JOURNAL OF EXPERIMENTAL ZOOLOGY (MOL DEV EVOL) 310B:24–40 (2008)

Investigating Divergent Mechanisms of Mesoderm Development in Arthropods: The Expression of *Ph-twist* and *Ph-mef2* in *Parhyale hawaiensis*

ALIVIA L. PRICE[1,2†] AND NIPAM H. PATEL[2,3*]
[1]*Department of Molecular Genetics and Cell Biology, Committee on Developmental Biology, University of Chicago, Chicago, Illinois*
[2]*Department of Molecular Cell Biology, Center for Integrative Genomics, and HHMI, University of California—Berkeley, Berkeley, California*
[3]*Department of Integrative Biology, Center for Integrative Genomics, and HHMI, University of California—Berkeley, Berkeley, California*

ABSTRACT The evolution of mesoderm was important for the development of complex body plans as well as key organ systems. Genetic and molecular studies in the fruitfly, *Drosophila melanogaster*, have provided the majority of information concerning mesoderm development in arthropods. In *Drosophila*, *twist* is necessary for the specification and correct morphogenesis of mesoderm and *myocyte enhancing factor 2* (*mef2*) is involved downstream of *twist* to activate muscle differentiation. In *Drosophila*, mesoderm is defined by positional cues in the blastoderm embryo, while in another arthropod group, the amphipod crustaceans, cell lineage plays a greater role in defining the mesoderm. It is not known how different mechanistic strategies such as positional information vs. cell-lineage-dependent development affect the timing and use of gene networks. Here we describe the development of the mesoderm in a malacostracan crustacean, *Parhyale hawaiensis*, and characterize the expression of *Parhyale twist* and *mef2* orthologues. In *Parhyale*, the mesoderm of the post-mandibular segments arises mainly through the asymmetric division of mesoteloblasts as the germband elongates. *Ph-twist* expression is seen in a subset of segmental mesoderm during germband development, but not during early cleavages when the specific mesodermal cell lineages first arise. *ph-mef2* expression starts after the segmental mesoderm begins to proliferate and persists in developing musculature. While the association of these genes with mesoderm differentiation appears to be conserved across the animal kingdom, the timing of expression and relationship with different mechanisms of mesoderm development may give us greater insight into the ancestral use of these genes during mesoderm differentiation. *J. Exp. Zool. (Mol. Dev. Evol.) 310B:24–40, 2008*.

How to cite this article: Price AL, Patel NH. 2008. Investigating divergent mechanisms of mesoderm development in arthropods: the expression of *Ph-twist* and *Ph-mef2* in *Parhyale hawaiensis*. J. Exp. Zool. (Mol. Dev. Evol.) 310B:24–40.

One of the primary events of cell differentiation during embryogenesis is the formation of the three germ layers: ectoderm, mesoderm, and endoderm. These three populations of cells are principally defined by the derivatives that they will eventually give rise to and their precursors can be defined by their position within the developing embryo during gastrulation. The mesoderm is defined as the ''middle'' germ layer, and gives rise to the musculature, blood, heart, and various organs. In addition to position and fate, molecular work on model and non-model organisms has uncovered a ''genetic identity'' for germ layers, where germ layers from different organisms may be compared based on the expression of

†Present address: The Salk Institute for Biological Studies, La Jolla, CA 92186.
*Correspondence to: N.H. Patel, Department of Integrative Biology, University of California—Berkeley, 3060 VLSB #3140, Berkeley, CA 94720-3140. E-mail: nipam@uclink.berkeley.edu.
Received 21 April 2006; Revised 11 July 2006; Accepted 10 August 2006
Published online 6 December 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21135

germ-layer-specific genes. A group of transcriptions factors associated with mesoderm development across the Metazoa has been emerging including bHLH proteins such as Twist and MyoD, zinc-finger proteins such as Snail, MADS box proteins such as Mef2, GATA factors, and T-box proteins such as Brachyury. While gene expression can be correlated with mesoderm development in various organisms, it is difficult to identify what the original role of these genes may have been during mesoderm development in the ancestral bilaterian. One way of gaining further understanding is to analyze the expression of an identified group of genes in two related organisms with similar bauplans but different ontogenies, or ways of achieving their body plan.

The major arthropod groups include the Insecta, the Crustacea, the Myriapoda, and the Chelicerata. The exact relationship between these groups is contentious, but it is widely accepted that insects and crustaceans are closely related groups within the Arthropoda, to the exclusion of the chelicerates and myriapods. We have developed a new crustacean system, *Parhyale hawaiensis*, as an organism for use in comparative studies with insect model systems, such as *Drosophila* and *Tribolium*, as well as among arthropods more generally (Browne et al., 2005). *Parhyale* is an ideal system for studying development because animals are easy to raise in the laboratory, eggshells and embryo tissues are optically clear allowing for visualization of development in vivo, and a variety of techniques are available for injection, cell-labeling, ablation, germline transformation, and characterization of gene expression. Notably, the cell lineage from early cleavage stages has been determined in *Parhyale* (Gerberding et al., 2002) and this provides a powerful tool that can be combined with gene expression to study germ layer development. In addition, a major benefit of using *Parhyale* to study developmental mechanisms in arthropods is that the early embryogenesis of *Parhyale* differs significantly from other arthropod model systems while later embryonic stages can easily be compared. Comparisons of diverse ontogenies allow one to understand the interplay between life history and development. This is especially important to consider in the group Arthropoda that comprises the most diverse group of animals on the planet and has come to fill countless niches with myriad forms and life histories.

There are several major differences between the early embryonic development of *Parhyale* and *Drosophila*, which is by far the most studied arthropod system. *Parhyale* develops by holoblastic cleavage and adds segments sequentially from anterior to posterior as the germband forms, while in *Drosophila* a syncitial blastoderm stage is followed by the simultaneous subdivision of the embryo into segments. In *Drosophila*, the syncytial blastoderm is created through 13 rounds of nuclear division unaccompanied by cell division. Towards the end of these divisions, the nuclei rise to the surface of the embryo and cell membranes form to give rise to a cellular blastoderm. The mesoderm is specified by the nuclear localization of Dorsal protein in the ventral blastoderm cells; this localization is controlled by earlier morphogen gradients (Roth et al., '89; Jiang et al., '91; Pan et al., '91; Ray et al., '91; Thisse et al., '91). In this way, the mesoderm in *Drosophila* is fated based on positional information within the blastoderm. Positional information also patterns the entire anterior–posterior axis of the embryo such that by the time of gastrulation all of the segments of the body have been established. These extremely fast patterning mechanisms allow *Drosophila* to complete embryogenesis in about 22 hr.

In holoblastic cleaving crustaceans, initial cleavages of the embryo are total and the development of mesoderm can be linked to cell lineage (Hertzler et al., '94; Gerberding et al., 2002; Wolff and Scholtz, 2002; Browne et al., 2005). Precursors of the mesoderm can be identified at a time in development when the embryo is composed of only a handful of cells as opposed to the approximately 6,000 cells present in the *Drosophila* blastoderm. Thus the composition of the embryo in terms of cell numbers, in addition to a syncytial vs. cellular environment, is very different when mesoderm, and indeed germ layers, are specified in *Drosophila* as compared to crustaceans. The ectoderm and mesoderm of the post-mandibular segments in crustaceans are formed sequentially from a posterior zone that elongates from anterior to posterior in a process more similar to short germ development in insects. *Parhyale* embryos hatch in about 10 days (250 hr; Browne et al., 2005). During the evolution of these different ontogenies, the use of molecular pathways in specifying mesoderm must have changed to accommodate heterochronic shifts in the timing of germ layer specification in relation to the cellular composition of the embryo. Analyzing the expression of genes associated with mesoderm development in systems with different styles of early development will provide a broader under-

standing of the ancestral role of these genes in mesoderm development.

twist was first discovered because of its role in mesoderm development in *Drosophila melanogaster* (reviewed in Castanon and Baylies, 2002). Subsequently, Twist family genes, which encode basic helix–loop–helix (bHLH) transcription factors, were shown to be involved in mesoderm development in animals as diverse as cnidarians, annelids, vertebrates, and insects (Sommer and Tautz, '94; Soto et al., '97; Linker et al., 2000; Spring et al., 2000; Tavares et al., 2001; O'Rourke and Tam, 2002; Martindale et al., 2004). The Twist proteins can act as both positive and negative regulators of transcription depending on dimerization partners and the cellular context of their expression (Spicer et al., '96; Harfe et al., '98; Castanon et al., 2001). In *Drosophila*, *twist* is regulated by nuclear localization of the Dorsal protein in the ventral region of the blastoderm fated to become mesoderm (Thisse et al., '88; Roth et al., '89; Jiang et al., '91; Pan et al., '91; Ray et al., '91; Thisse et al., '91). In *twist* mutants, gastrulation morphogenesis is disrupted and mesoderm does not form (Leptin, '91). Later in development, the modulation of levels of *twist* expression is important for subdivision of the mesoderm (Baylies and Bate, '96). Areas that maintain high levels of *twist* expression differentiate into somatic muscle and heart, while mesoderm expressing low levels of *twist* will form visceral mesoderm and fat body. Subsets of mesoderm cells that express high levels of *twist* are maintained through larval stages and are the precursors to the adult muscles. *twist* expression is then down-regulated when cells enter myogenesis.

The Mef2 family of genes plays an important role in the development of all muscle types, including somatic, visceral, and cardiac musculature, in a variety of animals (Lilly et al., '94; Olson et al., '95; Gunthorpe et al., '99; reviewed in Black and Olson, '98). Mef2 genes encode MADS-box transcription factors that act in combination with other factors to activate transcription of proteins including muscle-specific enzymes and structural proteins, as well as other transcription factors (Lin et al., '96; reviewed in Black and Olson, '98). Expression of *myocyte enhancing factor 2* (*mef2*) is downstream of *twist* in *Drosophila* (Cripps et al., '98), while the opposite may be the case in vertebrates (Spicer et al., '96; Corsi et al., 2000) The expression of Twist and Mef2 gene families throughout the Metazoa, in combination with functional studies in *Drosophila* and vertebrates, strongly suggest an ancient role in the development of mesoderm.

In order to use the great amount of information gleaned from *Drosophila* to understand the ancestral mode of mesoderm development in arthropods, we must first appreciate the relationships between the cellular and molecular mechanisms underlying mesoderm development in *Drosophila* and those of other arthropod groups. To further understand the development of mesoderm in crustaceans, we examined cellular and molecular aspects of mesoderm development in an amphipod, *Parhyale hawaiensis*. The cell lineage from the eight-cell stage has been described for *Parhyale* (Gerberding et al., 2002; Browne et al., 2005). In *Parhyale* the mesoderm arises from three cells at the eight-cell stage, and here we provide a more detailed description of mesoderm development including cell-lineage analysis of the segmental mesoderm precursor cells, the mesoteloblasts, and the first divisions of their segmental mesoderm progeny. Furthermore, to investigate the molecular biology of mesoderm development in *Parhyale*, we characterize the expression of *Parhyale* homologs of the genes *twist* and *mef2*. We find that expression of *Parhyale twist* and *mef2* correlates with later mesoderm patterning and development but not during the initial formation of mesoderm.

MATERIALS AND METHODS

Parhyale hawaiensis cultures

Embryos were obtained from a breeding culture of *Parhyale hawaiensis* raised in this laboratory as described (Browne et al., 2005). Following extraction from brood pouches of gravid females, embryos were cultured in artificial seawater sterilized by filtration through a 0.2 μm filter.

Preparation of constructs and cell labeling

Cell lineages were traced in live animals using either rhodamine-conjugated dextrans (TRITC-dextran) or the red fluorescent protein (DsRed.T1) as described in Gerberding et al. (2002). A nuclear localization signal was appended to the 3′ end of the coding sequence for DsRed in pSP-DsRed.T1 by ligation of oligonucleotide linker designed after Kalderon et al. ('84) and Lanford et al. ('86). Capped mRNA for nuclear localized DsRed (DsRed-NLS) was generated using the SP6 Ambion mMessageMachine kit. DsRed proteins can be visualized with rhodamine filter sets 2–4 hr

after injection of mRNA. TRITC-dextran and DsRed proteins are non-toxic and embryos develop to hatching.

Injection and time-lapse microscopy

Specific blastomeres were injected with mRNA at the eight-cell stage to target DsRed-NLS to germ layers (Gerberding et al., 2002). Time-lapse microscopy was performed with a Hamamatsu ORCA-ER camera using Openlab^{TM} 3.1.5 software (Improvision) on a Zeiss Axiovert 200M. The DsRed protein was visualized through either a Zeiss Plan-NeoFLUAR $5\times/0.15$ or Zeiss Plan-NeoFLUAR $10\times/0.3$ objective with light output from a 100-W mercury arc lamp (Zeiss AttoArc2 HBO 100W) through a Zeiss rhodamine filter set. Both ambient and fluorescent frames were captured at 3 or 5 min intervals. Embryos were visualized through a coverslip affixed over a hole in the bottom of a $10\times35\,mm^2$ Petri dish with aquarium sealant. The lid of the Petri dish was covered on the inside with black velvet to eliminate reflection and a seal of petroleum jelly placed around the edge of the Petri dish eliminated evaporation problems. Live embryos were immobilized in a slurry of 0.5% low melt agarose (SeaPlaque GTG agarose, FMC BioProducts) in filtered seawater. Embryos maintained in slurry remain viable through hatching.

PCR and cloning

Total RNA was isolated from a pool of *Parhyale* embryos of varying ages using TRIzol Reagent (Gibco BRL). First-strand cDNA was then synthesized from this pool of RNA using the SuperScript Preamplification System (Gibco BRL).

Degenerate primers for PCR were designed to the conserved regions within the bHLH domain of the Twist family proteins. Degenerate PCR was carried out with semi-nested primers (forward primer 5′-ATG GCN AAY GTN MGN GA-3′; reverse primer 1 5′-CCY TCC ATN CKC CAN AC-3′; reverse primer 2 5′-RCA NAR RAA RTC DAT RTA-3′). Fourteen identical clones were isolated of a 112 bp fragment of the conserved bHLH domain of *twist* by degenerate PCR. This sequence was used to design primers for both 5′- and 3′-RACE to clone the entire coding sequence plus the 5′ and 3′UTRs of *Ph-twist* mRNA (GenBank accession number DQ827719; Frohman, '93).

Degenerate primers for PCR were designed to the conserved MADS box and Mef2 domain of the Mef2 family proteins (Lilly et al., '94). Degenerate PCR was carried out with nested primers (forward primer 5′-ATH TCN CGN ATH CAN GAY GA-3′ and reverse primer 5′-NGT NAG NGA YTC RTG NGG-3′; forward primer 5′-CGN AAY CGN CAR GTN CAN TT-3′ and reverse primer 5′-YTC RTT RTA YTC NGT RTA YTT-3′). A 140 bp fragment of *Ph-mef2* was isolated by degenerate PCR. Additional 3′ and 5′ sequence of the mRNA transcript for *Ph-mef2* was obtained as described above for twist. Three 5′ RACE fragments were obtained that each had the same start and protein coding sequence, but different variants of the 5′UTR (GenBank accession numbers: DQ827720, DQ827721, and DQ827722). The longest 5′ RACE product, which shares 645 bp of identical sequence with the other two fragments, was used to perform in situ hybridization as it will recognize and bind to all three splice variants. 3′RACE was performed to isolate a fragment containing the entire conserved MADS-box and Mef2 domains (GenBank accession number DQ827723; Figure 3.5B).

In situ hybridization

Eggshells and extra-embryonic membranes were removed from embryos by hand dissection with tungsten needles during fixation in 1 part 37% formaldehyde:1 part $10\times$ PBS (18.6 mM NaH_2PO_4; 84.1 mM Na_2HPO_4; 1.75 M NaCl; pH7.4): 8 parts PEM (0.1 mM PIPES; 1 mM $MgSO_4$; 2 mM EGTA). After 30 min, embryos were washed with $1\times$ PBS, dehydrated through a series of 50%, 70%, 90%, 100% methanol in PBS and stored at $-20^{\circ}C$. Riboprobes were made according to (Patel, '96), stored in 300 uL hybridization buffer (50% Formamide; $5\times$ SSC, pH 4.5; 50 ug/ml heparin; 0.25% Tween-20; 1% SDS; 100 ug/ml sonicated salmon sperm DNA), and used at a 1:100 dilution. For hybridization, embryos were rehydrated through a series of 75%, 50%, 25% methanol in Ptw ($1\times$ PBS; 0.1%Tween). Embryos were washed several times in Ptw, post-fixed in 3.7% formaldehyde in Ptw for 20 min, washed in Ptw, permeabilized with detergent solution (1%SDS; 0.5% Tween; 50 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0; 150 mM NaCl), washed in Ptw, and prehybridized in hybridization buffer. Hybridization was performed in hybridization buffer+ denatured probe at $63^{\circ}C$ for 24–36 hr. Following hybridization, embryos were washed in hybridization buffer for 2 hr at $63^{\circ}C$, washed in $2\times$ SSC 30 min at $63^{\circ}C$, then washed at room temperature in PT ($1\times$ PBS; 0.2% Triton X-100), and blocked

in PT+5% new goat serum. Incubation for digoxygenin detection was performed overnight with a 1:3,000 dilution of Boehringer–Mannheim Sheep anti-Dig-AP. The next morning, embryos were washed for 4 hr in PT, reacted in Alkaline Phosphatase Buffer pH 9.5+NBT+BCIP, washed in PT, dehydrated through a methanol series, washed in methanol for several hours, rehydrated, and processed for antibodies or cleared in 50% glycerol+DAPI in PBS followed by 70% glycerol in PBS. Embryos were counterstained with the nuclear dye DAPI to clearly visualize all cells of the developing germbands.

RESULTS

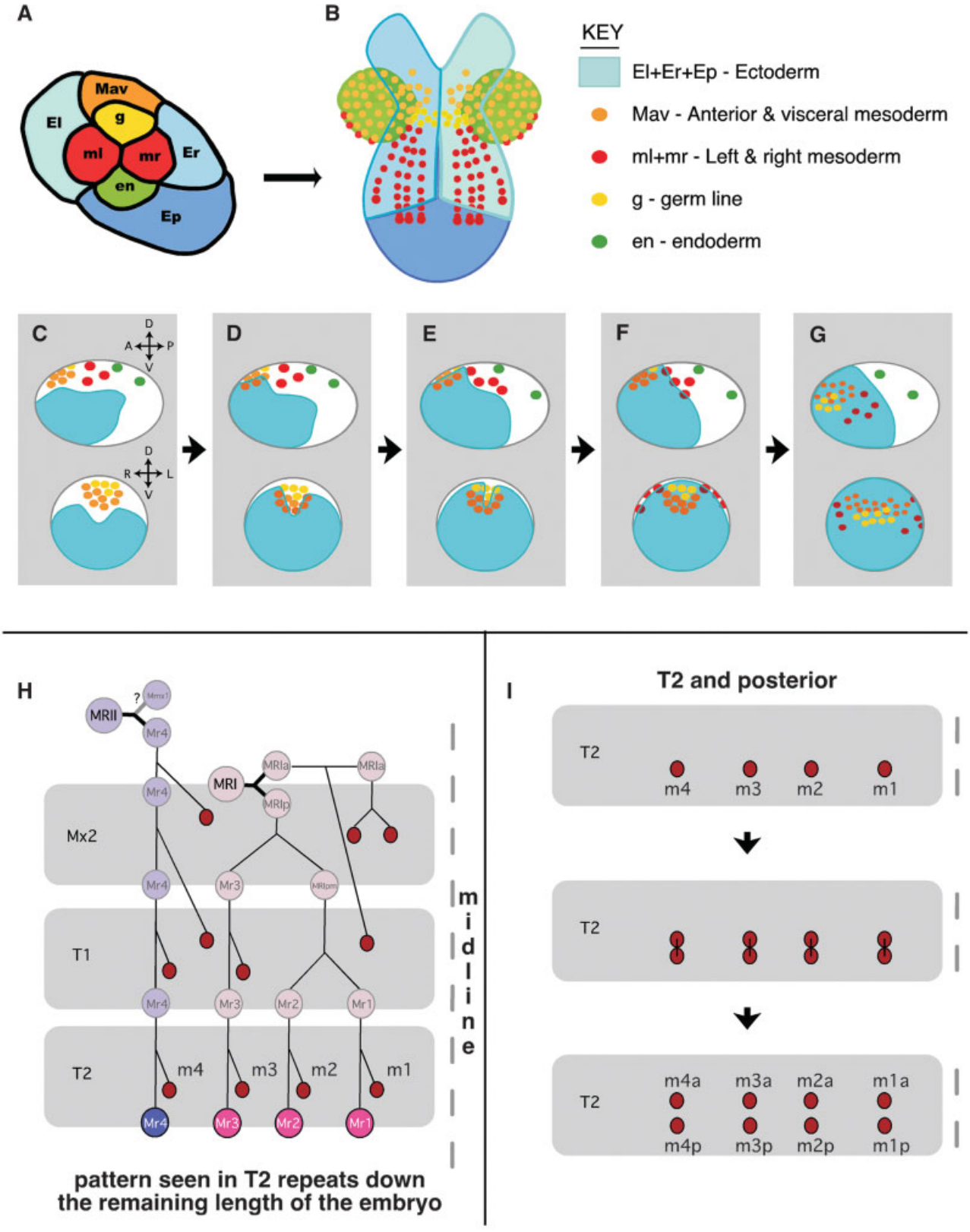
Gastrulation of mesoderm, germ line, and endoderm cell lineages

Germ layer fate in amphipods has been correlated to cell lineage (Gerberding et al., 2002; Wolff and Scholtz, 2002). We have used live flourescent imaging combined with time-lapse microscopy to follow the migrations of cell lineages from the eight-cell stage, stage 4 (st4), to diagram the process of gastrulation in *Parhyale* and to further define the origins of different mesodermal populations (Supplementary movies 1 and 2).

Early cell divisions in *Parhyale* lead to a blastula stage where the cells of the embryo are similar in size and evenly distributed around the embryo. As the *Parhyale* blastula begins to gastrulate, cells shunt their yolk towards the center of the embryo, and become localized to the periphery of the embryo. In a related amphipod, *Orchestia cavimana*, this correlates with the formation of a membrane between the cell body and yolk (Scholtz and Wolff, 2002). Our cell labeling experiments give additional evidence that the segregation of yolk towards the center of the embryo may occur by cell division, through the formation of vitellophages, large yolky cells, in addition to the smaller yolk-free cells at the periphery of the blastula that will form the embryo (Supplementary movie 2). This happens in various cell lineages, including the ''E'' macromere lineages and ''m'' micromere lineages.

At the transition from blastula to gastrula, cells on the surface of the embryo form two distinct cell aggregates (Fig. 1C; Browne et al., 2005). The larger of these aggregations is the coalescence of the ectoderm anlagen, made up of descendants of the ''El'', ''Er'', and ''Ep'' macromeres. Ectoderm cells become increasingly smaller and appear more tightly packed together as a cap of cells condensing towards the prospective anterior of the embryo. The smaller group of cells is termed the ''rosette'' and marks the future anterior pole of the embryo. The rosette is composed of progeny of the ''Mav'' macromere, which will form visceral and anterior segmental mesoderm, and the ''g''

Fig. 1. Cell lineages and gastrulation in *Parhyale*. (**A**) Representation of the eight-cell embryo with blastomeres colored to indicate their eventual germ layer fate. Dorsal view of embryo with anterior up and posterior down. (**B**) Representation of the early germband embryo labeled to show the contribution of blastomere lineages to the different germ layers. Ventral view of embryo with anterior up and dorsal down. (C–G) Panels represent time points during gastrulation. In each panel the top picture shows a lateral view of the embryo: anterior is to the left; posterior to the right; dorsal is up; ventral is down. The bottom picture shows an anterior view of the developing embryo: dorsal is up; ventral is down; left–right axis is indicated in the figure. (**C** and **D**) Formation of the two epithelial condensations: the ectoderm anlagen (blue) and the rosette (multicolored circles). (**E**) The ectoderm anlagen covers the rosette by epiboly and the gastrulation center closes. (**F**) The segmental mesoderm migrates under the lateral edges of the forming germ disc. (**G**) Cells of the rosette and ''m'' progeny migrate towards their appropriate positions within the germ disc. (A–G) Blue: ectoderm anlagen; Orange: anterior and visceral mesoderm; Yellow: germ-line; Red: segmental mesoderm; Green: endoderm. (**H**) The cell lineage of the mesoteloblasts and the formation of mesoblast cells in the second maxillary segment (Mx2) through the second thoracic segment (T2) is shown as a ventral-side view with the embryonic right lateral edge to the left and the midline denoted by the dashed line to the right. The pattern seen in the rest of the germband is the same as illustrated here for T2. The mesoteloblast lineage on the left side of the embryo is the same with mirror image symmetry. Circles represent cells and are named as follows: MRI and MRII are the two progenitors of right segmental mesoderm; Mr4 is the most lateral mesoteloblast; Mmx1 is occasionally produced by cleavage of MRII before assuming Mr4 fate, the question mark and light gray branch symbolize the infrequency of this event; MRIa and MRIp are the first two progeny of MRI, ''a'' and ''p'' denote anterior or posterior position, respectively; Mr3 and MRIpm are progeny of MRIp, Mr3 is the third mesoteloblast and MRIpm is denoted with ''m'' because of its medial position; Mr2 and Mr1 are the progeny of MRIpm, Mr1 is the first, most medial, mesoteloblast and Mr2 is the second mesoteloblast. Gray outlines represent mother cells and black outlines are cells present at the represented stage. Black lines show cell divisions; dark red circles are mesoblasts; Purple circles represent the MRII lineage; pink circles represent the MRI lineage. (**I**) The first division of the mesoblasts within each segment is anterior-to-posterior. The diagram shows three stages in the development of the right segmental mesoderm of T2. Mesoblasts are named based on their cell lineage and position (anterior or posterior) following division of the mother mesoblast cell. For example, a mesoblast progeny of mesoteloblast Mr1 is named m1 and divides in an anterior–posterior direction to give rise to mesoderm cells m1a and m1p.


A
Mav
El
g
ml
mr
Er
en
Ep
B
KEY
El+Er+Ep - Ectoderm
Mav - Anterior & visceral mesoderm
ml+mr - Left & right mesoderm
g - germ line
en - endoderm
C
D
E
F
G
H
MRII
MRI
MRIa
MRIp
Mx2
T1
T2
m4
m3
m2
m1
midline
pattern seen in T2 repeats down
the remaining length of the embryo
I
T2 and posterior
m4a
m3a
m2a
m1a
m4p
m3p
m2p
m1p

micromere, which will produce the germ line. The rosette first appears as a loosely organized band of cells, which gradually becomes tightly organized to finally form a circular rosette-like structure (Fig. 1C). At this time, descendants of ''ml'' and ''mr'', which will form mesoderm, flank the rosette posterior to the left and right, respectively. Finally, ''en'' progeny, the endoderm precursors, appear to spread as large squamous-like cells and occupy the space on the surface of the embryo left by the migration of the other cells (Fig. 1C–G).

Gastrulation progresses by a combination of epiboly and ingression in which the ectoderm anlagen slides up to and over the rosette followed by the ingression of the descendants of ''ml'' and ''mr'' around the lateral edges of the germ disc (Fig. 1C–F). As this takes place, the ectoderm migrates and incorporates more anterior cells into the condensing ectodermal sheet to gradually close over the rosette in a zipper-like movement that progresses from posterior to anterior (Fig. 1D–F; Supplemental movie 1). As the ectoderm enfolds it, the rosette slides under the anterior-most aspect of the ectoderm cap. Once covered by ectoderm, the cells of the rosette actively migrate posteriorly beneath the ectoderm to their final position (Fig. 1G; Supplemental movie 1). The ''g'' progeny of the rosette will migrate past and come to lie just posterior to the ''Mav'' progeny. At the same time ''ml'' and ''mr'' progeny actively migrate to the lateral edges of the ectoderm cap, left and right respectively, and ingress beneath the ectoderm to occupy a position lateral and posterior to the ''g'' and ''Mav'' progeny (Fig. 1G). The ''en'' progeny remain on the dorsal surface of the embryo until the ingression of the mesoderm is completed. Then, ''en'' cells migrate across the dorsal surface of the embryo and under the anterior edge of the germ disc to the paired midgut anlagen (Fig. 1B). The midgut anlagen are composed of two layers: a outer layer of ''Mav'' progeny and a inner layer of ''en'' progeny. These anlagen give rise to the gut and the associated digestive cecae. At the time of the formation of the midgut anlagen, mesoteloblasts that will form the segmental mesoderm are born from ''ml'' and ''mr'' progeny.

Mesodermal lineages, Mav, ml, and mr

The ''Mav'' macromere gives rise to the visceral mesoderm and head mesoderm of the first and second antennae as well as the mandibular segment. Following st4 (eight-cell stage), the ''Mav'' macromere divides more slowly than the ''E'' macromeres. By the beginning of gastrulation at st7, ''Mav'' has undergone only three divisions and these eight descendants cluster with the ''g'' descendants to create the rosette (Fig. 1C; Supplemental movie 1). Just after the ectoderm cap migrates over the rosette, ''Mav'' progeny undergo another round of division, and these 16 cells migrate posterior and ventrally to lie just below the level of the presumptive antennal segments (Fig. 1G). From this position, cells continue to proliferate through several more cell cycles as a single-layered structure that gradually extends further posterior to the maxillary segments. As cells spread posterior and dorsal, they create two distinct circles of cells on either side of the ventral midline and overlapping medially with the forming head lobes. These are the midgut anlagen and are composed of two layers of cells that make them readily visible by bright field microscopy at the beginning of germband extension, st11. The outer layer cells are descendant mainly from the ''Mav'' lineage combined with some descendants of the ''ml'' and ''mr'' lineages, and the inner layer is composed of ''en'' descendants. While the majority of ''Mav'' descendants form the midgut anlagen, a subset of cells migrates anterior under the developing head segments to form the antennal and mandibular mesoderm. Thus, at st11 the progeny of the ''Mav'' macromere contribute to the midgut anlagen and the head segmental mesoderm (Fig. 1B; Supplemental movie 1).

The progeny of the ''ml'' and ''mr'' micromeres from the eight-cell embryo will give rise to the prospective left and right segmental mesoderm, respectively, as well as some visceral mesoderm associated with the midgut. In an eight-cell embryo, the ''ml'' and ''mr'' micromeres are distinguished as the only two micromeres that make direct contact across the midline (Fig. 1A). The ''ml'' and ''mr'' micromeres undergo two–three divisions to give rise to between six and eight progeny prior to gastrulation. At the beginning of gastrulation, the ''ml'' and ''mr'' progeny flank the rosette to the left and right distal from the condensing ectoderm (Fig. 1C). As gastrulation proceeds, the ectoderm anlagen migrate over the rosette, and the prospective left and right mesoderm cells migrate to the anterior-lateral edges of the ectoderm anlagen. At this time, approximately eight descendants of a single mesoderm micromere (a total of 16 cells from both ''ml'' and ''mr'') lie to the left or right of the developing visceral and head mesoderm (Fig. 1E). These cells then

migrate to a position under the ectoderm anlagen posterior to the ''Mav'' progeny. From each side, two of these descendants will give rise to the mesoteloblasts and the mesoderm of the first and second maxillary segments. The other descendants of the ''ml'' and ''mr'' micromeres will contribute to the developing midgut anlagen as visceral mesoderm or remain associated with the yolk as vitellophage cells (Supplemental movie 2). In some rare cases, ''ml'' and ''mr'' progeny also give rise to head segmental mesoderm in a well-defined manner and experimental manipulations reinforce the idea that there are specific patterns of compensation among blastomeres that give rise to mesoderm (Price and Patel, in preparation).

Cell lineage of the mesoteloblasts

From each pool of the ''mr'' and ''ml'' micromere descendants in the germdisc (st9), two progenitor cells (MRI and MRII on the right; MLI and MLII on the left) give rise to the founders of the segmental mesoderm from the second maxillary segment to the end of the abdomen on each side of the embryo (Supplemental movie 3). Cleavages of these two founder cells produce the four mesoteloblasts on each side of the embryo for a total of eight mesoteloblasts (Mr4, Mr3, Mr2, Mr1, Ml1, Ml2, Ml3, Ml4) that generate the segmental mesoderm complements of the thoracic and abdominal segments (red cells in Fig. 1H,I). These cells are called mesoteloblasts because they undergo asymmetric, or teloblastic, division which produces one smaller daughter and one larger mesoteloblast cell. The smaller cell is a mesoblast and will divide to create segmental mesoderm, while the mesoteloblast cell will migrate posteriorly and continue dividing asymmetrically to give rise to more mesoblasts. The eight mesoteloblasts are arranged in a bow-shaped line across the midline of the embryo and are slightly staggered in their position such that the most medial mesoteloblasts are more posterior than the most lateral mesoteloblasts. As the germband elongates, the mesoteloblasts divide asymmetrically beneath the forming ectoderm segments to give rise to one row of eight mesoblast cells per segment. In the following description of the cell lineages of mesoteloblasts, we refer to Fig. 1H that shows a ventral view of the right-side mesoteloblasts, Mr1, Mr2, Mr3, and Mr4. The development of the mesoteloblasts on the left-hand side is the same, i.e., descriptions of Mr4 are the same for Ml4, but in a mirror-image orientation across the midline.

A medial ''m'' micromere descendant, MRI, gives rise to the three medial mesoteloblasts (Mr3, Mr2 & Mr1), two mesoblasts of the second maxillary segment (Mx2) and the most medial mesoblast of the first thoracic segment (T1; Fig. 1H; Supplemental movie 3). The first division of MrI results in the progeny MrIa and MrIp, located anterior and posterior, respectively. MrIa undergoes one teloblast-like division to give rise to a mesoblast that migrates posteriorly to populate the first thoracic segment, and a cell that divides symmetrically to give rise to two mesoblasts that will produce the mesoderm of the second maxillary segment. MrIp divides to form Mr3 and MrIpm. Mr3 then undergoes its first teloblastic division to give rise to a smaller mesoblast daughter cell, whose progeny will subsequently populate the first thoracic segment, and the stem-cell-like mesoteloblast Mr3 which will continue to produce more daughter mesoblasts. MrIpm divides to give rise to Mr2 and Mr1. Thus, when the germband begins extension at S11, the three most medial mesoteloblasts have been formed from a single precursor (Fig. 1H).

A lateral ''m'' descendant , MrII, most often begins dividing asymmetrically directly as the Mr4 mesoteloblast. In a minority of embryos, it divides once to give rise to a mesoblast that produces mesoderm of the first maxillary segment and the Mr4 mesoteloblast (Fig. 1H). The first two divisions of Mr4 are not in alignment with the other mesoteloblasts. The first Mr4 division gives rise to a mesoblast that generates mesoderm of the second maxillary segment, and the second to a mesoblast that populates the first thoracic segment. The third division is in register with the Mr3 teloblast and produces a mesoblast whose descendants also populate the first thoracic segment. All divisions after this give rise to one cell per segment in register with the progeny of the three more medial mesoteloblasts as Mr4 migrates posteriorly under the elongating germband.

For all segments posterior to the first thoracic segment, all eight mesoteloblasts (four on each side of the embryo) divide asymmetrically to produce a row of eight mesoblast cells under each segment (four on each side of the midline, called m1, m2, m3, and m4 with m1 closest to the midline; Fig. 1I). The four mesoteloblasts on each side do not divide synchronously, but the relative timing of divisions displays a regular pattern. The order of their division is established during the first teloblastic division cycle in which they all participate at the level of the second thoracic

segment. As in previous descriptions, the order of timing for the right side is described here but also holds for divisions of the left side mesoteloblasts. Mr2 always divides first and Mr4 always divides last for each segments worth of mesoblasts (9 of 9 embryos). Either Mr1 or Mr3 divide second. The relative timing of division of Mr1 and Mr3 is established during the first coordinated teloblast division cycle and is retained for the duration of their teloblastic divisions such that if Mr1 divides second and Mr3 divides third (3 of 9 embryos; in 6 of 9 embryos Mr3 divides first), they proceed in that order for the remainder of their divisions. The division of Mr4 lags roughly a segment behind the division of the other three mesoteloblasts, which corresponds with the medial to lateral development of the overlying ectoderm germ layer. Mr1, Mr2, and Mr3 are closely associated in a row, with Mr2 contacting both Mr1 and Mr3. In contrast, Mr4 does not contact the other mesoteloblasts and there is no contact of mesoteloblasts across the midline.

The first division of the eight mesoblasts (segmental daughter cells of the mesoteloblasts) in each segment is oriented to generate anterior/posterior pairs of mesoderm daughters, and thus leads to the formation of two rows of mesoderm under each ectoderm segment (Fig. 1I). These first daughter pairs from the mesoblasts on each side of the embryo are called m1a/m1p, m2a/m2p, m3a/m3p, and m4a/m4p (see Fig. 1I). After this first division it is difficult to follow individual cells, but using time-lapse microscopy to visualize later development of the mesoderm, we observe that most mesoblast progeny remain within the segments where they were born (Supplemental movie 2).

Cloning Ph-twist and Ph-mef2

Parhyale homologs of *twist* and *mef2* were isolated from embryonic cDNA by degenerate PCR followed by 3′ and 5′ RACE (Fig. 2). Alignment of the *Ph-twist* homolog shows high conservation of the bHLH domain as well as the WR-motif (Fig. 2A) both of which are important for dimerization and binding to DNA. Splicing variants of the Mef2 family proteins are known to produce different isoforms in *Drosophila* and

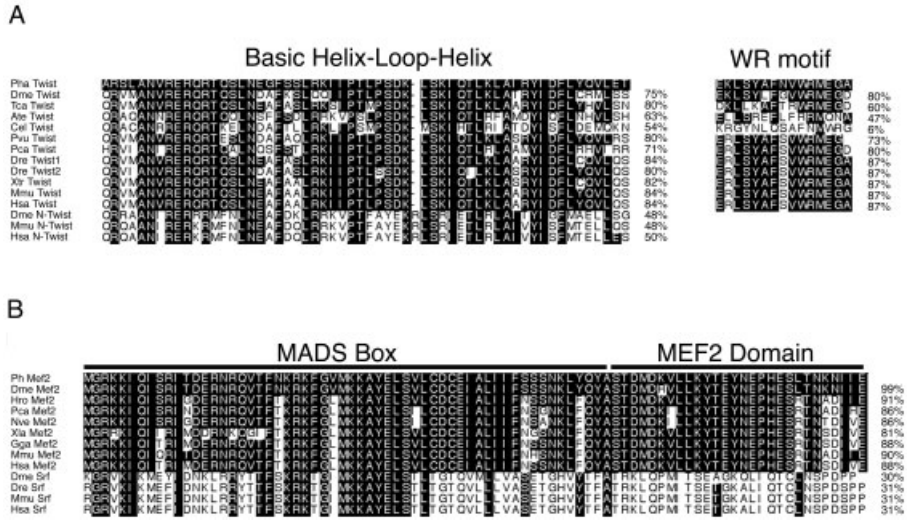


Fig. 2. *Parhyale* Twist and mef2 conserved domains. Amino acids identical to *Parhyale* proteins are indicated by black shading. Percentage identity to *Parhyale* proteins are shown to the right of corresponding sequences. (**A**) Alignment of the basic helix–loop–helix domains of Twist family members and the next most closely related protein, N-Twist, for comparison. (**B**) Alignment of the MADS box and Mef2 domains of Mef2 family members and the next most closely related proteins, the Serum response factors (Srf), for comparison. Sources and accession numbers: PhaTwist (*Parhyale hawaiensis*) DQ827719; DmeTwist (*Drosophila melanogaster*) CAA31024; AteTwist (*Achaearanea tepidariorum*) BAD51393; CeTwist (*Caenorhabditis elegans*) Q11094 O76255; PvuTwist (*Patella vulgata*) AAL15167; BbeTwist (*Branchiostoma belcheri*) AAD10038; DreTwist1 (*Danio rerio*) AAF17606; DreTwist2 (*Danio rerio*) AAF17605; HsaTwist (*Homo sapiens*) CAA71821; MmuTwist (*Mus musculus*) P26687; PcaTwist (*Podocoryne carnea*) CAC12667; XtrTwist (*Xenopus tropicalis*) AAD53290; DmeN-Twist (*D. melanogaster*) AAN04087; MmuN-Twist (*Mus musculus*) AAN04085; HsaN-Twist (*Homo sapiens*) AAN04086; PhaMef2 (*Parhyale hawaiensis*) DQ827723; DmeMef2 (*D. melanogaster*) AAF06817; HroMef2 (*Halocynthia roretzi*) BAA08722; PcaMef2 (*Podocoryne carnea*) CAD21522; NveMef2 (*Nematostella* vectensis) AAR24454; XlaMef2 (*Xenopus laevis*) AAB3304; GgaMef2A (*Gallus gallus*) AJ010072; MmuMef2 (*Mus musculus*) AAB29974; HsaMef2 (*Homo sapiens*) CAA48517; DmeSrf (*D. melanogaster*) AAF47195; DreSrf (*Danio rerio*) AAH50480; MmuSrf (*Mus musculus*) NP_065239; HsaSrf (*Homo sapiens*) P11831.

vertebrates (reviewed in Black and Olson, '98). In *Drosophila*, the function of different isoforms appears redundant and they have the ability to substitute for one another in rescue experiments (Gunthorpe et al., '99). 5′ and 3′ RACE for *Parhyale mef2* revealed that this gene encodes conserved MADS-box and mef2 domains (Fig. 2B), but we found three different 5′ UTR variants. Additional sequence fragments containing the entire conserved MADS-box and Mef2 domains were isolated by 3′ RACE (Fig. 2B). The longest 5′ RACE product shares 645 bp of identical sequence with the other two 5′ fragments and was used to perform in situ hybridization because it should recognize and bind to all three 5′ splice variants.

Expression of Ph-twist

twist is a bHLH transcription factor necessary for gastrulation and mesoderm formation in *Drosophila*. *Ph-twist* expression, however, is not detected during gastrulation in *Parhyale* by in situ hybridization, but is expressed later in developing mesoderm (Fig. 3A–F,M). *Ph-twist* expression is first detected around 75 hr (st13) in a subset of developing mesoderm of the maxillary and first antennal segments (Fig. 3A). Expression in the maxillary and first thoracic segments is broader and more transient than expression of *Ph-twist* in the mesoderm of all other segments. As the expression domain of *Ph-twist* expands in the head, expression in more posterior segmental mesoderm appears in an anterior to posterior progression as segments mature (Fig. 3B–E). Segmental *Ph-twist* is first expressed in each segment in the m2a mesoderm cell following the first division of mesoblast rows (Fig. 1I; Fig. 3M). The m2 mesoblast directly underlies the forming limb field in thoracic and abdominal segments (Fig. 3B,C), and subsequent expression of *Ph-twist* is detected in a larger subset of the mesoderm at the base of, and migrating into, the developing limbs throughout later development (Fig. 3D–F).

Expression of Ph-mef2

The Mef2 family of proteins are MADS box transcription factors necessary for myogenesis, and has also been shown to play a role in brain development in *Drosophila* and vertebrates. *Ph-mef2* expression is first detected in the anterior head lobes at early germband stages (st11-12). This region most likely gives rise to areas of the brain in *Parhyale*. Expression of *Ph-mef2* is next detected in a stripe in the posterior ectoderm of the first maxillary segment (st13; Fig. 3G). As embryogenesis proceeds, bands of expression in the head correlate with developing head segments (Fig. 3H) and then disappear as expression in the mesoderm begins (st17; Fig. 3I). *Ph-mef2* is expressed in a broader subset of the mesoderm than *Ph-twist* throughout development. Eventually, based on the location and arrangement of cells, expression of *Ph-mef2* appears to correlate with all cells that will eventually give rise to muscle (Fig. 3I–L).

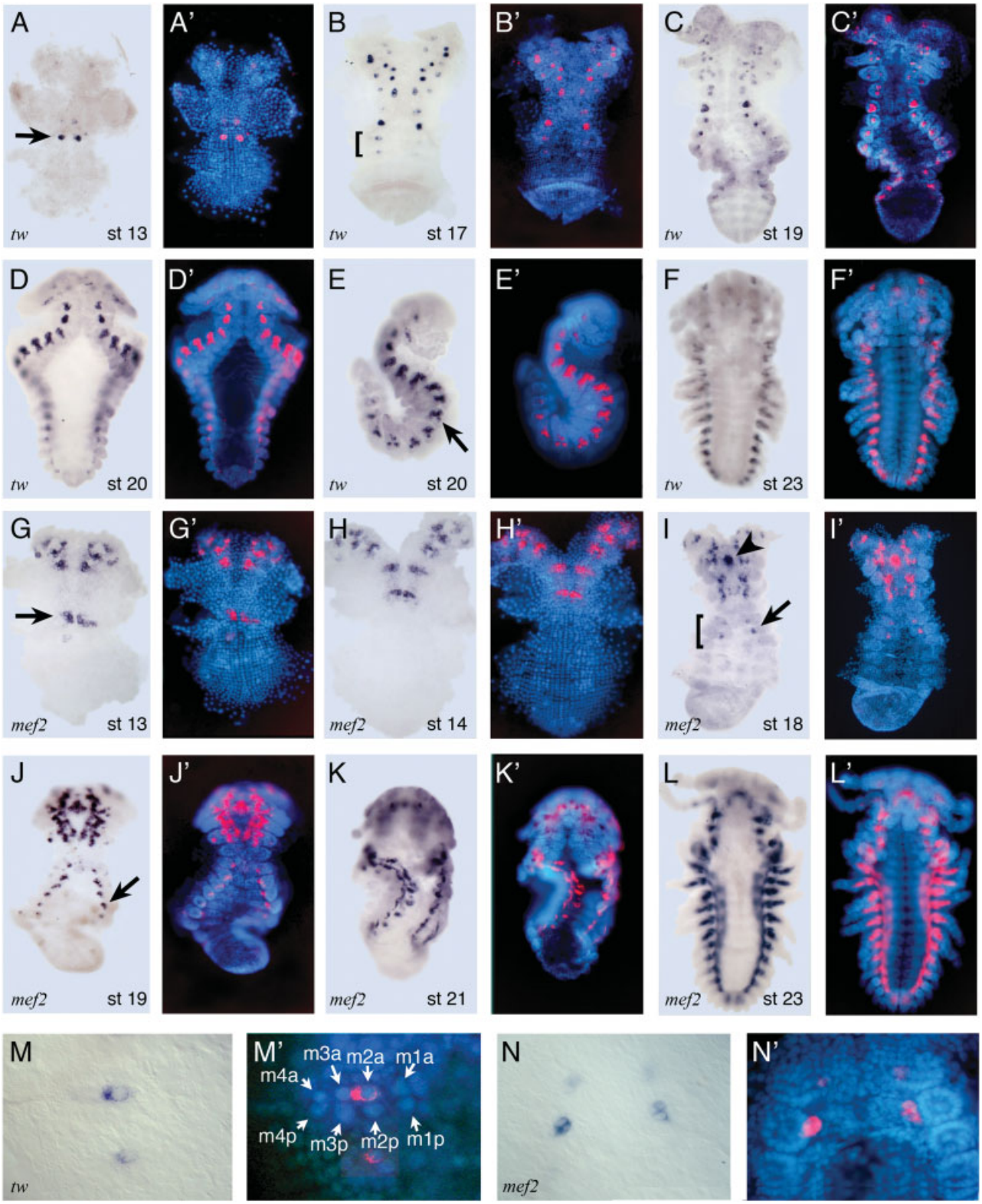
Mesodermal expression of *Ph-mef2* is first seen in the maxillary segments after expression of *Ph-twist*. Like *Ph-twist,* this expression is not typical of expression of *Ph-mef2* in other segments and does not persist. Later, expression persists in developing head mesoderm as bands of cells medial to the base of the developing antennal and mandibular segments and in the forming stomodaeum (Fig. 3I,J).

Expression in thoracic segments begins after the first division of the segmental mesoblasts in both anterior and posterior progeny of the most medial mesoblast (Fig. 3I,J,N). More mesoblast progeny express *Ph-mef2* as they begin to cluster at the base of limbs and form the segmental musculature (Fig. 3J,K). As *Ph-mef2* begins to be expressed in abdominal segments, a subset of mesoderm cells associated with the antennal and mandibular limbs also begin to express *Ph-mef2* (Fig. 3I,J). These cells appear to be forming the musculature for the head segments, and indeed, *Ph-mef2* expression persists in the muscle of the head segments late into development (Fig. 3L). Expression of *Ph-mef2* in thoracic and abdominal segments persists in mesoderm cells as they form bands medial to the developing limbs (Fig. 3J,K). These bands of cells go on to form longitudinal body wall musculature. Expression of *Ph-mef2* in limb mesoderm begins as the bands of body wall musculature become more apparent (Fig. 3K), and is seen in the limbs as muscle development proceeds (Fig. 3L). Expression is also apparent in bands of visceral muscle associated with the gut.

DISCUSSION

Studies on the origin of mesoderm in arthropods are not numerous and the sampling is especially small compared to the diversity that this taxon presents. Part of this is due to difficulty in rearing many arthropods in a laboratory environment, which makes it complicated to procure embryos at very early stages in most species. Another major

issue with arthropod eggs is that many have tough opaque egg shells which obscure the development of early embryos and are often impossible to remove without damaging the embryo. Many arthropods, including, chelicerates, myriapods, insects, and some crustaceans develop an early



syncytium during embryogenesis (Kume and Dan, '68). For those arthropods with early syncytial development, the number of nuclear divisions before the transition from syncytial to cellular blastoderm varies between species and the origins of the mesoderm, if investigated, is really only described after cellularization. It is not known to what extent maternal determinants might play a role in mesoderm specification throughout the arthropods. It is possible that determinants of mesoderm may be retained in transitions between holoblastic and syncitial modes of cleavage during evolution, and that, in extreme situations of heterochrony, elements that are normally at play only after cellularization may be used in a syncytial environment. Thus, it will be of interest to determine what aspects of early patterning of germ layers are retained between syncytial and holoblastic cleaving arthropods.

Among holometabolous insects, initial development of the mesoderm anlage appears to follow the archetypal mode seen in *Drosophila*, such that within a syncytial blastoderm, mesoderm is localized to a ventral stripe of cells that bisects the germ anlage (for discussion see Roth, 2004). The flour beetle, *Tribolium castenaeum*, has been shown to share some aspects of the molecular patterning at early gastrula stages with *Drosophila* including elements of *dorsal* patterning as well as *twist* expression (Sommer and Tautz, '94; Chen et al., 2000). However, later development of mesoderm in *Tribolium*, indeed in most insects, proceeds as posterior segments are added and, thus, these later portions of the mesoderm within the germband differentiate sequentially (Handel et al., 2005). The origin of the mesoderm in the chelicerates and myriapods is less certain. Recent studies have suggested that *twist* expression may be correlating with a stochastic ingression of presumptive mesoderm from all regions of the germ disc in the spider *Achaeranea tepidariorum* (Yamazaki et al., 2005). Nonetheless, early fate maps of mesoderm in chelicerates and myriapods are not available and new labeling techniques will need to be developed to provide fate maps of the mesoderm in these major arthropod groups.

In contrast to mesoderm development in insects, the development of the mesoderm in amphipods is largely tied to the pattern of cell lineage (Gerberding et al., 2002; Scholtz and Wolff, 2002; Wolff and Scholtz, 2002). Lineage-associated development of the mesoderm has also been shown in other crustaceans such as the tiger shrimp *Sicyonia* (Hertzler and Clark, '92; Hertzler et al., '94) and the barnacle *Mitella* (Shiino, 1957). Fate mapping in *Sicyonia* shows an early lineage distinction between mesoderm cells that will populate the naupliar larvae vs. the adult in these indirect developing crustaceans (Hertzler and Clark, '92). In *Parhyale*, the head and trunk mesoderm most often come from different blastomeres suggesting that lineage restriction may play a role, although we observe that it is not uncommon for these lineages to mix in some instances. This deviation in the lineage patterns that give rise to head vs. trunk mesoderm suggests that positional information may also play a role in directing the identity of these lineages during formation of the germ anlage. The naupliar larvae of many crustaceans represent a precocious

Fig. 3. Expression of *Ph-twist* and *Ph-mef2* in *Parhyale*. Embryos were in situ hybridized with an anti-sense riboprobe to show *Ph-twist* (A–F, M) or *Ph-mef2* (G–L, N) mRNA expression and companion photos show DAPI counterstain to visualize germband with a false color overlay of the mRNA expression in red (A′–N′). Stages of development (Browne et al., 2005) are indicated in the lower right-hand corner of bright-field images. (**A**, **A′**) *Ph-twist* is expressed in mesoderm underlying the maxillary (arrow) and first antennal segments at st13 (75 hr); (**B**, **B′**) in increasing numbers of mesoderm cells in the head segments, strongly in segmental mesoderm of the second maxillary segment and is beginning to appear in thoracic segments (brackets) at st17 (87 hr; note the asymmetry in the development of the left and right side which is sometimes observed; hemisegments in brackets are shown at higher magnification in M,M′); (**C**, **C′**) in subsets of segmental mesoderm and beginning in the abdominal segments at st19 (96 hr). (**D**, **D′**) Cells expressing *Ph-twist* make limb musculature at st20 (112 hr). (**E**, **E′**) Lateral view of embryo in (D, D′) to show anterior-to-posterior progression of *Ph-twist* expression in limbs (arrow); (**F**, **F′**) Expression is seen in limb mesoderm at st23 (144 hr). (**G**, **G′**) *Ph-mef2* is expressed in the anterior head lobes and a first maxillary stripe (arrow) at st13 (75 hr). There is faint expression in mesoderm cells underlying maxillary segments. (**H**, **H′**) *Ph-mef2* is expressed in stripes in the head ectoderm at st14 (78 hr); (**I**, **I′**) in the head and thoracic segmental mesoderm (arrow) as well as in the forming stomodeum (arrowhead) at st18 (90 hr; segments in brackets are shown at higher magnification in N,N′); (**J**, **J′**) through the head and thoracic segmental mesoderm (arrow) as well as in the forming stomodeum at st19 (96 hr); (**K**, **K′**) in bands of mesoderm at the base of the limbs and cells forming musculature of the limbs at st21 (120 hr); (**L**, **L′**) in musculature throughout the body at st23 (144 hr). (**M**, **M′**) Close up of panels (B) and (B′) showing *Ph-twist* expression in the mesoderm cell m2a. The mesoderm cells (daughters formed from the first division of the mesoblasts) are labeled according to the nomenclature shown in Figure 1I. (**N**, **N′**) Close up of panels (I) and (I′) showing expression of *Ph-mef2* in the most medial mesoderm cells at the inner base of the developing limbs.

development of the head segments of the animal that will live as a free-swimming larva gathering food while the remaining body segments form. In amphipods, the naupliar larva stage has been completely lost (for discussion see Scholtz, 2000). Still, the early segregation of mesoderm fates into head vs. body populations may be a common mechanism for mesoderm development in crustaceans. In *Parhyale* this trait may have been retained during descent from an indirect developing ancestor and the potential for interchange seen between the two populations may be the result of the obligatory alignment in space and time of these two populations during direct development. However, following the establishment of identity at early germband stages, ablation evidence suggests once mesoderm cells become committed they cannot be replaced by other lineages (Price and Patel, in preparation).

The position of presumptive mesoderm in relation to ectoderm and the anterior–posterior axis prior to gastrulation is very different in *Parhyale* compared to insects: in *Parhyale* the position of presumptive mesoderm is anterior-dorsal, while in holometabolous insects the mesoderm arises in a posterior-ventral area. The tardigrades are a minor group that is considered to branch in a basal position within the arthropods and may retain some ancestral developmental characters (Giribet et al., '96). In the one tardigrade whose cell lineage has been determined, the mesoderm, endoderm, and germline lineages gastrulate at the anterior of the forming germband at the position of the prospective mouth and the mesoderm forms by the posterior elongation and segmentation of mesodermal bands on either side of the body (Hejnol and Schnabel, 2005). Although within tardigrades development may vary quite extensively, these observations suggest that a change in spatial origin of the mesoderm at gastrulation may have occurred several times in the arthropod lineages, although subsequent anterior-to-posterior mesoderm development and segmentation is conserved.

Similarly, within crustaceans, the position of mesoderm precursors is not straightforward. In the amphipod, presumptive mesoderm first arises in a dorsal region of the embryo and it is only through migration under the anterior and lateral edges of the ectodermal anlage that it takes residence under the ectoderm of the germband. In the tiger shrimp, the gastrulation of the mesendoderm and naupliar mesoderm occurs at a posterior position during the formation of the naupliar larvae (Hertzler and Clark, '92). The origin of mesoderm from posterior and ventral regions of the germ anlage has been described for other crustaceans (reviewed in Gerberding, 2004); however, many of these crustaceans develop indirectly through a naupliar larva making it difficult to be confident of the spatial relationship of the germ layers to the final body plan and, for those that do develop directly, most of these lineage maps were generated before the advent of live cell markers that make it possible to trace lineages from the earliest stages of development. The origin of mesoderm from dorsal regions may be a synapomorphy shared by amphipods that may have occurred through the displacement of the mesoderm and endodermal precursors by the larger population of developing ectoderm at early germ stages. Alternatively, the application of new techniques for tracing early cell lineages in other direct developing crustaceans may lead to the development of more refined early fate maps, potentially suggesting that these crustaceans are not so different from amphipods in their organization of germ layers. Additional evidence for the origin of mesoderm in other direct developing crustaceans as well as in myriapods and chelicerates is necessary in order to advance the understanding of how spatial organization of presumptive germ layers in the blastula has evolved in arthropods.

Mesoderm genes in arthropods

We describe the first evidence of conserved gene expression in the developing mesoderm of crustaceans. In *Parhyale,* expression of *Ph-twist* and *Ph-mef2* begins after mesoderm specification and does not correlate with a requirement for these genes during initial specification of mesoderm. However, expression during development of the mesoderm suggests a role in mesoderm patterning and differentiation. Expression of these genes is later than might have been expected by comparison to *Drosophila*, but is consistent with timing of expression in other animals. Additionally, expression of *Ph-mef2* in developing musculature suggests that its role in differentiating musculature may be conserved in *Parhyale*.

In *Drosophila* the expression of *twist* marks the formation of mesoderm from the ventral blastoderm (Fig. 4; Thisse et al., '88; Jiang et al., '91; Leptin, '91) and expression of *mef2* closely follows that of *twist* (Lilly et al., '94; Cripps et al., '98). Expression of *twist* is modulated as its role

changes during segment maturation in *Drosophila* (Fig. 4; Baylies and Bate, '96). This is evident both from the dynamic changes in *twist* expression during mesoderm maturation and the changing roles of Twist in gene regulation dependent upon its cofactor Daughterless (Castanon et al., 2001; reviewed in Castanon and Baylies, 2002). In the flour beetle *Tribolium*, an intermediate germ insect, expression of *twist* at the late blastoderm stage is seen in the presumptive mesoderm in a similar pattern to that in *Drosophila* (Fig. 4; Sommer and Tautz, '94; Handel et al., 2005). Later, during elongation of the germband, the mesoderm that derives from the posterior region begins expression of *twist* only after it has populated formed segments (Fig. 4; Handel et al., 2005). It is possible that the early role of *twist* during gastrulation seen in *Tribolium* and *Drosophila* is not present in *Parhyale* because the mode of mesoderm internalization is not the same and does not require *twist*.

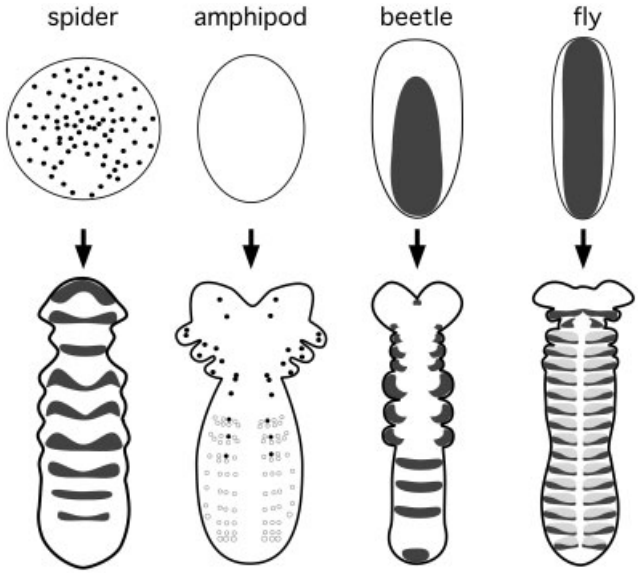


Fig. 4. Comparison of *twist* expression in arthropods. Shown are cartoon representations of *twist* mRNA expression at early developmental stages in the spider, amphipod, beetle, and fly. In the spider, *Achaearanea tepidariorum*, *At-twist* expression begins stochastically in cells, which may be invaginating mesoderm, throughout the germ disc just after migration of the cumulus mesenchymal cells to the periphery marks the establishment of the embryonic axes; later expression of *At-twist* initiates in the mesoderm of segments as they form (cartoon shows early germ disc stage 6 and a germband elongation stage 9 embryos; Yamazaki et al., 2005). In the amphipod crustacean, *Parhyale hawaiensis*, *Ph-twist* expression is not seen before the germband begins to elongate, but begins in a subset of segmental mesoderm as division of mesoblast precursors populate formed segments (cartoon shows beginning gastrula stage 7 and germband elongation stage 17 embryos; in the germband elongation stage cartoon, the cell lineage of the segmental mesoderm from T2 and posterior is shown in gray; for reference see Fig. 1I). In the flour beetle, *Tribolium castaneum*, expression of *Tc-twist* initiates at early blastula stages and is localized to the prospective mesoderm before gastrulation; in later segments formed during elongation of the germband, expression of *Tc-twist* is initiated in each segment as it forms (cartoon shows later blastoderm stage and germband elongation stage embryos; Handel et al., 2005). In the fruitfly, *Drosophila melanogaster*, *twist* expression initiates at blastoderm stages and marks the mesoderm that will invaginate during gastrulation; at later stages *Dm-twist* expression becomes more and more refined within segments as the subtypes of mesoderm differentiate (cartoon shows pregastrula stage 5 and flat-mounted germband extension stage 10 embryos; Baylies and Bate, '96).

Following widespread expression in the mesoderm during gastrulation, high *twist* expression domains are necessary for the differentiation of somatic and heart muscle in *Drosophila* (Borkowski et al., '95; Baylies and Bate, '96). The later role of *twist* in subdividing the mesoderm seen in *Drosophila* may be conserved in *Parhyale*, as *Ph-twist* expression is limited to a subset of cells in the developing mesoderm. Variation in the levels of *Ph-twist* expression in different subsets of mesoderm is not observed in *Parhyale*. However, the mesoderm cells that express *Ph-twist* are associated with the developing limb musculature that also express *Ph-mef2*. *mef2* is directly regulated by *twist* in *Drosophila* (Cripps et al., '98). The timing of expression of these two genes in limb musculature and the early maxillary segments suggests that this direct regulation may be conserved in *Parhyale*. However, Twist can act as a positive or negative regulator of mesodermal genes depending on cofactors as well as the level of expression in the maturing mesoderm in *Drosophila* (Baylies and Bate, '96; Castanon et al., 2001). Instead of promoting myogenesis, Ph-Twist may be keeping mesoderm cells in an undifferentiated state until they reach their final position in the limb-bud, more akin to the negative regulation of myogenesis by Twist seen in vertebrate somites and the muscle pioneer cells of *Drosophila*. As Daughterless is known to act with Twist to negatively regulate mesoderm differentiation in *Drosophila* (Castanon et al., 2001), characterization of a *daughterless* homolog in *Parhyale* would help to predict whether *Ph-twist* is activating or repressing transcription of myogenic genes at different times in development. Co-expression of *Ph-twist* and *daughterless* in the same cells would suggest a repressive role for *Ph-twist* in myogenesis,

whereas lack of expression of *daughterless* in *Ph-twist* positive cells would suggest an instructive role. In addition, the isolation of more markers for mesodermal fates in *Parhyale* will help to determine if the later role of *twist* in subdividing the mesoderm is a conserved trait in arthropods.

Expression of *Ph-mef2* is strongly associated with developing musculature which suggests a conserved role in the differentiation of muscle, as seen with Mef2 family members from organisms throughout the Metazoa. *mef2* is known to be upregulated in *Drosophila* by Twist (Cripps et al., '98). The precocious strong and dynamic expression of both genes in the gnathal regions strongly implies the possibility of regulation of *Ph-mef2* by *Ph-twist* in *Parhyale*. Later expression *Ph-mef2* is not always coincident with expression of *Ph-twist*, suggesting there may also be *Ph-twist* independent regulation of *Ph-mef2*.

The expression patterns of both *Ph-twist* and *Ph-mef2* in *Parhyale* indicate a role for these genes in patterning the mesoderm in crustaceans. Additionally, while the expression patterns do not closely follow the very initial development of the mesoderm that would be predicted from work done in *Drosophila*, this study highlights the types of changes you could expect when asking how conserved genes might function in different developmental contexts, even within a phylum. In this example, *Drosophila* develops in 1 day by an extremely long germ mode facilitated by a syncytial blastoderm while in *Parhyale* embryogenesis proceeds through holoblastic cleavage, germ disc condensation, and the sequential addition of segments, taking 10 days to complete. The observed temporal shift in the expression of these genes between these two animals in relationship to overall development may be a consequence of extremely fast development in *Drosophila* where the dorsal–ventral and anterior–posterior axes are patterned within the first few hours of development as opposed to formation of the germ disc in *Parhyale* which occurs over the course of several days followed by several more days to complete the anterior–posterior body axis. In this case, the expression of *twist* and *mef2* before gastrulation may be precocious in *Drosophila* reflecting the extensive patterning that has already occurred within the sycytial blastoderm, while in *Parhyale* expression of these genes in the mesoderm is seen only as segments are progressively developed over time. Further investigation of these and other genes involved in establishment and patterning of the mesoderm will provide us with a more complete understanding of mesoderm development in arthropods and how this germ layer has evolved throughout the Metazoa.

The evolution of mesoderm allowed for the elaboration of more extensive body plans in multicellular organisms. The arthropods are one of the most specious groups on the planet, encompassing many embryonic and adult forms that have evolved to exist in an enormous variety of niches. Investigations into comparative developmental mechanisms among arthropods will give us great insight into the ways evolution may act upon development during the creation of new life forms and life history strategies. Future studies of mesoderm development in the vast diversity of arthropods will provide greater appreciation for the role that evolutionary changes in developmental mechanisms play in the evolution of diversity on earth.

ACKNOWLEDGMENTS

We thank all members of the Patel group for discussions of this work, William Browne for introducing *Parhyale* to the lab, Matthias Gerberding for his expertise in amphipod embryology and development, Roberta Hannibal for making comments on this manuscript and Greg Davis and Courtney Babbitt for many helpful discussions about arthropod relationships and life histories. NHP is an Investigator of the Howard Hughes Medical Institute.

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