



Evolution of Developmental Control Mechanisms

The functional relationship between ectodermal and mesodermal segmentation in the crustacean, *Parhyale hawaiiensis*Roberta L. Hannibal^{a,1}, Alivia L. Price^{a,b}, Nipam H. Patel^{a,*}^a Departments of Molecular and Cell Biology and Integrative Biology, University of California - Berkeley, CA 94720, USA^b Department of Molecular Genetics and Cell Biology, Committee on Developmental Biology, University of Chicago, IL 60637, USA

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ABSTRACT

In arthropods, annelids and chordates, segmentation of the body axis encompasses both ectodermal and mesodermal derivatives. In vertebrates, trunk mesoderm segments autonomously and induces segmental arrangement of the ectoderm-derived nervous system. In contrast, in the arthropod *Drosophila melanogaster*, the ectoderm segments autonomously and mesoderm segmentation is at least partially dependent on the ectoderm. While segmentation has been proposed to be a feature of the common ancestor of vertebrates and arthropods, considering vertebrates and *Drosophila* alone, it is impossible to conclude whether the ancestral primary segmented tissue was the ectoderm or the mesoderm. Furthermore, much of *Drosophila* segmentation occurs before gastrulation and thus may not accurately represent the mechanisms of segmentation in all arthropods. To better understand the relationship between segmented germ layers in arthropods, we asked whether segmentation is an intrinsic property of the ectoderm and/or the mesoderm in the crustacean *Parhyale hawaiiensis* by ablating either the ectoderm or the mesoderm and then assaying for segmentation in the remaining tissue layer. We found that the ectoderm segments autonomously. However, mesoderm segmentation requires at least a permissive signal from the ectoderm. Although mesodermal stem cells undergo normal rounds of division in the absence of ectoderm, they do not migrate properly in respect to migration direction and distance. In addition, their progeny neither divide nor express the mesoderm segmentation markers *Ph-twist* and *Ph-Even-skipped*. As segmentation is ectoderm-dependent in both *Parhyale* and holometabola insects, we hypothesize that segmentation is primarily a property of the ectoderm in pancrustacea.

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Introduction

Arthropods, annelids and chordates all display segmented trunk regions where segments are formed from derivatives of both the ectoderm and mesoderm. Trunk segmentation has best been studied in the vertebrates and in the arthropod *Drosophila melanogaster*. In vertebrates, trunk mesodermal segments, or somites, are added sequentially from a posterior growth zone of paraxial mesoderm (Dequéant and Pourquié 2008). As patterning genes are still cyclically expressed in paraxial mesoderm explants, mesoderm segmentation is autonomous (Correia and Conlon, 2000; Palmeirim et al., 1998). Ectoderm-derived axons also develop in a segmental pattern in the vertebrate trunk, as each axon runs through the anterior half of each somite (Keynes and Stern, 1984). Unlike somites however, trunk axons do not display intrinsic segmental pattern. (Detwiler, 1934; Keynes and Stern, 1984; Lewis et al., 1981). Thus vertebrate trunk mesoderm is autonomously

patterned and also induces segmental arrangement of the adjacent ectoderm-derived nervous system.

In contrast to the vertebrate trunk, segments form almost simultaneously along the anterior–posterior axis of *Drosophila* (Akam, 1987). During the blastoderm stage, the entire embryo is subdivided into progressively smaller units by a molecular cascade, ultimately giving rise to the characteristic pattern of larval segments. As ectodermal segmental pattern is still apparent in mesoderm-deficient embryos, *Drosophila* ectoderm segments autonomously (Rao et al., 1991).

The question of whether *Drosophila* mesoderm segments autonomously has been difficult to address. Mesoderm forms from the most ventral region of the blastoderm that invaginates during gastrulation. Before gastrulation, stripes of segmental gene expression extend around the entire circumference of the embryo and are present in both the ectoderm and the mesoderm before the mesoderm is in contact with the ectoderm (Akam, 1987). In support of a model in which mesoderm is segmented independent of ectoderm, weak staining of the mesoderm-specific marker *bagpipe* (*bap*) can be seen before gastrulation in a segmental pattern (Azpiazu et al., 1996). However, functional experiments have shown that mesoderm segmentation is at least partially dependent on the ectoderm. The *Drosophila* heart is a segmented dorsal vessel that forms after gastrulation from the dorsal

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most mesoderm (Frasch, 1999). Several secreted molecules are involved in patterning the heart, and these molecules are expressed in both the ectoderm and the mesoderm. However, mesodermal expression of these molecules gradually fades away and experiments using genetic mosaics suggest that the ectodermal contribution is critical for normal patterning and pattern maintenance (Azpiazu et al., 1996, Frasch, 1999).

In summary, in *Drosophila*, ectoderm segments autonomously and provides at least some patterning information to the mesoderm. This also appears to be true for other holometabola insects. Ablation studies in the lacewing *Chrysopa perla* and the potato beetle *Leptinotarsa decelneata* found that the ectoderm can segment autonomously (Bock, 1942; Haget, 1953). These studies also suggest that mesoderm segmentation requires the ectoderm. As only morphological segmentation was examined, however, mesoderm in ectoderm-ablated embryos could still have segmental pattern on a molecular level.

The trait of segmentation in vertebrates and arthropods has been proposed to be inherited from a common, segmented ancestor (Peel and Akam, 2003; Peel, 2004; Pourquié, 2003; Tautz, 1994). Evidence for a segmented common ancestor lies in comparisons of shared molecular mechanisms of segmentation between groups, such as the Notch pathway (Gibb et al., 2010; Peel, 2004; Tautz, 1994), as well as the apparent difficulty of separately evolving such a complex trait as segmentation in different lineages (Telford and Budd, 2003). While these are valid arguments to consider, the distinction between which tissue layer contains the primary segmental information in vertebrates versus *Drosophila* makes it difficult to resolve whether the primary segmented tissue was the ectoderm or the mesoderm in a common segmented ancestor. One way of resolving this issue is to examine other arthropods, since the initial step of *Drosophila* segmentation utilizes a derived mechanism and may not accurately represent segmentation mechanisms in all arthropods (Sander, 1976). In *Drosophila*, much of segmentation takes place before gastrulation. In contrast, in most insects and arthropods, segments are added progressively from anterior to posterior only after gastrulation (Sander, 1976). Therefore, *Drosophila* may not reflect the ancestral mode of arthropod segmentation. It has even been hypothesized that ectoderm segmentation in crustaceans relies on the mesoderm as it does in vertebrates (Budd, 2001; Scholtz, 1990).

There are two lines of evidence supporting the hypothesis that, in arthropods, mesoderm segments autonomously and induces segmentation in the overlying ectoderm. First, comparative studies between fossil and extant arthropods suggest that mesoderm–ectoderm interactions in extant arthropods recapitulate the evolution and development of segmentation in arthropod ancestors. By examining fossil arthropods, Budd (2001) notes that the arthropod ancestor had an unsegmented ectoderm, but a segmented mesoderm. These animals had short, stumpy legs with unsynchronized movement. As arthropod ancestors evolved longer legs, Budd (2001) suggests that it became critical to synchronize leg movement; in order to gain better control of limb movement, ectodermal epidermal plates for muscle attachment evolved. Therefore, Budd (2001) concludes that ectoderm segmentation evolved secondarily and was based upon segmental pattern already established in the mesoderm. Finally, Budd (2001) suggests that the development of ancestral arthropods would reflect this evolutionary scenario, in that the evolutionarily older segmental mesoderm would induce the evolutionarily younger ectoderm to segment. Mesoderm may still segment autonomously and induce segmentation in the ectoderm in extant arthropods.

Second, Scholtz (1990) suggests that conservation of development of the segmental mesoderm amongst Malacostracan crustaceans implies a critical role for the mesoderm during segmentation. Malacostracans utilize a teloblastic mechanism of growth to produce segments where large stem cells, teloblasts, undergo repeated divisions to create smaller segmental founder cells, blast cells. Comparative studies of Malacostracan crustaceans found that eight mesodermal stem

cells, mesoteloblasts, produce segmental trunk mesoderm in all species examined. In contrast, formation of trunk ectoderm segments varies between species. Some species utilize ectodermal stem cells, ectoteloblasts, while others do not. Moreover, the number of ectoteloblasts varies between species. Conservation of mesoderm segmentation is consistent with a critical developmental function, such as providing an instructive signal to the segmental ectoderm.

To test the hypothesis that the mesoderm segments autonomously and induces the ectoderm to segment, we investigated the functional relationship between the segmental ectoderm and mesoderm in the Malacostracan crustacean *Parhyale hawaiiensis*. While Malacostracans utilize a teloblastic mechanism of growth to produce segments that has not yet been found outside this group, subsequent segmentation steps are similar to other, non-Malacostracan crustaceans (Davis et al., 2005). In addition, *Parhyale* is a particularly excellent model system for arthropod segmentation because they are easily raised in the laboratory, have a short generation time, and are amenable to various experimental manipulations (Browne et al., 2005; Liubicich et al., 2009; Pavlopoulos et al., 2009; Price and Patel, 2008; Vargas-Vila et al., 2010). Molecular markers of the segmental ectoderm and mesoderm have been identified in *Parhyale*. The transcription factor Engrailed (En) provides a visual marker for ectoderm segmentation, as it is expressed in the anterior row of cells of each parasegment (Browne et al., 2005; Martinez-Arias and Lawrence, 1985; Patel et al., 1989). *Ph-twist* (*Ph-twi*; Price and Patel, 2008) and *Ph-Even-skipped* (*Ph-Eve*; Vargas-Vila et al., 2010) are the earliest known molecular markers of segmentation in the mesoderm, and continue to be expressed in a subset of the segmental mesoderm throughout later development. In addition, *Parhyale* embryos have early stereotypical cleavage patterns that allow targeting of reagents to each segmental germ layer (Fig. 1; Gerberding et al., 2002).

To investigate interactions between the segmental ectoderm and mesoderm in *Parhyale*, we first observed how the formation of one segment's worth of ectoderm and mesoderm is coordinated during normal development. Then, we asked whether segmentation is an intrinsic property of one or both germ layers by ablating one layer and assaying for segmentation in the other. Contrary to the hypothesis that the mesoderm drives segmentation in arthropods, we find that, in *Parhyale*, the ectoderm both segments autonomously and supplies at least a permissive signal for mesoderm segmentation.

Materials and methods

Injection, photoablation and timelapse microscopy

P. hawaiiensis rearing and staging were performed as described in Browne et al., 2005. To follow lineages, specific blastomeres were injected following Gerberding et al., 2002 and Rehm et al., 2009 with approximately 50 picoliters of 5 mg/mL fluorescein isothiocyanate (FITC) covalently linked to dextran (250,000 MW; Sigma), or capped mRNA (SP6 and T7 Ambion mMessageMachine kits) encoding a nuclear localized version of either DsRed red fluorescent protein (called DsRed-NLS; Price and Patel, 2008) or a tandem version of Tomato red fluorescent protein (called tdTomato-NLS). Since both DsRed-NLS and tdTomato-NLS produce similar red, nuclear-localized, fluorescence, both are referred to as “DsRed-NLS”. While higher amounts of FITC-dextran are toxic to cells when exposed to UV light (see below), the amount of FITC-dextran used as a lineage tracer (approximately 50 μ l of 5 mg/mL) is non-toxic to cells, even when combined with UV light (Gerberding et al., 2002).

Photoablation of targeted cells were performed as described in Price et al., 2010. First, approximately 100 μ l of 25–50 mg/ml FITC-dextran was injected into the appropriate cell(s) at the eight-cell stage (Stage 4; 8 h). Second, to ablate these cells or their lineages at later stages, the entire embryo was exposed to the fluorescein excitation wavelength ($\lambda = 488$ nm) for 10–20 min. Cell death following

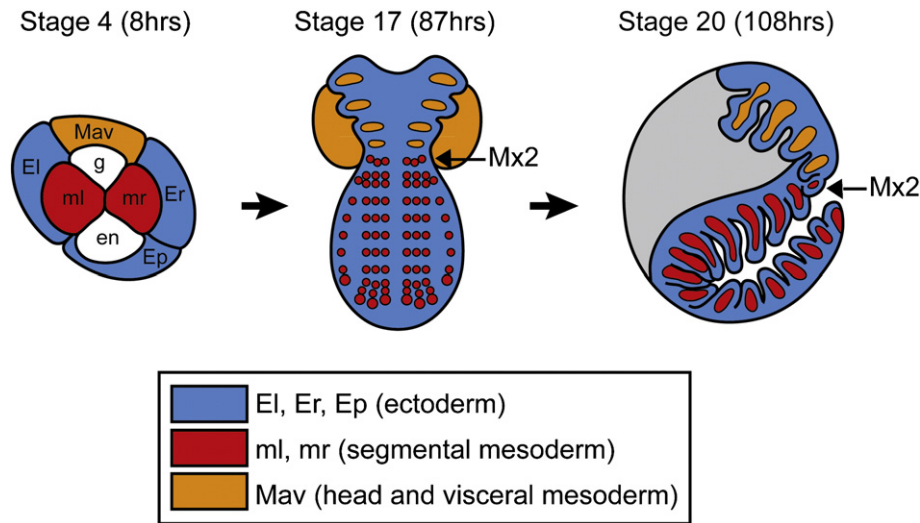


Fig. 1. Schematic of mesoderm and ectoderm lineages in *Parhyale*. Dorsal view of an eight-cell embryo (Stage 4; 8 h), ventral view of a mid-germband embryo (Stage 17; 87 h), and lateral view, with dorsal to the left, of an embryo with limb buds (Stage 20; 108 h). Anterior is to the top. The ectoderm is derived from three macromeres, El, Er and Ep, which will form the anterior left, anterior right and posterior ectoderm, respectively. The segmental mesoderm is derived from two micromeres, ml and mr, and one macromere, Mav. Typically, ml and mr will form the left and right segmental mesoderm posterior to and including maxilla 2 (Mx2), while Mav will form segmental mesoderm anterior to Mx2. Mav will also give rise to the visceral mesoderm (large orange oblongs at Stage 17), which is internalized by Stage 20. The remaining cells, g and en, will give rise to the germ line and the endoderm, respectively (Gerberding et al., 2002).

irradiation was confirmed by lack of cell division followed by presence of cellular debris. This method causes cell death only in FITC-dextran containing cells.

Timelapse microscopy was performed with a Hamamatsu ORCA-ER camera using Volocity software (PerkinElmer) on a Zeiss Axiovert 200 M. Embryos were visualized through the glass bottom of a 10 × 35 mm Petri dish (MatTek Corporation) filled with artificial sea water. The lid of the Petri dish was covered on the inside with black velvet to eliminate reflection. Fluorescent frames were captured at five or six-minute intervals. Each mesoteloblast as well of mesoblast was carefully followed to determine the number of divisions or lack of divisions during the duration of the movie. To capture both the ectoderm and the mesoderm, photos on two focal planes were taken at each time point, one focused on the ectoderm and one focused on the mesoderm. To make the movie, the in-focus planes of the ectoderm and the mesoderm were combined. Adobe After Effects was used in order to track individual cells in a movie.

PhHsp70-DsRed-NLS line and heat shock

To visualize all of the cells in *Parhyale* embryos, we used embryos transgenic for nuclear localized DsRed (DsRed-NLS) driven by the *Parhyale* heat shock 70 promoter (PhHsp70; generated by Modrell and Patel, unpublished, after Pavlopoulos and Averof, 2005). Embryos were heat shocked at approximately Stage 11 (60 h) for 1 h at 37 °C with pre-warmed artificial sea water in an air incubator and then cultured at standard conditions (Browne et al., 2005) until an appropriate stage for filming, approximately Stage 15 (80 h).

Antibody staining, in situ hybridization and visualization

Parhyale embryo fixation and histochemical in situ were performed as described in Rehm et al., 2009. Fluorescent in situ was performed as described in Rehm et al., 2009 with the following modifications: PT solution contained 0.3% Triton X-100, blocking solution consisted of 1:5 Roche blocking reagent to PT, and the TSA with fluorescein system was used to visualize staining (PerkinElmer). Antibody staining for *Parhyale* was performed as described in Patel (1994). Primary antibody incubations were overnight at 4 °C with MAb 4D9 anti-Engrailed (Patel et al., 1989) at a 1:30 dilution, rat anti-*Ph-Eve*

(Kwan, Parchem and Patel, unpublished) at a 1:1000 dilution, and rabbit anti-DsRed (Clontech) at a 1:1000 dilution. Secondary antibody incubations were at 2 h room temperature with Alexa 546 conjugated goat anti-mouse, Alexa 488 conjugated goat anti-rat, and Alexa 546 conjugated goat anti-rabbit, (Invitrogen) each at a 1:600 dilution. Antibody staining following in situ hybridization for *Ph-mef2* was performed as described above with the following modifications: embryos were dehydrated with methanol after in situ hybridization and rehydrated with PT before antibody staining, Mab 4D9 was used at a 1:2 dilution, and secondary antibody incubation was overnight at 4 °C with HRP conjugated goat anti-mouse (Jackson ImmunoResearch). Embryos were counterstained with 2.5 μM Draq5 (Biostatus Limited) and/or 0.1–1 μg/ml DAPI in 50% glycerol and transferred to 70% glycerol for clearing and mounting. Photographs were taken on either a Zeiss Axiophot with a Spot digital camera or a Zeiss Axio ImagerA1 with a ProgRes digital camera. Confocal images were taken on a Leica DMRXE. To combine multiple focal planes, Volocity software (confocal images; PerkinElmer) or Helicon Focus software (brightfield images; Helicon Soft Ltd., Kharkov, Ukraine) was used to generate a single, focused image.

For the “ $n = x/y$ embryos” values shown in the results, y represents the number of embryos in which injection and ablation were successful and the embryos survived to the stage required, and x represents the number of embryos that showed the indicated result. For the “ $n = x/y$ mesoteloblasts in w/z embryos” and “ $n = x/y$ mesoblasts in w/z embryos” values shown in the results, x represents the number of mesoteloblasts or mesoblasts, out of y , the total number of mesoteloblasts or mesoblasts observed, displaying a certain behavior; z represents the number of embryos in which injection and ablation were successful and the embryos survived to the stage required, and w represents the number of embryos that showed the indicated mesoteloblast or mesoblast behavior.

Results

Relationship between the segmental ectoderm and mesoderm

Before addressing whether segmentation is an intrinsic property of the ectoderm and/or mesoderm in *Parhyale*, we first characterized the coordination of the ectoderm and mesoderm within a given

segment. In order to address this relationship, we examined fixed embryos stained with DAPI via confocal microscopy. In addition, we fluorescently labeled the mesoderm and ectoderm in live embryos, and monitored segmentation via timelapse microscopy. We filmed embryos when segmental tissue is both generated and patterned, Stage 15 (80 h) to Stage 19 (96 h).

Our analysis corroborated previous research that separately characterized the formation of the segmental ectoderm and mesoderm in *Parhyale*. At the eight-cell stage (Stage 4; 8 h), three cells will form the anterior left, anterior right, and posterior ectoderm (El, Er and Ep, respectively; Fig. 1), and two cells will form the left and right segmental trunk mesoderm (ml and mr, respectively; Fig. 1; Gerberding et al., 2002). Segmental trunk ectoderm, from the posterior compartment of the mandible on, is formed by the organization of ectodermal cells into Parasegment Precursor Rows (PSPRs; Supplementary Movies 1, 2; Browne et al., 2005). With the exception of rows 0 and 1, which give rise to parts of the mandible and first maxillae, the rest of the PSPRs divide in a stereotypical manner. First, the PSPRs divide along the anterior–posterior axis to give rise to rows a/b and c/d. Then, rows a/b and c/d divide along the anterior–posterior axis to give rise to rows a and b, and c and d, respectively. PSPRs form and divide in a progressive manner in the embryo, both anterior to posterior, and medial to lateral. Segmental trunk mesoderm, from the second maxilla on, forms in a progressive manner from anterior to posterior through the migration and division of eight mesodermal stem cells (derived from ml and mr) called mesoteloblasts (Supplementary Movies 1, 3, 4; Browne et al., 2005). The mesoteloblasts are arranged in four columns on either side of the midline and are designated one through four (M1–M4), with column one being closest to the midline. The mesoteloblasts give rise to the mesoblasts, which are the segmental founder cells, as each row of mesoblasts will give rise to one segment's worth of mesoderm.

By examining segmentation simultaneously in both the ectoderm and the mesoderm, we found that, prior to approximately Stage 13 (72 h), the mesoteloblasts begin to migrate and divide before the first four PSPRs have begun to divide ($n = 8/8$ embryos; Figs. 2A, A'). As development progresses, and the PSPRs begin to divide, the relationship between the mesoteloblasts and the PSPRs gradually changes. After approximately Stage 14 (77 h), the mesoteloblasts (mesoderm) are either beneath or slightly anterior to the parasegment precursor row (PSPR; ectoderm) that is actively dividing ($n = 9/9$ embryos; Fig. 2; Supplementary Movies 1–6). In summary, although the mesoteloblasts begin to migrate and divide before the PSPRs begin to divide, PSPR division catches up to the position of the mesoteloblasts. The dynamic relationship between the mesoteloblasts and PSPRs suggests at least some autonomy for segmenting mesoderm and ectoderm.

As the mesoteloblasts migrate, they undergo a series of divisions to produce the mesoblasts, the segmental founder cells ($n = 7/7$ embryos; Fig. 2; Supplementary Movies 1–6). Since the mesoteloblasts produce mesoblasts concurrent with PSPR divisions, we hypothesized that one parasegment's row of mesoblasts would be born under and remain associated with that parasegment's ectoderm. Surprisingly, we found that the mesoblasts do not stay associated with the ectodermal PSPR that they are born under ($n = 5/5$ embryos; Fig. 2D; Supplementary Movies 1–6). Rather, a mesoteloblast initially generates two to three mesoblasts, mesodermal parasegments, under only one PSPR,

ectodermal parasegment. After birth, these mesoblasts migrate posteriorly to associate with the progeny of one PSPR, and position themselves under ectodermal rows c and d. While mesoblasts appear to migrate to a specific PSPR all along the anterior–posterior axis, the greatest distance between where the mesoblast is born and where it ultimately ends up is in the anterior of the embryo. After mesoblast migration, one discrete parasegment of mesoderm remains associated with one ectodermal parasegment. The mesoblasts always remain in birth order, so that earlier born mesoblasts are associated with more anterior ectodermal parasegments and later born mesoblasts are associated with more posterior ectodermal parasegments. Our finding that ectodermal and mesodermal progenitors undergo independent stereotypic divisions is consistent with a model in which mesoderm and ectoderm have autonomous segmental mechanisms.

Ectoderm and mesoderm have regional identity

Since the mesoderm and ectoderm present in a given parasegment may not be born in alignment, we hypothesized that ultimate position may be based on a shared regional cue. To test this hypothesis, we examined Hox gene expression in the mesoblasts and ectodermal parasegments. Hox genes specify regional identity in many animals, including *Parhyale* (Liubicich et al., 2009; McGinnis and Krumlauf, 1992). We focused on the Hox gene *Ph-Ubx*, as it is expressed in the easily visualized anterior thoracic region (Liubicich et al., 2009). By performing fluorescent in situ hybridization for *Ph-Ubx*, we found that *Ph-Ubx* is expressed in PSPRs 4–8 and their progeny, similar to previously published results (Liubicich et al., 2009; Figs. 3A, B). Supporting our hypothesis, *Ph-Ubx* is expressed in mesoblasts that are initially out of register with ectodermal parasegments expressing *Ph-Ubx*, and continues to be expressed in mesoblast progeny after mesoblasts have moved into register with the ectoderm and have divided (Figs. 3C–D').

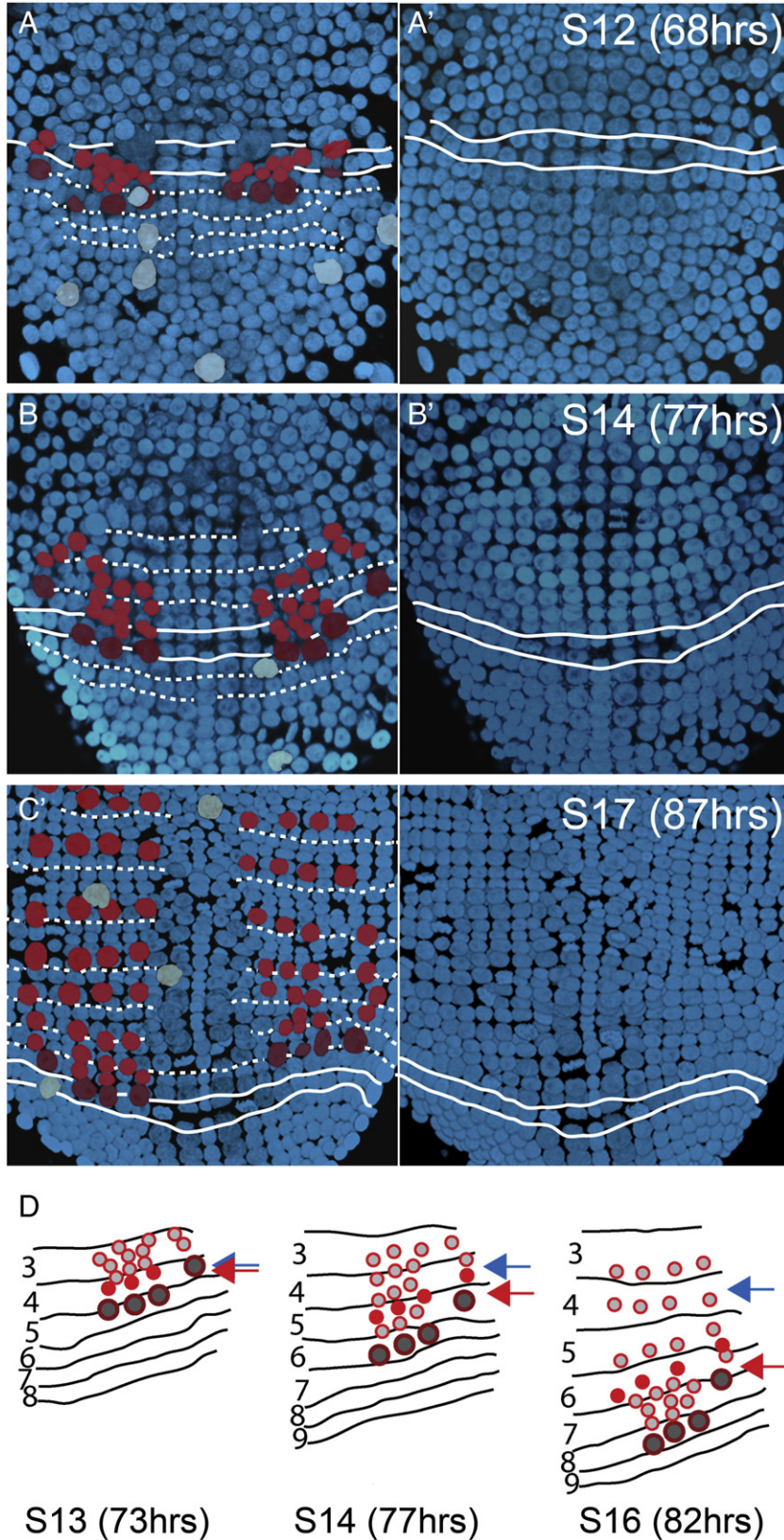
Ectoderm segmentation does not require mesoderm

To test whether the ectoderm requires a signal from the mesoderm to segment properly, we photo-ablated all three mesodermal precursor cells (Mav, ml and mr) at the eight-cell stage (Stage 4; 8 h). Then we cultured these embryos until Stage 17 (87 h) and visualized segmentation in the ectoderm both morphologically, by staining nuclei with DAPI, and molecularly, by staining for En, a marker of ectoderm segmentation (Browne et al., 2005; Patel et al., 1989). At this stage in control embryos, multiple PSPRs have formed and many have undergone multiple rounds of division (Figs. 4A, A'). PSPR and progeny division are from both anterior to posterior and medial to lateral, producing a chevron pattern (Fig. 4A; Browne et al., 2005). Rows a and b can be distinguished from rows c and d by their smaller cell size (Fig. 4A'). Additionally, En is expressed in the anterior row, row a, of each parasegment following two rounds of division (Figs. 4A, A'). Segmental En expression is also apparent in ectoderm anterior to the PSPRs that does not form via PSPR division (Fig. 4A; Browne et al., 2005). This produces a reiterated, segmental, pattern of En-positive and En-negative rows of cells along the anterior–posterior axis of the embryo.

Fig. 2. Mesoderm segmentation is not initially in register with ectoderm segmentation. (A–C') Confocal stacks of the posterior region of DAPI stained embryos, anterior to the top. (A, B, C) Dorsal view. Ectoderm is blue; mesoteloblasts, mesoblasts and vitellophages are false colored dark red, red, and light blue, respectively. Dashed white lines delineate ectodermal parasegment boundaries and solid white lines delineate the most posterior PSPR that is actively dividing. (A', B', C') Ventral view of ectoderm, flipped horizontally to facilitate comparison with the dorsal view. Solid white lines delineate the most posterior PSPR that is actively dividing. (A, A') Stage 12 (68 h) embryo. Mesoteloblasts are not yet aligned with the PSPR that is actively dividing; the PSPR that is actively dividing is anterior to the mesoblasts. (B, B') Stage 14 (77 h) embryo. (C, C') Stage 17 (87 h) embryo. The anterior ectodermal parasegments that consist of at least four rows of cells associate with one parasegment's worth of mesoderm. (D) Diagram of three timepoints from timelapse movie (Supplementary Movies 2–3, 5–6). Mesoteloblasts are large, dark gray circles outlined in dark red, while mesoblasts are either small, light gray circles outlined in red or are small, solid red circles. One row of mesoblasts is represented by solid red circle to highlight the relationship between the mesoblasts and the ectoderm. The row of solid red mesoblasts is born under ectodermal parasegment four, but then migrates to become associated with ectodermal parasegment six. Red arrow denotes present position of the row of solid red mesoblasts in each panel, blue arrow denotes birth position (ectodermal parasegment four) of this row. Note that the fourth mesoblast in this row is not yet born in the first panel. Lines indicate boundaries of ectodermal parasegments; ectodermal parasegments are numbered following Browne et al., 2005.

Previously, we had found that mesoderm-ablated embryos form morphologically normal ectodermal germbands (Price et al., 2010). However, these embryos died during early stages of segmentation (by Stage 13; 70 h) before molecular segmental pattern could be examined. By improving our injection technique (Rehm et al. 2009),

we were able to culture mesoderm-ablated embryos through Stages 17–20 (87–112 h), where the extent of ectodermal segmentation could be examined in detail. Following mesoderm ablation, we cultured embryos until Stage 17 (87 h). Nuclear staining revealed that the ectoderm does form normal morphological segments in the



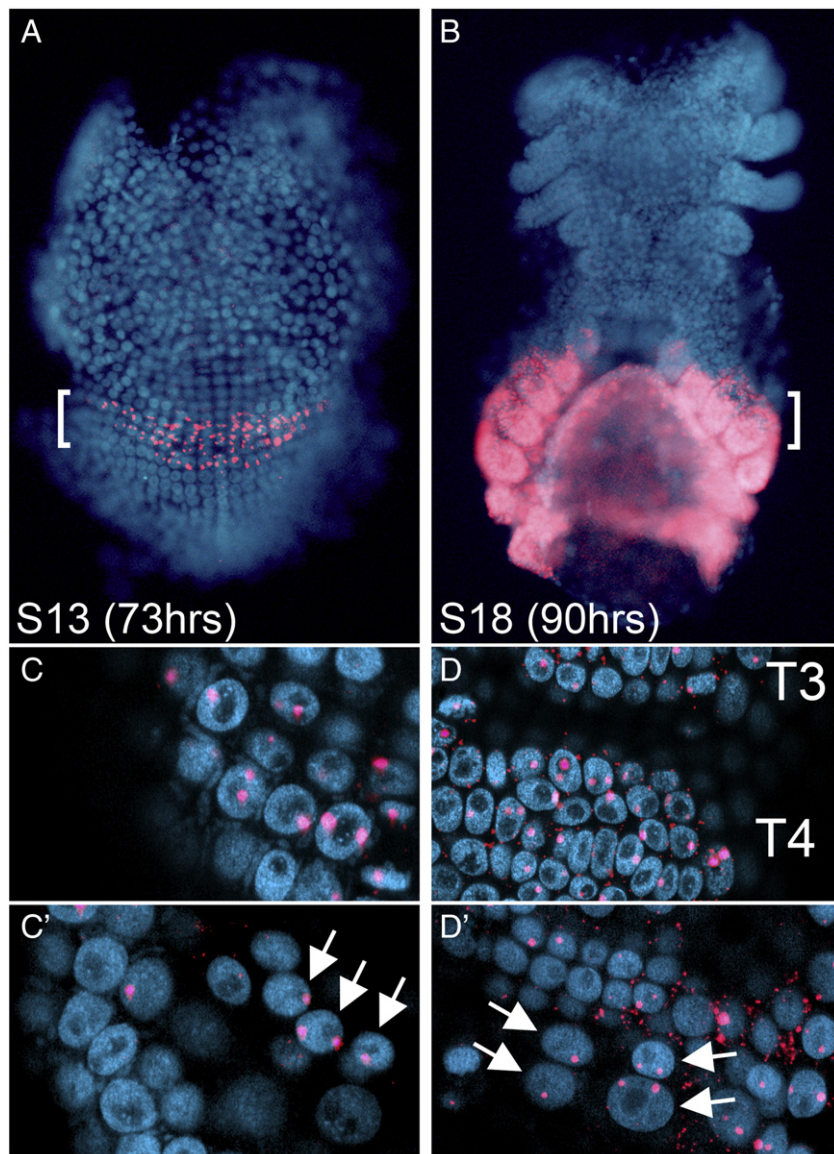


Fig. 3. *Ph-Ultrabithorax* (*Ph-Ubx*) is expressed in the ectoderm and the underlying mesoderm. (A–D') Ventral views; anterior to the top. Overlay of *Ph-Ubx* (red) and DAPI nuclear stain (blue). Nuclear dots (red dots) indicate active transcription. (A) Stage 13 (73 h). *Ph-Ubx* is expressed in PSPRs 4–8. (B) Stage 18 (90 h). *Ph-Ubx* is expressed in thoracic segments 2–8 (T2–T8). (C, C') Confocal image of bracketed area in (A). (D, D') Confocal image of bracketed area in (B); bracketed area includes the posterior of T3 and all of T4. (C, D) *Ph-Ubx* is expressed in the ectoderm. (C', D') *Ph-Ubx* is also expressed in the underlying mesoderm. Arrows point to mesoblasts (C') and mesoblast progeny (D') expressing *Ph-Ubx*. Mesoblasts are not yet aligned with a specific PSPR in (C'), while mesoblast progeny are aligned with the progeny of a specific PSPR, PSPR 7 (posterior of T4), in (D').

absence of the mesoderm ($n = 13/13$ embryos; Figs. 4C–D; Supplementary Movies 7 and 8). Mesoderm-ablated embryos displayed the same pattern of PSPRs and subsequent PSPR divisions as control embryos. PSPRs formed and divided in a progressive manner, from both anterior to posterior and medial to lateral (Figs. 4A, C). Additionally, similar to controls, rows a and b could be distinguished from rows c and d based on their smaller cell size (Figs. 4A', C'). To molecularly confirm normal ectoderm segmentation we examined En expression in mesoderm-ablated embryos. Antibody staining revealed proper expression of En in the anterior row, row a, of each parasegment ($n = 13/13$ embryos; Figs. 4C, C'). En was expressed in the same reiterated, segmental, pattern of En positive and negative rows of cells as controls. These data show that ectoderm segmentation does not require the mesoderm.

While the ectoderm does not require the mesoderm for initial segmental pattern, it could be required for later pattern maintenance. As our mesoderm-ablated embryos died by Stage 20 (112 h), likely due

to the lack of visceral mesoderm crucial for the formation and function of the digestive system, we used an alternative ablation strategy to assay for the dependence of ectoderm segmentation on the mesoderm later in development. In order to selectively ablate the trunk segmental mesoderm, while keeping the visceral mesoderm intact, we photo-ablated the progeny of the left and/or right mesodermal precursor cells (ml and/or mr) right after the mesoteloblasts formed (Stage 10; 56 h). At the time of ablation, the progeny of ml/mr consists of all four mesoteloblasts as well as 0–2 mesoblasts per mesoteloblast, since some mesoteloblasts have already begun to divide by the time all four mesoteloblasts have formed. In addition, the progeny of ml/mr also consists of approximately 5–30 cells that will form head and visceral mesoderm (Price and Patel, 2008). This is the earliest stage possible to ablate the progeny of ml and/or mr without progeny from the remaining mesodermal cell (Mav) compensating for their loss (Price et al., 2010). We then cultured these embryos until Stage 23 (142 h). At this stage, control embryos have developed all of

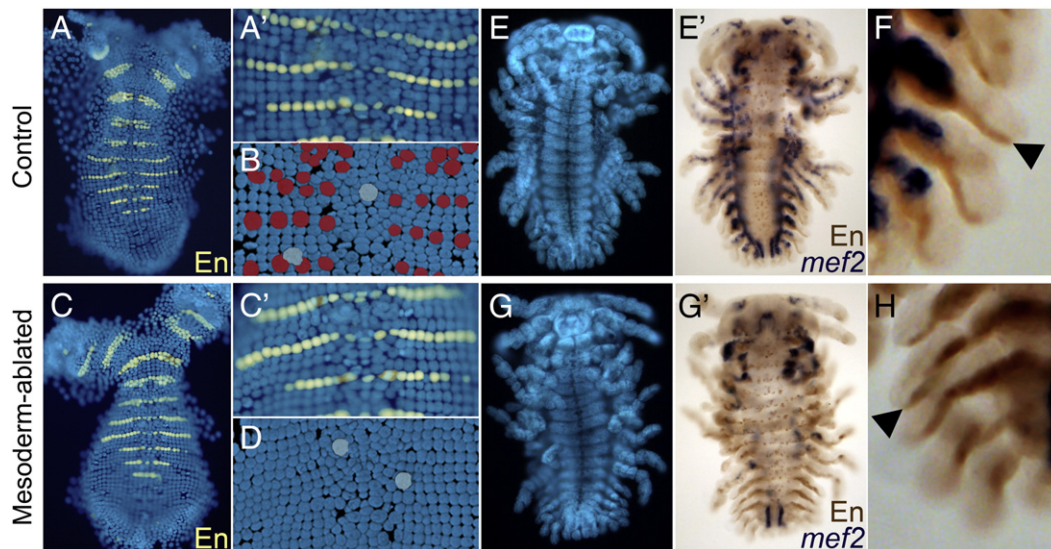


Fig. 4. The ectoderm segments autonomously. (A–D) Stage 15 (80 h) embryos, anterior to the top. Nuclear DAPI staining in blue. (A–B) Control embryo. (A, A') Ventral view. (A') Thoracic segments of embryo(A). Engrailed (En) protein (yellow) is present in the most anterior row (row a) of each parasegment once each PSPR has undergone two rounds of division. (B) Dorsal view; confocal stack of (A'), showing mesoderm (false colored red cells) and vitellophages (false colored light blue cells) under the segmental ectoderm. (C–D) Mesoderm-ablated embryo. (C, C') Ventral view. (C') Thoracic segments of embryo (C). Just as in the control embryo, En protein is present in the most anterior row (row a) of each parasegment once each PSPR has undergone two rounds of division. (D) Dorsal view; confocal stack of (C'), showing the lack of mesoderm in this embryo, although vitellophages are still present. Dorsal views (B) and (D) were flipped horizontally to facilitate comparison with the overlying ectoderm. (E–H) Ventral view of Stage 23 (142 h) embryos, anterior to the top. Nuclear DAPI staining in blue (E, G). (E–F) Control embryo. (G–H) Segmental trunk mesoderm-ablated embryo. (F, H) Posterior region of embryos (E') and (G'), respectively. To generate segmental trunk mesoderm-ablated embryo, the mesoteloblasts, which produce the segmental trunk mesoderm, were ablated at their birth (Stage 10; 56 h). Head segmental mesoderm and visceral mesoderm, derived from Mav, are still present in this embryo. Ablation was confirmed by absence of *Ph-mef2* (purple) in the trunk body walls and limbs (compare E' to G'). Residual *Ph-mef2* staining in (G', H) is visceral mesoderm derived from Mav. En protein (brown) is present in the posterior of each ectodermal segment (arrowheads in (F, H)) in both control and mesoderm-ablated embryos.

their limbs, which are clearly organized in a segmental manner, and these embryos also display distinct grooves between each trunk segment (Fig. 4E). In addition, En is expressed both in the posterior of each ectodermal segment and in a subset of neurons in the trunk (Browne et al., 2005). To confirm ablation of the segmental mesoderm, we performed in situ hybridization for a marker of differentiated mesoderm, *Ph-myocyte enhancing factor 2* (*Ph-mef2*; Price and Patel, 2008). *Ph-mef2* was largely absent in the ablated regions, except for trace staining in visceral mesoderm derived from Mav (Fig. 4G'). We found that mesoderm-ablated embryos displayed normal segmentation ($n=6/6$ embryos; Fig. 4). Similar to controls, mesoderm-ablated embryos developed segmented limbs and distinct grooves between each trunk segment (Fig. 4G). In addition, mesoderm-ablated embryos displayed proper expression of En in the posterior of each limb (Fig. 4H) and in the nervous system (Fig. 4G'). These data show that the ectoderm does not require the mesoderm to form morphological segments, to molecularly pattern these segments, or to maintain segmental pattern late in development.

Mesoteloblasts can divide autonomously, but mesoblast division requires the ectoderm

To test whether the mesoderm requires a signal from the ectoderm to segment properly, we ablated the ectoderm and assayed for segmentation in the mesoderm. Because the ectodermal precursor cells (Er, El and Ep) compose most of the volume of the embryo, ablation of all three, or even two, of these cells results in death of the entire embryo within 1 day of injection, 2 days before segmentation would normally begin (Price et al., 2010). Ablation of only one ectodermal precursor cell at the eight-cell stage is also impractical for this study because progeny of the remaining ectodermal precursor cells compensate for the ablated cell by the time segmentation begins (Price et al., 2010). To overcome these limitations, we photo-ablated the progeny of either

the left or right anterior ectodermal precursor cell (El or Er, respectively) after the mesoteloblasts formed, when the embryo can no longer compensate (Stage 10; 56 h; Price et al., 2010). Additionally, in order to track the mesoderm on the ectoderm-ablated side of the embryo, we labeled the ipsilateral mesodermal precursor cell (either ml or mr) with the nuclear localized fluorescent lineage tracer DsRed-NLS.

To assay for mesoderm segmentation, we made movies of labeled mesoteloblasts in ectoderm-ablated embryos (Figs. 5A–F'; Supplementary Movies 9 and 10). In control embryos, mesoteloblasts migrated posteriorly, progressing 1/3 the length of the embryo in a 10 h period ($n=16/16$ mesoteloblasts in 4/4 embryos). During this migration, mesoteloblasts went through a series of asymmetric divisions along their anterior–posterior axis, producing one mesoblast every 2 h ($n=16/16$ mesoteloblasts in 4/4 embryos). In ectoderm-ablated embryos, however, most mesoteloblasts migrated in both posterior and lateral directions ($n=10/11$ mesoteloblasts in 3/3 embryos) instead of only posteriorly ($n=1/11$ mesoteloblasts in 1/3 embryos). In addition, mesoteloblasts traveled less than 1/4 the length of the embryo in a 10 hour period ($n=8/11$ mesoteloblasts in 3/3 embryos). If the mesoteloblasts came into contact with ectoderm from one of the remaining ectodermal lineages, El or Er, and/or Ep, they resumed posterior migration and traveled 1/3 the length of the embryo in a 10 hour period ($n=3/11$ mesoteloblasts in 1/3 embryos; Fig. 5G). Similar to controls, mesoteloblasts in ectoderm-ablated embryos produced one mesoblast every 2 h ($n=11/11$ mesoteloblasts in 3/3 embryos; Figs. 5A–F'; Supplementary Movies 9 and 10). Additionally, mesoteloblasts in ectoderm-ablated embryos divided in the same anterior–posterior orientation as they did in controls ($n=11/11$ mesoteloblasts in 3/3 embryos; Supplementary Movies 9 and 10). These data show that the mesoteloblasts do not require the ectoderm for proper division, but do require the ectoderm for proper migration. Proper migration may require the physical substrate provided by the ectoderm, and/or could require a posterior migration cue secreted from the ectoderm.

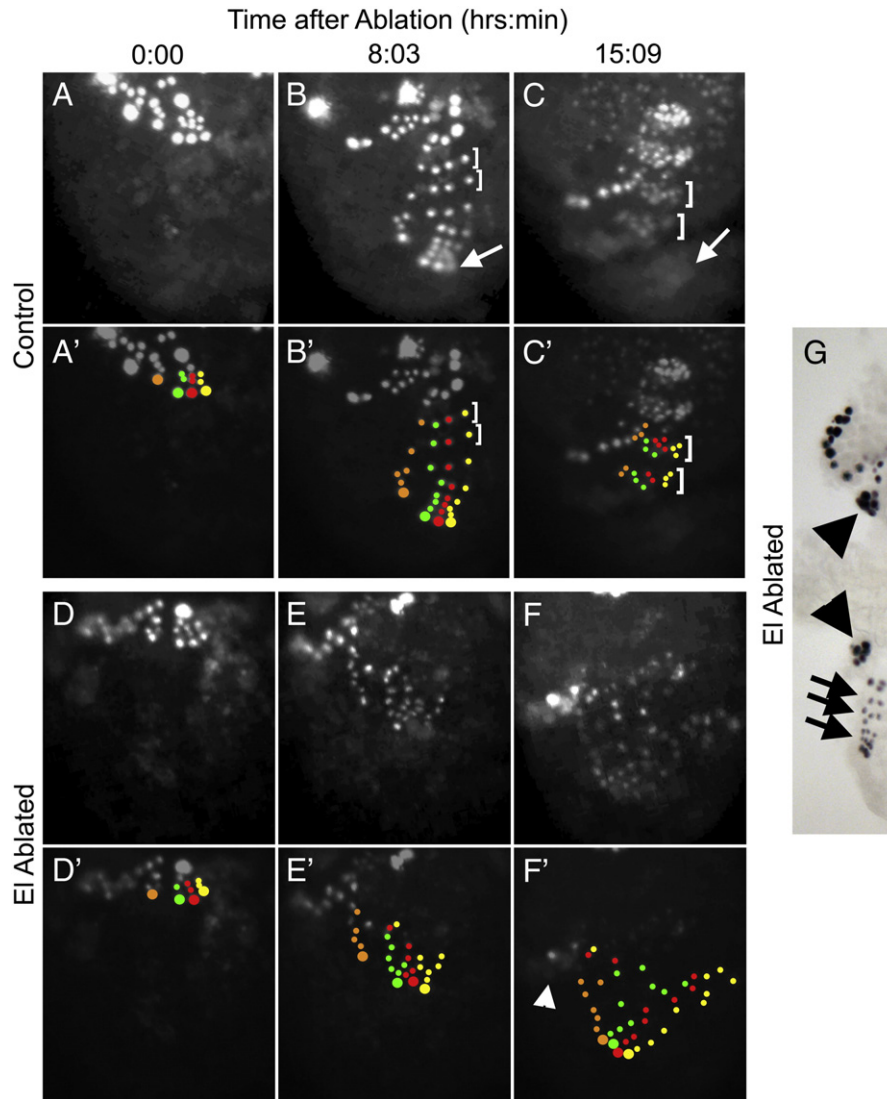


Fig. 5. Mesoteloblast division is autonomous, but mesoblast division requires the ectoderm. (A–F′) Stills from timelapse movie of mesoderm development in control and left ectoderm-ablated embryos Stage 14 (77 h) through Stage 18 (92 h). Ventral–lateral view; anterior to the top. Nuclei of left mesoderm are labeled with DsRed-NLS (white). (A–C′ and D′–F′) In focus nuclei are false-colored to highlight mesoteloblasts and their progeny. Large circles denote mesoteloblasts, small circles denote mesoblasts. Mesoblasts are the same color as the mesoteloblast from which they originated. (A–C′) Control embryo. (A–B′) Mesoteloblasts divide to give rise to mesoblasts while migrating posteriorly. Mesoblasts then undergo subsequent divisions (C, C′). Each bracket in C and C′ indicates one segment's worth of mesoderm where the mesoblasts have undergone multiple divisions. Arrows in B and C indicate the formation of a tailfold in the control embryo, which obscures more posterior mesoteloblasts and mesoblasts. (D–F′) Left ectoderm-ablated embryo. Mesoteloblasts continue to undergo divisions to give rise to mesoblasts, but do not migrate properly. In addition, mesoblasts born both before and after ectoderm ablation do not divide (F, F′). While more mesoblasts are in focus in the left ectoderm-ablated embryo compared to the control embryo, these cells have not divided. Arrowhead in (F′) indicates approximate location of an orange lineage mesoblast that has gone out of focus. (G) Left side of fixed left ectoderm-ablated embryo; left mesoderm is labeled with and stained for DsRed-NLS (purple). Ventral view, anterior to the top. Mesoderm in the ablated region is in unorganized clumps (arrowheads). Once mesoderm from the ablated side comes into contact with the posterior ectoderm, however, mesoderm is again spatially organized into rows (arrows; compare to control mesoderm in B, B′).

In contrast to the mesoteloblasts, the mesoblasts do not divide in the absence of the ectoderm. Mesoblasts born both before and after ectoderm ablation never divide ($n = 9/9$ and $15/15$ mesoblasts, respectively, in 3/3 embryos; Figs. 5F, F′; Supplementary Movies 9 and 10), indicating that a signal from the ectoderm is required later in mesoblast development, or for a longer period of time. We conclude mesoteloblast division is autonomous, but division of their progeny, the mesoblasts, requires the ectoderm.

In the absence of ectoderm, mesoblasts do not express segmentation markers

Although mesoblast division requires the ectoderm, we hypothesized that mesoblasts may still have intrinsic segmental gene expression without the ectoderm. For example, mesoblasts might still be

able to express segmental genes with the correct developmental timing even if they are unable to divide. To test this hypothesis, we examined expression of molecular markers of mesoderm segmentation, *Ph-twi* and *Ph-Eve* (Fig. 6; Price and Patel, 2008; Vargas-Vila et al., 2010), in ectoderm-ablated embryos. Not only are *Ph-twi* and *Ph-Eve* the earliest known molecular markers of mesoderm segmentation, but they continue to be expressed in a subset of the segmental mesoderm throughout later development. In control embryos, *Ph-twi* and *Ph-Eve* are expressed in the anterior daughters of mesoblasts in the second and fourth columns of mesoderm, respectively, in T2 and more posterior segments. The second column of mesoderm is underneath the developing limb field and the fourth column is the most dorsal mesoderm. *Ph-twi* and *Ph-Eve* are also expressed in a similar segmental pattern in the mesoderm of Mx2 and T1. Later in development, *Ph-twi* and *Ph-Eve* are

expressed in clusters of mesodermal cells in roughly the same positions as in early development (Figs. 6A, B). *Ph-twi* is expressed at the base of limb and in cells moving into the limbs (Price and Patel, 2008). *Ph-Eve* is expressed in a cluster of the dorsal-most mesodermal cells (Vargas-Vila et al., 2010). Based on comparison with similar *Eve* positive cells in *Drosophila* (Frasch et al., 1987), *Ph-Eve* positive cells likely contribute to the heart and dorsal somatic mesoderm.

In ectoderm-ablated embryos, however, we found that neither *Ph-twi* nor *Ph-Eve* was expressed in the mesoderm on the ectoderm-ablated side ($n = 6/6$ embryos for *Ph-twi* and $5/5$ embryos for *Ph-Eve*; Fig. 6). These data are consistent with two potential scenarios (Figs. 7 and 8). First, the ectoderm is required for both division and subsequent segmental patterning of the mesoderm. Alternatively, the mesoderm may contain intrinsic segmental pattern, but the pattern is not visible until after the mesoblasts have divided, and this division requires a signal from the ectoderm.

Discussion

Ectoderm segmentation does not require the mesoderm

We have characterized the functional relationship between the segmenting ectoderm and mesoderm in the crustacean *P. hawaiiensis*. We asked whether segmentation is an intrinsic property of the ectoderm and/or the mesoderm by ablating one layer and assaying both

for morphological and molecular segmentation in the remaining layer. We found that the ectoderm does not require the mesoderm to form morphological segments, to molecularly pattern these segments, or to maintain segmental pattern. Our data refute a hypothesis from arthropod literature that mesoderm segmentation evolved first, followed by ectoderm segmentation, and that this evolutionary scenario could be reflected in the developmental order and importance of this tissue in extant animals (Budd, 2001). This evolutionary scenario is not reflected in the development of *Parhyale*.

Data from *Parhyale* also argue against another potential signaling source of segmental pattern to the epidermal–ectoderm. Besides suggesting that ectoderm segmentation could be dependent on the mesoderm, Budd (2001) suggests that the neural-ectoderm could induce segmental pattern in the rest of the ectoderm. Similar to the mesoderm, a segmented nervous system evolved prior to a segmented epidermis and this evolutionary scenario could be reflected in developmental mechanisms of extant animals. In *Parhyale*, however, segmental pattern in the epidermal–ectoderm is apparent before nervous system formation, arguing against induction of pattern from the neural-ectoderm to the epidermal–ectoderm (Browne et al., 2005).

Although our data argue against Budd's evolutionary models, we have only examined the functional relationship between the ectoderm and mesoderm in *Parhyale*. *Parhyale* is an attractive system for this study as its mesodermal and ectodermal lineages are separated very early in development, and are thus amenable to ablation and

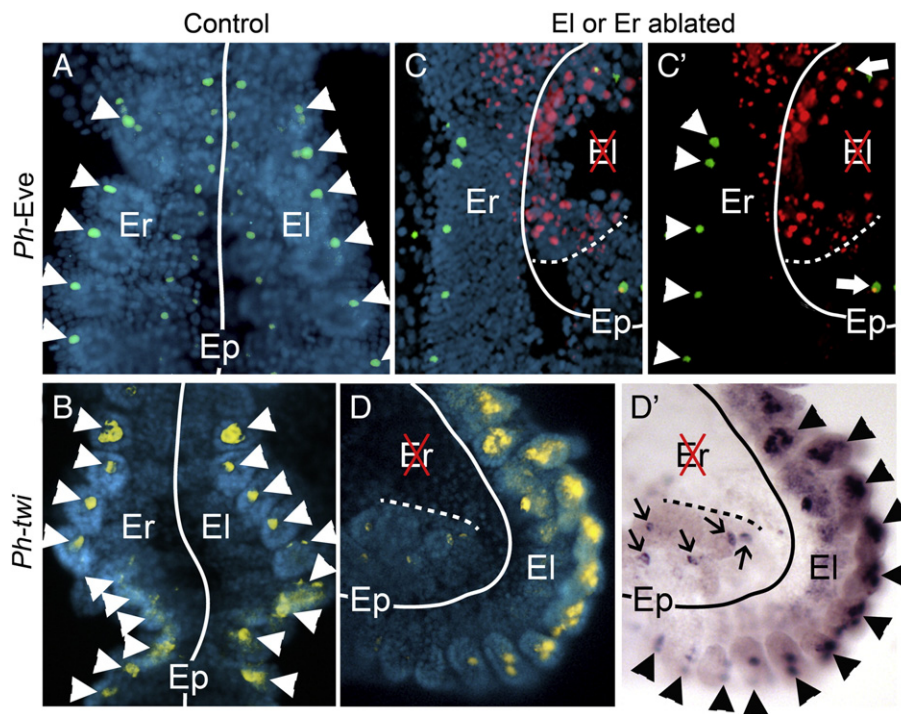


Fig. 6. In the absence of ectoderm, mesoblasts do not express markers of mesoderm segmentation. Ventral view; anterior to the top. (A, B) Control embryos bilaterally express segmentation markers in a subset of the mesoderm throughout development. Overlay of *Ph-Eve* protein (green; A) and false color overlay of *Ph-twi* (yellow; B) over DAPI (blue) at Stage 18 (90 h). Solid white lines delineate midline; arrowheads point to bilateral expression in maxilla 2 (Mx2) and thoracic mesoderm. Left, right and posterior ectoderm are labeled EI, Er and Ep, respectively. (C–D') EI or Er ablated embryos. Solid white lines delineate midline; dashed white lines delineate the boundary between the ablated ectoderm and the posterior ectoderm. Left, right and posterior ectoderm are labeled EI, Er and Ep, respectively. Ablated ectoderm is crossed out with a red "X". (C, C') Left ectoderm-ablated embryos Stage 17 (87 h). The mesoderm on the ablated side of the embryo is labeled with DsRed-NLS (red). (C) Overlay of *Ph-Eve* expression (green) and left mesoderm (red) over DAPI (blue). (C') On the unablated (Er) side, *Ph-Eve* is expressed in a subset of the progeny of the mesoblasts in Mx2 and thoracic segments (arrowheads). On the ablated side (EI), *Ph-Eve* is not expressed in mesoblasts. *Ph-Eve* is expressed in one mesoteloblast near the posterior of the embryo, as well as in one mesoteloblast in the anterior of the embryo that has not migrated properly (arrows). Medial neural expression of *Ph-Eve* in the ectoderm is absent on both the ablated and the control side of this embryo (compare (A) to (C, C')), although is present on the control side of older ectoderm-ablated embryos (data not shown), suggesting a slight delay in nervous system formation. (D, D') Right ectoderm-ablated embryo Stage 18 (90 h). While this embryo is orientated with anterior to the top, it is curved so that posterior is to the left due to differential growth caused by ablation of the right ectoderm. (D) False color overlay of *Ph-twi* (yellow) over DAPI (blue). (D') On the unablated side (EI), *Ph-twi* (purple) is expressed in a subset of the progeny of the mesoblasts in Mx2 and thoracic segments (arrowheads). On the ablated side (Er), *Ph-twi* is not expressed in the ablated region of the embryo. However, *Ph-twi* is expressed in a subset of the mesoderm from the ablated side that now lies under the posterior ectoderm (arrows).

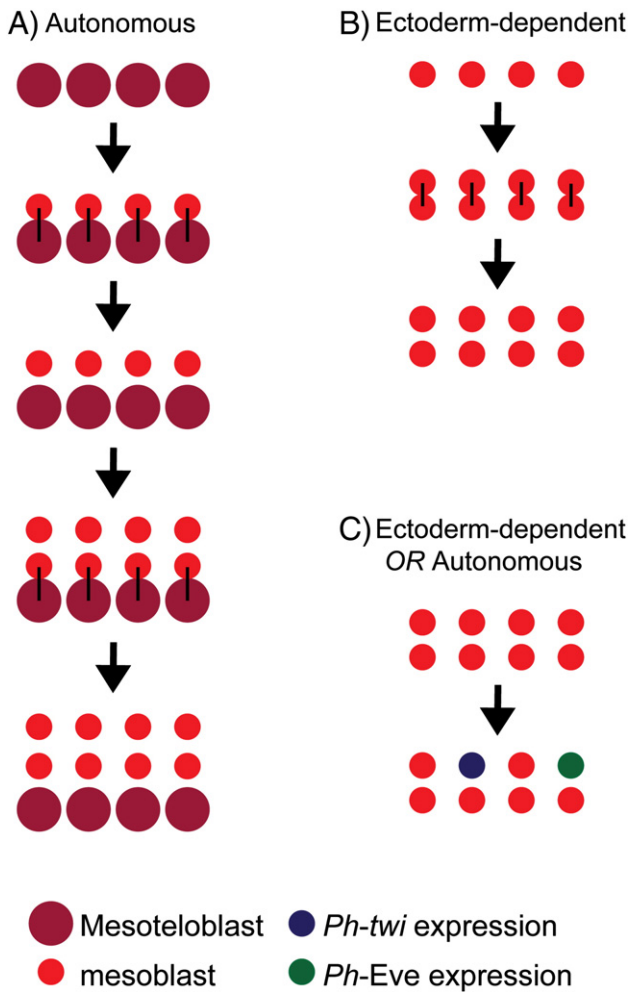


Fig. 7. The development of the segmental mesoderm involves an autonomous and an ectoderm-dependent phase. Ventral view of left mesoderm (right side is a mirror image); anterior to the top, midline to the left. (A) Mesoteloblast division is not dependent on the ectoderm. The mesoteloblasts divide asymmetrically to give rise to the mesoblasts. Each row of mesoblasts will give rise to one segment's worth of mesoderm (the production of two segment's worth of mesoderm is depicted here). (B) Mesoblast division is dependent on the ectoderm. The mesoblasts divide symmetrically, generating anterior and posterior daughters. (C) Generation of segmental pattern in the mesoderm may be dependent on the ectoderm. After the mesoblasts divide, the first known molecular markers of mesoderm segmentation are expressed. The second anterior daughter from the midline expresses *Ph-twi* and the fourth anterior daughter from the midline expresses *Ph-Eve*, while their posterior sisters do not.

lineage-tracing experiments. Also, the necessary experimental tools were available for this study, as *Parhyale* is an emerging model crustacean. However, the relationship between the ectoderm and mesoderm may have changed over evolutionary time and may no longer reflect the evolution of the segmental ectoderm and mesoderm (Budd, 2001). Additionally, the ectoderm–mesoderm relationship in *Parhyale*, a Malacostracan, might not be the same as in non-Malacostracan crustaceans that do not utilize teloblasts to produce segments. While this is a concern, the absence of teloblasts in crustaceans outside of the Malacostracans could be due to a scarcity of developmental data versus an actual lack of teloblasts in these species. Moreover, subsequent steps of segmentation are relatively similar in species with and without teloblasts (Davis et al., 2005), suggesting that the relationship between the segmenting ectoderm and mesoderm could be the same as well. Even if the ectoderm–mesoderm relationship is similar amongst all pancrustaceans, it may differ from the other arthropod groups, the myriapods and chelicerates. In order to form a more comprehensive picture of the relationship between the ectoderm and mesoderm in arthropods, more species should be examined.

Scholtz also uses comparative data to suggest that the mesoderm is the primary segmental germ layer in Malacostracan crustaceans (Scholtz, 1990). By comparing Malacostracans, Scholtz notes that segmental trunk mesoderm development is conserved, while segmental trunk ectoderm development varies, between species. This conservation could suggest a greater importance of the segmental mesoderm during development, perhaps by inducing ectoderm segmentation. Instead, our data from the Malacostracan *Parhyale* suggest that the developmental conservation of the segmental mesoderm and the developmental plasticity of the segmental ectoderm in Malacostracans do not correlate with their relative contributions to segmentation.

Development of segmental mesoderm in Parhyale involves both an autonomous and an ectoderm-dependent phase

We found that the ectoderm and mesoderm composing each parasegment are not initially associated with one another. After birth, one parasegment of mesoderm migrates to associate with its corresponding parasegment of ectoderm. This raises the possibility that the mesoderm has some autonomy from the ectoderm, a hypothesis we have confirmed with ablation experiments. When we ablated either the left or right anterior ectoderm in *Parhyale*, we found that the mesoteloblasts continued to divide at a normal rate and in the correct anterior–posterior orientation. Although the mesoteloblasts often failed to migrate properly, this may be due to the lack of a physical substrate, rather than intercellular signals.

After this first, autonomous production of the segmental mesoderm (Fig. 7A) a second, ectoderm-dependent, phase of mesoderm segmentation occurs (Fig. 7B). We found that the progeny of the mesoteloblasts, the mesoblasts, only divide after they are associated with one parasegment's worth of ectoderm. Our ablation experiments show that the mesoblasts fail to divide and do not express known markers of later mesoderm segmentation in the absence of the ectoderm. While we found that the mesoderm requires a signal from the ectoderm to form mature segments, future experiments are needed to address whether this ectodermal signal is instructive or permissive (Fig. 7C). A permissive signal would allow the mesoblasts to divide, but the mesoblasts themselves would contain segmental patterning information, and would express markers of segmentation autonomously after division occurred (Fig. 8A). Alternatively, an additional instructive signal from the ectoderm could impart segmental pattern to the mesoderm (Fig. 8B). One way to distinguish between these possibilities would be to examine a protein expressed in either the anterior or posterior membrane of the mesoblasts, if such a protein were to be discovered. If mesoblasts have intrinsic segmental pattern, this marker would still be present in its proper anterior–posterior location in ectoderm-ablated embryos.

Evolution of segmentation

There is considerable debate on whether trunk segmentation in the arthropods, annelids and chordates is homologous, since each phylum is more closely related to unsegmented phyla than to each other (Davis and Patel, 1999; Peel and Akam, 2003; Seaver, 2003). One way to approach this question of homology is to compare development of trunk segmentation amongst these groups. If the common ancestor of these groups was segmented, perhaps the relationship between the two segmental layers, the ectoderm and mesoderm, would be the same. For example, the mesoderm could segment autonomously and induce segmental pattern in the ectoderm in all three groups.

In vertebrates, trunk mesoderm segments autonomously and induces segmental arrangement of the overlying ectoderm-derived nervous system (Correia and Conlon, 2000; Detwiler, 1934; Keynes and Stern, 1984; Lewis et al., 1981; Palmeirim et al., 1998). This method of segmentation is conserved amongst all vertebrates examined. In contrast, in the

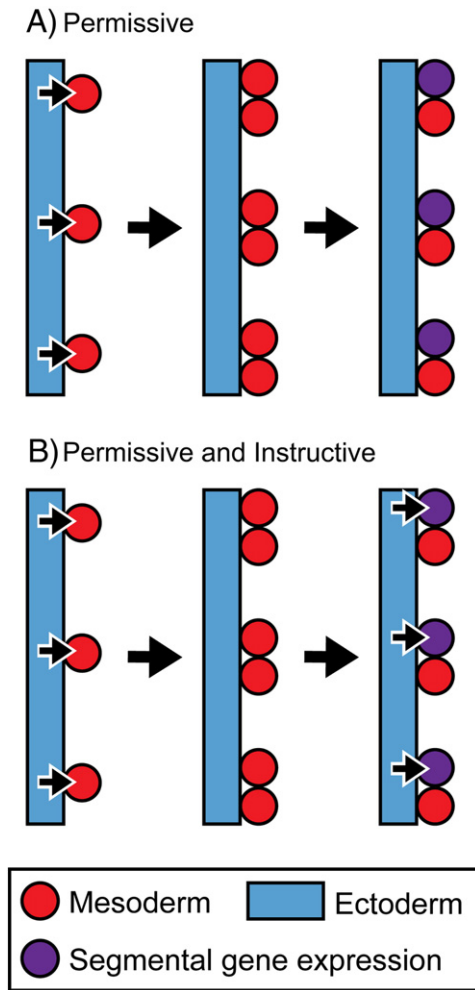


Fig. 8. The ectoderm provides the mesoderm with a permissive, and possibly instructive, signal. Transverse view of the ectoderm and underlying mesoderm. Anterior to the top, dorsal to the right, ventral to the left. Small arrows represent signals from the ectoderm to the mesoderm. Large arrows represent developmental progression. Mesoblasts require a permissive signal from the ectoderm in order to divide. (A) Mesoblast progeny may have intrinsic segmental pattern. (B) Mesoblast progeny may require an instructive signal from the ectoderm for segmental pattern.

holometabola insects *Drosophila*, *Chrysopa* and *Leptinotarsa*, the ectoderm segments autonomously and is at least partially required for proper mesoderm segmentation (Azpiazu et al., 1996; Bock, 1942; Frasch, 1999; Haget, 1953; Rao et al., 1991). Our ablation experiments show that this is not restricted to the holometabola insects. In *Parhyale*, ectoderm can also segment autonomously and, at the least, supplies a permissive signal for mesoderm segmentation. Together, these data suggest that segmentation is primarily a property of the ectoderm in the pancrustacea (crustaceans plus insects; Regier et al., 2010). It will be interesting to investigate segmentation in the other arthropod groups, specifically the myriapods and chelicerates, to define whether segmentation is primarily a property of the ectoderm in all arthropods.

Unlike vertebrates and pancrustaceans, in annelids, there does not appear to be one germ layer that has intrinsic segmental pattern and induces the other to segment. A caveat to this statement is that these interactions have only been studied in two annelids, so a common method may emerge if more species were studied. In the leech *Helobdella* and the slug worm, *Tubifex*, segments are produced by the asymmetrical divisions of ectodermal and mesodermal stem cells (Goto et al., 1999; Nakamoto et al., 2000; Weisblat and Shankland, 1985). In *Helobdella*, mesoderm and ectoderm are dependent on each other for segmental organization and pattern, but not for the production

and division of segmental precursor cells (Blair, 1982). Similar to *Helobdella*, in *Tubifex*, ectoderm segmental precursor cells are produced without the mesoderm, but these cells are not arranged in a segmental manner (Nakamoto et al., 2000). However, in contrast to *Helobdella*, *Tubifex* mesoderm segmentation is autonomous (Goto et al., 1999). As only morphological segmentation was examined in these studies, molecular segmental pattern could still be intrinsic.

Together, these data show that the germ layer containing segmental information differs between arthropods and vertebrates, and even amongst different species of annelids. This suggests that the tissue layer that imparts segmental information might be easily acquired, modified and lost, and therefore may not be a suitable developmental feature for inferring instances of the independent evolution of segmentation.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.09.033

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