CHAPTER SEVEN

# Putting in the Erk: Growth factor signaling and mesoderm morphogenesis

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## Contents



## Abstract

It has long been known that FGF signaling contributes to mesoderm formation, a germ layer found in triploblasts that is composed of highly migratory cells that give rise to muscles and to the skeletal structures of vertebrates. FGF signaling activates several

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pathways in the developing mesoderm, including transient activation of the Erk pathway, which triggers mesodermal fate specification through the induction of the gene *brachyury* and activates morphogenetic programs that allow mesodermal cells to position themselves in the embryo. In this review, we discuss what is known about the generation and interpretation of transient Erk signaling in mesodermal tissues across species. We focus specifically on mechanisms that translate the level and duration of Erk signaling into cell fate and cell movement instructions and discuss strategies for further interrogating the role that Erk signaling dynamics play in mesodermal gastrulation and morphogenesis.

## 1. Introduction

"Time is a flat circle" in developmental signaling (Fukunaga, 2014): a small number of signaling pathways are used recurrently to dictate cell fates and movements throughout embryogenesis. Despite a relatively complete molecular parts list, there is still much to learn about how cell signaling pathways are activated at just the right positions and developmental times, and how pathway activity is interpreted into the gene expression programs required for each developmental fate choice.

One of the earliest and most fundamental developmental fate choices is germ layer specification during gastrulation. A collection of initially pluripotent cells differentiates into endoderm, ectoderm or mesoderm in a manner that depends on their position in the embryo and exposure to signaling cues. While the cues that establish each germ layer are well-established, it is still unclear how these cues are organized dynamically in space and time to control different aspects of cell fate specification and cell movements. Here, we explore these dynamics in one specific context: the role of transient Erk signaling in early mesoderm development. We note that we cover the first steps of mesoderm induction, prior to somitogenesis, which is already covered by excellent reviews (Aulehla & Pourquié, 2010).

Activation of the kinases Erk1 and Erk2 (henceforth grouped together as Erk) in the mesoderm is primarily triggered by activation of fibroblast growth factor receptor (FGFR), a receptor tyrosine kinase (RTK) that has long been implicated in mesoderm induction in vertebrates. Early experiments showed that ectopically injected fibroblast growth factor (FGF) is sufficient to induce mesoderm in *Xenopus* (Smith, 1989), and over the next decade, specific FGFs and FGFRs that participate in mesoderm induction were identified (Christen & Slack, 1997; Golub et al., 2000; Isaacs, Pownall, & Slack, 1995; Lombardo, Isaacs, & Slack, 1998). One of the

primary roles FGF plays in mesoderm specification is induction of *brachyury* (Isaacs, Pownall, & Slack, 1994), a gene that marks the mesodermal precursor population in vertebrates. Experiments with small molecule inhibitors and morpholinos against Erk revealed that FGF triggers *brachyury* expression through Erk signaling in a manner that is conserved across vertebrates (Christen & Slack, 1999; Hardy, Yatskievych, Konieczka, Bobbs, & Antin, 2011; Krens, Corredor-Adámez, He, Snaar-Jagalska, & Spaink, 2008; LaBonne & Whitman, 1994; van Boxtel, Economou, Heliot, & Hill, 2018; Yao et al., 2003). Such experiments also revealed that FGF-induced Erk signaling plays a role in bringing the newly specified mesoderm to its proper location in the embryo (Hardy et al., 2011; Krens, He, et al., 2008; Sivak, Petersen, & Amaya, 2005).

Interestingly, Erk signaling dynamics in the mesoderm are also well-conserved. Early antibody stainings suggested that Erk signaling is transient in the vertebrate mesoderm (Christen & Slack, 1999; Krens, He, et al., 2008; Lunn, Fishwick, Halley, & Storey, 2007), as well as in certain invertebrate mesodermal subtypes (Mandal, Dumstrei, & Hartenstein, 2004; San Martin & Bate, 2001). Recently developed biosensors for live imaging of embryonic signaling have captured the dynamics of such signals at high temporal resolution (Wong, Akiyama, Bessho, & Matsui, 2018) and have detected transient Erk signals in tissues where older staining techniques failed (Corson, Yamanaka, Lai, & Rossant, 2003; Morgani et al., 2018). The fact that transient Erk signaling is so well-conserved begs the question: are the dynamics of Erk signaling playing a functional role in mesoderm specification?

Historically, this hypothesis has been difficult to test because Erk plays other important roles prior to gastrulation. In mouse, for example, Erk signaling is required for epiblast specification (Nakamura, Goto, Kondo, & Aoki, 2021), and in zebrafish, Erk signaling is required for epiboly, a morphogenetic movement that precedes mesodermal ingression (Krens, He, et al., 2008). With the advent of advanced signaling perturbation techniques like optogenetics, however, it is finally possible to test specific hypotheses regarding dynamic Erk signaling requirements for the induction of specific cell types and morphogenetic behaviors. Optogenetic tools developed to control Erk signaling in invertebrates have revealed that the *Drosophila* embryo depends on Erk signaling dynamics for several germ layer choices, including the choice between endoderm and neurogenic ectoderm and the choice between caudal visceral mesoderm and hindgut ectoderm (Johnson & Toettcher, 2019; McFann, Dutta, Toettcher, & Shvartsman, 2021). It is

likely that vertebrates, too, possess molecular mechanisms for decoding Erk signaling dynamics during germ layer specification. Indeed, recent optogenetic experiments in zebrafish have shown that varying the duration of Erk signaling during germ layer specification affects cell movements during gastrulation (Patel et al., 2019).

In this review, we discuss what is known about the roles that Erk and its dynamics play in mesodermal specification. We draw examples from lower invertebrates (*Xenopus*, zebrafish) and higher vertebrates (chick, mouse) to highlight conserved features of Erk signaling during the earliest moments of mesodermal specification, like mechanisms for ensuring Erk transience at the margins and primitive streaks of embryos. Additionally, we include the *Drosophila* caudal visceral mesoderm as an example to highlight how some features of mesodermal Erk signaling are more widely conserved, like Erk's role in inducing and maintaining *brachyury* expression and its importance in highly migratory tissues.

We are also interested in contexts where mesodermal development diverges, like in gastrulation where different organisms employ different types of cell movement to position the mesoderm. Erk's continued presence in these divergent contexts suggests a flexibility in this signaling module that allows it to be coopted by evolution for a wide variety of morphogenetic purposes. On the other hand, it is important to note that Erk is only one process triggered by upstream RTK activation, and it remains possible that other RTK-dependent but Erk-independent processes are at least as important for coordinating cell movements. We spend the final section of this review discussing emerging techniques that may prove helpful in answering such questions going forward.

## 2. The fates of vertebrate mesodermal populations marked by *brachyury*

How can early mesodermal cells be identified across organisms and contexts? We focus particularly on the earliest cell populations that express *brachyury* and its homologs, as *brachyury* is expressed in every mesodermal subtype in vertebrates, and its expression marks the mesodermal precursor population (Amaya, Stein, Musci, & Kirschner, 1993; Burdsal, Flannery, & Pedersen, 1998; Griffin, Patient, & Holder, 1995; Isaacs et al., 1994; Kispert, Ortner, Cooke, & Herrmann, 1995; Krens, Corredor-Adámez, et al., 2008; LaBonne & Whitman, 1994; Schulte-Merker & Smith, 1995; Smith, Price, Green, Weigel, & Herrmann, 1991; Yao et al., 2003). This population is highly migratory. Upon receiving pro-mesodermal cues, mesodermal precursors

loosen connections to their cellular neighbors and traverse great distances to reach their final embryonic positions. Even in invertebrates like *Drosophila*, in which mesoderm specification is initiated through different means than in vertebrates, the caudal visceral mesoderm, the only mesodermal sub-population to express *brachyury*, migrates the greatest distance of any *Drosophila* embryonic cell type (Bae, Trisnadi, Kadam, & Stathopoulos, 2012).

In lower vertebrates like *Xenopus* and zebrafish, mesodermal precursors arise from the margins of spherical embryos. Meanwhile, in higher vertebrates, the geometries of embryos vary from the cup-like mouse gastrula to the disc-like chick gastrula, with mesodermal precursors arising from a structure called the primitive streak. In this section, we discuss mesodermal gastrulation in *Xenopus*, zebrafish, chick, and mouse to set the groundwork for discussing general principles regarding Erk-dependent programming of mesodermal induction and morphogenesis across species.

In *Xenopus* and zebrafish, a *brachyury* homolog is expressed at the embryonic margin, a region that borders the blastopore (Fig. 1A, green region). Cells at the margin descend over the embryo through a process called epiboly. In *Xenopus*, epiboly involves the vegetal movement of cells over the endodermal precursor population, while in zebrafish, epiboly involves vegetal movement over the yolk (Solnica-Krezel & Sepich, 2012). Concurrent with the expression of *brachyury*, mesodermal precursors undergo gastrulation. In *Xenopus*, cells expressing *Xbra*, the *Xenopus brachyury* homolog, form the mesodermal layer by folding beneath the ectodermal layer, a process termed involution (Smith et al., 1991; Solnica-Krezel & Sepich, 2012). In zebrafish, cells expressing *no tail* (*ntl*), the zebrafish *brachyury* homolog, push past cells in the epiblast to form a mesoendodermal layer via ingression (Rodaway et al., 1999; Solnica-Krezel & Sepich, 2012).

In both *Xenopus* and zebrafish, the type of mesoderm each cell will become is determined by its dorsal-ventral location along the margin. The dorsal margin gives rise to axial mesoderm, while cells medially localized along the dorsal-ventral axis give rise to paraxial and lateral mesoderm (Dale & Wardle, 2015; Warga & Nüsslein-Volhard, 1999). In all vertebrates, the axial mesoderm goes on to form the prechordal plate and notochord. Meanwhile, paraxial mesoderm gives rise to the somites and tailbud, whereas lateral mesoderm contributes to the appendicular skeleton, heart, and other organs. In both *Xenopus* and zebrafish, upon entering the mesodermal layer, the axial mesoderm extends anteriorly. Meanwhile, the paraxial and lateral mesoderm extend anteriorly while also converging toward the dorsal midline (Solnica-Krezel & Sepich, 2012).

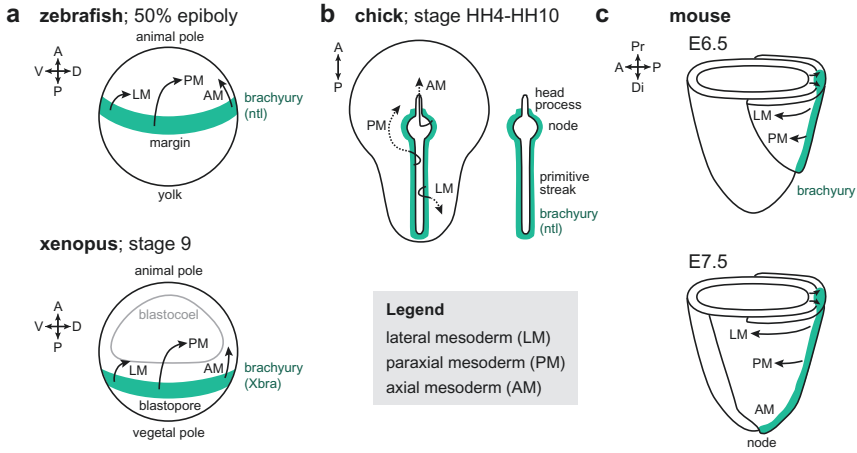


**Fig. 1** Mesodermal cell populations across vertebrate species. (A) Mesodermal gastrulation in zebrafish and *Xenopus*. The dorsal-ventral and anterior-posterior axes are marked along with the animal cap, the yolk (zebrafish only) and the vegetal pole (*Xenopus* only). A *brachyury* homolog (*ntl* in zebrafish, *Xbra* in *Xenopus*) is expressed in a ring, marked in green, at the margin. Mesodermal precursors ingress at the margin. Axial mesoderm arises from the dorsal margin while paraxial and lateral mesoderm arise from medial regions along the dorsal-ventral axis. The axial mesoderm extends anteriorly, while the paraxial and lateral mesoderm converge dorsally while extending anteriorly. These cell movements are indicated by arrows. (B) Mesodermal gastrulation in chick. The anterior-posterior axis is marked. *brachyury*, marked in green, is expressed around the primitive streak and Hensen's node. Mesodermal precursors ingress in an anterior-to-posterior fashion, beginning at Hensen's node. Ingression is indicated by the solid portions of arrows, while migration occurring in the newly formed mesodermal layer is indicated by the dotted portions of arrows. Cells that ingress through Hensen's node migrate anteriorly to form the prechordal plate and head structures. As the primitive streak begins to retreat posteriorly, mesodermal precursors continue to ingress through the node and migrate anteriorly to form the notochord. Paraxial mesodermal precursors ingress through the anterior streak between HH4 and HH7 and travel a path first away from the streak and then toward the prechordal plate and head structures. Lateral mesoderm ingresses through the posterior streak between HH8 and HH10 and migrates away from the streak. (C) Mesodermal gastrulation in mouse. The anterior-posterior and proximal-distal axes are marked. *brachyury* is expressed along the primitive streak. Ingression into the streak is marked by the most proximal pair of arrows. Mesodermal precursors that ingress through the proximal streak migrate anteriorly away from the streak (marked by an arrow) and form lateral mesoderm. Precursors that ingress through the distal streak migrate anteriorly, but not as far as the lateral mesoderm, and form paraxial mesoderm (migration marked by an arrow). Axial mesoderm arises from cells that ingress through the node and migrate anteriorly.

In contrast to the circular margins of *Xenopus* and zebrafish, the primitive streak of birds and mammals resembles a slit and is built in a progressive manner. Primitive streak ingression in chick begins as a thickening of cells in the posterior blastodisc. The streak then extends anteriorly through convergence and extension. Mesodermal precursors enter the streak in an anterior-to-posterior manner and, upon internalization, travel to their final location via migration (Gilbert, 2000) (Fig. 1B). All mesodermal precursors that enter the streak express *brachyury* (Kispert et al., 1995). The most anteriorly positioned mesodermal precursors, the axial mesoderm population, enter Hensen's node and migrate anteriorly to become the head mesoderm and notochord. The next group of mesodermal precursors then enters the anterior streak to become paraxial mesoderm. Upon internalization, these cells are first directed away from the embryonic midline during migration and then anteriorly toward the head process. The final group of mesodermal precursors then enters the posterior streak. Upon internalization, these cells migrate posteriorly and away from the midline to form the lateral mesoderm (Gilbert, 2000).

In mouse, primitive streak ingression is initiated in the proximal posterior (Fig. 1C, top-right arrows), and the streak is extended throughs progressive induction of epithelial to mesenchymal transitions (EMT) along the proximal-distal axis (Williams, Burdsal, Periasamy, Lewandoski, & Sutherland, 2012). Upon ingression, mesodermal precursors migrate anteriorly from the midline to form paraxial and lateral mesoderm. Lateral mesoderm arises from the proximal streak and extends further anteriorly than the paraxial mesoderm, which arises from the distal streak and remains closer to the midline. Mesodermal precursors that remain at the midline form the axial mesoderm (Takada et al., 1994). All the mesodermal precursors express *brachyury* (Kispert & Herrmann, 1994).

Thus, while the specifics surrounding mesoderm induction and internalization differ among vertebrates, features like *brachyury* expression, internalization of the mesoderm through an opening, and patterning of the various mesodermal subtypes along that opening are ubiquitous.

## 3. FGF expression in the mesoderm: An upstream cue for Erk-dependent gene induction

The mechanisms by which *brachyury* expression is instantiated in the mesoderm are also well-conserved among vertebrates. At the time the embryo is being patterned for gastrulation, mesodermal precursors express

receptor tyrosine kinases (RTKs), including members of the FGFR family. RTKs function by binding to extracellular ligands, which induce a conformational change that allows for phosphorylation of the RTK kinase domains and C-terminal tails, leading to recruitment of adaptor proteins and activation of multiple intracellular signaling pathways, including Erk. Depending on the type of RTK, the ligand to which the RTK binds, and which intracellular components are present to mediate adaptor recruitment, a wide range of different intracellular signaling pathways can be activated (Brewer, Mazot, & Soriano, 2016).

One RTK expressed by the mesodermal precursors of every model vertebrate we discuss here is FGFR1 (Golub et al., 2000; Lunn et al., 2007; Ota et al., 2010; Yamaguchi, Conlon, & Rossant, 1992). In addition to being present at the right place and right time to be involved in mesoderm induction and morphogenesis, inhibition or removal of FGFR downregulates *brachyury* expression in *Xenopus*, zebrafish, and chick (Chung et al., 2004; Hardy et al., 2011; Rodaway et al., 1999) and early mesodermal cell movements are disrupted in all four model vertebrates (Amaya et al., 1993; Griffin et al., 1995; Yamaguchi, Harpal, Henkemeyer, & Rossant, 1994; Yang, Dormann, Münsterberg, & Weijer, 2002).

Nevertheless, it is important to note that FGFR-to-Erk signaling is not one-to-one, but many-to-many. FGFRs activate a variety of canonical targets, including the Erk, PI3K, PLC$\gamma$, STAT, and Jnk pathways, and also participate in non-canonical interactions with cell-adhesion molecules, like cadherin and integrins (Brewer et al., 2016; Clark & Soriano, 2022). Conversely, there are many roads to Erk, including other RTKs.

In the lower vertebrates *Xenopus* and zebrafish, FGFR activation triggers Erk signaling at the margin (Christen & Slack, 1999; van Boxtel et al., 2018) and inhibiting Erk signaling via small molecule inhibitors or morpholinos disrupts both *brachyury* expression and mesodermal cell movements (Krens, Corredor-Adámez, et al., 2008; LaBonne & Whitman, 1994). Erk appears to control *brachyury* via phosphorylation of its canonical targets, Ets family transcription factors (Kawachi, Masuyama, & Nishida, 2003; Kjolby, Truchado-Garcia, Iruvanti, & Harland, 2019; McFaul, Hart, & Draper, 2020; Znosko et al., 2010). A similar situation is also found in the chick embryo, where FGFR activity induces *brachyury* through Erk in mesodermal precursors as they enter the primitive streak, as well as in mesodermal cells as they exit the primitive streak to migrate anteriorly (Lunn et al., 2007). Inhibiting Erk signaling during primitive streak formation disrupts *brachyury* expression and the expression of other genes required for proper cell movement (Hardy et al., 2011).

In mouse, the relationship between FGFRs, Erk pathway activation, and mesodermal development is less straightforward. Once again, the Erk pathway is transiently activated in cells entering the primitive streak and is activated a second time in mesodermal precursors as they migrate away from the streak to position themselves in the embryo (Morgani et al., 2018). Knockdown of Erk2, the predominant Erk isoform in the early embryo, also prevents both *brachyury* expression and mesoderm formation in the mouse, although Erk1 is able to compensate during *in vitro* mesoderm differentiation (Yao et al., 2003). However, some *brachyury* induction and initial mesoderm formation persists in the absence of a functional FGFR1, and a combination of FGFR1/2 variants that lack signal transduction to Erk are still able to produce *brachyury*-expressing mesoderm (Ray et al., 2020; Yamaguchi et al., 1994), suggesting that other upstream processes are involved in Erk pathway activation during mesoderm induction and primitive streak formation. In higher vertebrates, it has yet to be shown whether Erk signaling induces *brachyury* expression through phosphorylation of Ets family transcription factors as in lower vertebrates, although Ets family transcription factors are present in the proper location (the pre-mesodermal epiblast) for this to be the case (Chotteau-Lelievre et al., 2001; Lunn et al., 2007).

For an RTK to initiate signaling it must typically be stimulated by a ligand, and FGFRs are no exception to this rule. Vertebrate genomes tend to contain many different FGFs: the mammalian genome contains 22 and the zebrafish genome contains 31 (Ornitz & Itoh, 2001). Similarly, in *Xenopus*, at least 13 FGF ligands are expressed by stage 40 (Lea, Papalopulu, Amaya, & Dorey, 2009). However, only a few FGFs are expressed during mesodermal specification, and even fewer FGFs appear to be required. In mouse, for example, FGF3, FGF4, FGF5, and FGF8, and FGF17 are all expressed in the primitive streak at the time of gastrulation, but only FGF8 and FGF4 are required for embryonic development to proceed normally (Ciruna & Rossant, 2001; Maruoka et al., 1998; Sun, Meyers, Lewandoski, & Martin, 1999).

As touched on briefly above, mesodermal precursors in lower vertebrates experience a single pulse of Erk signaling as they pass through the margin, which induces *brachyury* in these cells and informs their convergence and extension movements. Conversely, many mesodermal precursors in higher vertebrates experience two pulses of Erk signaling: one as they enter the primitive streak and a second as they migrate away from the streak. These signaling events are triggered by FGFR activation in response to the spatiotemporally controlled release of FGF ligands. Interestingly,

patterns of FGF expression and mechanisms for controlling the duration of FGF release differ among organisms, especially between lower and higher vertebrates.

Lower vertebrates with blastopores, like *Xenopus* and zebrafish, express FGF around the blastopore during mesodermal gastrulation, closely matching the domain of *brachyury* expression and mesoderm induction. In *Xenopus*, FGF3, FGF4, and FGF8 are expressed in a broad strip around the margin (Christen & Slack, 1997; Isaacs et al., 1995; Lombardo et al., 1998) (Fig. 2A). It is unclear exactly how these three FGFs are initially induced, as a complex cocktail of maternally deposited factors and signaling from the vegetal sphere are required for full FGF expression (Fletcher & Harland, 2008; Vonica & Gumbiner, 2002). The way in which FGF

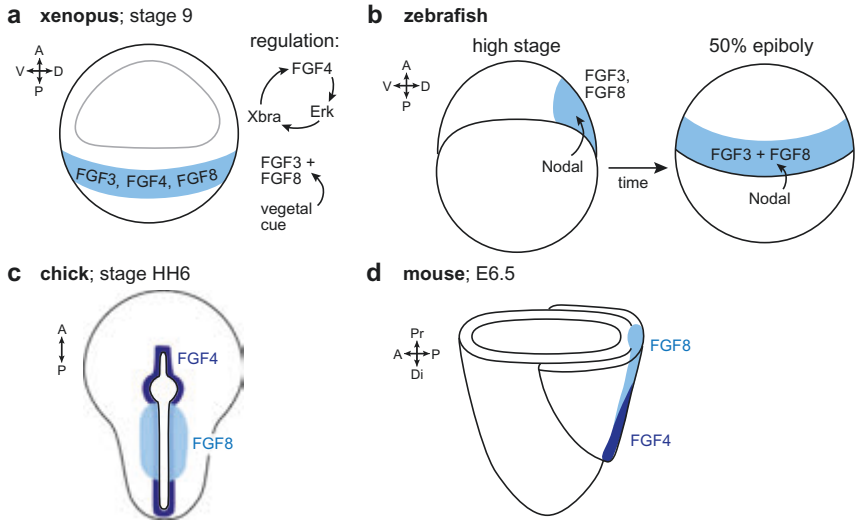


**Fig. 2** FGF expression for mesoderm specification in vertebrates. (A) FGF expression in *Xenopus*. FGF3, FGF4, and FGF8 are expressed in similar patterns at the *Xenopus* margin, marked in light blue. FGF4 induces *Xbra* via Erk activation, and FGF4 expression is sustained by a positive feedback loop with *Xbra*. Meanwhile, FGF8 is sustained by signaling from the vegetal embryo. (B) FGF expression in zebrafish. FGF3 and FGF8 are expressed in similar patterns, first in the dorsal embryo and then at the margin in zebrafish downstream of Nodal signaling. (C) FGF expression in chick. FGF8 (light blue) and FGF4 (dark blue) are expressed in distinct patterns, with FGF8 expressed in the medial primitive streak and FGF4 expressed extending more anteriorly and more posteriorly along the streak. (D) FGF expression in mouse. FGF8 and FGF4 are expressed in distinct patterns, with FGF8 expressed in a proximal-to-distal gradient along the primitive streak and FGF4 in a distal-to-proximal gradient.

expression is sustained is better understood. FGF4 is maintained through a positive feedback loop with *Xbra*: maternally deposited FGF4 helps induce *Xbra*, which induces the zygotic transcription of FGF4. The zygotically induced FGF4 then induces more *Xbra* (Fletcher & Harland, 2008; Isaacs et al., 1994). Despite displaying a similar expression pattern to that of FGF4, FGF8 is sustained independently of prior FGFR activation (Fletcher & Harland, 2008), which suggests that FGF8 is not involved in a positive feedback loop with *Xbra*. Instead, FGF8 appears to be maintained by a separate signal, likely emitted by the vegetal hemisphere (Fig. 2A).

While similar in broad strokes, FGF expression in zebrafish differs in some important ways from that of *Xenopus*. First, FGF expression in the zebrafish embryo is initially asymmetric along the dorsoventral axis (Reifers et al., 1998): FGF is initially expressed only in dorsal regions of the embryo, and then its expression spreads to encompass the rest of the margin (Fürthauer, Reifers, Brand, Thisse, & Thisse, 2001) (Fig. 2B). This early dorsal-ventral asymmetry is important for specifying the axial mesoderm apart from the paraxial mesoderm, with high levels of FGF signaling at dorsal positions promoting axial mesodermal fates (Fürthauer, Van Celst, Thisse, & Thisse, 2004; Maegawa, Varga, & Weinberg, 2006). This is in marked contrast to *Xenopus*, in which FGF signaling promotes paraxial mesoderm specification at the expense of axial mesoderm (Fletcher & Harland, 2008).

A second difference involves the way in which FGF signaling is induced and maintained. In zebrafish, no positive feedback loop exists between FGF and expression of the *brachyury* homolog *ntl*, as exists in *Xenopus* (Lolas, Valenzuela, Tjian, & Liu, 2014). Instead, an orthogonal Nodal signal induces and sustains FGF3 and FGF8 at the margin (Mathieu et al., 2004; Rodaway et al., 1999; van Boxtel et al., 2018) (Fig. 2B). Thus, while FGF expression in *Xenopus* takes the cell's dynamic history of FGFR activation into account through positive feedback between FGF4 and *Xbra*, FGF expression in zebrafish does not, relying instead upon the continued supply of Nodal signaling. It is unknown whether these distinct modes of FGF maintenance are functionally equivalent or confer different downstream signaling properties or functions.

In both *Xenopus* and zebrafish, the different FGF subtypes are expressed in overlapping domains. This is not the case in vertebrates with primitive streaks, like chick and mouse. Both FGF8 and FGF4 are required for proper mesodermal development in these species, and in their absence, mesodermal precursors accumulate in the streak (Hardy et al., 2011; Sun et al., 1999).

In both chick and mouse, FGF4 is dependent upon FGF8 expression, so removing FGF8 in either organism is sufficient to prevent FGF4 expression as well (Hardy et al., 2011; Sun et al., 1999). Yet despite being dependent upon FGF8, FGF4 and FGF8 are expressed in different patterns. In chick, FGF8 is only expressed in the middle streak, while FGF4 is expressed in the middle streak, posterior streak, and anterior streak, including Hensen's node and the head process (Karabagli, Karabagli, Ladher, & Schoenwolf, 2002) (Fig. 2C). In mouse, FGF8 is expressed in a proximal-to-distal gradient along the streak, while FGF4 is expressed in a mirroring distal-to-proximal gradient (Sun et al., 1999) (Fig. 2D). It is still unclear how these non-overlapping domains of FGF4/8 expression are achieved. Two immediate models may be envisioned: a cell-migration model, where FGF4-expressing cells originate from the FGF8-expressing region but have migrated away from that region by the time FGF4 expression is in full swing; or a cell-communication model, where FGF8 expression drives FGF4 expression in a neighboring cell population through diffusion of a signal, perhaps FGF8 itself.

Although higher vertebrates share many common features with lower vertebrates, like FGF induction of *brachyury* in mesodermal precursors, one notable difference is that higher vertebrates express different FGF ligands in distinct, non-overlapping domains. An intriguing hypothesis is that FGF plays different morphogenetic roles in the two vertebrate classes and that these different roles require different ligand expression patterns. While lateral and paraxial mesodermal precursors employ cell intercalation to position themselves in *Xenopus* and zebrafish embryos, directed cell migration is used in chick and mouse (Solnica-Krezel & Sepich, 2012; Sun et al., 1999; Yang et al., 2002). It may be that distinct spatial patterns of FGF8 and FGF4 are required to direct migration, while overlapping FGF patterns are sufficient to program cell intercalation. In addition to evolutionary questions regarding how FGF expression patterns have evolved across species, questions remain regarding whether various FGF subtypes function redundantly. Can mesodermal precursors in chick or mouse still migrate properly if the expression patterns of FGF8 and FGF4 are switched but all else remains the same? We will further explore these questions in later sections of the review.

## 4. Generation and interpretation of transient Erk signaling in vertebrate mesoderm

We have already seen that mesodermal precursors are transiently localized to the margin or primitive streak before they pass through to a new layer

to embark on their journey across the embryo. Prior to passing through the margin/streak, they are transiently exposed to FGF. Once they have passed through and left the margin/streak, this exposure ceases, implying that Erk may only be activated transiently in mesodermal precursors because of their highly mobile nature. This kind of transient Erk signaling during transit between tissues can be inferred from fixed staining of the chick primitive streak, where Erk phosphorylation is present in the FGF-expressing pre-ingression epiblast but absent in the primitive streak itself (Lunn et al., 2007). Likewise, in the mouse primitive streak, Erk signaling is only present in cells actively undergoing EMT, just as they enter the streak (Morgani et al., 2018). Further supporting this view, expression of Erk negative regulators is high in mesodermal precursors across vertebrate species, suggesting that Erk activity decreases rapidly as cells leave regions of continued FGF stimulation, which raises the question of how expression of specific negative regulators alters the dynamics of Erk activity (Branney, Faas, Steane, Pownall, & Isaacs, 2009; Fürthauer et al., 2001; Gómez et al., 2005; Hanafusa, Matsumoto, & Nishida, 2009; Hardy et al., 2011; Lolas et al., 2014; Lunn et al., 2007; Molina, Watkins, & Tsang, 2007; Morley et al., 2009; Sivak et al., 2005; Tsang et al., 2004). In this section, we will explore the origin and role of such negative regulation.

Several classes of negative regulators have been implicated in FGF signal interpretation in the mesoderm. Dual specificity phosphatases (DUSPs), which directly dephosphorylate Erk (Fig. 3A), are one such class of negative regulators (Caunt & Keyse, 2013; Theodosiou & Ashworth, 2002). In *Xenopus*, zebrafish, and mouse, a cytoplasmic DUSP, DUSP6, is induced downstream of *brachyury* in embryonic regions where FGF signaling is active (Gómez et al., 2005; Lolas et al., 2014; Molina et al., 2007; Morley et al., 2009; Tsang et al., 2004). This DUSP appears to primarily affect the amplitude of active Erk signaling in mesodermal tissues (Li, Scott, Hatch, Tian, & Mansour, 2007; Maillet et al., 2008; Tsang et al., 2004; Umbhauer, Marshall, Mason, Old, & Smith, 1995), as opposed to eliminating Erk signaling altogether or modulating Erk signal timing.

In contrast to DUSP6, other DUSPs have been implicated in the complete attenuation of Erk signaling. While Ntl is inducing DUSP6 at the zebrafish margin, Nodal signaling from the yolk induces DUSP4, a nuclear DUSP, in a subset of marginal cells. The presence of DUSP4 prevents Erk signaling from reaching the threshold for mesodermal gene induction, conferring an endodermal fate to cells expressing *DUSP4* (van Boxtel et al., 2018) (Fig. 3B). It is unclear whether a difference in localization, timing, or expression level is what confers a different fate to cells expressing *DUSP6* as

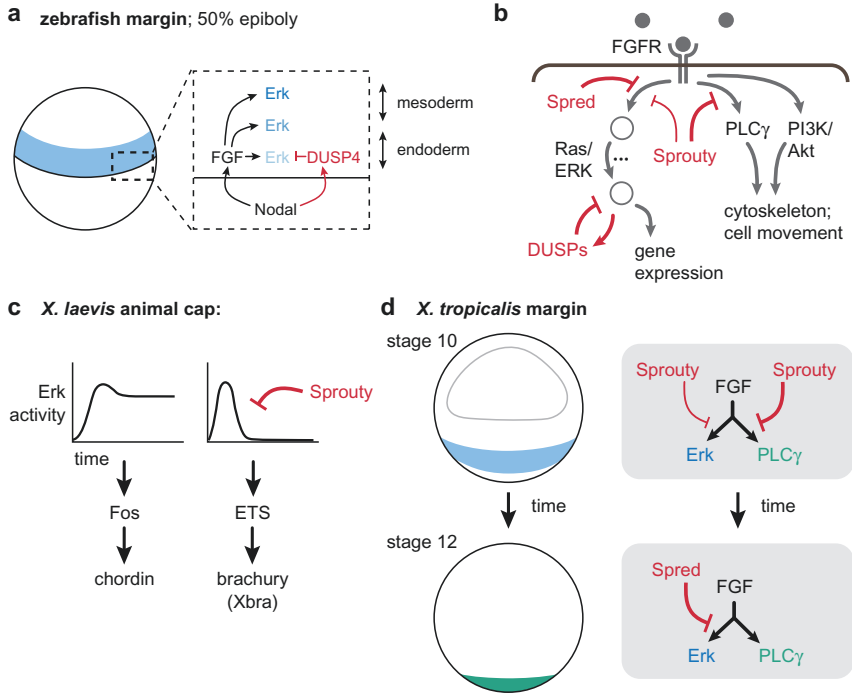


**Fig. 3** Feedback loops on FGFR signaling regulate mesoderm specification. (A) DUSP4 downregulation of Erk for endoderm specification. In zebrafish, Nodal signaling from the yolk induces FGF and DUSP4 expression at the margin. While FGF as a ligand can spread significantly beyond where it is produced at the margin, DUSP4 only negatively regulates Erk signaling where DUSP4 is expressed. Different shades of blue represent the resulting dorsal-to-ventral Erk gradient at the margin. Endoderm is specified cell autonomously where DUSP4 is expressed (low Erk region), while mesoderm (high Erk region) is specified in a non cell-autonomous manner by FGF released from endoderm precursors. (B) Induction of Erk, PI3K, and PLC$\gamma$ signaling by FGFR activation. FGFR is activated by FGF. Erk phosphorylation is initiated downstream of FGFR activation through the Ras/Erk signaling cascade. Phosphorylated Erk induces gene expression of pro-mesodermal genes and DUSPs, negative regulators that de-phosphorylate Erk. Ras/Erk signaling can be inhibited further upstream by negative regulators like Sprouty and Spred. Sprouty also inhibits PLC$\gamma$ signaling. PI3K and PLC$\gamma$ signaling promote cytoskeleton rearrangement and cell movement. The different widths of the Sprouty and Spred negative regulation arrows indicate that they inhibit different pathways to different extents. (C) Injecting *sprouty* into the *Xenopus* animal cap makes Erk signaling transient and prevents expression of genes like chordin. The *Xbra* enhancer contains Ets binding sites, and Erk induces *Xbra* expression via Ets phosphorylation. Meanwhile, the chordin enhancer contains Fos binding sites. FOS acts similarly in *Xenopus* as in mammalian cells, acting as an Erk duration sensor. (D) A switch from Erk to PLC$\gamma$ signaling downstream of Sprouty and Spred in *X. tropicalis*. Sprouty expression blocks PLC$\gamma$ signaling during gastrulation, causing FGF signaling to act through the Erk (blue) pathway. Around stage 12, Sprouty expression ceases and Spred is induced, blocking Erk signaling downstream of FGFR activation and allowing PLC$\gamma$ (green) signaling to proceed.

opposed to those expressing *DUSP4*. Likewise, it has proven difficult to interpret what role individual DUSPs play in mesodermal development, as it is common for multiple DUSPs to be expressed in a cellular population at the same time. In *Xenopus*, for example, expression of *DUSP6* at the margin is accompanied by expression of the nuclear DUSPs *DUSP1* and *DUSP5* (Branney et al., 2009).

A second class of negative regulators, Sprouty-family proteins, function at the FGF receptor level to alter the balance of downstream signaling pathways, including the Ras/Erk pathway (Fig. 3A). While DUSPs have been implicated in controlling the level of Erk activity in cells, members of the Sprouty-family have been implicated in controlling Erk signal timing. The protein *Xsprouty2* is present in *Xenopus* and is itself induced by FGF and Erk signaling, forming a negative feedback loop (Nutt, Dingwell, Holt, & Amaya, 2001). When *Xenopus* animal caps are continuously exposed to FGF ligand, a transient Erk signal is produced. Injecting *Xsprouty2* makes the Erk signal more transient, while injecting a dominant negative version of *Xsprouty2* converts the Erk signal into a sustained one. Taken together, these results suggest that *Xsprouty2* helps dictate the duration of the native Erk signal (Hanafusa et al., 2009) (Fig. 3C).

Differences in Erk signaling dynamics may also contribute to differences in gene expression observed between different regions in the embryo. Active Erk is present in a dorsal-to-ventral gradient at the *Xenopus* margin, with sustained Erk signaling at the dorsal margin and more transient signaling at the ventral margin (Branney et al., 2009; Christen & Slack, 1999; Hanafusa et al., 2009). While *Xbra* is expressed throughout the margin, the gene *chordin* is only expressed dorsally. However, injecting a morpholino against *Xsprouty2* into the ventral margin induces *chordin* ventrally, suggesting that long duration Erk signaling is required for *chordin* induction, in contrast to *Xbra*, which can be induced by long or short duration Erk signals (Hanafusa et al., 2009). This difference in Erk-duration requirement can be explained by differences between the transcription factors that activate these genes. *Xbra* is induced by Ets-family transcription factors, while *chordin* is induced by XFos, a transcription factor whose mammalian homolog c-Fos is known to be stabilized through post-translational modification downstream of sustained Erk signaling (Hanafusa et al., 2009; Kawachi et al., 2003; Murphy & Blenis, 2006; Murphy, Smith, Chen, Fingar, & Blenis, 2002). This suggests a model where only long-duration Erk signaling is sufficient to both induce and stabilize XFos, leading to *chordin* induction.

Another example of Sprouty-family regulation of Erk signal timing is found in *X. tropicalis*. Sproutys are expressed at the *X. tropicalis* margin beginning in stage 10 during epiboly. At stage 12, however, *sprouty* expression is attenuated just as the expression of *spred*, another Sprouty-family member, begins (Sivak et al., 2005). As discussed above, Sprouty is a negative regulator of Erk signaling (Hanafusa et al., 2009). Spred, however, is a stronger repressor of Erk signaling than Sprouty (Sivak et al., 2005). Thus, the switch from *sprouty* so *spred* expression downregulates Erk signaling. Meanwhile, Sproutys are more effective than Spreds at negatively regulating PLC$\gamma$ signaling. Thus, the switch from *sprouty* to *spred* expression marks a change in FGF signal interpretation; prior to the switch, FGFR activation preferentially triggers Erk signaling at the margin, but after the switch, FGFR activation preferentially triggers PLC$\gamma$ signaling (Sivak et al., 2005) (Fig. 3D).

Sproutys and Spreds are expressed during mesodermal development in other species as well. In the chick preingression epiblast, Sprouty*s* and Spreds are expressed downstream of FGF/Erk signaling in the primitive streak (Hardy et al., 2011), and in zebrafish, Sproutys are expressed downstream of FGF signaling at the margin (Fürthauer et al., 2001). It is currently unclear, however, whether Sproutys and Spreds in these scenarios are performing the same function as in *X. tropicalis*—regulating Erk signal timing and controlling the ratio of Erk to PLC$\gamma$ signaling—or a different function entirely.

As we have just seen, Erk signaling can be made transient by negative regulation that targets the Ras/Erk pathway despite the presence of sustained FGFR activation upstream. Regulation of FGFR expression, however, is also commonly employed by mesodermal precursors in modulating the duration of Erk activation. In chick, DUSP6 is conspicuously absent from the primitive streak at HH3, but FGFR1 expression is actively regulated at this time; FGFR1 is not expressed in the primitive streak but is present in the pre-mesodermal epiblast. Erk signaling follows this pattern of FGFR1 expression, with active Erk present in the epiblast layer of the primitive streak but not in the emerging mesoderm (Lunn et al., 2007). Thus, it appears that controlled FGFR1 expression can dictate Erk signaling dynamics without the need for downstream negative regulation. In fact, tight spatiotemporal control of FGFR expression is common among gastrulation-stage embryos, with FGFR regulation present in *Xenopus*, chick, and mouse gastrulas as well as in the *Drosophila* caudal visceral mesoderm (Golub et al., 2000; Lunn et al., 2007; Mandal et al., 2004; Ota et al., 2010; San Martin & Bate, 2001; Yamaguchi et al., 1992).

To recap, Erk signaling dynamics during early mesoderm development are dictated by upstream patterns of FGF and FGFR expression, as well as through the induction of negative regulators. Negative regulation via DUSPs can modulate the level of Erk signaling or suppress signaling altogether. Other forms of negative regulation, like Sproutys and Spreds, can switch the mode through which FGF signaling operates, allowing for the separation of Erk signaling from other FGF-dependent signals in time. Each vertebrate species discussed here exhibits a unique combination of regulatory features during mesoderm induction, suggesting that regulatory features can be mixed and matched through evolution to elicit different cellular behaviors. In the sections that follow, we will explore these distinct cellular behaviors in greater depth.

## 5. The role of Erk signaling in initiating and sustaining *brachyury* expression

One recurring theme in mesoderm specification is the expression of a critical transcription factor, *brachyury*. In the preceding sections we presented evidence that Erk triggers *brachyury* expression, but that Erk activity is only transiently encountered prior to mesoderm precursors leaving the margin or primitive streak. These observations present interesting questions for *brachyury* dynamics. Is *brachyury* expression transient or sustained in mesodermal precursor cells, and how might it be maintained after Erk signaling is no longer present? In this section, we discuss the various Erk-dependent and Erk-independent feedback loops that have evolved for controlling the duration of *brachyury* expression and speculate on distinct functions that different durations of *brachyury* expression may play in mesodermal development.

In zebrafish, mesodermal *brachury* expression is sustained through a series of positive feedback loops. The *brachyury* homolog *no tail* (*ntl*) is expressed first in the dorsal epiblast and then at the margin (Schulte-Merker & Smith, 1995), closely tracking the timing and location of FGF signaling in Fig. 2B. Although Erk signaling induces *ntl* in both regions (Krens, Corredor-Adámez, et al., 2008), different mechanisms are responsible for sustaining it. At the margin, Ntl participates in a feedback loop with various Wnts. Ntl induces expression of Wnt3a and Wnt8 (Fig. 4A), with Wnt8 induced during early gastrulation at the time when the margin is giving rise to the anterior paraxial mesoderm, and Wnt3a induced during late gastrulation at the time when the tailbud, which will give rise to the posterior

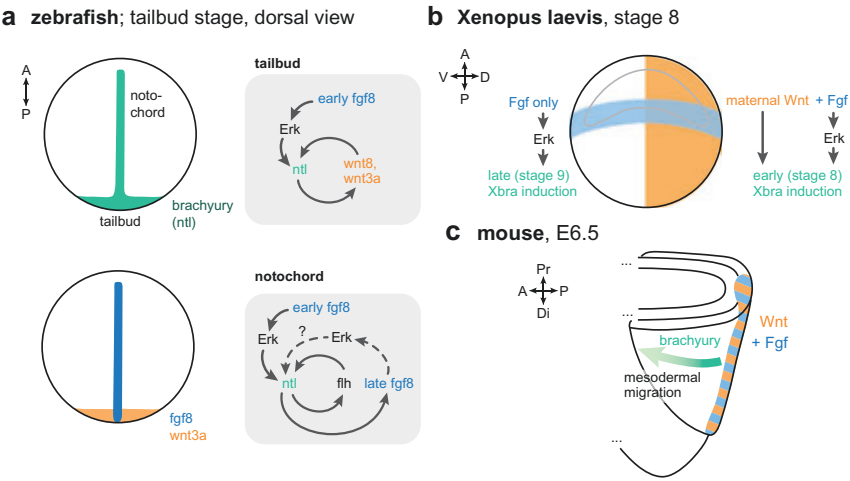


**Fig. 4** Crosstalk between Erk and Wnt signaling regulates brachyury expression. (A) Mechanisms for *brachyury* induction and maintenance. Positive feedback loops maintain *ntl* (green) expression in the zebrafish notochord and future tailbud. In both the notochord and future tailbud region, FGF8 initially induces *ntl* via Erk activation. In the future tailbud region, *ntl* then induces *wnt8* during early gastrulation and *wnt3a* during late gastrulation (orange). Both Wnts maintain *ntl* expression through positive feedback loops. In the notochord, *ntl* induces the notochord-specific gene *flh*, maintaining *ntl* expression in a positive feedback loop. *ntl* also induces FGF8 (blue) in the notochord, which may help sustain *ntl* expression. (B) *Xbra* induced asynchronously in the dorsal vs. ventral *Xenopus* embryo due to Wnt signaling differences. While FGF (blue) is sufficient to induce *Xbra* (green) via Erk signaling in *Xenopus*, *Xbra* can also be induced via canonical Wnt signaling. The presence of maternal Wnt ligands (orange) in the dorsal embryo results in faster *Xbra* induction there (early, stage 8) than in the ventral embryo (late, stage 9). (C) Induction and maintenance of a posterior-to-anterior *brachyury* gradient in mouse. FGF (blue) and Wnt (orange) work synchronously to induce *brachyury* (green) expression. *brachyury* levels drop in mesodermal precursors as they migrate anteriorly away from the streak.

paraxial mesoderm, is beginning to form. Both ligands activate the canonical Wnt/β-catenin signaling pathway (Martin & Kimelman, 2008). In addition to the Ets sites discussed previously, the *ntl* enhancer possesses sites for Tcf, a transcription factor that is activated by Wnt/β-catenin signaling (van Noort & Clevers, 2002). Thus, through Tcf activation, Ntl-induced Wnt signaling goes on to induce more Ntl at the margin as paraxial mesoderm precursors ingress through it (Martin & Kimelman, 2008).

By contrast, *wnt3a* and *wnt8* expression is excluded from the zebrafish dorsal epiblast, which goes on to form axial mesoderm and then the notochord (Fig. 4A). Instead, *ntl* in the future axial mesoderm is induced

by Erk signaling downstream of FGF and then maintained through a positive feedback loop with a notochord-specific transcription factor *floating head* (*flh*). It is noteworthy that FGF8 expression is itself a target of *ntl* in the notochord (Martin & Kimelman, 2008). It is thus tempting to speculate that this FGF8 might itself trigger additional *ntl* expression, similar to earlier embryonic stages and reminiscent of the Xbra/FGF4 feedback loop found in *Xenopus*. A Ntl/FGF8 feedback loop may thus exist that, alongside Flh, helps maintain notochordal *ntl* expression (Martin & Kimelman, 2008).

While Wnt signaling does not appear to play a role in sustaining *Xbra* expression in *Xenopus*, it does help control the timing of *Xbra* induction along the dorsal-ventral axis (Fig. 4B). As discussed previously, *Xbra* is sustained in *Xenopus* through a positive feedback loop involving *FGF4* (Isaacs et al., 1994). However, *Xbra* also possesses Tcf binding sites that make it sensitive to Wnt signaling. While Wnt signaling is not required for induction of *Xbra*, the presence of maternal Wnt signaling on the dorsal side of the embryo speeds up induction of *Xbra* there relative to the ventral embryo, resulting in early gastrulation of the dorsal margin relative to the ventral margin (Vonica & Gumbiner, 2002).

In contrast to the sustained *brachyury* expression observed in the vertebrate notochord and tailbud, *brachyury* expression in the lateral and trunk paraxial is transient, decaying with distance from the primitive streak. Like the *Xenopus* and zebrafish *brachyury* homologs, mouse *brachyury* possesses Tcf binding sites. Wnt signaling synergizes with FGF signaling in mouse to initiate *brachyury* expression (Ciruna & Rossant, 2001; Yamaguchi, Takada, Yoshikawa, Wu, & McMahon, 1999) (Fig. 4C), with *brachyury* expression being highest in the lateral mesoderm precursors, which express both *FGF8* and *wnt3a* (Yamaguchi et al., 1999). *wnt3a* is induced downstream of BMP signaling from the extraembryonic ectoderm and is locally enhanced by FGFR1 activation (Ben-Haim et al., 2006; Ciruna & Rossant, 2001). As lateral mesoderm cells migrate away from the streak, their *brachyury* levels decrease (Morgani et al., 2018), presumably because they are moving away from the source of FGF and Wnt ligands (Fig. 4C). Conversely, axial mesoderm develops under the continued influence of Wnt signaling, which maintains *brachyury* expression in the notochord (Yamaguchi et al., 1999). *wnt3a* maintains its own expression in the primitive streak through a self-sustaining positive feedback loop (Tortelote et al., 2013). While the role of Wnt signaling in *brachyury* maintenance is less clear in chick, superficially, the situation appears to be similar to that in mouse. *brachyury* in the migrating paraxial and lateral mesoderm is dependent on FGF signaling

and attenuates once mesodermal precursors ingress, concomitant with the attenuation of FGFR expression in these cells (Hardy et al., 2011; Kispert et al., 1995; Lunn et al., 2007). In the notochord and Hensen's node, however, *brachyury* expression is sustained independently of FGF signaling (Hardy et al., 2011; Kispert et al., 1995).

To summarize, among vertebrates, *brachyury* is transiently induced in cells of the lateral and trunk paraxial mesoderm but sustained in cells of the axial mesoderm and future tailbud. While Erk signaling is ubiquitously involved in initiating *brachyury* expression among mesodermal subtypes, it is common for *brachyury* regulation to be handed off to other signaling pathways, particularly the Wnt pathway, in regions where *brachyury* expression will become sustained. Whether *brachyury* serves a different function when it is induced transiently as opposed to when it is expressed in a sustained manner remains an open question.

## 6. A role for Erk and a *brachyury* homology in *Drosophila* mesoderm specification

Widening the lens beyond vertebrates reveals dramatic differences in how signaling pathways control mesoderm specification. The *Drosophila* embryo presents a fascinating variation on our theme of spatial FGF patterns, Erk signaling, *brachyury* induction, and mesoderm specification that helps shed light on some shared principles. We will focus here on the signaling and morphogenetic mechanisms employed specifically by a mesodermal precursor population termed the caudal visceral mesoderm (CVM), a tissue that employs FGF signaling to embark on the longest migration in all of *Drosophila* embryogenesis, and the only mesodermal tissue subtype that the Drosophila *brachyury* homolog helps specify.

In *Drosophila*, mesoderm specification and invagination are triggered by the NF-κB homolog Dorsal (Dl) along the ventral surface of the embryo. This region will give rise to several mesodermal populations, including the trunk visceral mesoderm (TVM). Most *Drosophila* mesodermal cells do not express *brachyenteron* (*byn*), the *Drosophila* homolog of *brachyury* (Fig. 5A). Rather, mirroring the vertebrate scenario, *byn* expression is under control of the Erk pathway. At the stage at which the mesoderm is specified, Erk is active at the posterior pole, and *byn* expression takes the form of a ring in a subregion of the pole where Erk is active at a moderate level (Fig. 5A). Thus, while most *byn*-expressing cells do not form mesoderm, the ventral-most population of *byn*-expressing cells receive both a

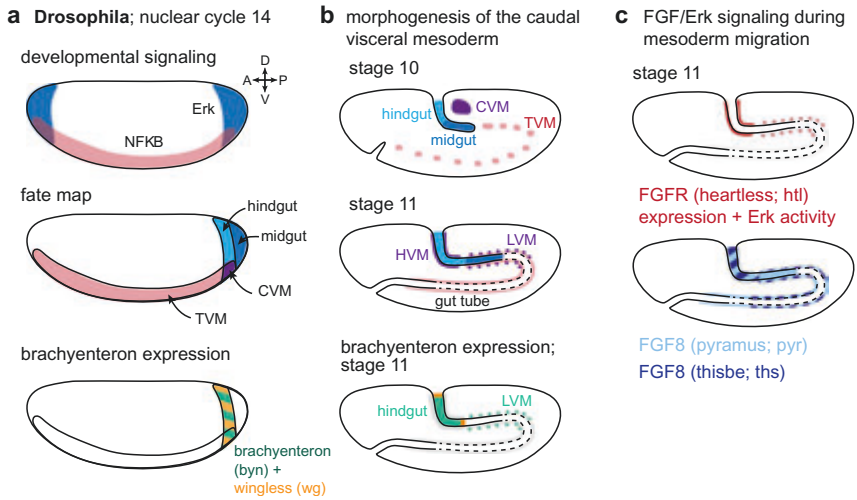


**Fig. 5** Making invertebrate mesoderm: recurrent roles for *byn* and FGFR signaling. (A) NF-κB (pink) and Erk (blue) signaling specify the CVM in *Drosophila*. TVM (pink) is specified in the ventral embryo, where only NF-κB signaling is present. Hindgut (light blue) and midgut (dark blue) are specified in the posterior in regions where only Erk signaling is present. CVM (purple) is induced in the ventral posterior where Erk and NF-kB signaling intersect. *byn* (green) and *wg* (orange) are expressed in both the CVM and hindgut where Erk signaling is low, but not in the midgut where Erk signaling is high. (B) The CVM splits into HVM and LVM. The TVM (pink blobs at stage 10, pink midgut casing at stage 11) and CVM (purple blob) are internalized via ventral furrow invagination. The hindgut (light blue) and midgut (dark blue) are internalized via posterior midgut invagination (stage 10). Germband extension positions the CVM adjacent to the hindgut and midgut, and positions the TVM such that it marks where the gut tube will form (stage 10). At stage 11, the midgut uses the TVM as a guide for gut tube formation. Dotted lines represent regions where the gut tube has yet to form. The TVM forms circular fibers around the gut tube. The LVM (purple dots) migrates over the TVM, forming longitudinal fibers. The HVM (purple hindgut casing) forms circular fibers around the hindgut as the hindgut extends. *byn* (green) expression persists in the hindgut and LVM, but not in the HVM. *wg* (orange) persists at the anterior and posterior ends of the hindgut. (C) FGFR and FGF expression during HVM/LVM development. *htl* (red), an FGFR, is expressed strongly in the HVM and weakly in the LVM. Erk is active in the HVM and LVM where *htl* is expressed. FGFs *pyr* (light blue) and *ths* (dark blue) are expressed throughout the midgut, hindgut, and TVM. Each ligand is expressed in a distinct pattern. Stripes of *ths* and *pyr* represent regions where both ligands are expressed.

mesoderm-inducing NF-κB cue and posteriorizing Erk cue. These cells go on to form the CVM, a tissue that is internalized through ventral furrow invagination along with the rest of the mesoderm (Kusch & Reuter, 1999).

What is the fate of CVM cells? Around Stage 10, CVM cells begin migrating and differentiating into a tissue that gives rise to the circular

muscles that encapsulate the *Drosophila* hindgut, termed the hindgut visceral mesoderm (HVM), as well as the longitudinal muscles that encapsulate the midgut, termed the longitudinal visceral mesoderm (LVM) (Frasch & Sink, 2006; San Martin & Bate, 2001) (Fig. 5B). Although it is unclear what cues the CVM to split into these distinct populations, *byn* in particular is expressed in the LVM at this stage but not in the HVM (Kusch & Reuter, 1999), suggesting that *byn* may serve as a marker for LVM specification.

In contrast to *byn*, FGF signaling is not involved in *Drosophila* mesoderm specification at all, but it does direct the migration of several mesodermal subpopulations, including the HVM and the LVM (Mandal et al., 2004; San Martin & Bate, 2001; Sun, Macabenta, Akos, & Stathopoulos, 2020). This makes *Drosophila* an excellent model system for exploring how FGF signaling dictates mesoderm morphogenesis independently of mesoderm induction. Expression of *heartless* (*htl*), the FGFR expressed in the CVM, coincides with the initiation of Erk activity and the beginning of migration in both the LVM and HVM (Mandal et al., 2004; San Martin & Bate, 2001). However, the mode of migration and level of FGFR expression varies between the two populations. In the HVM, migration is dependent on Connectin (Gould & White, 1992; Nose, Mahajan, & Goodman, 1992), a homophilic cell adhesion molecule that is induced downstream of *htl* and allows the HVM to spread over the hindgut. Conversely, cells in the LVM do not express Connectin and instead employ a different, yet-to-be-identified set of cell-cell adhesion mechanisms to travel in two, neat lines over the TVM via directed migration.

Cells in the HVM express a significantly higher level of *htl* than cells in the LVM (San Martin & Bate, 2001) (Fig. 5C). While the reason for this is still unclear, one possibility is that crosstalk between *byn* and *wingless* (*wg*), a *Drosophila* Wnt ligand, is responsible. *wg* is expressed downstream of Torso signaling in the posterior embryo in a similar ring pattern to that of *byn* (Hoch & Pankratz, 1996; Kispert, Herrmann, Leptin, & Reuter, 1994; Lengyel & Iwaki, 2002; Wu & Lengyel, 1998). *wg* continues to be expressed in the hindgut upon its invagination. Once the hindgut begins to extend, however, *wg* is restricted to the hindgut's anterior and posterior ends (Hoch & Pankratz, 1996; Lengyel & Iwaki, 2002; Wu & Lengyel, 1998). Wg released from the hindgut signals to the HVM, resulting in enhanced expression of *htl* through the induction of *twist* (*twi*) (San Martin & Bate, 2001), a gene associated with mesodermal development in both vertebrates and invertebrates (Castanon & Baylies, 2002).

In contrast to the HVM, the LVM does not express *twi*. Evidence from other organisms suggests this may be due to the LVM-specific presence of *byn*. In mouse, *twi* is not expressed in the notochord, where *brachyury* expression is sustained. *twi* is, however, induced in notochord-derived cells in *brachyury*-knockdown mice, which possess an abnormally loose and mesenchymal morphology (Zhu, Kwan, & Mackem, 2016). It is possible, then, that *brachyury* plays a conserved role in vertebrates and invertebrates, downregulating *twi* expression to prevent loose, mesenchymal morphologies. Unlike in vertebrates, however, where *wnt* and *brachyury* expression often enhance one another through positive feedback loops, the putative interaction between *byn* and *wg* in the *Drosophila* LVM is antagonistic, with *byn* expression appearing to protect cells from the downstream effects of Wnt signaling.

The mode through which FGF expression directs LVM migration resembles that found in avian and mammalian mesodermal migration (Fig. 2C-D). Just as FGF8 and FGF4 activate FGFR in chick and mouse, the FGF ligands Pyramus (Pyr) and Thisbe (Ths) activate Htl in *Drosophila* (Stathopoulos, Tam, Ronshaugen, Frasch, & Levine, 2004). The spatiotemporal expression pattern of each ligand is distinct (Fig. 5C). *pyr* is expressed in the TVM from stage 10 through stage 11 and in the midgut throughout LVM migration (Reim, Hollfelder, Ismat, & Frasch, 2012). Conversely, *ths* is expressed in the TVM from stage 10 through stage 13, with expression in stages 11 and 12 being strongest in the posterior 2/3 of the TVM (Kadam, Ghosh, & Stathopoulos, 2012; Reim et al., 2012). *ths* is never expressed in the midgut (Reim et al., 2012). In a later section, we will discuss potential mechanisms by which FGF may be guiding migration in both cells of the *Drosophila* LVM and chick mesodermal precursors.

In summary, although *byn* expression and FGF signaling are not generally required for mesodermal induction in *Drosophila*, both are required for proper CVM development. Unlike in vertebrates, however, where *brachyury* expression is dependent on FGF activation, *byn* is expressed independently of FGF signaling in *Drosophila*, relying on the activation of Torso, a different RTK, instead. Thus, the roles of *byn* and FGF have been "remixed" in the CVM relative to their roles in the vertebrate mesoderm, making the CVM a useful alternative model for studying *byn* and FGF function in mesodermal specification and morphogenesis. Intriguingly, although *byn* expression and FGF activation are decoupled from one another in *Drosophila*, *byn* is still expressed downstream of Erk signaling at the

embryonic poles, and FGF activation still triggers Erk activity in the migrating LVM (Mandal et al., 2004; San Martin & Bate, 2001). It is not yet known whether the Erk signaling dynamics triggered by Torso differ from those triggered by FGFR. Furthermore, it is not yet known how Erk signaling dynamics translate to *byn* expression dynamics, or how *byn* expression dynamics translate to cell specification and morphogenesis.

## 7. Moving mesoderm: Erk-dependent control of convergence and extension

The final sections of this review will be concerned with the processes by which newly-specified vertebrate mesoderm travels to its final location. While the details of mesodermal precursor travel vary among vertebrates, two of the most common morphogenetic modes employed are (1) convergence and extension and (2) epithelial-to-mesenchymal transition followed by directed cell migration. As in the *Drosophila* CVM, Erk signaling plays a role in orchestrating these movements. In this section, we cover how Erk signaling helps coordinate CE through the regulation of protrusions and cell-cell adhesion.

In both *Xenopus* and zebrafish, convergence and extension (CE) is required for lateral and paraxial mesoderm precursors to reach their final embryonic positions (Solnica-Krezel & Sepich, 2012). In these cells, CE occurs through a crawling mode in which the migrating tissue behaves as a loosely associated mesenchyme (Fig. 6A). Cells initiate movement by inserting protrusions between neighboring cells and establishing weak cell-cell adhesions, which allows cells to crawl between their neighbors. Tissue-level movement then proceeds through cellular rearrangements (Shindo, 2018).

Erk signaling plays a role in regulating both cell protrusion and cell-cell adhesion. Protrusions in the paraxial mesoderm are stabilized through noncanonical Wnt signaling, which is stimulated by the binding of noncanonical Wnt ligands to receptors (Davey & Moens, 2017; Heisenberg et al., 2000; Shindo, 2018; Tada & Smith, 2000; Ulrich et al., 2003; Wallingford et al., 2000). One such ligand, Wnt11, is induced and released in *Xenopus* and zebrafish embryos at the margin downstream of FGFR and Erk signaling (Conlon & Smith, 1999; Ku & Melton, 1993; Makita, Mizuno, Koshida, Kuroiwa, & Takeda, 1998; Saka, Tada, & Smith, 2000; Tada & Smith, 2000). While canonical Wnt signaling proceeds through localization of β-catenin to the nucleus, noncanonical Wnt signaling can initiate signaling

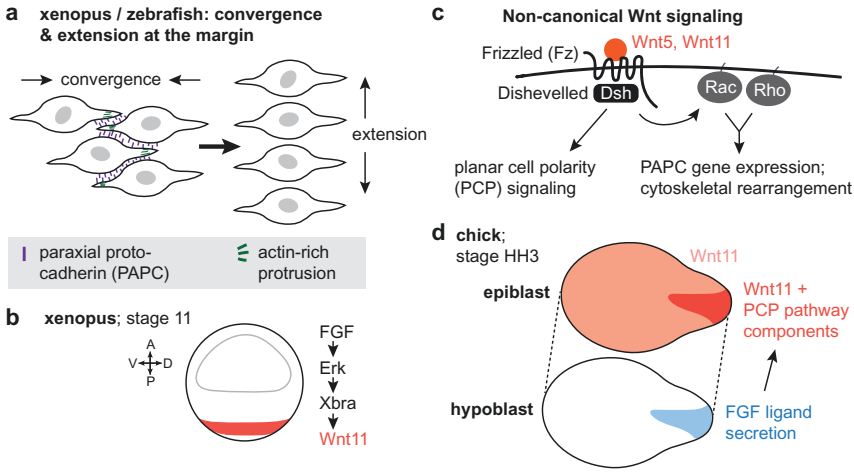


**Fig. 6** Signaling control over cell rearrangements in convergent extension (CE) and primitive streak elongation. (A) Crawling processes coordinated by FGF signaling during CE in *Xenopus* and zebrafish. CE occur through a crawling mode in which cells insert actin-rich protrusions (green) between neighboring cells and establish new adhesions through PAPC (purple), a weak cell-cell adhesion molecule. The result of CE is convergence of cells along one axis and extension along an orthogonal axis. (B) Wnt11 expression (orange) downstream of FGF/Erk/Xbra at the *Xenopus* margin (stage 11). (C) Noncanonical Wnt signaling for cell rearrangement. Noncanonical Wnt ligands (orange), including Wnt5 and Wnt11, bind to Fz receptors. Noncanonical Wnt signaling through Dsh can trigger PCP signaling and activate Rho and Rac. Rho and Rac influence cytoskeletal rearrangement directly and indirectly through PAPC induction. (D) Primitive streak elongation through CE in chick. Localized noncanonical Wnt signaling is required for streak elongation. All cells of the epiblast express Wnt11 (light orange). FGF secreted by the hypoblast (blue) localizes downstream components of the Wnt/PCP pathway (dark orange) to the future streak.

through the planar cell polarity (PCP) pathway and through Rho and Rac activity downstream of Disheveled activation. Together, these pathways coordinate cytoskeletal rearrangements to generate protrusions (Shindo, 2018; Tada & Kai, 2009) (Fig. 6B).

In addition to regulating protrusions, Erk signaling also regulates cell-cell adhesion in the paraxial mesoderm through control of paraxial protocadherin (PAPC) expression. PAPC is a weakly adhesive molecule expressed in the paraxial mesoderm of *Xenopus* and zebrafish throughout gastrulation. It is required for paraxial mesoderm CE and is unique in its ability to promote cell movements while also providing cell-cell adhesion (Kim, Yamamoto, Bouwmeester, Agius, & Robertis, 1998; Yamamoto et al., 1998). This is likely due to PAPC's role in modulating Rho and JNK in a way that is

non-redundant to Wnt/PCP signaling (Medina, Swain, Kuerner, & Steinbeisser, 2004; Unterseher et al., 2004). In zebrafish, PAPC expression is induced in an Erk-dependent manner downstream of the *ntl* target *spadetail* (Yamamoto et al., 1998). In *Xenopus*, noncanonical Wnt signaling induces PAPC, and FGF signaling induces a functional partner of PAPC, potentially through Erk signaling, which aids PAPC both in its adhesion and signaling roles (Chung, Yamamoto, & Ueno, 2007; Schambony & Wedlich, 2007).

Taken together, these observations indicate that Erk signaling helps coordinate CE through induction of Wnt/PCP signaling and control of PAPC activity (Fig. 6B). Although Wnt/PCP signaling and PAPC adhesion are conserved between *Xenopus* and zebrafish, it is interesting that the gene networks connecting Erk signaling to these processes differ, and that paraxial cell movement trajectories are distinct in the two organisms (Solnica-Krezel & Sepich, 2012), which raises the question: Does the timing of Wnt/PCP signaling relative to that of PAPC activation matter for dictating cell trajectories? Furthermore, does the transient Erk pulse that precedes these processes help to coordinate their relative timing?

Although CE is not used for mesodermal morphogenesis in chick and mouse, the chick embryo does employ CE in establishing its primitive streak (Fig. 6C). It is speculated that the chick primitive streak evolved from the ancestral blastopore through the acquisition of an additional cell-intercalation event (Chuai, Serrano-Najere, Serra, Mahadavan, & Weijer, 2021; Voiculescu, Bertocchini, Wolpert, Keller, & Stern, 2007). Like the CE movements initiated at the blastopores of *Xenopus* and zebrafish, lengthening of the primitive streak in chick depends upon FGF control of Wnt/PCP signaling through Wnt11. Unlike in *Xenopus* and zebrafish, *wnt11* is expressed globally throughout the chick epiblast. In order to localize PCP signaling to the future streak, it is thought that the hypoblast, which underlies the epiblast, releases FGF at positions corresponding to the future primitive streak. FGF signaling then induces expression of three PCP pathway components in the epiblast, allowing Wnt11 to locally activate PCP signaling in the region where the streak will form (Chuai et al., 2021; Voiculescu et al., 2007). It is likely that FGF induces these components via Erk signaling (Hardy et al., 2011).

In summary, CE is required for lateral and paraxial mesoderm morphogenesis in anamniotes and is regulated by Erk-dependent gene induction. Although different mechanisms are used for mesodermal gastrulation in amniotes than in anamniotes, CE and the mechanisms underlying it have been coopted by some amniotes for other morphogenetic events, like

primitive streak extension. It remains to be seen whether the transient pulse of Erk signaling experienced by migratory mesodermal precursors in vertebrates coordinates the timing of protrusion and cell-cell adhesion events relative to one another. If this is the case, Erk coordination of mesodermal morphogenesis might prove a useful lens through which the evolution of mesodermal gastrulation in vertebrates can be examined.

## 8. Erk regulation of epithelial-to-mesenchymal transition in the primitive streak

In mouse and chick, mesodermal precursors that travel through the streak undergo an epithelial-to-mesenchymal transition (EMT) before migrating to their final embryonic positions (Nakaya & Sheng, 2009; Nakaya, Sukowati, Wu, & Sheng, 2008; Williams et al., 2012). EMT is a process through which cells organized as a closely adhered, two-dimensional sheet de-adhere from one another and lose their polarity to become migratory, loosely associated cells capable of navigating three-dimensional space. It is characterized by a change in cadherin expression, with mesodermal precursors expressing E-cadherin prior to transition and N-cadherin after (Gheldof & Berx, 2013). FGF signaling has been linked to both processes. In mouse, FGF signaling downregulates E-cadherin levels through the induction of Snail, a transcription factor that is thought to repress E-cadherin expression (Ciruna & Rossant, 2001). In chick, however, FGF signaling at the primitive streak affects neither Snail nor E-cadherin levels. Instead, FGF signaling initiates N-cadherin upregulation through induction of PDGFR at the streak (Hardy et al., 2011). In both cases, cadherin regulation is tied closely to the regulation of other mesoderm-related processes: Erk-dependent *brachyury* expression and canonical Wnt signaling in mouse and PI3K signaling in chick. In this section, we discuss how FGF-control of EMT can either be coupled to or decoupled from mesodermal induction through Erk-dependent mechanisms.

The primitive streak is formed through progressive EMT in mouse (Williams et al., 2012) (Fig. 7A). FGF signaling orchestrates this process through induction of *snail* (Ciruna & Rossant, 2001), an event that is Erk-dependent in the chick epiblast (Hardy et al., 2011) and many human cell lines, and is likely Erk-dependent in the mouse epiblast as well. FGF induction of *snail* subsequently downregulates E-cadherin in the mouse primitive streak (Ciruna & Rossant, 2001). A side effect of this E-cadherin

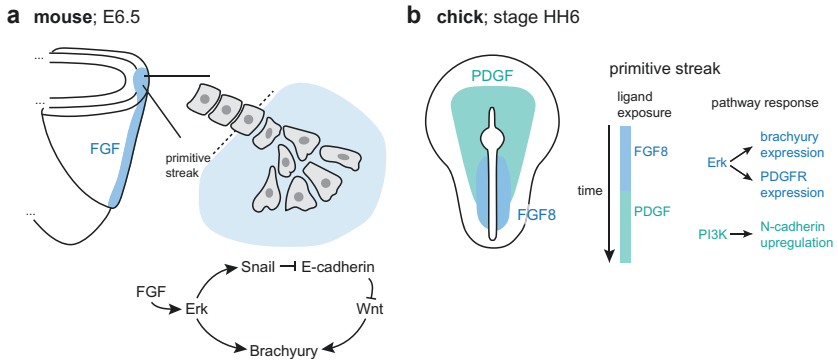


**Fig. 7** FGF signaling for epithelial-to-mesenchymal transition (EMT). (A) FGF signaling downregulates E-cadherin in mouse mesodermal precursors, allowing for ingression via EMT through the primitive streak. FGF is expressed in the primitive streak (blue). Downregulation of E-cadherin, likely through FGF/Erk/Snail, upregulates Wnt signaling. FGF is also thought to induce *brachyury* directly through Erk phosphorylation of Ets family transcription factors. Thus, there are two Erk-dependent arms, one direct and one indirect, through which FGF signaling can upregulate *brachyury*. (B) FGF signaling promotes EMT through PDGFR expression. FGF8 (blue) is expressed by mesodermal precursors along the primitive streak, and PDGF (green) is expressed at the streak and more broadly. Cells from the streak that receive FGF8 activate Erk and express PDGFR. Binding of PDGF to PDGFR triggers PI3K signaling, which upregulates N-cadherin expression, triggering EMT. This gene circuit allows cell fate specification through *brachyury* to be decoupled from PDGF/PI3K-triggered EMT. An arrow representing time indicates the order in which signaling and gene expression events occur. FGF8 triggers the first set of events (blue) and PDGF triggers the second set (green).

downregulation is that canonical Wnt signaling is upregulated (Ciruna & Rossant, 2001); E-cadherin is potently capable of β-catenin sequestration, so high levels of E-cadherin sequester β-catenin at the membrane and limit its ability to induce transcription downstream of Wnt stimulation (Orsulic, Huber, Aberle, Arnold, & Kemler, 1999).

As discussed previously, Erk signaling induces *brachyury* in the mouse primitive streak (Yao et al., 2003), potentially via phosphorylation of Ets family transcription factors. It has been observed that mesodermal cells with high *brachyury* levels migrate further from the primitive streak than those with lower levels (Kispert & Herrmann, 1994; Wilson & Beddington, 1996; Yamaguchi et al., 1999). While this observation could be interpreted to mean that Brachyury promotes migration, a more likely scenario is that E-cadherin downregulation enhances *brachyury* expression through the upregulation of Wnt signaling. Thus, in the mouse migratory mesoderm, mesodermal specification and morphogenesis appear to be linked through

Erk signaling, which induces *brachyury* and downregulates E-cadherin simultaneously. Notably, this genetic architecture also provides two mechanisms through which Erk can modulate *brachyury* expression: a direct pathway and an indirect pathway through E-cadherin and Wnt signaling (Fig. 7A).

By contrast, the upregulation of N-cadherin in chick is decoupled from *brachyury* expression over the course of mesodermal gastrulation (Fig. 7B). Initially, Erk signaling in the primitive streak induces expression of a wide range of mesoderm-related genes including *brachyury*, noncanonical Wnt ligands, and PDGFR (Hardy et al., 2011). PDGFR is activated by PDGF ligands released by the surrounding epiblast. Like FGFR, PDGFR is an RTK capable of initiating both Erk and PI3K signaling. Unlike FGFR, in the context of mesodermal specification and morphogenesis, PDGFR preferentially stimulates the PI3K pathway. This PI3K signaling results in N-cadherin upregulation, which allows cells to migrate away from the streak (Yang, Chrisman, & Weijer, 2008). When FGF, Erk, PDGF, or PI3K signaling is inhibited, migration becomes impossible (Hardy et al., 2011; Yang et al., 2008).

Erk's ability to induce PDGFR expression in the primitive streak allows for the separation of mesoderm specification from mesodermal morphogenesis. While *brachyury* expression is Erk-dependent, N-cadherin upregulation is PI3K-dependent. By passing control of N-cadherin regulation to PDGFR, which only signals through PI3K and is activated by ligands that are expressed in a different spatiotemporal pattern than FGFs, EMT can be initiated in a spatiotemporally distinct manner from mesodermal specification. It remains unclear whether there is a functional significance to separating these events in chick. Similarly, it remains unclear whether coupling these events in mouse serves a function.

## 9. A potential role for Erk signaling in mesodermal directed cell migration

While CE is central to mesodermal morphogenesis in *Xenopus* and zebrafish, in mouse and chick, internalized cells do not undergo CE. Instead, mesodermal precursors that have traveled through the primitive streak migrate to their final positions using directions supplied by FGF signaling (Hardy et al., 2011; Sun et al., 1999; Yang et al., 2002). Mutant experiments in mouse have shown that mesodermal precursors are unable to migrate away from the primitive streak in the absence of FGF4 and

FGF8 (Sun et al., 1999). While it is unknown which pathways FGF signaling activates in migratory mesoderm, imaging experiments in mouse show that Erk activity increases in cells as they migrate away from the streak (Morgani et al., 2018), supporting a model where FGF signaling serves as a directional cue, potentially mediated by Erk.

In chick, tissue transplant experiments more directly support a chemotactic model of mesodermal migration. In wild type embryos FGF8 is released at the primitive streak, and FGF4 is released anterior to the primitive streak downstream of the FGF8 cue (Hardy et al., 2011) (Fig. 2C). Paraxial mesoderm precursors ingress through the anterior primitive streak, moving in an arc first anteriorly and away from the midline and then anteriorly and toward the midline (Fig. 1B). Experiments with FGF4 and FGF8-releasing beads show that middle streak cells are repelled by FGF8 release and attracted to FGF4 release, suggesting that the arc that paraxial mesoderm precursors travel is directed by the localized release of FGF8 and FGF4 (Yang et al., 2002). As in the mouse streak, mesodermal migration in chick also coincides with Erk activation. FGF8 triggers both Erk and PI3K activity, the latter of which induces FGF4 (Hardy et al., 2011). Erk levels decrease as mesodermal cells enter the streak and then increase again as cells emerge to migrate anteriorly (Lunn et al., 2007), presumably in response to FGF4. Thus, this mesodermal population experiences two waves of Erk signaling, the first prior to ingression through the streak and the second during anterior migration.

Although the mechanism by which different FGFs direct mesodermal migration in chick is still unclear, considerable progress has been made toward dissecting migration of the LVM in *Drosophila*, a superficially similar system. Normally, LVM cells migrate along the TVM in two parallel, synchronous lines. In the absence of the FGFR *htl*, however, cells from the two lines merge and lose their synchronicity (Kadam et al., 2012; Reim et al., 2012). They also lose Erk signaling (Mandal et al., 2004). In embryos lacking both FGFs (*pyr* and *ths*), ectopically providing either FGF is sufficient to cause LVM cells to migrate toward the ligand source. Conversely, when *pyr* and *ths* are expressed together ectopically, LVM cells stall (Kadam et al., 2012).

One interpretation of these results is that heterodimeric binding of *pyr* and *ths* to *htl* induces different cellular behaviors than the homodimeric binding of either (Kadam et al., 2012). Other experiments, however, have shown that high ectopic levels of either *pyr* or *ths* alone are sufficient to stall LVM migration, supporting the interpretation that it is the overall level of

FGF ligand present, regardless of identity, that determines whether cells migrate toward the ligand source or stall (Reim et al., 2012). This view supports a permissive model of FGF signaling, in which FGF signaling promotes association among mesodermal cells. In the presence of native levels of *pyr* and *ths*, cells of the LVM associate with one another but are primarily guided by signals emitted from the TVM. In the presence of excess *pyr* and *ths*, however, mesoderm-mesoderm contacts become too strong relative to mesoderm-substrate contacts and motility is hindered (Bae et al., 2012). Because Erk signaling is present in the migrating LVM and lost in the absence of *htl* (Mandal et al., 2004), it is tempting to speculate that Erk mediates this process.

It is unclear whether directed migration in chick is governed by a similar mechanism to that of the *Drosophila* CVM. In any case, intuition garnered from the *Drosophila* model can be used going forward to make testable predictions about directed migration of mesoderm in chick. For example, the chick paraxial mesoderm moves cohesively relative to the individually driven cell movements of the lateral mesoderm (Sweetman, Wagstaff, Cooper, Weijer, & Münsterberg, 2008). Furthermore, cells of the paraxial mesoderm are guided by FGF8 and FGF4 while cells of the lateral mesoderm appear to be guided by FGF8 alone (Yang et al., 2002). A model of migration based on what is seen in the *Drosophila* CVM would suggest that cells of the paraxial mesoderm experience higher levels of signaling in response to being exposed to more FGF than the lateral mesoderm, potentially leading to stronger cell-cell contacts within the paraxial mesoderm than the lateral mesoderm. It also suggests that Erk signaling dynamics in the paraxial mesoderm would differ from those in the lateral mesoderm due to differences in the timing and amount of FGF these two populations receive.

Alternatively, it is possible that a difference in timing between paraxial and lateral mesoderm induction is primarily responsible for the different cell migration patterns. Chick embryos gastrulate in an anterior-to-posterior manner, with the paraxial mesoderm migrating through the streak at an earlier stage than the lateral mesoderm (Frasch & Sink, 2006). As observed in *Xenopus*, where changes in negative regulator expression over time change the way in which FGFR activation is interpreted (Sivak et al., 2005), there may be a switch in regulation that occurs at some point in chick developmental time, causing the lateral mesoderm to interpret FGFR activation differently than the paraxial mesoderm. It is thus possible that Erk signaling mediates migration in one mesodermal population but not the other.

FGF may also be acting through Erk to activate the noncanonical Wnt and PCP pathways in the chick mesoderm. The noncanonical Wnt pathway is active in cells of the lateral mesoderm (Sweetman et al., 2008), and at least one of its ligands is induced by Erk signaling in the posterior streak (Hardy et al., 2011). Meanwhile, canonical Wnt induced in the anterior streak by the nearby neural plate represses noncanonical Wnt signaling in the paraxial mesoderm, resulting in a less migratory phenotype for the paraxial mesoderm than the lateral mesoderm (Sweetman et al., 2008). Thus, Erk signaling may attempt to trigger the noncanonical Wnt pathway in both the paraxial and lateral mesoderm but only succeeds in the lateral mesoderm due to the presence of repressors against noncanonical Wnt activity in the paraxial mesoderm.

In truth, it is likely that all three mechanisms—regulation of cell-cell adhesion, ingression timing, and noncanonical Wnt signaling—are employed to some extent in directing paraxial and lateral mesoderm migration in chick. More work, however, is required to determine exactly how directed migration is being achieved in each streak population and whether Erk signaling has more control over one mechanism than the others.

## 10. Open questions and new tools for Erk control over mesoderm specification and movement

Many open questions remain regarding the role of Erk signaling in the mesoderm. The most central question raised in this review revolves around the role that Erk dynamics play in mesoderm induction and morphogenesis. It has long been known that Erk signaling is involved in mesoderm induction in vertebrates, but in recent years, combined observations of signaling activity and cell movements have brought increased attention to the fact that Erk signaling is transient in mesodermal precursors. This transience poses interesting questions both from the perspective of germ layer induction as well as from that of morphogenetic programming.

In vertebrates, mesodermal and endodermal precursor cells arise from the same progenitor population (Grapin-Botton & Constam, 2007). As seen in the case of endoderm induction in zebrafish, neighboring cells at the margin can take on different germ layer fates depending on whether or not they activate Erk, despite the fact that both populations receive FGF ligand stimulation (van Boxtel et al., 2018). Perhaps the widespread observation of transient Erk activity during mesoderm specification is due to the high pre-existing expression level of negative regulators in mesendodermal

precursors (e.g., DUSP6 in zebrafish) that is required to tune the Erk threshold such that FGF signaling is only able to trigger Erk activity in the presumptive mesoderm and not neighboring endodermal precursor cells. In this scenario, a DUSP6-expressing mesoderm precursor cell would be predicted to rapidly turn off Erk signaling upon exit from the margin, leading to a transient pathway response.

From the perspective of mesodermal morphogenesis, on the other hand, transient Erk signaling can be interpreted as playing a functional role in coordinating diverse morphogenetic processes. In organisms like *Xenopus* and zebrafish where convergence and extension drive morphogenesis, Erk signaling is upstream of processes that polarize cells and enable cell intercalation through protrusion generation and cadherin recycling. It is plausible that these events must occur in a precise order to properly position mesodermal tissues, necessitating an Erk signal that is present for a specific period in developmental time. Similarly, in organisms like chick and mouse where FGF ligands direct mesodermal cell migration, more than one sort of cell movement is required for cells to achieve their final position. A dynamic Erk signal would allow the level of active Erk in cells to rise and fall as cells' modes of migration evolve over developmental time.

## 10.1 New tools: Probing the function of signaling dynamics using optogenetic control

The recent development of optogenetic tools for controlling cell signaling pathways offers opportunities to further test these hypotheses. Optogenetic stimulation is especially powerful for interrogating developmental signaling because both the spatial range and dynamics of a light input can be easily controlled. In contrast, chemical stimuli typically diffuse over a broad range (limiting the ability to apply them to specific tissues) and often bind tightly, limiting dynamic control. Excellent light-based stimuli are now available for studying growth factor signaling through Erk, with optogenetic variants of growth factor receptors (Dine, Gil, Uribe, Brangwynne, & Toettcher, 2018; Grusch et al., 2014; Kim et al., 2014), Ras (Toettcher, Weiner, & Lim, 2013), Raf (Zhang et al., 2014), and MEK (Patel et al., 2019; Zhou, Fan, Li, Shen, & Lin, 2017). Thus, it is now possible to stimulate at virtually every node of the FGFR/Erk signaling cascade, controlling the timing and location of pathway activity, and measuring cells' resulting responses.

A roadmap for these sorts of experiments has also begun to emerge, primarily through studies in *Drosophila*. In the early fly embryo, activation

of the Torso RTK produces a long-lived gradient of Erk at the anterior and posterior poles; later, during nuclear cycle 14, EGFR activation along two lateral stripes produces a 30 min pulse of Erk activity at these positions (Lim et al., 2015). These two RTK patterns are responsible for distinct cell fate outcomes, with Torso driving gut endoderm formation and EGFR producing neurogenic ectoderm. One may thus ask several questions with analogies to mesoderm specification. First, is Erk sufficient to recapitulate all functions downstream of RTK activation? Second, might varying the timing or duration of Erk signaling be sufficient to switch between ectoderm and endoderm cell fates?

Optogenetic control of Ras/Erk signaling proved an ideal technology for addressing these questions. Light-based membrane recruitment of a constitutively active Ras activator SOS ($SOS^{cat}$) (Gureasko et al., 2008) is sufficient to trigger rapid, reversible, and spatially localized Erk activation, producing an optogenetic tool termed the OptoSOS system (Johnson et al., 2017; Toettcher et al., 2013). We found that light-based OptoSOS stimulation could be used to test for a causal role for Erk signaling dynamics *in vivo*, revealing that increasing the duration of Erk signaling could produce neurogenic ectoderm after 30 min of illumination, or gut endoderm after 60 min of light (Johnson & Toettcher, 2019). Additionally, we found that light pattern at the termini can rescue complete genetic loss of the Torso RTK to rescue the entire fly life cycle, demonstrating that localized activation of the Ras/Erk pathway can indeed recapitulate all essential properties of a developmental RTK (Johnson, Djabrayan, Shvartsman, & Toettcher, 2020).

The above studies were successful in relating pathway activity to an eventual phenotypic response, but many details are missing from the intermediate layers of pathways, gene networks, and cell movements that link signaling to cell fate. These intermediate layers have begun to be filled in through a growing number of studies that combine precise light stimuli with quantitative biosensors of cell movement and gene expression (Guglielmi, Barry, Huber, & De Renzis, 2015; Huang, Amourda, Zhang, Tolwinski, & Saunders, 2017; Singh et al., 2021; Viswanathan et al., 2019). These approaches have already proven useful for dissecting how cells interpret incoming Erk signals to choose between mesodermal and endodermal fates in *Drosophila* mesendoderm (McFann et al., 2021). We found that mesendodermal cells are sensitive to two distinct Erk durations: a short duration is sufficient to inhibit mesoderm invagination along the ventral furrow through a dynamic gene network involving the terminal gap gene *hkb*

and the EMT regulator *snail*, whereas longer Erk durations are needed to override the mesoderm fate and drive endoderm-associated morphogenetic movements. With the recent development of perturbation tools for vertebrate model systems (Čapek et al., 2019; LaBelle et al., 2021; Patel et al., 2019; Reade et al., 2017; Sako et al., 2016), as well as a rapidly growing arsenal of live biosensors for signaling and transcription, we will likely soon see similar approaches used to address questions of germ layer specification in other organisms.

## 10.2 New tools: ERK biosensors may reveal additional dynamics during mesoderm differentiation

Currently, Erk activity dynamics in the early mesoderm are inferred from the observation of a fixed region of high Erk activity through which mesoderm cells pass (e.g., the margin in lower vertebrates; the primitive streak in higher vertebrates). However, a key challenge for the field is the lack of direct visualization of early mesodermal single-cell Erk dynamics in any model system. This lack of direct observation raises the possibility that even more complex dynamics might be present but have simply gone unnoticed in the single snapshots afforded by fixed staining.

Supporting this possibility, live-cell Erk activity biosensors are now available in cultured cells and *in vivo* and have revealed that complex Erk dynamics—such as pulses, oscillations, or traveling waves—are widespread across cellular contexts. In general, these biosensors are based on engineering a synthetic, fluorescent Erk substrate whose phosphorylation can be read out in a change in fluorescence intensity or localization. For example, the EKAR family of biosensors takes advantage of a "clamshell" design, in which two fluorophores are appended to the N- and C-terminal ends of the substrate and are brought into proximity by a conformational change induced by Erk phosphorylation (Harvey et al., 2008; Komatsu et al., 2011). FRET-based biosensors have been used to generate transgenic mice in which Erk waves have been visualized in the skin, cochlea, and tumors (Hiratsuka et al., 2015; Ishii, Tateya, Matsuda, & Hirashima, 2021), and in zebrafish embryos with a particular focus on traveling Erk waves in a later mesodermal population, the presomitic mesoderm (Sari et al., 2018; Wong et al., 2018). An alternative to FRET-based designs, the "kinase translocation reporter" (KTR) was developed to encode Erk activity in a change in the synthetic substrate's localization, shifting from nuclear to cytosolic localization upon Erk activation (Regot, Hughey, Bajar, Carrasco, & Covert, 2014). The Erk KTR and

subsequent variants have been used to visualize pulses and waves of Erk activity in mammalian cell lines (Hino et al., 2020; Regot et al., 2014), primary epidermal cells (Goglia et al., 2020), and *in vivo* in *C. elegans* (de la Cova, Townley, Regot, & Greenwald, 2017), the *Drosophila* pupal notum (Moreno, Valon, Levillayer, & Levayer, 2019), zebrafish scales (De Simone et al., 2021), and the early mouse embryo (Pokrass et al., 2020; Simon, Rahman, Raina, Schröter, & Hadjantonakis, 2020). Additionally, recent progress in biosensor multiplexing has made it possible to follow the dynamics of many signaling pathways at once downstream of RTK stimulation (Yang et al., 2021). We thus look forward to a detailed characterization of Erk signaling and other FGF-dependent signals across additional developmental transitions, including early mesodermal specification and migration during gastrulation.

## 10.3 New tools: CRISPR editing to define precise roles for components with overlapping function

A third class of questions relates to the identity and levels of different FGFs in controlling Erk activation across various mesodermal fates and movements. We have repeatedly seen examples of FGFs presented in distinct or overlapping regions, where genetic evidence suggests that they each play essential roles. Are all FGFs molecularly equivalent, but simply differ in their genetic control, thus leading to distinct expression domains and timing? Alternatively, are certain FGFs interpreted in specific ways, perhaps because of their affinity for cognate receptors, other ligands, or the extracellular matrix? Recent evidence from BMP ligands and receptors suggests a complex code, where even simple differences in binding affinity can lead to complex combinatorial logic (Antebi et al., 2017; Su et al., 2020). It remains to be seen whether similar logic holds *in vivo* or can be generalized to other ligand-receptor systems such as FGFs.

Excitingly, it is also now possible to envision addressing these types of questions experimentally. The recent advent of efficient CRISPR knock-in techniques (Gu, Posfai, & Rossant, 2018) opens the door to rapidly "swapping" coding sequences for different ligand or receptor isoforms. We can now envision replacing FGF8 with FGF4 in mouse (or *pyramus* and *thisbe* in *Drosophila*) and assessing phenotypic differences compared to wild-type embryos. Applying similar logic to sequential CRISPR

knock-out of EGF family ligands has already shed light on the molecular requirements for Erk dynamics in epithelial cell lines (Lin et al., 2022), demonstrating that the future is bright for sophisticated ligand deletion and replacement studies.

Another class of questions revolves around how Erk-dependent processes translate to cell movement. While some mesodermal subpopulations move in a loosely associated manner, others move as a cohesive group. It is still unclear what morphogenetic processes are required for each mode of movement. FGF signaling through Erk has been implicated in cell polarization, protrusion generation, cadherin recycling, and induction of cadherins with varying adhesion strengths. An appealing hypothesis is that different modes of movement require different combinations of features, for example, the presence of different adhesion molecules or different levels of dynamism in adhesion molecule recycling. It has yet to be seen whether the level or duration of Erk signaling controls this balance of features, or whether additional, independent signaling cues are interpreted alongside Erk at different times and places to define specific migratory states. For example, both PLC$\gamma$ and PI3K signaling play important roles during vertebrate morphogenesis around the same time that Erk signaling is also active (Böttcher & Niehrs, 2005; Carballada, Yasuo, & Lemaire, 2001; Geary & LaBonne, 2018; Hardy et al., 2011; Montero, Kilian, Chan, Bayliss, & Heisenberg, 2003; Sivak et al., 2005; Yang et al., 2008). Further investigation is needed to understand how specific pathways are triggered downstream of FGFR activation, as well as how these pathways work together to regulate cadherin turnover and other processes required for cytoskeletal rearrangement and morphogenesis.

In conclusion, Erk signaling is involved in mesoderm specification and morphogenesis across species, but the specific roles the pathway plays in gene induction, cell rearrangement, EMT, and migration remain unclear. In particular, the implications of Erk signaling being transient are still poorly understood. With modern tools for signaling and genetic perturbations, it is finally possible to manipulate the timing, level, and location of Erk signaling at nearly every node in the pathway, and to do the same for other signaling pathways downstream of FGFR. In the coming years, we hope to see these tools applied toward uncovering the Erk signaling requirements for the many processes involved in building a proper mesoderm, from genetic regulation for germ layer fate specification to cytoskeletal rearrangement and cadherin turnover.

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