FGF signaling induces mesoderm in the hemichordate *Saccoglossus kowalevskii*

Stephen A. Green1,*, Rachael P. Norris2, Mark Terasaki2 and Christopher J. Lowe1,3,*

**SUMMARY**

FGFs act in vertebrate mesoderm induction and also play key roles in early mesoderm formation in ascidians and amphioxus. However, in sea urchins initial characterizations of FGF function do not support a role in early mesoderm induction, making the ancestral roles of FGF signaling and mechanisms of mesoderm specification in deuterostomes unclear. In order to better characterize the evolution of mesoderm formation, we have examined the role of FGF signaling during mesoderm development in *Saccoglossus kowalevskii*, an experimentally tractable representative of hemichordates. We report the expression of an FGF ligand, *fgf8/17/18*, in ectoderm overlying sites of mesoderm specification within the archenteron endomesoderm. Embryological experiments demonstrate that mesoderm induction in the archenteron requires contact with ectoderm, and loss-of-function experiments indicate that both FGF ligand and receptor are necessary for mesoderm specification. *fgf8/17/18* gain-of-function experiments establish that FGF8/17/18 is sufficient to induce mesoderm in adjacent endomesoderm. These experiments suggest that FGF signaling is necessary from the earliest stages of mesoderm specification and is required for all mesoderm development. Furthermore, they suggest that the archenteron is competent to form mesoderm or endoderm, and that FGF signaling from the ectoderm defines the location and amount of mesoderm. When considered in a comparative context, these data support a phylogenetically broad requirement for FGF8/17/18 signaling in mesoderm specification and suggest that FGF signaling played an ancestral role in deuterostome mesoderm formation.

**KEY WORDS:** FGF, Deuterostome, Hemichordate, Mesoderm, Mesoderm induction

**INTRODUCTION**

Fibroblast growth factors (FGFs) and Nodals play crucial roles in mesoderm formation in many vertebrates (Ciruna and Rossant, 2001; Kimelman, 2006). In *Xenopus laevis*, FGF signaling is necessary for the initiation of *Xbra* expression (Fletcher and Harland, 2008), which marks early dorsal and posterior mesoderm (Smith et al., 1991). FGF signaling also maintains mesodermal fate (Schulte-Merker and Smith, 1995; Casey et al., 1998), and loss of FGF signaling leads to reductions in posterior mesoderm (including somites and notochord) (Amaya et al., 1991; Conlon et al., 1996; Kumano and Smith, 2000; Kumano and Smith, 2002). The ligand FGF8 is particularly important for this process (Fletcher et al., 2006). FGF8 signaling is also necessary for mesoderm formation in zebrafish, and loss of both Fg8 and its paralog Fgf24 reduces posterior mesoderm (Draper et al., 2003). FGF signaling is involved in mesoderm formation in invertebrate chordates. In amphioxus, FGF signaling is necessary for development of anterior somites (Bertrand et al., 2011), and in ascidians it is necessary for mesenchyme, notochord and secondary muscle development (Kim and Nishida, 1999; Kim et al., 2000; Darras and Nishida, 2001; Kim and Nishida, 2001; Imai et al., 2002; Miya and Nishida, 2003; Yasuo and Hudson, 2007). The widespread roles of FGF signaling in chordate mesoderm specification suggest that FGF signaling probably acted to specify mesoderm in stem chordates.

Although mesoderm induction is increasingly well-characterized in chordates, comprehensive studies in other deuterostomes are lacking and experimental analyses are limited to echinoderms. The limited functional studies of FGFs in sea urchins do not support an early role in mesoderm induction (McCoon et al., 1996; McCoon et al., 1998; Röttinger et al., 2008), and instead implicate Delta/Notch signaling (Sherwood and McClay, 1999; Range et al., 2008). Outside of deuterostomes, studies are limited to major ecdysozoan model systems. In *Drosophila melanogaster*, mesoderm is specified by high activity of the transcription factor Dorsal, which is activated by the Spatzle ligand and Toll receptor (Jiang et al., 1991; Ip et al., 1992), and FGFs act in mesoderm migration, but not in mesoderm induction (Stathopoulos et al., 2004; Kadam et al., 2009; McMahon et al., 2010; Tulin and Stathopoulos, 2010). In *Caenorhabditis elegans*, mesoderm specification requires Notch signaling (Good et al., 2004), and FGF signaling is involved only in the specification of larval sex myoblasts, a small subset of mesoderm (DeVore et al., 1995; Burdine et al., 1998; Goodman et al., 2003; Lo et al., 2008). Currently, there are no functional data on FGF signaling from any lophotrochozoan phyla. A rigorous test of the evolution of developmental mechanisms regulating mesoderm formation requires broader sampling at key phylogenetic positions.

As sister group to echinoderms and closely related to chordates (Turbeville et al., 1994; Bourlat et al., 2006; Dunn et al., 2008), hemichordates are in a key phylogenetic position to test hypotheses of the early evolution of deuterostome developmental mechanisms (Cameron et al., 2000; Lowe et al., 2006; Brown et al., 2008; Cannon et al., 2009). Despite differences in body plan organization between hemichordates and chordates, they share very similar anteroposterior (Lowe et al., 2003) and dorsoventral patterning (Lowe et al., 2006) and endomesoderm specification (Darras et al., 2011), suggesting that molecular comparisons can provide insights into early deuterostome evolution. In enteropneust hemichordates, a group of solitary, burrowing marine worms, mesoderm derives...
from five pouches that evaginate from the archenteron in a process called enterocoely (Bateson, 1884); this trait is shared with echinoderms and amphioxus (Conklin, 1932) and is suggested to be primitive for deuterostomes (Remane, 1963; Valentine, 2004). These morphogenetic similarities with echinoderms and basal chordates, and close similarities with chordates in early body plan patterning, suggest that analysis of mesoderm specification in enteropneusts could help reconstruct ancestral deuterostome developmental mechanisms for mesoderm induction.

To investigate a potentially conserved role of FGF signaling in deuterostome mesoderm induction, we examined the role of FGF signaling during early development of the direct-developing hemichordate Saccoglossus kowalevskii (Bateson, 1884; Bateson, 1886; Colwin and Colwin, 1953; Lowe et al., 2004; Gerhart et al., 2005; Röttinger and Lowe, 2012). We tested the function of the FGF ligand FGFR-B (Röthscher et al., 2009) in hemichordate mesoderm formation, and our work demonstrates that FGFR8/17/18 signals from ectoderm to the underlying archenteron to induce mesoderm. These findings suggest that an ortholog of the fgfr subfamily was essential for mesoderm induction in the deuterostome common ancestor and have important implications for the evolution of mesoderm induction.

MATERIALS AND METHODS

Embryonic culture

Adult hemichordates were collected from Waquoit Bay, MA, USA. Fertilization and embryonic maintenance were performed as described by Lowe et al. (Lowe et al., 2004). For chemical treatments, embryos were immersed in filtered seawater containing either SU5402 (Mammadi et al., 1997) or U0126 (Favata et al., 1998) (both Calbiochem) dissolved in DMSO. Control embryos were treated with DMSO. Inhibitor treatments were changed approximately every 12 hours.

Surgery

Fertilization envelopes were removed with forceps and embryos were cultured in filtered seawater supplemented with 50 μg/ml gentamicin sulfate (FSW + GS). Embryos at the flat-plate gastrula stage were dissected on clay dishes in FSW + GS. The vegetal explants include archenteron endomesoderm and surrounding (posterior) ectoderm. Animal explants are composed only of ectoderm.

Alignment and phylogenetic trees

Protein sequences were obtained from Pubmed, and aligned using MegAlign ver. 8.1.4 (DNASTar), using a BLOSUM series and default CLUSTALW alignment parameters. Neighbor-joining cladograms for mPrx, mesp, six1, snail, mLm, mlca2, zic and foxj1 trees were made using MEGA 4.0 with a JTT model of molecular evolution, bootstrapped with 2000 iterations, and are shown in supplementary material Fig. S1 (Tamura et al., 2007). The fgf8/17/18 gene tree was made with MrBayes (Ronquist et al., 2003). Accession numbers for all sequences are shown in supplementary material Table S1.

Small interfering RNAs (siRNAs)

siRNA targets were identified using the siDESIGN Center (Dharmacon) and purchased from Ambion (Applied Biosystems). Coding domains of fgf8/17/18 were subcloned into pcS2+, and capped mRNAs were made with the mMessage Machine Kit (Ambion). siRNAs or mRNAs were injected as described by Lowe et al. (Lowe et al., 2006). fgf8/17/18 siRNA-a had the sense sequence 5′-AAAAACCGCUUACAAUUUAUGA-3′, and siRNA-b had the sense sequence 5′-AACTGGAGATATTTACGCTAGA-3′. FGFR-B siRNA-a had the sense sequence 5′-CUAUAACUAGGAACACUATT-3′ and FGFRB-siRNA-b had the sense sequence 5′-GGAUUACCGGAAAAACGUGATT-3′. siRNA was injected to a final dose of 100 pM, and mRNA was injected at ~50 pg per embryo.

RESULTS

Mesoderm is specified during gastrulation

Mesoderm arises as enterocoelic evaginations from the archenteron in each of the three body regions: the proboscis, collar and trunk (Bateson, 1884) (Fig. 1, top row). To determine when mesoderm is specified in S. kowalevskii, we examined the expression patterns of hemichordate orthologs of genes with conserved roles in bilaterian mesoderm and endomesoderm formation (see supplementary material Fig. S1 for gene trees).

The expression patterns of four transcription factors associated with early mesoderm development in other bilaterian phyla suggest that mesoderm is specified prior to the morphological segregation of mesoderm and endoderm. We examined mesoPrx (mPrx), a homolog of prx1 and prx2 (Cserjesi et al., 1992; de Jong and Meijlink, 1993; Leussink et al., 1995; Norris et al., 2000; Jones et al., 2001; Doufexi and Mina, 2008); mesp, a paralog to the vertebrate mesp and mesogenin (Sage et al., 1996; Sage et al., 1997; Sawada et al., 2000; Satou et al., 2004; Imai et al., 2006; Saga and Takahashi, 2008); six1 (Kozmik et al., 2007; Beaster-Jones et al., 2008; Gillis et al., 2012); and snail (Hammerschmidt and Nüsslein-Volhard, 1993; Erives et al., 1998; Fujisawa et al., 1998; Langeland et al., 1998; Cano et al., 2000; Wu and McClay, 2007; Rahimi et al., 2009). We did not detect expression of any of these genes in blastula-stage embryos (Fig. 1A,G,M,S). We first detected expression during gastrulation in anterior endomesoderm, in a site that later separates to become the proboscis mesoderm (Fig. 1B,H,N,T). Additional expression domains later appear in the presumptive trunk mesoderm and collar mesoderm (Fig. 1C,D,I,O,P,U,V). These early sites of expression coincide with sites of mesoderm structural gene expression (see below), suggesting that the expression of mPrx, mesp, six1 and snail are good markers of early mesoderm specification.

Expression patterns of mesoderm structural genes corroborate this view of mesoderm specification (Fig. 1Y-JJ). We examined expression of myosin light chain alkali 2 (mlca2) (Holland et al., 1995; Thézé et al., 1995; Thiébaud et al., 2001), an ortholog of other myosin light chain, and muscle Lim (mLim) (Arber et al., 1994; Stronach et al., 1996; Stronach et al., 1999; Martindale et al., 2004). These genes are expressed in a pattern that closely matches the spatial pattern of transcription factor expression. However, the timing of structural gene expression is slightly delayed relative to the expression of transcription factors, consistent with structural genes acting downstream. mLim and mlca2 expression persists in...
mesoderm after enterocoely (Fig. 1L), indicating that these genes are excellent markers of late mesoderm.

Two other genes, though not expressed exclusively in mesoderm at early stages, are useful markers. Zic, a transcription factor with diverse developmental roles (Layden et al., 2010) is expressed in early mesoderm, in the vegetal plate and in anterior ectoderm (Fig. 1KK-PP), foxA is expressed in the vegetal plate (prospective endoderm and mesoderm) prior to gastrulation (Fig. 1QQ), similar to its expression in sea urchins (Harada et al., 1996; Oliveri et al., 2006) and frogs (Suri et al., 2004). During gastrulation, as endomesoderm invaginates to become the archenteron, foxA is maintained in endoderm, but downregulated in mesoderm (Fig. 1RR-VV; supplementary material Fig. S3B). It is not clear whether the vegetal plate cells marked by early foxA expression are endomesodermal precursors to both endoderm and mesoderm, or whether they are endodermal by default, but for the purposes of discussion we refer here to foxA-expressing cells from late blastula to early post-gastrula stages as endomesoderm. Once mesoderm markers are expressed, we describe internal foxA-positive cells as endoderm. The expression patterns of early transcription factors, structural genes and foxA suggest that mesoderm forms after vegetal archenteron endomesoderm contacts overlying ectoderm.
Ectoderm induces mesoderm

To test the hypothesis that hemichordate mesoderm requires ectodermal contact, we carried out surgical experiments on gastrulating embryos. When embryos begin gastrulation, their vegetal plate flattens, making the animal-vegetal axis obvious (Fig. 2A). Embryos at that stage were bisected into animal and vegetal explants (Fig. 2C). Blastomere isolation experiments (Colwin and Colwin, 1950), studies of vegetal β-catenin signaling (Darras et al., 2011) and gene expression (Fig. 1) indicate that animal explants are composed entirely of ectoderm, whereas the vegetal explants contain both vegetal plate endomesoderm and some posterior ectoderm. The vegetal plate normally invaginates to form the entire archenteron, and after surgery vegetal explants go through apparently normal cell movements to make an archenteron (Fig. 2D). In uncut embryos, the mesoderm marker snail is strongly expressed in anterior endomesoderm by mid-gastrula stage, following contact with anterior ectoderm (Fig. 2B). However, vegetal explants incubated to mid-gastrula stage lack snail expression (Fig. 2D). These data suggest that mesoderm does not form autonomously, but instead requires interaction with ectoderm.

To test whether ectoderm is capable of inducing mesoderm, we grafted small pieces of ectoderm cut from animal explants onto vegetal explants of equivalent stage, making animal/vegetal conjugates that were incubated until mid-gastrula stage (Fig. 2E). Ectoderm from the animal pole induced snail expression in the vegetal tissue directly underlying it (Fig. 2F). Precise placement of animal tissue varied, but snail expression was always adjacent to the animal tissue graft (supplementary material Fig. S3C), suggesting that ectoderm induces endomesoderm to become mesoderm. To test whether mesoderm can be induced by contact with any embryonic tissue, we made vegetal-vegetal conjugates (Fig. 2G) and examined them for mesodermal expression. However, vegetal endomesoderm could not induce snail expression (Fig. 2H). These data are consistent with the model that ectoderm provides a signal(s) to the underlying endomesoderm to induce mesoderm.

Expression of fgf8/17/18 and fgfr-B are consistent with roles in mesoderm formation

To examine the possible roles of FGF signaling in hemichordate mesoderm formation, we investigated the expression of FGF ligands and FGF receptors. We isolated fgf8/17/18 (Pani et al., 2012), an ortholog of the FGFD family, which is implicated in mesoderm development in several groups (see Discussion). We identified two splice forms (fgf8/17/18.1 and fgf8/17/18.2) that differ only in the predicted N-terminal signal peptide, but are not orthologs of vertebrate fgf8 splice variants (Fletcher et al., 2006) (supplementary material Fig. S2B). Expression of fgf8/17/18 was examined by in situ hybridization using a probe for fgf8/17/18.1, which hybridizes to both splice forms. Expression is detected at blastula stage (Fig. 3A; supplementary material Fig. S3A), and during gastrulation becomes increasingly restricted to the anterior ectoderm, the region that is contacted by the anterior tip of the archenteron (Fig. 3B). Following gastrulation, additional expression domains are detected at intermediate positions along the anterior-posterior (A/P) axis, in the collar region (Fig. 3C,D). Here, fgf8/17/18 is expressed in two slim, lateral, ectodermal bands immediately overlying sites of mesoderm specification in the collar. Further posterior, fgf8/17/18 is expressed in a weak circumferential ring overlying the site of the trunk mesoderm (Fig. 3D,E). Thus, the expression of fgf8/17/18 in the ectoderm is associated with the induction of all three regions of mesoderm in the archenteron.

S. kowalevskii has two FGF receptors, FGFR-A and FGFR-B, which arise from a hemichordate-specific duplication (Rebscher et al., 2009) (Fig. 3F-J). fgfr-B is expressed in endomesoderm of the archenteron at early gastrula stage and is later upregulated in nascent mesoderm. It is also expressed in ectoderm beginning at late gastrula (not shown) and persisting into later stages (Fig. 3H-J). Expression of fgfr-B is both temporally and spatially coincident with upregulation of mesoderm specification markers. Thus, the expression of both fgf8/17/18 and fgfr-B are consistent with the model that FGF8/17/18 is secreted from ectoderm to induce mesoderm in underlying endomesoderm (Fig. 3K-O).
Endomesodal fgfr-B is necessary for mesodermal specification
To test whether fgfr-B is necessary for mesodermal specification, we injected zygotes with custom-designed siRNAs. Expression of fgfr-b is reduced following siRNA injection (supplementary material Fig. S4A; ~40% reduction relative to controls). Expression of the mesodermal specification markers snail and mLim is strongly reduced at early gastrula stages (compare Fig. 4A,F with 4C,H), suggesting that fgfr-B is necessary for mesodermal specification. Mesodermal zic expression is also reduced, but expression in anterior ectoderm is unaffected (Fig. 4B,G), indicating that ectodermal expression is not FGF dependent. At later juvenile stages, the differentiated mesodermal marker mLim is strongly reduced (Fig. 4E,J), suggesting a failure of muscle formation. Furthermore, the endodermal epithelium extends abnormally far into the proboscis and contacts the anterior-most ectoderm (Fig. 4E,J, arrow), suggesting that anterior endomesoderm differentiates into endoderm in the absence of FGF signaling. A second siRNA for fgfr-B gave a consistent but milder phenotype, supporting the specificity of the knockdown (supplementary material Fig. S3D). Similarly, treating embryos with 20 μM of the FGFR inhibitor SU5402, (Mohammadi et al., 1997) or 8 μM of the MEK inhibitor U0126 (Favata et al., 1998) beginning at late blastula stages (compare Fig. 3. Expression of FGF signaling components in relation to endomesodermal and mesodermal gene expression. (A-J) Expression of fgf8/17/18 (A-E) and fgfrB (F-J) during early stages of S. kowalevskii development. The animal/anterior pole is to the upper left. All images are dorsal views of median optical sections of cleared embryos, except for D, which is a lateral surface view of an uncleared embryo. (K-O) Model of hypothetical interactions during mesoderm formation. White arrows indicate direction of FGF signaling from ectoderm to archenteron endomesoderm. Mesoderm is red, ectoderm blue, endoderm yellow, and endomesoderm is mixed yellow and red.

fgfr-B in the underlying endomesoderm leads to complete loss (4/8) or reduction (4/8) of snail expression (Fig. 4W). This suggests that mesoderm induction requires reception of FGF signals by endomesodal FGFR-B.

Ectodermal fgf8/17/18 is necessary for mesodermal specification
In order to test the hypothesis that FGFR/B induces mesodermal specification, we injected embryos with either of two siRNAs (fgf8a, fgf8b), each targeting both fgf8/17/18 splice variants. RT-PCR analyses of fgf8/17/18 levels demonstrate that expression is strongly reduced following siRNA injection (supplementary material Fig. S5; ~77% reduction relative to controls). Embryos fixed during enterocoely show reduced expression of the mesodermal markers zic, mLim and myosin (Fig. 4K-M,P-R), and the endomesodal/endodermal marker foxA continues to be expressed at the sites where mesoderm would normally form (Fig. 4N,S). At the juvenile stage, mLim expression is reduced, proboscis muscle is largely absent and the anterior endodermal epithelium expands into the proboscis (Fig. 4O,T; supplementary material Fig. S3E,F). This is similar to the fgfr-B siRNA phenotype, and consistent with FGF8/17/18 inducing mesoderm.

To test whether fgf8/17/18 is required in ectoderm or endomesoderm for mesodermal specification, we made conjugates by combining animal and vegetal explants from siRNA-injected and un.injected embryos as already described for FGFR-B. Following knockdown of fgf8/17/18 in vegetal tissue, ectodermal explants induce snail expression normally (Fig. 4Y), but if fgf8/17/18 is knocked down in the animal explants and grafted onto normal endomesoderm, mesodermal specification fails (Fig. 4X). This is consistent with the model that FGF8/17/18 secreted from ectoderm is required to specify mesoderm in underlying endomesoderm.

fgf8/17/18 is sufficient for mesodermal specification
To test whether fgf8/17/18 is sufficient to induce mesoderm, we injected capped fgf8/17/18 mRNA or a control tdTomato mRNA into zygotes. At early developmental stages, fgf8/17/18 mRNA expanded mesodermal markers (snail and zic) (Fig. 5A,C,I,K) and reduced expression of the endoderm/endomesodermal marker foxA (Fig. 5B,J). In juveniles, mesoderm is expanded (mLim; Fig. 5D,L) and endoderm is significantly reduced (foxA; Fig. 5E,M), suggesting that fgf8/17/18 can induce archenteron endomesoderm to become mesoderm. If FGF8/17/18 signals through FGFR-B, then mesoderm
expansion should require \textit{fgfr-B} expression. In embryos co-injected with \textit{fgf8/17/18} mRNA and \textit{fgfr-B} siRNA, archenteron expression of the mesoderm marker \textit{zic} is reduced (Fig. 5F,N) relative to its expression in embryos injected with \textit{fgf8/17/18} mRNA alone (Fig. 5K), suggesting that \textit{fgfr-B} is necessary to transduce the inductive effect of the exogenous \textit{fgf8/17/18} mRNA. Furthermore, if \textit{fgf8/17/18} induces mesoderm directly, then it should have the capacity to do so in the absence of ectoderm. In order to test the sufficiency of \textit{fgf8/17/18}, we cut vegetal explants from embryos injected with \textit{fgf8/17/18} mRNA. The resulting explants expressed \textit{zic} throughout most of the endomesoderm (Fig. 5O). By contrast, vegetal explants made from embryos injected with control 	extit{tdTomato} mRNA did not show any \textit{zic} expression (Fig. 5G). Next, we tested whether overexpression of \textit{fgf8/17/18} in vegetal explants conveys mesodermal inductive properties. To this aim, we made vegetal-vegetal conjugates between explants from uninjected embryos and \textit{fgf8/17/18} mRNA-injected embryos. Resulting conjugates show mesoderm induction in the half composed of the injected explant (3/3), and either partial (1/3) or complete (2/3) mesoderm induction in the half composed of the uninjected explant (Fig. 5P). By contrast, injection of 	extit{tdTomato} mRNA failed to induce \textit{zic} expression in either explant (Fig. 5H). These data suggest that \textit{fgf8/17/18} signaling is sufficient to induce mesoderm from endomesoderm.

**DISCUSSION**

**Hemichordate mesoderm is specified from endomesoderm**

Expression analyses indicate that the mesodermal markers \textit{snail}, \textit{mLim}, \textit{mlcal2}, \textit{mesopr}, \textit{six1} and \textit{mesp} are co-expressed in tissues clearly identifiable as mesoderm. Expression analyses also show that \textit{foxA} is expressed first throughout the vegetal plate at early gastrula, and that its expression is lost in cells that initiate expression of mesodermal markers. It remains to be experimentally determined whether this early \textit{foxA}-positive domain represents endomesoderm or an endodermal default. The mesodermal expression arises in a characteristic pattern towards the end of gastrulation, first anteriorly in the proboscis mesoderm, then posteriorly in the trunk and collar mesoderm (Fig. 1). We do not detect expression of any gene at blastula stage. We cannot rule out the possibility that there is earlier expression of other genes, but based on these data it appears that hemichordate mesoderm is specified during gastrulation, later in development compared with chordates and sea urchins.

In \textit{S. kowalevskii}, mesoderm arises as subsets of the vegetal, endomesodermal tissue that forms the archenteron. These tissues are visible at early gastrula stage as a thick vegetal plate that is marked by expression of \textit{foxA} and \textit{zic}. As mesoderm specification begins, \textit{foxA} is restricted to the remaining endomesoderm/endoderm, whereas \textit{zic} is first restricted to the proboscis mesoderm, but later is expressed in the trunk and collar mesoderm. The endomesoderm might be either endoderm by default, or a distinct precursor cell type that requires signaling to adopt either fate. In either model, these data imply that the vegetal endomesoderm has competence to form mesoderm or endoderm.

**zic and mesoderm specification**

In \textit{S. kowalevskii}, \textit{zic} is the only gene in our study expressed throughout the vegetal plate and then restricted to mesoderm at later stages. It is possible that vegetal plate expression of \textit{zic} might be necessary for appropriate mesoderm specification. This possibility deserves special consideration, as \textit{zic} is a homolog of the ascidian gene \textit{macho-1} (Layden et al., 2010), which acts in ascidian cell-autonomous mesoderm specification (Nishida and Sawada, 2001; Sawada et al., 2005). However, \textit{S. kowalevskii} \textit{zic} is expressed throughout the vegetal plate, unlike ascidian \textit{macho-1}. This means...
that hemichordate zic is also expressed in precursors of all endoderm, indicating that zic expression in the vegetal plate cannot be sufficient for mesoderm specification, but the subsequent loss of zic expression in endoderm might be necessary for endoderm differentiation. Loss of zic expression following knockdown of fgf8/17/18 (Fig. 4K-M,P-R) and expanded zic expression in response to fgf8/17/18 misexpression (Fig. 5C,K,G,H,P) suggests that late zic expression is actively induced or maintained by ectodermal FGF signaling. We believe that the expression of zic in the vegetal plate probably represents a distinct role, perhaps in specification of endomesoderm, or patterning of the animal-vegetal axis. Analyses of zic loss of function and misexpression will be necessary to test this hypothesis.

**Hemichordate FGF8/17/18 induces endomesoderm to become mesoderm**

Three kinds of evidence presented here indicate that FGF signaling induces mesoderm in the hemichordate *S. kowalevskii*. First, fgf8/17/18 expression in the ectoderm oversees regions of the archenteron with upregulated expression of the FGF receptor gene fgfr-B (Fig. 3) and a battery of mesodermal markers (Fig. 1). Second, in loss-of-function experiments, knockdown of either fgf8/17/18 or fgfr-B greatly reduces or eliminates mesoderm formation, leaving endoderm formation intact or expanded (Fig. 3). Surgical experiments indicate that mesoderm can be induced in archenteron presumptive endomesoderm by signals released from attached pieces of ectoderm (Fig. 2) that express fgf8/17/18 (Fig. 3). Similar experiments that combine surgical grafting with fgf8/17/18 overexpression induces archenteron endomesoderm to become mesoderm, even in the absence of ectoderm (Fig. 5), and this mRNA gives endomesoderm the capacity to induce mesoderm in other explants (Fig. 5). These data provide strong evidence that FG8/17/18 signals from the ectoderm to adjacent endomesoderm to induce mesoderm, and also imply that spatially regulated fgf8/17/18 expression in the ectoderm determines the location and amount of mesoderm induced from the archenteron.

**FGF8/17/18 and deuterostome mesoderm specification**

Our findings contribute to a growing body of evidence indicating that FGF8/17/18 signaling is necessary for mesoderm specification in many deuterostomes. FGF signaling is required for posterior mesoderm (somites, notochord) in *Xenopus laevis* and the zebrafish *Danio rerio* (Amaya et al., 1991; Draper et al., 2003; Fletcher et al., 2006; Fletcher and Harland, 2008), for induction of mesenchyme, notochord and some tail muscles in ascidians (Kim and Nishida, 1999; Kim et al., 2000; Darras and Nishida, 2001; Kim and Nishida, 2001; Imai et al., 2002; Miya and Nishida, 2003), and for anterior somites in amphioxus (Bertrand et al., 2011). fgf8/17/18 orthologs are crucial for these signaling events in frog (Fletcher et al., 2006), zebrafish (Draper et al., 2003) and ascidians (Yasu and Hudson, 2007), but the FGF ligand acting in amphioxus is unknown. Echinoderms are the only deuterostome phylum without experimental data supporting a role of FGF in mesoderm specification. However, our study suggests that a more comprehensive experimental analysis of the FGF complement in echinoderms will be required before it can be conclusively ruled out as a regulator of early mesoderm fate in this group. We can now state that FGF8/17/18 signaling is also essential in mesoderm specification in a representative of another deuterostome phylum, the hemichordates. It will now be interesting to test further the requirement of FGF signaling more broadly in hemichordates by investigating its role during the development of enteropneust species characterized by indirect development and a distinct larval body plan, more similar to echinoderm early developmental strategies. Nevertheless, comparison of experimental data between deuterostome species suggests that there is a widespread requirement for FGF8/17/18 signaling in mesoderm specification.

**Hemichordate FGF8/17/18 ligand signals from ectoderm to prospective endomesoderm**

Our data suggest that FGF8/17/18 is produced in ectoderm and transfers patterning information from ectoderm to the underlying prospective endomesoderm. This is different from vertebrates, in which FGF signals required for mesoderm fate are instead produced within the mesoderm itself in response to signaling from endoderm. However, in both cases FGF signaling is received within the
endomesoderm, suggesting that this is a conserved feature of deuterostomes. The site of signal production might be more evolutionarily flexible than the site where signaling is received, and so it is possible that hemichordates lost mesoderm-specific FGF gene expression. Alternatively, chordates might have gained FGF expression in mesoderm. One informative outgroup, the dipteran D. melanogaster, requires FGF signaling for mesoderm migration, and, as in hemichordates, FGF ligands are produced in ectoderm (Stathopoulos et al., 2004; Kadam et al., 2009; McMahon et al., 2010; Tulin and Stathopoulos, 2010). It is possible that FGF signals to mesoderm originally signaled from ectoderm to endomesoderm, but shifted to a production site in the mesoderm during the evolution of early chordates. Testing this hypothesis will require examination of other deuterostome invertebrates and other protostome phyla.

Inductive signaling in deuterostome mesoderm specification

In vertebrates, several other signaling pathways have particularly important roles in mesoderm induction. Nodal signaling is generally required for vertebrate mesoderm (Kimelman, 2006). However, there is limited comparative support for a conserved role of this pathway outside of vertebrates. Nodal does not induce mesoderm in amphioxus (Onai et al., 2010) or sea urchins (Duboc et al., 2005).

Another major signaling pathway, Delta/Notch signaling, appears to have at least two distinct and relevant roles in deuterostome early endomesoderm development. There is a widespread requirement for Delta/Notch signaling in endomesoderm segregation in frog, zebrafish and sea urchins (Kikuchi et al., 2004; Contakos et al., 2005; Revinski et al., 2010; Sethi et al., 2012), but it generally seems to promote endoderm at the expense of mesoderm. In sea urchins, Delta/Notch signaling also plays a key role in mesoderm induction (Sherwood and McClay, 1999; Range et al., 2008; Materna and Davidson, 2012). This would appear to be more comparable to the role of hemichordate FGF signaling that we observe here, but the inductive role of Delta/Notch signaling has not been observed in other animals. It does not have this role in the closely related asteroid echinoderms (Hinman and Davidson, 2007) or in mouse and zebrafish (Sherwood and McClay, 1999; Shi et al., 2005; Range et al., 2008).

The general distribution of Nodal and Notch requirements is consistent with those pathways acquiring roles in mesoderm induction during the evolution of stem vertebrates and echinoderms, respectively. Examining these signaling pathways within hemichordates will help to test these hypotheses. In contrast to Nodal and Delta/Notch signaling, FGF signaling has phylogenetically broad support for a role in mesoderm specification. We propose that FGF8/17/18-mediated mesoderm specification was a primitive trait of deuterostomes and that FGF signaling might have acted non-autonomously to induce mesoderm, as it does in S. kowalevskii.

We propose two broad evolutionary models of the roles of FGF8/17/18 signaling in deuterostome mesoderm specification. First, FGF signaling might have been required for formation of all or most mesoderm. This scenario implies that other signaling pathways, including Nodal (Kimelman, 2006) in vertebrates and Notch signaling in echinoderms (Sherwood and McClay, 1999; Range et al., 2008), gained additional prominence during later evolution, but it is also consistent with multiple signals being required for mesoderm specification. It would also imply lineagespecific secondary loss of FGF-dependent induction in subpopulations of mesoderm in different chordate groups, and potentially complete loss in echinoderms. A second possibility is that FGF signaling was ancestrally associated with induction of a specific subset of mesoderm (either a specific tissue or axial level), but gained additional importance during hemichordate evolution.

We consider the first model, that FGF8/17/18 signaling was required for all mesoderm in early deuterostomes, to be the best fit for the available comparative developmental data, for several reasons. First, FGF signaling acts broadly in mesoderm induction in both hemichordates and ascidians, where it is required in multiple mesodermal tissues at all axial levels (Lemaire et al., 2008). Second, in chordates, in which FGF signaling is required in only a subset of mesoderm, there is little commonality in the precise location of the requirement: FGF signaling is required for posterior mesoderm in frogs (Kumano and Smith, 2000; Kumano and Smith, 2002; Kimelman, 2006; Bertrand et al., 2011) and zebrafish (Draper et al., 2003), but is required for anterior somites in amphioxus (Bertrand et al., 2011). This condition seems more likely to result from a selective loss of FGF function than from multiple independent gains of function, suggesting that FGF signaling might have had ancestral roles in mesoderm specification.

FGF signaling and the origins of mesoderm

Mesoderm evolved prior to the divergence of deuterostomes and protostomes, but the changes in gene regulation that led to the innovation of a distinct mesodermal germ layer remain unknown. Our data suggest that FGF signaling acted to induce mesoderm in early deuterostomes, but determining whether this was an ancestral bilaterian trait requires additional data from protostomes. Currently, studies from ecdysozoan model systems do not support this model; FGFs act in protostome mesoderm patterning and migration, but we are not aware of any evidence of a role in mesoderm induction. Data from the other main protostome lineage, the lophotrochozoans, will be important for testing this hypothesis. Currently there are no experimental analyses of lophotrochozoan FGF signaling, but experiments from gastropod molluscs have implicated MAPK signaling in formation of larval mesoderm derived from the 3D lineage (Lambert and Nagy, 2001; Lambert and Nagy, 2003; Koop et al., 2007), and FGFs are good candidates for this signaling event.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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