlike most annelids [21] but similar to molluscs [3, 52], 4d contributes to both endoderm/hindgut and mesoderm in *O. fusiformis*. Fourth, FGF/ERK1/2 activity also upregulates the Notch ligand *delta* in 4d, as well as a set of mesodermal and posterior genes, Wnt ligands and TGF- β modulators in its surrounding cells, most of which establish a direct cell-to-cell contact with 4d (Figure 4A, E; Figure 6A). Therefore, 4d instructs cell fates in the D and C quadrant at the vegetal pole, probably through the Notch signalling pathway, as also

proposed for the mollusc *Tritia obsoleta* [53]. Altogether, our study demonstrates the organising properties of the 4d cell (endomesodermal precursor) in *O. fusiformis*, defining a comprehensive, testable model to study the establishment of the body plan in this and other spiral cleaving organisms.

Enrichment of di-phosphorylated ERK1/2 in the 4d micromere is common to *O. fusiformis* and *H. hexagonus*, the two conditional spiral cleaving annelids studied so far [17], which contrasts with the variability in ERK1/2 activity observed among autonomous cleaving annelids [25-27]. Likewise, the 4d cellular identity of the D-quadrant organiser in *O. fusiformis* differs from the autonomous annelid species *C. teleta* and *T. tubifex*, which concentrate the organising activity in the 2d and 2d¹¹ + 4d cells respectively [26, 28]. However, the 4d cell identity observed in *O. fusiformis* resembles the molluscan condition, where ERK1/2 is active in 3D and maintained in 4d, both of which can exert organising activity [9, 12]. Moreover, *cdx* and *delta*, the downstream targets of ERK1/2 in the 4d of *O. fusiformis*, are also expressed in 4d in the mollusc *Tritia obsoleta* [52, 53], further suggesting conservation in both signalling cascades and transcriptional outputs between D-quadrant organisers in annelids and molluscs. Therefore, our study supports that ERK1/2 activity in the cell acting as embryonic organiser is a shared homologous trait between Annelida and Mollusca and given our current understanding of Spiralia phylogeny [6, 7], a probable ancestral character to at least the internal node Lophotrochozoa. Under this scenario, heterochronic shifts to confer organising activity to 2d micromeres as seen in *C. teleta* and *T. tubifex* [26, 28] and the use of Activin/Nodal signalling instead of ERK1/2 to establish the body plan [24, 29] are thus derived conditions.

More broadly, our study uncovers a role of the FGF signalling pathway, an evolutionarily conserved upstream regulator of mesoderm development in bilaterians [47, 54, 55], in the specification of 4d, the D-quadrant organiser and endomesodermal precursor in *O. fusiformis* (Figure 5B–D). FGFR activity in 4d results in the activation of *cdx*, Notch signalling (*delta*) and Wnt signalling (*wnt1*, *wntA*) in vegetal micromeres of the D-quadrant (Figure 4A) that will occupy the posterodorsal blastoporal lip as gastrulation begins, supporting that blastopore-associated axial organisers also occur during spiral cleavage [4]. Furthermore, FGF, Wnt and Notch signalling, together with *cdx* are all conserved gene modules regulating posterior axis elongation in an array of bilaterian embryos (Figure 6B) [39, 46, 47, 56–58]. Classically, axial elongation in spiralian embryos has been proposed to occur by proliferation of the 2d clone [59, 60], which express *delta* and *wntA* in *O. fusiformis* (Figure 4A), further suggesting that specification of 4d also results in the deployment of broadly conserved genetic mechanisms involved in posterior growth that eventually promote axial (and blastopore) elongation in *O. fusiformis* (Figure 2C; Figure 6B). Altogether, our study demonstrates that the establishment of the axial identities (A–D quadrants), endomesoderm specification, posterodorsal patterning and axial elongation are all intimately connected events that depend on the instruction of a single cell fate (the 4d cell) and use evolutionarily conserved mechanisms in *O. fusiformis*, a conditional spiral cleaving annelid. Decoupling these embryonic organising processes through maternal determinants (e.g., as in autonomous spiral cleavers), heterochronic shifts in organiser specification and/or signalling pathway rewiring (e.g., co-option of Activin/Nodal) might have thus contributed to the emergence of the vast diversity of body plans observed today in Spiralia.

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Author Contributions

O.S., A.M.C.-B. and J.M.M.-D. conceived and designed the study. O.S., A.M.C.-B., Y.L. and J.M.M.-D. performed all experimental approaches and critically analysed the data. O.S., A.M.C.-B. and J.M.M.-D. wrote the manuscript. All authors read and commented on the manuscript.

Declaration of Interests

The authors declare no competing interests.

Materials and Methods

Animal husbandry and embryo collections

Adult specimens of *O. fusiformis* Delle Chiaje, 1844 were collected from the coast near the Station Biologique de Roscoff (France) during the reproductive season (May to July). In the lab, animals were kept in artificial seawater (ASW) at 15°C. *In vitro* fertilizations were conducted as previously described [31, 39] and embryos develop in glass bowls with filtered ASW at 19 °C until the desired embryonic stage.

Drug treatments

Embryos were treated with either brefeldin A (BFA; Sigma-Aldrich, #B7651), U0126 (Sigma-Aldrich, #U120), or SU5402 (Sigma-Aldrich, #572630). All drug stocks were made in dimethyl sulfoxide (DMSO) and diluted in ASW prior use, with optimal working concentrations (10 µM for BFA and U0126 and 30 µM for SU5402) established after initial titration. For all drug treatments, equivalent volumes of DMSO were used as negative control, and in all cases, treatments were initiated after ~0.5 hours post fertilisation (hpf), right after fertilisation and sperm removal. Treatments were stopped by washing out the drug with at least three washes in ASW and embryos were either collected for downstream analyses or raised until larval stage. Samples collected for immunohistochemistry and gene expression analyses were fixed in 4% paraformaldehyde (PFA) in ASW for 1h at room temperature (RT). Larvae were relaxed in 8% magnesium chloride (MgCl₂) prior fixation and fixed in 4% PFA in 8% MgCl₂ for 1h at RT. After fixation, samples were washed several times with 0.1% Tween-20 phosphate-buffered saline (PTw), and either stored in PTw at 4°C for immunohistochemistry or dehydrated to 100% methanol and stored at -20°C for whole mount *in situ* hybridisation. Treated and control embryos and larvae collected for RNA-seq analyses were snap frozen in liquid nitrogen and stored at -80°C prior total RNA extraction.

449

450 ***Immunohistochemistry***

451 F-actin labelling, and antibody staining were conducted as previously described [31], with the
 452 modification that samples collected for anti-di-P-ERK1/2 staining were washed in PTw
 453 supplemented with 1:100 dilution of phosphatase inhibitors (Cell Signalling, #5872) after
 454 fixation. The primary antibodies mouse anti-double phosphorylated ERK1/2 (Sigma-Aldrich,
 455 #M8159, 1:200) and mouse anti-acetylated α -tubulin (clone 6-11B-1, Millipore-446 Sigma,
 456 #MABT868, 1:500) were diluted in 5% normal goat serum (NGS) in 0.1% Triton X-100
 457 phosphate-buffered saline (PTx) and incubated overnight (ON) at 4 °C. After several washes
 458 in 1% bovine serum albumin (BSA) in PTx, samples were incubated with either an anti-
 459 mouse peroxidase (POD) conjugated antibody (Millipore-Sigma, #11207733910, 1:100) or an
 460 AlexaFluor594 conjugated antibody (ThermoFisher Scientific, #A32731) diluted in 5% NGS
 461 in PTx ON at 4°C. For samples incubated with mouse anti-double phosphorylated ERK1/2
 462 and anti-mouse POD conjugated antibody, signal was developed using either
 463 diaminobenzidine (DAB) (Vector Laboratories, #SK-4100) or Tyramide Signal Amplification
 464 (TSA) kit (Akoya Biosciences, #NEL742001KT) following manufacturer's
 465 recommendations. DAB-developed samples were stored in 70% glycerol and counterstained
 466 with 4',6-diamidino-2- phenylindole (DAPI, ThermoFisher Scientific, #D3571, 1:1000)
 467 nuclear marker. Samples for laser scanning confocal microscopy (LSCM) were
 468 counterstained with DAPI diluted in 1% bovine serum albumin (BSA) in PTx for 1h at RT
 469 and mounted in 70% glycerol.

470

471 ***Gene isolation and gene expression analyses***

472 Candidate genes were amplified by two rounds of nested PCR using gene specific primers and
 473 a T7 universal primer on cDNA from mixed developmental stages as initial template (Table

S9). Riboprobes were synthesized with the T7 enzyme (Ambion's MEGAscript kit, #AM1334) and stored in hybridisation buffer at a concentration of 50 ng/μl. Colorimetric and fluorescent whole-mount *in situ* hybridisation were performed following an established protocol [31, 39]. After probe washes, samples for fluorescent *in situ* hybridisation were incubated with anti-DIG-POD Fab fragments (ROCHE, #11633716001) and a mouse anti-acetylated α -tubulin antibody (clone 6-11B-1, Millipore-446 Sigma, #MABT868, 1:500) to co-stain cell boundaries. After signal development with the TSA kit (Akoya Biosciences, #NEL742001KT), these samples were washed several times in PTx and treated for secondary antibody incubation as described above.

Genome sequencing, gene annotation and RNA-seq developmental time course

High molecular weight genomic DNA was obtained from a single *O. fusiformis* male following the agar plug extraction method and sequenced on a PacBio Sequel II platform to a ~200x coverage. Initial assembly was conducted with Canu [61], subsequently scaffolded with BioNano optical mapping data generated on an Irys platform and finally de-haploidised with purge_haplotigs [62]. To annotate protein coding genes, we used available transcriptomic data (SRA accession number SRR1222288) and generated replicated stage-specific RNA-seq data covering twelve developmental time-points, from the unfertilized active oocyte until the mature mitraria larva. RNA-seq reads were mapped with STAR [63] and gene models were generated combining Mikado v.2.1 [64], Augustus [65] and PASA [66] pipelines. Gene completeness was assessed with BUSCO [67] (97.5% complete, 1.5% fragmented, 1% missing). Trinotate [68] and Panther [69] were used to functionally annotate gene predictions and obtain initial gene orthology assignments. To identify gene expression dynamics during development, we used the stage specific RNA-seq generated for annotation, representing the average expression of the two replicates with the package pheatmap v.1.0.12

available in R, where colour intensity shows the z-score value for each gene. All raw sequencing data is available under the project with accession number **XXXXXX** in the European Nucleotide Archive (ENA).

Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)

Two replicated ATAC-seq libraries were generated from >50,000 cells at 5 hpf (850-900 coeloblastulae) following the Omni-ATAC protocol [70], employing a home-made Tn5 transposase [71] and amplifying for 12 cycles. Libraries were sequenced on an Illumina HiSeq4000 platform, 75 base paired-end output mode. After adaptor removal with cutadapt v.2.5 [72], reads were mapped with NextGenMap v.0.5.5 [73] and duplicates removed with samtools v.1.9 [74]. BAM files were shifted using deepTools2 v.3.4.3 [75] and MACS2 v2.2.7.1 [76, 77] was used for peak calling with parameters -f BAMPE --min-length 100 --max-gap 75 -q 0.01. Reproducible peaks ($p < 0.05$) were identified applying Irreproducible Discovery Rate package v2.0.4.2 [78]. Transcription factor motif enrichment was performed with HOMER v.4.11 [79] and transcription factor footprinting with TOBIAS v.0.12.0 [80]. To identify bound transcription factors on peaks of *cdx* and *delta*, we intersected the genomic positions of peaks and bound transcription factors with BEDTools v.2.29.2 [81]. Genomic tracks were plotted with pyGenomeTracks v.2.1 [82].

RNA-seq profiling and differential gene expression analyses

Total RNA was extracted using the Monarch total RNA Miniprep Kit (New England Biolabs, #T2010) and used for Illumina stranded mRNA library prep. Sequencing was performed on an Illumina HiSeq 4000 platform in 75 bases paired-end output mode, generating ~20 million reads per sample. Adapter sequences and low-quality bases were removed using Trimmomatic v.0.36 [83]. Cleaned reads were then mapped to *O.*

fusiformis reference genome annotation using Kallisto v.0.44.0 to generate gene expression counts [84]. Differential gene expression analyses were performed independently for each drug and computed in pairwise comparisons of different stages and conditions using the R package DESeq2 v.1.38.0 [85]. Significance threshold was adjusted to a p-value equal or lower than 0.05 and a Log2 fold change greater than 1.5 or lower than -1.5 [86]. PCAs and hierarchical clustering were plotted using ggplot2 and pheatmap R packages, respectively [87]. Volcano plots were obtained with the package EnhancedVolcano, available in R. Candidate genes for further gene expression analyses were selected based on being differentially expressed in both drug treatments (U0126 and BFA) or highly differentially expressed at 5.5 hpf in U0126 treated embryos. We further refined our selection based on gene ontology (GO) categories, focusing on transcription factors and developmental genes (Table S7).

GO and KEGG enrichment analyses

For GO mapping, the GO terms of differentially expressed genes (DEGs) associated with each comparison were extracted from *O. fusiformis* reference genome annotation. GO enrichment analyses were implemented using the GOrse R package, correcting for gene length bias [88], and analysing up-regulated and down-regulated genes independently. GO terms with corrected p-values lower than 0.05 were considered to be significantly enriched (Table S7). A similar approach was followed for Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses, mapping differentially represented KO numbers to their respective KEGG pathway [89] (Table S8).

Orthology assignment and domain architecture analyses

Generally, gene orthology was based on the functional annotation of *O. fusiformis* gene repertoire, based on BLAST searches against SwissProt database and Panther searches [69, 90]. Protein sequences for ERK1/2 and ERK5, WNT ligands, RHOX and related homeoboxes, DLL and JAGGED, FOXH and FOXF genes and FGFR and VEGFR were retrieved by mining publicly available transcriptomes and databases. Multiple protein alignments (Supplementary File 1) were constructed with MAFFT v.7 in automatic mode [91] and poorly aligned regions were removed with gBlocks version 0.91b [92]. Maximum likelihood trees were constructed with either RAxML v.8.2.11 [93] or FastTree [94] and visualised with FigTree (<https://github.com/rambaut/figtree/>). InterProScan 5 [95] was used to perform the protein domain composition of DLL.

Imaging

Representative embryos and larvae from colorimetric analyses were imaged with a Leica DMRA2 upright epifluorescent microscope equipped with an Infinity5 camera (Lumenera), using bright field and differential interference contrast optics. Fluorescently stained samples were scanned with a Leica SP5 LSCM. Confocal stacks were analysed with Fiji and brightness/contrast and colour balance were adjusted in Pixelmator Pro (v. 2.0.3) or Photoshop (Adobe), applying changes always to the whole image and not parts of it. Final figures were designed using Illustrator (Adobe).

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Figures

Figure 1. Di-phosphorylated ERK1/2 is enriched in the 4d cell in *O. fusiformis*. (A)

Spiralia is a major bilaterian lineage exhibiting spiral cleavage as the most probably synapomorphy. Differently from other bilaterian lineages, body patterning and embryonic organisation is controlled by a cell in the D-quadrant, which in molluscs exhibits and requires active di-phosphorylated ERK1/2. Whether the molluscan condition is ancestral to Spiralia is unclear. **(B)** *O. fusiformis* is a member of Palaeoannelida, the sister group to all remaining annelids, and undergoes typical equal spiral cleavage **(C)**. The transition from the 4-cell to the 8-cell stage is characterised by a dextral shift of the mitotic spindle at a 45° angle relative to the animal–vegetal axis and generates a quartet of slightly larger animal cells (the micromeres, 1q) and a quartet of vegetal cells (the macromeres, 1Q). **(D)** Active di-phosphorylated-ERK1/2 (yellow) is enriched in four animal cells (1q¹¹¹) at 4 hours post fertilisation (hpf), in a single cell (the 4d micromere) at 5hpf, and in six cells of the 2a-c lineage and 4d at 6 hpf. DAPI: cyan. **(E)** Dynamics of cleavage and quadrant identities during the onset and early division of 4d. The fourth micromere quartet emerges asymmetrically (early 4 hpf), which is soon followed by the asymmetric division of one of the 3q micromeres (arrowheads) (late 4 hpf). The vegetal pole is however quadri-radial at 5 hpf, with the 4d arresting its division with respect to 4a–c, and only cleaving into MR and ML after ingresson at 7 hpf. F-actin (grey) shows cell boundaries and DAPI stains the nuclei. False colouring indicates cell or quadrant identities. Asterisks in **(C)** and **(D)** point to the animal pole, and to part of the 4d progeny in **(E)**. Descriptions for **(D)** and **(E)** are based on at least 10 embryos per stage, except at 7 hpf (n = 3). Drawings are not to scale. Scale bars are 50 µm.

Figure 2. ERK1/2 signalling controls axial polarity in *O. fusiformis*. (A) ERK1/2

signalling cascade responds to cell-cell communication and involves the di-phosphorylation

of ERK1/2 (di-P-ERK1/2) via MEK. Brefeldin A (BFA), a protein trafficking inhibitor, and U0126, a specific MEK inhibitor, can block ERK1/2 di-phosphorylation. **(B)** Immunostaining against di-P-ERK1/2. BFA and U0126 treatments from 0.5 hours post fertilisation (hpf) to 5 hpf prevent di-P-ERK1/2 enrichment in the 4d cell at 5 hpf. **(C)** BFA and U0126 treatment (0.5 hpf to 5 hpf) result in anteroventrally radialised larvae that fail to form posterior structures (e.g., hindgut, mesodermal bands and chaetae). Main panels are lateral z-stack projections of confocal images and insets in treated embryos are ventral views. Cilia is labelled with anti-acetylated tubulin. **(D)** Whole mount *in situ* hybridisation on control and U0126 treated (0.5 to 5 hpf) larvae. Treated radial larvae show reduced apical markers (*six3/6*, and *syt-1* in the inset), radially expanded anterior markers (*gsc*), and absent posterior endomesodermal (*cdx*, *twist*) but not a midgut gene (*GATA4/5/6b*). **(E)** Schematic representation of drug treatment windows and observed larval phenotypes (WT, wild type; R, radial). **(F)** Morphological analysis of the compressed larval phenotype obtained after BFA treatment from 0.5 hpf until 3–4 hpf. Treated larvae have a reduced apical organ and apical tuft (first row) and reduced expression of apical gene markers (*six3/6*, and *syt-1* in the inset), but other tissues are not affected. The larval blastocele is somehow obliterated. Asterisks point to the apical pole. Schematic drawings are not to scale. ao: apical organ; an: anus; ch: chaetae; mg: midgut; mo: mouth; pt: prototroch. Scale bars are 50 μ m.

Figure 3. ERK1/2 signalling activates posterodorsal and mesodermal genes. (A)

Experimental design for RNA-seq collections. Solid colour bars show period of control (DMSO), BFA or U0126 treatments. Red circles show sample collection, at the coeloblastula stage (5.5 hpf) and at the larval stage (24 hpf). **(B)** Number of differentially expressed (DE) genes in different conditions and comparisons, where red bars are downregulated genes and blue bars are upregulated genes. **(C)** Volcano plots for BFA and U0126 treated coeloblastula.

Red dots show DE genes, with candidate genes labelled for each comparison. **(D)** Temporal and spatial time course of expression of candidate genes affected by 4d misspecification and associated with apical/anterior, chaetae/posterior, hindgut or mesoderm development. The heatmap depicts the normalised z-score value of expression for each gene throughout development, with red blocks indicating high levels of expression and blue blocks indicating low levels of expression. The horizontal dotted line highlights the timing of 4d specification. On the right, whole mount *in situ* hybridisation of a subset of these 22 candidate genes at the coeloblastula (5 hpf), gastrula (9 hpf) and larval stages (24 hpf). **(E)** Only three of the candidate genes (*cdx*, *foxH* and *fer3*) are downregulated in all stages and treatment comparisons. Validation via *in situ* hybridisation demonstrates the expression is lost after drug treatment. Pictures for *cdx* at the coeloblastula and larva in **(D)** and **(E)** are the same. Asterisks point to the animal/apical pole, and arrowheads to the domains of expression. Schematic drawings are not to scale. bp: blastopore; mo: mouth.

Figure 4. ERK1/2 signalling specifies and patterns the D-quadrant. **(A)** Whole mount *in situ* hybridisation of a subset of genes (ERK1/2-dependent and independent) patterning the vegetal pole at (5 hpf) and after (5.5 hpf) 4d specification. The row below is a schematic representation of the vegetal pole depicting gene expression (in green) at 5.5 hpf. **(B)** The ParaHox gene *cdx* (yellow) is expressed in the 4d cell at the coeloblastula stage and in the progeny of MR and ML at the gastrula stage, corroborating the endodermal sublineage of 4d. **(C)** The Notch ligand *delta* (yellow) is expressed in 4d, multiple cells from the D-quadrant, and a few animal micromeres from the other three quadrants at the coeloblastula stage. In **(B, C)** Nuclei (DAPI) are shown in cyan and cell boundaries (acetylated tubulin) in magenta. Asterisks point to the animal pole. **(D)** Distribution of known bound transcription factors in open chromatin regions around *cdx* (upper part) and *delta* (lower part) loci at 5 hpf. Dark and

light blue depict the two ATAC-seq replicates. (E) Number of cellular contacts for each of the 4q micromeres at 5.5 hpf based on the analysis of eight embryos. While $4a^1-4c^2$ establish 8 direct cell contacts, 4d touches 13 cells of the D and C quadrants. (F) Inhibition of ERK1/2 di-phosphorylation by U0126 prevents D-quadrant specification, as shown by the expansion of the anterior marker *gsc* and loss of the posterior marker *AP2*. The 4d micromere is no longer recognised, as all 4q micromeres cleave at the same time (asterisks). However, *gsc* is lost in BFA treated embryos, and thus needs of inductive signals. Gene expression is shown in yellow, nuclei (DAPI) in cyan and cell boundaries (acetylated tubulin) in magenta or grey. In all panels, arrowheads point to expression domains. Schematic drawings are not to scale. gp: gastral plate.

Figure 5. FGFR signalling regulates ERK1/2 di-phosphorylation in 4d. (A) The drug SU5402 specifically inhibits receptors tyrosine kinase of the type FGF/VEGF, which can signal through the ERK1/2 signalling cascade. (B) Whole mount immunohistochemistry against di-phosphorylated ERK1/2 (di-P-ERK1/2). SU5402 treated embryos lose ERK1/2 enrichment in a vegetal micromere at 5 hours post fertilisation (hpf). (C) Schematic representation of drug treatment windows and observed larval phenotypes (WT, wild type). (D) Morphological and molecular patterning analysis of SU5402 treated embryos (4 to 6 hpf) fixed at 24 hpf. The first column shows diagrams of lateral views of the wild type in DMSO controls and radial larvae in treated embryos. Second and third columns show larvae labelled with DAPI (cyan) and F-actin (magenta) plus acetylated tubulin staining (yellow), respectively. Fourth column onwards are whole mount in situ hybridisations. SU5402 treatment from 4 hpf to 6 hpf results in phenocopies of U0126 and BFA radial larvae. Insets are ventral views. Asterisks point to the apical pole. Schematic drawings are not to scale. ao: apical organ; an: anus; ch: chaetae; mg: midgut; mo: mouth; pt: prototroch.

Figure 6. ERK1/2 activity in the D-quadrant embryonic organiser is an ancestral spiralian trait. (A) Schematic model of FGFR/ERK1/2 activity in the 4d micromere during late spiral cleavage in *O. fusiformis*. At 4 hours post fertilisation (hpf), di-phosphorylated ERK1/2 (di-P-ERK1/2) is enriched in the apical most micromeres (1q¹¹¹). At 5 hpf, an unidentified signal activates FGF receptor (FGFR) which di-phosphorylates ERK1/2 in the 4d micromere. This activates *cdx* and *delta* in 4d (becoming the endomesodermal precursor) and correlates with 4d arresting its cell cycle progression, as 4a–4c cleave. ERK1/2 activation in 4d is required to limit an anteriorising signal (Signal X) to the A–C quadrants, which activates *gsc* expression in these quadrants. In addition, ERK1/2 activity in 4d induces the expression of a battery of genes in the D-quadrant and part of the C-quadrant that probably induce lateral mesodermal and posterodorsal fates, ultimately controlling axial elongation. This inductive activity might happen via the Notch pathway. (B) The similarities in ERK1/2 activation patterns between *O. fusiformis* and other molluscan embryos suggest this is a shared ancestral trait to spiral cleavage. More broadly, ERK1/2 activity induces the expression of a set of genes and signalling pathways that are repeatedly involved in posterior specification and anteroposterior growth in bilaterian embryos. Drawings are not to scale.

Supplemental Information

Figure S1. ERK1/2 activity during *O. fusiformis* early embryogenesis. (A) Maximum likelihood orthology assignment for ERK1/2, using ERK5 as outgroup. Only bootstrap values for major nodes are shown. (B) DEseq2 normalised expression of *erk1/2* during *O. fusiformis* development, from the active oocyte to the mitraria larval stage. The time of the specification of 4d is highlighted with a schematic drawing. Coloured dots indicate values of expression for each replicate and the dotted red line shows the mean value of expression. (C) Whole mount immunohistochemistry against di-phosphorylated-ERK1/2 (dark orange) during spiral cleavage in *O. fusiformis*. ERK1/2 is active at low levels during early divisions, and later enriched in the four 1q¹¹¹ animal micromeres at 4 hours post fertilisation (hpf), the 4d micromere at the time of its specification (5 hpf) and finally in six vegetal cells forming a bilaterally symmetrical pattern (arrowheads) at 6 hpf. Insets in (C) show either nuclear staining (blue) or lateral views. Asterisks point to the animal pole.

Figure S2. The effect of BFA and U0126 treatment in *O. fusiformis* development. (A) Percentage of coeloblastula showing di-phosphorylated ERK1/2 (di-P-ERK1/2) enrichment in the 4d micromere after 0.5 to 5 hours post fertilisation (hpf) treatment with brefeldin A (BFA), U0126 or DMSO (control). (B) Percentage of radial phenotypes obtained when treating embryos from 0.5 to 5 hpf with a range of BFA and U0126 concentrations. (C) Whole mount in situ hybridisation of apical (*six3/6*), oral (*gsc*), posterior/hindgut (*cdx*), midgut (*GATA4/5/6b*) and trunk mesodermal (*twist*) gene markers in control and treated larvae. Radial phenotypes have a reduction of the apical marker, radial expansion of an oral gene, loss of posterior structures (chaetae) and posterior/trunk mesoderm gene expression and retain the expression of a midgut gene. Radial larvae treated with U0126 from the 2-cell stage to 3 hpf show scattered *twist* expression in some embryos (arrowheads). The compressed

larval phenotype after BFA treatment from 0.5 to 4 hpf have a reduced apical organ and apical tuft (first row) and reduced expression of apical markers (*six3/6*), but otherwise normal morphology besides an obliterated internal blastocele. Asterisks indicate the apical pole and arrowheads point to expression domains. Insets are ventral views. mo: mouth.

Figure S3. Differential expression analyses in BFA and U0126 treated embryos. (A)

Hierarchical pairwise correlation matrix between brefeldin A (BFA) and U0126 treated and control samples. **(B)** Principal component analysis (PCA) plot for BFA (left) and U0126 (right) RNA-seq analyses. **(C)** Volcano plots for BFA and U0126 treated larva. Red dots show differentially expressed (DE) genes and analysed candidate genes are labelled in each comparison. **(D)** Venn diagrams showing the number of up- (top) and downregulated (bottom) DE genes shared between different conditions. **(E)** Ten most overrepresented KEGG pathways in each of the four conditions analysed by RNA-seq. The size of the coloured dots is proportional to the number of overrepresented sub-categories nested into a given KEGG pathway.

Figure S4. Validation of the differential expression analyses. (A–G) Whole mount in situ hybridisation of control and treated embryos showing in addition a wild type gastrula stage to reconstruct the time course of expression of each gene. In all cases, the expression patterns disappear in BFA and U0126 treated embryos. Insets are ventral views. Asterisk point to the animal/apical pole. Arrowheads point to 4d in **(A)** and to the apical organ in **(F)**. bp: blastopore; gp: gastral plate; mo: mouth.

Figure S5. Genes patterning the D-quadrant after the specification of the 4d-organiser cell. (A)

Schematic drawing of the cellular arrangements and cell nomenclature of the vegetal

pole just after the specification of the 4d micromere in *O. fusiformis* embryo at 5.5 hours post fertilisation (hpf). Different colours depict different quadrants. **(B)** Time course of expression of *cdx*, *foxH* and *fer3* via whole mount *in situ* hybridisation from 6 hpf to 18 hpf during *O. fusiformis* embryogenesis. While *cdx* is expressed in the cells forming the hindgut, *foxH* appears to upregulate at the earliest stages of cells giving rise to mesodermal derivatives. The gene *fer3* is expressed in two posterior most cells of unknown function in the gastrula and larva. Insets are lateral views. **(C)** The protein domain architecture of the Notch ligand DELTA in *O. fusiformis* in comparisons with the domain structures of the Delta orthologs in *D. melanogaster* and *H. sapiens*. **(D–G)** Maximum likelihood orthology assignments of DELTA, FOXH, WNT ligands, and RHOX in *O. fusiformis*. Only bootstrap values supporting each major clade are shown. bp: blastopore; mo: mouth.

Figure S6. The FGF pathway and FGFR activity during 4d specification in *O.*

***fusiformis*.** **(A)** Maximum likelihood orthology assignment of fibroblast growth factor receptor (FGFR) and vascular endothelial growth factor receptor (VEGFR). Only bootstrap values for key nodes are shown. **(B)** Multiple protein alignment between *O. fusiformis* and *H. sapiens* FGFR and VEGFR orthologs indicating conserved residues for the interaction of the drug SU5402 with these receptors. **(C)** Temporal time course of expression of members of the FGF and VEGF pathways in *O. fusiformis*. The heatmap depicts the normalised z-score value of expression for each gene, with red blocks indicating high levels of expression and blue blocks indicating low levels of expression. The horizontal dotted line highlights the specification of the 4d micromere. **(D)** Whole mount *in situ* hybridisation for *FGFR* and *VEGFR* at the coeloblastula stage of *O. fusiformis*. *FGFR* is expressed weakly at the gastral plate at the time of 4d-instructed posterior re-patterning. **(E)** Proportion of treated and control embryos exhibiting di-phosphorylated ERK1/2 enrichment in the 4d cell at 5 hpf after

1022 SU5402 drug treatment from 0.5 to 5 hours post fertilisation (hpf). (F) Morphological and
 1023 molecular characterisation of SU5402 treated embryos fixed at the larval stage. The first
 1024 column shows diagrams of lateral views of the wild type, compressed and radial phenotypes.
 1025 From the second column onwards, whole mount *in situ* hybridisations of control and SU5402
 1026 treated larvae. The compressed larvae are phenocopies of brefeldin A (BFA) compressed
 1027 larvae, with reduced apical organ and apical tuft (second row) and reduced expression of
 1028 apical markers (*six3/6*), but other tissues are not affected. Radial larvae are phenocopies of
 1029 BFA and U0126 radial larvae with reduction of apical markers (*six3/6*), radial expansion of
 1030 oral genes (*gsc*), loss of posterior structures (chaetae) and expression of posterior
 1031 endomesodermal markers (*cdx*, *twist*), but normal expression of an endodermal gene
 1032 (*GATA4/5/6b*). Insets are ventral views. Asterisks point to the animal/apical pole. ao: apical
 1033 organ; an: anus; ch: chaetae; gp: gastral plate; mg: midgut; mo: mouth; pt: prototroch. Scale
 1034 bars are 50 µm.

1035 **Supplementary Tables and Files**

1036 Table S1. Di-P-ERK1/2 activation in spiralian.

1037 Table S2. Di-P-ERK1/2 activation at 5 hpf in control, BFA and U0126 treated embryos.

1038 Table S3. Larval phenotypes in control, BFA and U0126 treatments.

1039 Table S4. Larval phenotypes at different concentrations of BFA and U0126.

1040 Table S5. Larval phenotypes during staggered time windows of BFA and U0126 treatments.

1041 Table S6. List of all differentially expressed genes at 5.5 hpf and 24 hpf.

1042 Table S7. List of enriched gene ontology terms.

1043 Table S8. List of enriched KEGG pathways.

1044 Table S9. List of candidate genes and their gene IDs in differential gene expression analyses.

1045 File S1. Multiple protein alignments

A **B** **C**

A ● Coeloblastula Control ● Coeloblastula 10 μ M BFA
● Larva Control ● Larva 10 μ M BFA

B ● Coeloblastula Control ● Larva Control ● Coeloblastula 10 μ M BFA ● Larva 10 μ M BFA

C 10 μ M BFA treated (larva)

● Coeloblastula Control ● Coeloblastula 10 μ M U0126 ● Larva Control ● Larva 10 μ M U0126

10 μ M U0126 treated (larva)

D Upregulated

Downregulated

E