ERK1/2 is an ancestral organising signal in spiral cleavage

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Abstract

Animal development is classified as conditional or autonomous based on whether cell fates are specified through inductive signals or maternal determinants, respectively. Yet how these two major developmental modes evolved remains unclear. During spiral cleavage—a stereotypic embryogenesis ancestral to 15 invertebrate groups, including molluscs and annelids—most lineages specify cell fates conditionally, while some define the primary axial fates autonomously. To identify the mechanisms driving this change, we studied *Owenia fusiformis*, an early-branching, conditional cleaving annelid. In *Owenia*, ERK1/2-mediated FGF receptor signalling specifies the endomesodermal progenitor. This cell acts as an embryonic organiser, inducing mesodermal and posterodorsal fates in neighbouring cells and repressing anteriorising signals. The organising role of ERK1/2 in *Owenia* is shared with molluscs, but not with autonomous cleaving annelids. Together, these findings indicate that conditional specification of an ERK1/2⁺ organiser is ancestral in spiral cleavage, repeatedly lost in annelid lineages as they evolved autonomous development.

Introduction

The commitment of the first embryonic cells to more restricted developmental fates (e.g., endoderm, neuroectoderm and mesoderm) is a pivotal step in animal embryogenesis that leads to the establishment of body plans and influences subsequent development^{1–3}. To define this early spatial organisation, animal embryos combine conditional, inductive cell-cell interactions with the asymmetric inheritance of cell-autonomous maternal determinants^{1,2,4}. Frequently, one of these developmental strategies is predominant and thus animal embryogenesis is defined as either conditional or autonomous^{1,2,4}. During evolution, animal lineages have transitioned between these two major modes of cell fate specification⁵. However, how these developmental transitions occur is unclear because they often coincide with additional variation in early embryogenesis (e.g., in cleavage patterns^{6,7}) that makes the causes of cell fate specification shifts difficult to identify.

Spiral cleavage is an ancient and stereotypic early developmental programme characterised by alternating oblique cell divisions from the 4-cell stage onwards that is found among invertebrate groups within Spiralia, including molluscs and annelids^{8,9} (Fig. 1A). Spiral cleaving embryos organise cell fates around four embryonic quadrants — named A to D — that roughly correspond to the left, ventral, right and dorsal body sides, respectively^{8,9}. Although spiral cleavage is often depicted as a textbook example of autonomous development^{2,4}, spiral cleaving embryos specify their axial identities either conditionally or autonomously, without this affecting the overall conservation of the cleavage programme and the embryonic cell lineages^{8–10} (Fig. 1B). In conditional spiral cleavage, inductive signals between the cells in the animal pole and one vegetal blastomere at ~ 32–64-cell stage commit the latter to the dorsal D fate¹¹. This cell will act as embryonic organiser, instructing neighbouring cells towards certain fates and establishing the animal body plan (Fig. 1B). This mode of spiral cleavage is widespread and most likely ancestral to Spiralia^{11–13} (Fig. 1C). However, some molluscan and annelid lineages^{11,12} specify the axial

identities through the asymmetric segregation of maternal determinants to one blastomere in the 4-cell stage embryo^{14–16} (Fig. 1B). This blastomere will adopt the dorsal D fate, and one of its descendants will subsequently act as embryonic organiser. Therefore, the presence of both conditional and autonomous modes of development in spiral cleaving animals¹² is an ideal system to identify the cellular and molecular mechanisms underpinning cell fate specification transitions in animal embryos. Yet the mechanisms regulating spiral cleavage, and by extension the switches in developmental mode, are poorly understood.

The ERK1/2 signalling pathway – an evolutionarily conserved intracellular cascade of kinases¹⁷ – specifies the dorsal D quadrant identity and controls organising activity in Mollusca (Fig. 1C; Table S1). In many conditional cleaving molluscs, inductive interactions activate ERK1/2 in the blastomere that adopts the dorsal D fate and acts as embryonic organiser^{18–22} (Table S1). Likewise, active di-phosphorylated ERK1/2 is required in the D-quadrant blastomere functioning as embryonic organiser²³ in the otherwise autonomous cleaving mollusc *Tritia obsoleta*¹⁶. In Annelida however, autonomous cleaving species exhibit diverse patterns of ERK1/2 activity and do not require this signalling pathway to specify the dorsal D quadrant and the embryonic organiser^{24–27} (Fig. 1C; Table S1). Yet the role of ERK1/2 during spiral cleavage is unknown for conditional cleaving annelids, as well as for most other spiralian groups (Fig. 1C). This prevents inferring the ancestral mechanisms controlling body patterning in Annelida, and Spiralia generally (Fig. 1C), and thus it is unknown whether the axial organising role of ERK1/2 is a molluscan innovation or an ancestral cell fate specification mechanism lost in some autonomous annelid lineages.

To determine the role of ERK1/2 signalling in a conditional cleaving annelid, we strategically studied the species *Owenia fusiformis*^{28,29} (Fig. 1C). This marine annelid belongs to Palaeoannelida – the sister group to all remaining annelids³⁰ – and exhibits embryonic traits considered ancestral to Annelida²⁹. Therefore, the study of this species can, by comparison with other annelid and spiralian lineages, help to infer ancestral traits to this group, and Spiralia generally.

Results

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

O. fusiformis has a single ERK1/2 ortholog already expressed at low levels in active oocytes (Figure S1A, B). The expression of *erk1/2* is stable during early cleavage divisions and up to 4 hours post fertilisation (hpf; ~32 cell stage), increasing thereafter and plateauing at the larval stage (Figure S1B). A cross-reactive antibody against the active di-phosphorylated form of ERK1/2 (di-P-ERK1/2)^{19,23} shows weak ubiquitous signal in the zygote and up to 3 hpf (~16 cell stage) (Figure S1C). At 4 hpf, however, the four most animal/apical micromeres exhibit enriched di-P-ERK1/2 signal, which disappears at 5 hpf (coeloblastula) (Figure 2A, Figure S1C). At this stage, a single cell in the vegetal pole shows strong signal

for di-P-ERK1/2 (Figure 2A, Figure S1C). This cell is one of the fourth quartet of micromeres, or a 4q blastomere following the conventional spiralian nomenclature³¹ that uses a number to indicate the quartet and a lowercase or uppercase letter (q for quartet and a to d for the quadrant) to refer to the animal micromeres and the vegetal macromeres, respectively. In *O. fusiformis*, the fourth micromere quartet (4q) starts to form at 4 hpf, when the macromeres of the third quartet (3Q) sharing the vegetal cross furrow divide (Figure 2B). 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 2B). By 5 hpf however, the vegetal pole exhibits again a symmetrical arrangement (Figure 2B). At 6 hpf, with the onset of gastrulation²⁹, di-P-ERK1/2 signal is enriched in seven vegetal cells forming a bilaterally symmetrical pattern that includes one 4q micromere and two blastomeres of each of the other three embryonic quadrants (Figure 2A, Figure S1C). At this stage, the macromeres of the fourth quartet (4Q) have cleaved synchronously into the 5Q and 5q cells and all but the 4q micromere enriched in di-P-ERK1/2 have divided. This is the earliest morphological sign of bilateral symmetry in *O. fusiformis* embryos. The di-P-ERK1/2 positive 4q micromere divides into two large cells only after ingressión during early gastrulation at 7 hpf (Figure 2B), with the later progeny occupying a bilateral position on the posterodorsal side of the archenteron once gastrulation completes at 9 hpf (Figure 2B). The behaviour of this 4q micromere resembles the cellular dynamics described for the 4d micromere in other conditional spiral cleaving annelids³²⁻³⁵, which cell lineage analyses identify as the trunk mesodermal precursor in most annelid embryos³⁶⁻³⁸. Therefore, our data suggests that the di-P-ERK1/2 enriched 4q micromere at 5 and 6 hpf in *O. fusiformis* is the 4d cell (Figure 2C), resembling the condition observed in many gastropod molluscs^{18-21,23}.

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^{28,39}; and U0126, a selective inhibitor of MEK1/2 and ERK1/2 di-phosphorylation^{23,40} (Figure 3A). 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 3B; Figures S2A; Table S2) and causes the loss of bilateral symmetry, posterior structures (e.g., chaetae and hindgut) and larval muscles in a dosage-dependent manner up to 100% of the embryos at a 10 μ M concentration (Figure 3C; 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 *six3/6³* and just a few apical cells positive for the neuronal marker *synaptotagmin-1 (synt-1)*²⁹ (Figure 3D). In addition, treated embryos lack expression of hindgut (*cdx*) and trunk mesodermal (*twist*) markers³, exhibit expanded expression of the oral ectodermal marker gene *gsc³* around the single gut opening (Figure 3D; Figure S2C), and retain expression of the midgut endodermal marker *GATA4/5/6b³*

(Figure S2C). We deem this phenotype as antero-ventrally radialised (or Radial; Figure 3C, E). Therefore, activation of ERK1/2 signalling in the 4d cell at the coeloblastula stage relies on inductive cell-cell communication (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 3E; 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 3F; 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 3E, Figure S2C). All of 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 3E), with just slight differences between certain timepoints (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 suggests 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 next 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 4A; Figure S3A, B). Differential expression analyses revealed 90 and 268 differentially expressed genes (DEGs; $\log_2$ (fold change) < -1.5 and FDR-adjusted p-value < 0.05) in BFA treated coeloblastulae and larvae, respectively, and 132 (coeloblastula) and 373 (larva) DEGs after U0126 treatment (Figure 4B). 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 4B, C; Figure S3C, D) and only three DEGs (*cdx*, *fer3* and *foxH*) appeared systematically downregulated in both drugs and in the two developmental time points (Figure 4B; Figure S3D). Our approach thus revealed a confident and relatively small set of genes whose expression is strongly dependent on ERK1/2 activity and early inductive signals.

To validate that the DEGs are affected by 4d misspecification, we selected 22 DEGs for further gene expression analyses (Figure 4D, 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 the expression of all candidate genes upregulates at the time of or just after 4d specification and di-P-ERK1/2 enrichment in this cell (Figure 4D). 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 4D, 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 4E, Figure S4), thus validating our RNA-seq approach. Altogether, our findings uncover a set of co-regulated genes that act downstream of ERK1/2 signalling and/or early inductive signals, 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 4E, Figure S4). None of these genes are expressed at 5 hpf, at the onset of ERK1/2 activity in the 4d micromere (Figure 5A). Instead, the early endodermal marker *GATA4/5/6a*³, 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 5A; 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 5A). The ParaHox gene *cdx*, which is detected in the hindgut of *O. fusiformis* at the larval stage³ (Figure 4E;

Figure S5B), becomes expressed in 4d, and later on in two cells of the 4d progeny at the gastrula stage (Figure 5B), as expected from the behaviour of 4d at those 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 animal micromeres and ectodermal derivatives of the C and D quadrant at the vegetal pole (Figure 5A, 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 transcription factors bound to the accessible regions associated with these genes (Figure 5D). These include transcriptional regulators known to be modulated by ERK1/2 phosphorylation, such as ETS, RUNX and GATA factors¹⁷. Therefore, ERK1/2 di-phosphorylation in 4d seems to 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 5A). The transcription factor *foxH* (Figure S5E), which regulates mesoderm development during gastrulation in vertebrate embryos⁴¹⁻⁴⁴, is detected in two micromeres adjacent to 4d (Figure 5A), whose progeny contributes to lateral ectomesoderm in the annelid *Urechis caupo*³⁵. 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 S5F), expressed in the posterior region and D-quadrant during development in the annelid *Platynereis dumerilii*⁴⁵, are expressed in two bilaterally symmetrical columns of micromeres and *wntA* is also detected in two additional D-quadrant micromeres (Figure 5A). The homeobox *rhox* (Figure S5G), which is expressed in male and female primordial germ cells in vertebrates⁴⁶, is detected in two vegetal micromeres in the D-quadrant (Figure 5A) and thereafter in two small cells inside the blastoceles in the gastrula (Figure S4). The transcription factors *fer3* and *AP2* are expressed in two single micromeres and in a broader posterior ectodermal domain, 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 5E). Altogether, these results demonstrate the inductive role of the 4d in defining mesodermal and posterodorsal fates. Moreover, the upregulation of the Notch-ligand *delta* in 4d after ERK1/2 activation suggests that the organising role of 4d 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 a U-shape domain of micromeres of the A, B and C quadrants that occupy the prospective anterolateral blastoporal rim (Figure 5A). 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 5F). Indeed, in ERK1/2 inhibited embryos all 4q micromeres cleave into 4q¹ and 4q², as no 4q becomes the 4d cell (Figure 5F). Therefore, all quadrants adopt antero-ventral fates (*gsc*), preventing the expression of posterior marker genes such as *AP2* (Figure 5F). 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 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 trunk elongation⁴⁷. We thus hypothesised that FGF signalling might be the upstream regulator driving ERK1/2 activity in 4d in *O. fusiformis* (Figure 6A), which is also required to induce the expression of *cdx* and *delta* and specify the hindgut and trunk mesodermal progenitor. *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 7B). 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 also observed in brachiopods and phoronids⁴⁸. At this stage, FGF ligands are only weakly expressed (Figure S6C) and not detected by *in situ* hybridisation. Treatment with 30 µM SU5402, a selective inhibitor of FGFR phosphorylation and activation⁴⁹ (Figure 6A), prevents di-P-ERK1/2 enrichment at 4d cell in 96% of the treated embryos at 5 hpf (Figure 6B, 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 6C) 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 6D, 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 3C), 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 activity of the embryonic organiser in the conditional spiral cleaving annelid *O. fusiformis* (Fig. 7A). FGFR/ERK1/2 signalling at around 5 hpf is necessary for instructing one of the recently emerged 4q

micromeres into the dorsal D fate to become the embryonic organiser (Fig. 3E, Fig. 6C, Fig. 7A). However, 4q micromeres form already asymmetrically at 4 hpf in *O. fusiformis* (Fig. 2B, C), yet whether there is an exact correspondence between the order of formation and the fate of each of these cells remains elusive. Asynchronies in the cleavage of the third quartet of macromeres (3Q) have not been described in other conditional cleaving annelids^{32–34}, but are more common in molluscs, where the 4d cell can be either the first or the last micromere to form^{10,13,50}. 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) during early cleavage (Fig. 3E, Figure S2C) suggest that cell-autonomous ERK1/2-dependent mechanisms might act before the inductive specification of the embryonic organiser in *O. fusiformis*, thus challenging traditional views that conditional spiral cleaving species rely mostly on inductive signals to establish their body pattern.

The specification of embryonic organiser has at least four immediate consequences during *O. fusiformis* embryogenesis (Fig. 7A). First, and contrary to its role in promoting cell proliferation¹⁷, ERK1/2 activity correlates with 4d cell cycle arrest, breaking the quadri-radial symmetry of the embryo as the 4a–4c micromeres cleave (Fig. 2; Fig. 7A). In some molluscs, activation of ERK1/2 in the 3D blastomere also correlates with cleavage delays^{10,19}, 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 (Fig. 5F). These findings suggest that two 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 (Fig. 7A). 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 (Fig. 5A) or the apical organ (Fig. 3D). Third, FGF/ERK1/2 activity upregulates the hindgut gene *cdx* in 4d (Fig. 4C; Fig. 5A; Fig. 7A). Inhibition of both FGF and ERK1/2 activity affects hindgut but not foregut and midgut development (Fig. 3C; Fig. 6D), indicating that unlike most annelids³⁶ but similar to molluscs^{50,51}, 4d most likely 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 (Fig. 5A, E; Fig. 7A). Therefore, 4d instructs cell fates in the D and C quadrants at the vegetal pole, and this instructing role probably occurs through the Notch signalling pathway, as also proposed for the mollusc *Tritia obsoleta*⁵². Altogether, our data defines a comprehensive model of dorsal D fate specification and embryonic organiser activity in the conditional cleaving annelid *O. fusiformis*, which solves long-standing questions on the nature of the signals specifying this signalling centre in Spiralia and the genetic and cellular cascades this embryonic structures triggers to establish the spiralian body plan^{9,10,53}.

Our study demonstrates that the cell identities, signalling cascades, and transcriptional outputs associated with the specification of the dorsal D quadrant and the embryonic organiser are largely conserved between the annelid *O. fusiformis* and spiral cleaving molluscs^{9,10,51,52} (Fig. 7B), but differ with the array of conditions described in autonomous cleaving annelids^{25–27, 54}. The enrichment of active ERK1/2 in the 4d cell is also shared between *O. fusiformis* and the conditional cleaving annelid *Hydroides hexagonus*¹⁹. Therefore, our data supports that the axial organising role of ERK1/2 observed in *O. fusiformis* is ancestral to Annelida and a shared homologous trait between Annelida and Mollusca. Given our current understanding of the phylogeny of Spiralia^{55,56}, an ERK1/2-mediated embryonic organiser is a probable ancestral spiralian character, at least to the internal node Lophotrochozoa (Fig. 7B). This scenario reinforces the view that conditional spiral cleavage is the ancestral mode of development in Spiralia^{11–13} and thus its study is fundamental to understand the evolution of this major animal clade. In this context, our study uncovers striking similarities between the molecular cascade specifying the embryonic organiser in conditional cleaving spiralian and the gene modules regulating posterior trunk elongation in an array of bilaterian embryos^{3,47,48,57–59}, specially during tail elongation in vertebrates (Fig. 7B). This suggests that the evolution of the FGFR/ERK1/2-mediated embryonic organiser in spiral cleavage brought the instruction of axial identities and posterior trunk elongation temporally and spatially together in a single, conditionally specified, instructing cell. The dissociation of these two processes, e.g. through heterochronic shifts to confer organising activity to early D-quadrant micromeres^{26,54} and/or the loss of ERK1/2 activity to establish the trunk body plan^{24,60}, are then derived conditions evolved independently in certain annelids as they transitioned to an autonomous mode of cell fate specification.

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^{3,29} 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 (MgCl_2) prior to fixation and fixed in 4% PFA in 8% MgCl_2 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 before total RNA extraction.

Immunohistochemistry

F-actin labelling, and antibody staining were conducted as previously described²⁹, with the modification that samples collected for anti-di-P-ERK1/2 staining were washed in PTw supplemented with 1:100 dilution of phosphatase inhibitors (Cell Signalling, #5872) after fixation. The primary antibodies mouse anti-double phosphorylated ERK1/2 (Sigma-Aldrich, #M8159, 1:200) and mouse anti-acetylated α -tubulin (clone 6-11B-1, Millipore-446 Sigma, #MABT868, 1:500) were diluted in 5% normal goat serum (NGS) in 0.1% Triton X-100 phosphate-buffered saline (PTx) and incubated overnight (ON) at 4 °C. After several washes in 1% bovine serum albumin (BSA) in PTx, samples were incubated with either an anti-mouse peroxidase (POD) conjugated antibody (Millipore-Sigma, #11207733910, 1:100) or an AlexaFluor594 conjugated antibody (ThermoFisher Scientific, #A32731) diluted in 5% NGS in PTx ON at 4°C. For samples incubated with mouse anti-double phosphorylated ERK1/2 and anti-mouse POD conjugated antibody, signal was developed using either diaminobenzidine (DAB) (Vector Laboratories, #SK-4100) or Tyramide Signal Amplification (TSA) kit (Akoya Biosciences, #NEL742001KT) following manufacturer's recommendations. DAB-developed samples were stored in 70% glycerol and counterstained with 4',6-diamidino-2-phenylindole (DAPI, ThermoFisher Scientific, #D3571, 1:1000) nuclear marker. Samples for laser scanning confocal microscopy (LSCM) were counterstained with DAPI diluted in 1% bovine serum albumin (BSA) in PTx for 1h at RT and mounted in 70% glycerol.

Gene isolation and gene expression analyses

Candidate genes were amplified by two rounds of nested PCR using gene specific primers and 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^{3,29}. 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 Canu⁶¹, subsequently scaffolded with BioNano optical mapping data generated on an Irys platform and finally de-haploidised with purge_haplotigs⁶². 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 STAR⁶³ and gene models were generated combining Mikado v.2.1⁶⁴, Augustus⁶⁵ and PASA⁶⁶ pipelines. Gene completeness was assessed with BUSCO⁶⁷ (97.5% complete, 1.5% fragmented, 1% missing). Trinotate⁶⁸ and Panther⁶⁹ 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 protocol⁷⁰, employing a home-made Tn5 transposase⁷¹ 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.2.5⁷², reads were mapped with NextGenMap v.0.5.5⁷³ and duplicates removed with samtools v.1.9⁷⁴. BAM files were shifted using deepTools2 v.3.4.3⁷⁵ 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 v2.0.4.2⁷⁸. Transcription factor motif enrichment was performed with HOMER v.4.11⁷⁹ and transcription factor footprinting with TOBIAS v.0.12.0⁸⁰. 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.2.29.2⁸¹. Genomic tracks were plotted with pyGenomeTracks v.2.1⁸².

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.0.36⁸³. Cleaned reads were then mapped to *O. fusiformis* reference genome annotation using Kallisto v.0.44.0 to generate gene expression counts⁸⁴. 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.1.38.0⁸⁵. 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 -1.5⁸⁶. PCAs and hierarchical clustering were plotted using ggplot2 and pheatmap R packages, respectively⁸⁷. 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 Goseq R package, correcting for gene length bias⁸⁸, 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 pathway⁸⁹ (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 mode⁹¹ and poorly aligned regions were removed with gBlocks version 0.91b⁹². Maximum likelihood trees were constructed with either RAxML v.8.2.11⁹³ or FastTree⁹⁴ and visualised with FigTree (<https://github.com/rambaut/figtree/>). InterProScan 5⁹⁵ 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).

Declarations

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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.

Competing Interests

The authors declare no competing interests.

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Figures

Figure 1

Conditional and autonomous cell fate specification occur in spiral cleavage. (A) Spiral cleavage is a stereotypic developmental mode ancestral to Spiralia, one of the three major lineages of bilaterally symmetrical animals (Bilateria). Spiral cleavage is characterised by alternating oblique cell divisions (red arrows) along the animal vegetal axis from the 4-cell stage onwards. (B) Spiral cleaving embryos specify their axial identities and embryonic organiser either conditionally or autonomously. During conditional

spiral cleavage, the specification of the posterodorsal identity (D-quadrant; represented as a D) and the embryonic organiser occurs late in cleavage through inductive signals. On the other hand, autonomous spiral cleavage relies on differentially segregated maternal factors (red dots) that specify the D-quadrant already at the 4-cell stage (cell with D). Later in cleavage, one cell of the D-quadrant that presumably inherits part of those maternal factors will act as embryonic organiser. (C) The conditional mode is found in all spiralian lineages exhibiting spiral cleavage and the autonomous mode is only observed in Mollusca and Annelida, which suggests conditional spiral cleavage is ancestral. While conditional and autonomous spiral cleaving molluscs use the MAP kinase ERK1/2 to establish their body plan, all studied autonomous spiral cleaving annelids do not rely on this signalling cascade and knowledge outside Mollusca and Annelida is absent. The ancestral mechanisms controlling spiral cleavage are thus still unknown, which limits our understanding of the evolution of Spiralia. As an early-branching conditional spiral cleaving annelid, *Owenia fusiformis* can help to infer ancestral developmental characters to Annelida and Spiralia as a whole. Drawings are not to scale.

Figure 2

Di-phosphorylated ERK1/2 is enriched in the 4d micromere in *O. fusiformis*. (A) Active di-phosphorylated-ERK1/2 (di-P-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. (B) Dynamics of cleavage and quadrant identities during the onset and early division of 4d. All images are z-scan projections of the vegetal pole. 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 4a¹–c, and only cleaving into MR and ML (4d daughter cells) after ingressation at 7 hpf. F-actin (grey) shows cell boundaries and DAPI stains the nuclei. False colouring indicates cell or quadrant identities. (C) Schematic representation of the vegetal pole during birth and first division of the fourth micromere quartet in *O. fusiformis*. In red, enriched di-P-ERK1/2 signal. Asterisks in (A) point to the animal pole, and

to part of the 4d progeny in (B). Descriptions for (A) and (B) are based on at least 10 embryos per stage, except at 7 hpf (n = 3). Drawings are not to scale. Scale bars are 50 μ m.

Figure 3

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 (main panels are lateral views; insets are vegetal views). 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 and F-actin with phalloidin. (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 4

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 DEGs, 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. 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; CB), gastrula (9 hpf) and larval stages (24 hpf; L). (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 5

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 4d 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 4a¹–4c² 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 6

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 7

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 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 trunk growth in bilaterian embryos, suggesting that the integration of axial patterning and trunk elongation into a single developmental process of embryonic organising activity is an ancestral hallmark of spiral cleavage and Spiralia. Drawings are not to scale.

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