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ORIGINAL ARTICLE

Analysis of *snail* genes in the crustacean *Parhyale hawaiensis*: insight into *snail* gene family evolution

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Abstract The transcriptional repressor *snail* was first discovered in *Drosophila melanogaster*, where it initially plays a role in gastrulation and mesoderm formation, and later plays a role in neurogenesis. Among arthropods, this role of *snail* appears to be conserved in the insects *Tribolium* and *Anopheles gambiae*, but not in the chelicerates *Cupiennius salei* and *Achaearanea tepidariorum*, the myriapod *Glomeris marginata*, or the Branchiopod crustacean *Daphnia magna*. These data imply that within arthropoda, *snail* acquired its role in gastrulation and mesoderm formation in the insect lineage. However, crustaceans are a diverse group with several major taxa, making analysis of more crustaceans necessary to potentially understand the ancestral role of *snail* in Pancrustacea (crustaceans + insects) and thus in the ancestor of insects as well. To address these questions, we examined the *snail* family in the Malacostracan crustacean *Parhyale hawaiensis*. We found three *snail* homologs, *Ph-snail1*, *Ph-snail2* and *Ph-snail3*, and one *scratch* homolog, *Ph-scratch*. *Parhyale snail* genes are expressed after gastrulation, during germband formation and elongation. *Ph-snail1*, *Ph-snail2*, and *Ph-snail3* are expressed in distinct patterns in the neuroectoderm. *Ph-snail1* is the only *Parhyale snail* gene expressed in the mesoderm, where its expression cycles in the mesodermal stem cells, called mesoteloblasts. The mesoteloblasts go through a series of cycles, where each cycle is composed of a migration phase and a division phase. *Ph-snail1* is expressed during the migration phase, but not during the division phase. We found that as each mesoteloblast division produces one segment's worth of mesoderm, *Ph-snail1* expression is linked to both the cell cycle and the segmental production of mesoderm.

Keywords *Parhyale hawaiensis* · *snail* · *scratch* · Arthropod · Crustacean

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Introduction

Members of the *snail* superfamily of transcriptional repressors are found throughout the metazoa and play a crucial role in many cell and developmental processes (reviewed in Hemavathy et al. 2000). A C-terminal domain consisting of four to six zinc fingers characterizes this superfamily, which includes both the Snail and Scratch families. Although *snail* was first implicated in mesoderm specification and gastrulation, *snail* homologs are now known to have many functions, including in nervous system development, dorsal–ventral patterning, mesoderm segmentation, neural crest cell migration, cancer metastasis, and even left–right asymmetry (reviewed in Hemavathy et al. 2000;

reviewed in Nieto 2002). In contrast, homologs of the other member of the *snail* superfamily, *scratch*, are only involved in nervous system development (Marin and Nieto 2006; Nakakura et al. 2001; Roark et al. 1995).

The original *snail* gene was discovered in *Drosophila* via a large-scale screen for loci essential for larval patterning (Grau et al. 1984; Nusslein-Volhard et al. 1984). Embryos homozygous for loss-of-function *snail* mutations display defects in invagination of the presumptive mesoderm, the most ventral region of the embryo where *snail* is normally expressed (Alberga et al. 1991; Kosman et al. 1991; Leptin 1991). Not only does the presumptive mesoderm fail to invaginate in these mutants, but neuroectodermal genes are ectopically expressed in the ventral region of the embryo (Ip et al. 1992; Kosman et al. 1991; Leptin 1991). These data indicate that *snail* is essential for mesoderm formation. In addition, they suggest a role for *snail* in dorsal–ventral patterning, since *snail* represses neuroectodermal genes in the ventral region of the embryo and correctly positions them in the lateral regions of the embryo (Ip et al. 1992).

The *Drosophila* genome contains genes encoding two zinc-finger proteins closely related to *snail*, *escargot* and *worniu* (meaning "snail" in French and Mandarin, respectively), and one other related zinc finger containing gene, *scratch* (Ashraf et al. 1999; Roark et al. 1995; Whiteley et al. 1992). In addition, there are two predicted zinc-finger genes similar to *scratch*: *scratch-like1* and *scratch-like2* (Manzanares et al. 2001). While only *snail* is expressed in the mesoderm, *snail*, *escargot*, *worniu*, and *scratch* are all expressed in the developing nervous system, and both *snail* and *escargot* are expressed in the wing imaginal disc. In addition to overlapping expression patterns, some of the *snail* family genes have functional redundancy in the developing nervous system and wing imaginal disc (Ashraf et al. 1999; Fuse et al. 1996; Roark et al. 1995).

In contrast to the many functions of *snail*, *Drosophila scratch* is expressed and functions only in the nervous system (Nakakura et al. 2001; Roark et al. 1995). Named after a rough eye phenotype, homozygous *scratch* mutants are also weak and uncoordinated. When combined with mutants for other neural genes, such as *deadpan*, these *Drosophila* mutants have a detectable CNS phenotype. In support of a role in nervous system development, overexpression of *scratch* via heat shock increases neuron formation (Roark et al. 1995).

In arthropods, *snail* homologs have been characterized in insects besides *Drosophila* as well as in chelicerates, myriapods, and Branchiopod crustaceans. In the insects *Tribolium* and *Anopheles gambiae*, *snail* is expressed in the ventral mesoderm during gastrulation, similar to *Drosophila* (Sommer and Tautz 1994; Goltsev et al. 2007). Potential later expression in the nervous system, however, has not yet been examined. In the chelicerate *Cupiennius salei*, *snail* is expressed in the developing nervous system, although the possibility of early mesoderm expression requires further investigation (Weller and Tautz 2003). In the chelicerate *Achaearanea tepidariorum*, *snail* is expressed during gastrulation, but only in ectodermal cells (Yamazaki et al. 2005). *Achaearanea snail* is also expressed in neuroectodermal cells later in development (Yamazaki et al. 2005). In the myriapod *Glomeris marginata*, *snail* is first expressed in presumptive neural precursors and is later expressed in all neural precursors in the ventral neuroectoderm and in the ectodermal midline (Pioro and Stollewerk 2006). In the Branchipod crustacean *Daphnia magna*, *snail* is expressed in neuroblasts in the ventral neuroectoderm (Ungerer et al. 2011). These studies on non-Dipteran insects, chelicerates, myriapods, and Branchiopod crustaceans have found only one *snail* homolog per species, raising the question of whether *snail* duplicates are found only in *Drosophila*. Moreover, since crustaceans are a paraphyletic group with several diverse forms of major taxa, data from more species of crustaceans in necessary to determine the typical number and expression pattern of *snail* family members (Regier et al. 2010). Since crustaceans, together with insects, form the Pancrustacea, analysis in more crustacean species, especially from groups outside Branchipoda, would provide important information for understanding the ancestral role of *snail* in Pancrustacea and thus in the ancestor of all insects as well (Regier et al. 2010).

To expand our knowledge of the *snail* family, we are studying the *snail* superfamily in the Malacostracan crustacean *Parhyale hawaienesis*. *Parhyale* is an excellent model for studying the potential role of the *snail* family in gastrulation and mesoderm formation, as *Parhyale* mesoderm formation has been well characterized. In *Parhyale*, mesodermal fate is restricted to two cells at the eight-cell stage, ml and mr, through a currently unknown mechanism (Gerberding et al. 2002; Price et al. 2010). During blastula stages, ml and mr will go through two to three rounds of division; during gastrulation, these cells will become located under the ectoderm through a combination of their own migration as well as the migration of the overlying ectoderm (Price and Patel 2008). After gastrulation, the progeny of ml and mr will divide to give rise to eight mesoteloblasts, the mesodermal stem cells. These cells will form the trunk segmental mesoderm by undergoing a series of migrations and divisions (Supplementary Movie 1; Browne et al. 2005; Price and Patel 2008).

Here, we characterize the phylogenetic placement, protein sequence motifs and expression patterns of three *snail* homologs and one *scratch* homolog in *Parhyale*. We then discuss how these data add to our understanding of *snail* gene family evolution.

Materials and methods

Cloning *snail* genes and screening the *Parhyale* BAC Library

We generated cDNA from mixed-stage *Parhyale* embryos as previously described (Price and Patel 2008). We cloned one *Parhyale* homolog of *snail* (*Ph-snail1*) using the following degenerate primers: forward primer 5′-ATG GGM YTR WSY AAR CA-3′ and reverse primer 5′-NGC RTA YTT YTT NAC RTC-3′; nested forward primer 5′-GGA GCA YTG AAR ATG CA-3′ and nested reverse primer 5′-RTG NGT TYG YTT RTG NGC-3′; nested forward primer 5′-CAY CAN TTR CCN TGY AAR TG-3′ and nested reverse primer 5′-TGW GCT CKC AGA TTA SWN CKR TC-3′. Additional sequence was obtained using 5′ and 3′ RACE (GeneRacer, Invitrogen). We cloned two more homologs of *snail* (*Ph-snail2* and *Ph-snail3*) and one homolog of *scratch* (*Ph-scratch*) using low stringency 5′-RACE with the following primers: forward primer 5′-AAN GGY TTY TCN CCN GTR TG-3′ and reverse primer GeneRacer-5′-Outer (Invitrogen); nested forward primer 5′-TGN CCY TGN ARN ARC CAN GG-3′ and nested reverse primer GeneRacer-5′-Inner (Invitrogen). Additional sequence was obtained using 3′ RACE (Ambion FirstChoice RLM-RACE kit). New sequences were deposited in GenBank with the following accession numbers: *Ph-snail1* JN858902, *Ph-snail2* JN858903, *Ph-snail3* JN858904, and *Ph-scratch* JN858901. We screened the *Parhyale* BAC library for *Ph-snail1*, *Ph-snail2*, *Ph-snail3*, and *Ph-scratch* as previously described (Parchem et al. 2010).

Phylogenetic analysis

Species included in phylogenetic analysis have both *snail* and *scratch* homologs to provide approximately equal analysis of the two families. In addition, the finding of both *snail* and *scratch* homologs in a species indicates that a thorough search for all *snail* genes had been performed and thus prevents bias from being introduced to the tree in the form of a highly derived version of a *snail* homolog from a species that actually had other, more canonical *snail* genes as well. Sequence data were analyzed with MacClade version 4.05 OSX and PAUP version 4.0b10. The deduced amino-acid sequences were aligned using Clustal X version 1.62b followed by refinement by eye in an effort to maximize potential homology (Supplementary Fig. 1). Only zinc-finger domains were included in this alignment, as other domains (SNAG domain, NT box, and CtBP interaction motif) were not conserved amongst all *snail* genes. Ambiguous alignments and gaps were discarded. The aligned amino acid sequence was subjected to Bayesian inference (BI) based methods of phylogenetic reconstruction. ProtTest version 1.3 was used to estimate the evolutionary model that best fit the amino-acid data set. The Akaike Information Criterion (AIC) implemented in ProtTest selected RtRev + I + G evolutionary model. BI analyses were performed with MrBayes 3.12 by simulating a Metropolis-coupled Markov chain Monte Carlo (MCMCMC) with four simultaneous chains, each of 3 million generations (sampled every 100 generations) under the RtREV + I + G model. Trees sampled before the cold chain reached stationary (as judged by plots of ML scores) were discarded as "burn-in." The resulting BI consensus tree was rooted in the midpoint. Robustness of the resulting BI tree was evaluated using Bayesian posterior probabilities (BPPs).

Antibody production

We generated a polyclonal antibody in rat to an N-terminal portion of Ph-Snail1 protein (aa13–aa352) covering the region of protein from the NT-box through the first two zinc fingers. This N-terminal portion was fused to the product of the TrpE gene using pATH expression vectors (Koerner et al. 1991). Bacterial expression and purification, as well as affinity purification of anti-Ph-Snail serum using a column bound with a His-tagged version of the N-terminal portion of Ph-Snail1, were carried out as described (Patel et al. 1992).

In situ hybridization and antibody staining

We reared and staged *Parhyale* embryos as previously described (Browne et al. 2005). Embryo fixation, antibody staining, and in situ hybridization were performed as previously described (Rehm et al. 2009). Embryos were counterstained with 0.5 μg/ml DAPI in 50 % glycerol and transferred to 70 % glycerol for clearing and mounting. To combine multiple focal planes, Volocity software (confocal images; PerkinElmer) or Helicon Focus software (bright-field images; Helicon Soft Ltd., Kharkov, Ukraine) was used to generate a single, focused image. Segment identification and nomenclature are as previously described (Browne et al. 2005).

Parhyale injection and timelapse microscopy

We reared and staged *Parhyale hawaiensis* embryos as described (Browne et al. 2005). To label mesoteloblasts and progeny, we injected ml and/or mr at the eight-cell stage (Gerberding et al. 2002; Rehm et al. 2009) with RNA or DNA encoding a red fluorescent protein. To label the nucleus of cells, we injected capped mRNA (SP6 Ambion mMessageMachine Kit) encoding a nuclear localized version of DsRed red fluorescent protein (called DsRed-NLS;

Price and Patel 2008). To label the membrane of cells, we injected DNA encoding red fluorescent protein fused to a membrane-localized protein driven by the *P. hawaiensis* EF1α promoter (tandem dimmer Tomato fused to *Drosophila*-moesin; called tdTomato-Moesin). 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 6 or 4-min intervals (Supplementary Movies 1 and 2, respectively).

Results

There are tree *snail* homologs and one *scratch* homolog in *Parhyale*

We recovered three *snail* homologs and one *scratch* homolog in *Parhyale* as characterized by phylogenetic placement (Fig. 1). As we found a separate genetic locus for each homolog in the *Parhyale* BAC library (Supplementary Fig. 2), these homologs are separate genes, versus alleles of a single gene. We named these genes *Ph-snail1* (*Ph-sna1*), *Ph-snail2* (*Ph-sna2*), *Ph-snail3* (*Ph-sna3*), and *Ph-scratch* (*Ph-scrt*). We decided to use the name *snail*, as *snail* is commonly used for other *snail* homologs (Barrallo-Gimeno and Nieto 2009). In addition, we found that the three *Parhyale snail* genes grouped closer to *Drosophila snail* than to either *Drosophila escargot* or *worniu*, or even with the *snail* homolog from the other species of crustacean in our tree, *Daphnia pulex*.

Common Snail family N-terminal sequence motifs

To further characterize *Parhyale snail* family members, we examined them for protein sequence motifs found in other Snail homologs. Although the C-terminal zinc fingers are the most conserved sequence motifs in Snail homologs (Fig. 2; Supplementary Fig. 3), there are some common N-terminal motifs as well (reviewed in Hemavathy et al. 2000; Fig. 2; Supplementary Fig. 4). These motifs are the SNAG domain, NT box and CtBP interaction motif. The SNAG (**Sna**il/**G**fi-1) domain was first discovered in vertebrates and is possibly involved in transcriptional repression. The NT (**N-t**ermini) box was first discovered in the *Drosophila* proteins Snail, Escargot and Worniu, but its function is not yet known. Finally, the CtBP (**C-t**erminal **B**inding **P**rotein) interaction motif was first found in *Drosophila* and facilitates interaction with the co-repressor *Drosophila* C-terminal Binding Protein.

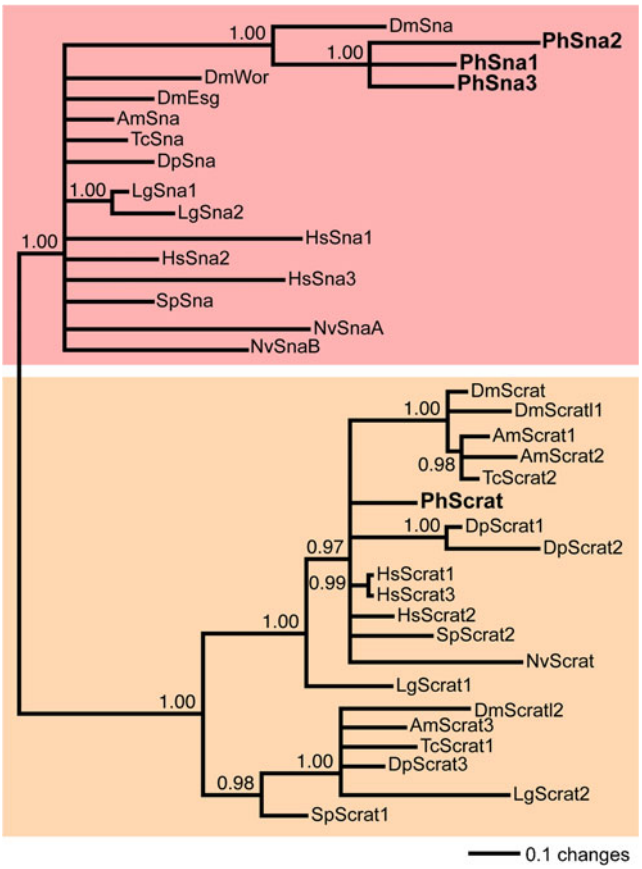

Fig. 1 Phylogenetic relationships among members of the *snail* superfamily. Fifty percent majority rule consensus post-burn in sampled tree from a Bayesian interference analysis of members of the *snail* superfamily (*snail* plus *scratch*). Branch lengths are mean estimates. Numbers in the nodes are Bayesian posterior probabilities (BPP; only BPP≥ 0.95 are shown). The *snail* clade is shaded *red*; the *scratch* clade is shaded *orange*. *Parhyale snail* (*Ph-sna1*, *Ph-sna2*, and *Ph-sna3*) and *scratch* (*Ph-scrat*) genes are *bold*. Abbreviations: *Am Apis mellifera*, *Dm Drosophila melanogaster*, *Dp Daphnia pulex*, *Hs Homo sapiens*, *Lg Lottia gigantea*, *Nv Nematostella vectensis*, *Ph Parhyale hawaiensis*, *Tc Tribolium castaneum*, *Sp Strongylocentrotus purpuratus*, *sna snail*, *wor worniu*, *esg escargot*, *scrat scratch*, *scratl scratch-like*. Sequence sources from GenBank: *Am-sna* XM_393944, *Am-scrat1* XM_001121064, *Am-scrat2* XM_394200, *Am-scrat3* XM_001120596, *Dm-sna* NM_057384, *Dm-esg* NM_057252, *Dm-wor* NM_057253, *Dm-scrat* NM_079187, *Dm-scratl1* NM_206275, *Dm-scratl2* NM_138196, *Hs-sna1* NM_005985, *Hs-sna2* NM_003068, *Hs-sna3* NM_178310, *Hs-scrat1* NM_031309, *Hs-scrat2* NM_033129, *Hs-scrat3* XM_001719366, *Nv-snaA* AY651960, *Nv-snaB* AY465179: *Nv-scrat* XM_001632193, *Sp-sna* NM_214660, *Sp-scrat1* XM_780320, *Sp-scrat2* XM_780886, *Tc-sna* XM_961369, *Tc-scrat1* XM_966236, *Tc-scrat2* XM_964647. Sequence sources from *Daphnia pulex* v1.0: *Dp-sna* Dappu1/scaffold_23:1247838–1249532, *Dp-scrat1* Dappu1/scaffold_110:221444–265987, *Dp-scrat2* Dappu1/scaffold_110:185485–202469, *Dp-scrat3* Dappu1/scaffold_39:946757–955938. Sequence sources from *Lottia gigantea* v1.0: *Lg-sna1* Lotgi1/sca_19:701190–704146, *Lg-sna2* Lotgi1/sca_19:671352–676199, *Lg-scrat1* Lotgi1/sca_56:1248565–1249143, *Lg-scrat2* Lotgi1/sca_56:716397–716972

Although Ph-Sna1 has a SNAG domain, NT box and CtBP interaction motif, Ph-Sna2 and Ph-Sna3 do not have

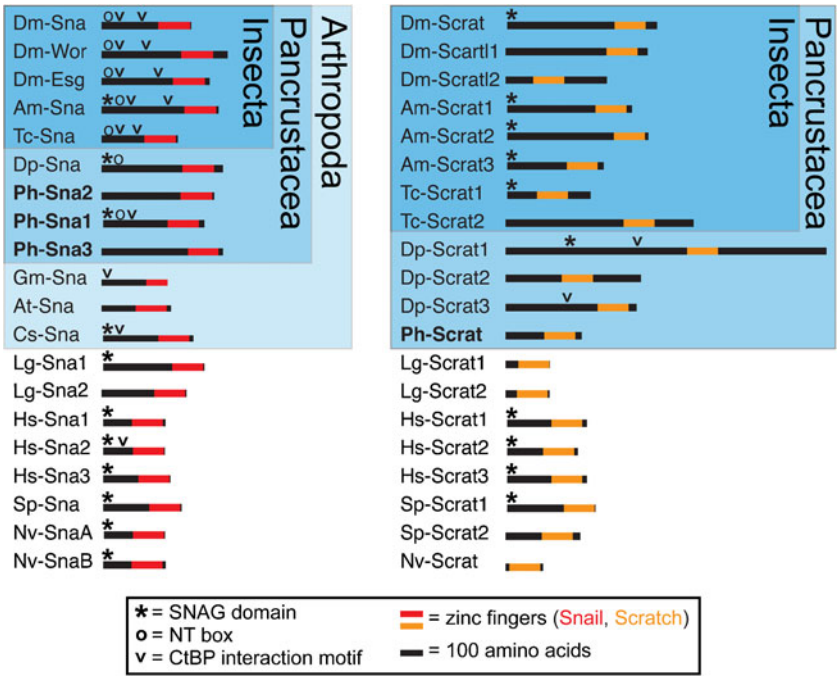

Fig. 2 Comparison of sequence motifs in the *snail* superfamily. All members of the Snail superfamily contain a zinc-finger domain (*red bar* for Snail, *orange bar* for Scratch). While SNAG domains (*asterisk*) are found in many family members, CtBP interaction motifs (*letter v*) are enriched for in arthropod Snail/Scratches and NT boxes (*circle*) are only found in pancrustacean Snail proteins. Due to close spacing of some of these domains, the position of the domains is not to scale. Abbreviations: *Am Apis mellifera*, *At Achaearanea tepidariorum*, *Cs Cupiennius salei*, *Dm Drosophila melanogaster*, *Dp Daphnia pulex*, *Gs Glomeris marginata*, *Hs Homo sapiens*, *Lg Lottia gigantean*, *Nv Nematostella vectensis*, *Ph Parhyale hawaiensis*, *Tc Tribolium castaneum*, *Sp Strongylocentrotus purpuratus*. Only the C terminus of Gs-Snail and Nv-Scrat is depicted/known. For amino acid sequences with domain annotation, see Supplementary Figure 3. *Scale bar*=100 amino acids

any of these motifs (Fig. 2, Supplementary Fig. 4). To investigate how common N-terminal motifs are in Snail homologs, we compared Snail proteins among multiple species, with particular focus on the arthropods (Fig. 2, Supplementary Fig. 4). Arthropod Snail proteins have the most sequence motifs, often having a SNAG domain, NT box, and CtBP interaction motif. The distribution of the SNAG domains amongst the Snail and Scratch proteins suggests that the ancestral bilatarian Snail/Scratch protein had these motifs and that subsequent losses occurred. The NT box is only found in arthropod Snail proteins, suggesting it may have evolved in the ancestral arthropod Snail protein. The CtBP interaction motif is enriched for in arthropod Snail and Scratch proteins. All insect Snail proteins examined have two CtBP interaction motifs (Dm-Snail, Dm-Worniu, Dm-Escargo, Am-Snail, and Tc-Snail). This may be an insect-specific feature, as only one *Parhyale* Snail protein has a single CtBP interaction motif, and CtBP interaction motifs are only found in *Daphnia* Scratch, but not *Daphnia* Snail proteins. In contrast to the arthropods, only one non-arthropod Snail protein examined, *Homo sapiens* Snail2, has a CtBP interaction motif. It will be interesting to see whether the presence of this motif in arthropods and humans is due to shared ancestry or to convergence.

Ph-sna1 is expressed in the mesoderm and neuroectoderm

Since *snail* homologs in insects are expressed during mesoderm specification and gastrulation, we hypothesized that one or all of the *Parhyale snail* homologs may have similar expression (reviewed in Hemavathy et al. 2000; Sommer and Tautz 1994; Goltsev et al. 2007). We determined the temporal and spatial expression pattern of *Ph-sna1*, *Ph-sna2*, *Ph-sna3*, and *Ph-scrt* by in situ hybridization. In addition, we also characterized the protein localization of Ph-Sna1 using a polyclonal antisera generated for this purpose. We examined expression from stage 1 (one cell, 0 h) to stage 23 (142 h), the latest stage where RNA probes and antibodies can be used on whole mount preparations). We will describe each expression pattern one at a time.

Ph-sna1 expression commences at stage 11 (60 h) in neuroectodermal cells in the ocular lobes and in the mesoteloblasts, the stem cells that generate the segmental trunk mesoderm (Fig. 3A, A'). However, the immediate progeny of the mesoteloblasts, the mesoblasts, do not express *Ph-sna1*. We identified mesoteloblasts and mesoblasts by both their morphology and their position in the embryo. Both cell types are located under the ectoderm, which is organized into a grid (Browne et al. 2005). The mesoteloblasts are

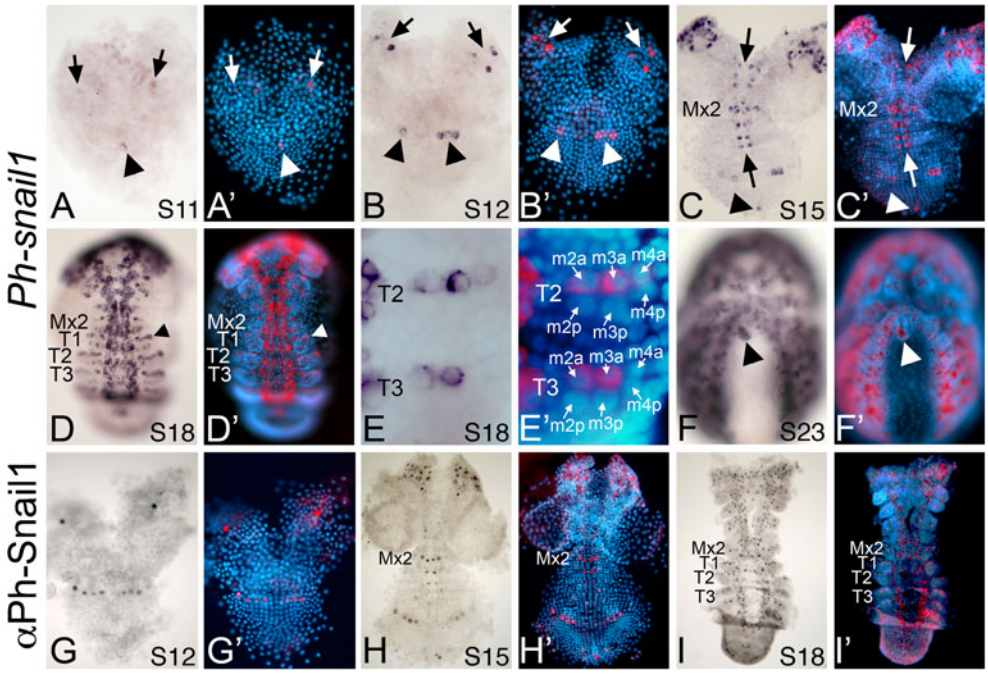

Fig. 3 *Ph-sna1* expression. Embryos stained for *Ph-sna1* mRNA (*A–F'*) and protein (*G–I'*). Ventral views, anterior to the top. False color overlays of *Ph-sna1* mRNA (*red*; *A'*, *B'*, *C'*, *D'*, *E'*, *F'*) and protein (*red*; *G'*, *H'*, *I'*) over nuclear DAPI stain (*blue*). *A*, *A'* Onset of *Ph-sna1* expression at stage 11 (60 h) in neuroectodermal cells in the anterior ocular lobes (*arrows*) and in the mesoteloblasts (*arrowhead*). *B*, *B'* Stage 12 (68 h) embryo. As development progresses, *Ph-sna1* is expressed in a greater number of cells in the ocular lobes (*arrows*) and continues to be expressed in the mesoteloblats (*arrowheads*). *C*, *C'* Onset of *Ph-sna1* expression in the ventral neuroectoderm on either side of the midline (*pair of arrows* point to midline). *Ph-sna1* is expressed in a larger number of cells in the second maxillary segment (Mx2). *Ph-sna1* is also expressed in a single cell that is associated with the proctodeum (*arrowhead*). *D*, *D'* Onset of *Ph-sna1* expression in the segmental mesoderm (*arrowhead* points to expression in the first thoracic segment, T1) at stage 18 (90 h). *Ph-sna1* is also expressed in clusters of ventral neuroectodermal cells that flank the midline. *E*, *E'* Close up of *Ph-sna1* in the segmental mesoderm of the second and third thoracic hemisegments (T2 and T3) of a stage 18 (90 h) embryo. After the mesoblasts divide, *Ph-sna1* is strongly expressed in the anterior daughters of the second and third mesoblasts (m2a and m3a, respectively), and is weakly expressed in the anterior daughter of the fourth mesoblast (m4a; T2 only at this stage). *Ph-sna1* is not expressed in the posterior daughters of the mesoblasts (m2p, m3p, and m4p). *F*, *F' Ph-sna1* is expressed in a number of cells associated with the proctodeum (*arrowhead*) at stage 23 (140 h). *G–I* Ph-Sna1 protein is expressed in the same cells as *Ph-sna1 mRNA*. Ph-Sna1 protein localization at stage 12 (68 h; *G*, *G'*), stage 15 (80 h; *H*, *H'*), and stage 18 (90 h; *I*, *I'*)

distinguished from the mesoblasts both because of their larger size and their position as the most posterior mesodermal cells (Browne et al. 2005). As development progresses, *Ph-sna1* continues to be expressed in the mesoteloblasts and is also expressed in a greater number of cells in the ocular lobes (Fig. 3B, B', G, G'). Around stage 15 (80 h), *Ph-sna1* commences expression in the ventral neuroectoderm and is expressed in a larger number of cells in the second maxillary segment (Mx2; Fig. 3C, C', H, H'). At this time, *Ph-sna1* is also expressed in a posterior cell associated with the proctodeum, the future anus (Fig. 3C, C', H, H'). Around stage 18 (90 h), *Ph-sna1* commences expression in a subset of the progeny of the mesoblasts, the segmental mesoderm that had been generated by the mesoteloblasts (Fig. 3D–E', I, I'). After the first, anterior-posterior, division of the mesoblasts, *Ph-sna1* is strongly expressed in the anterior daughters of the second and third mesoblasts, and weakly expressed in the anterior daughter of the fourth mesoblast. In addition, *Ph-sna1* is now expressed in clusters of ventral neuroectodermal cells that flank the midline. Late in development, around stage 23 (140 h), *Ph-sna1* is expressed in a number of cells associated with the proctodeum (Fig. 3F, F').

Upon closer examination, we discovered that *Ph-sna1* expression in the mesoteloblasts is dynamic, since only a subset of mesoteloblasts express *Ph-sna1* in each embryo, and the subset of *Ph-sna1* expressing mesoteloblasts varies between embryos. Moreover, *Ph-sna1* is expressed in a varying subset of mesoteloblasts at many stages of development, supporting a dynamic expression pattern (Figs. 3 and 4). Since each mesoteloblast does not divide at exactly the same time as either the other mesoteloblasts on the same side or the corresponding mesoteloblast on the other side, we hypothesized that the *Ph-sna1* expression in a subset of the mesoteloblasts at any given time correlates with the mesoteloblast's position in the cell cycle (Supplementary Movie 1; Fig. 4; Browne et al. 2005; Price and Patel 2008). To investigate this, we stained embryos for *Ph-sna1*

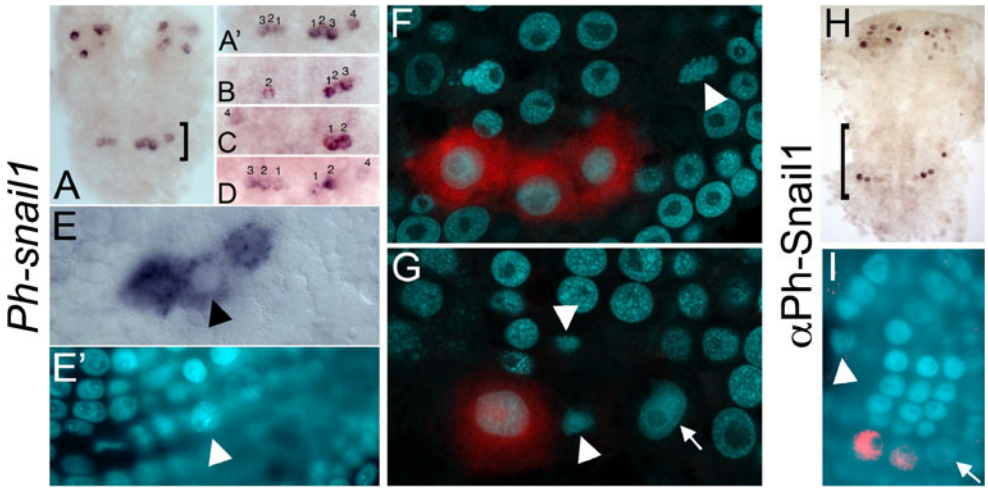

Fig. 4 *Ph-sna1* mRNA and protein cycle in the mesoteloblasts. Ventral view, anterior to the top, of stage 14–17 (77–87 h) embryos stained for *Ph-sna1* mRNA (*A–G*) and protein (*H*, *I*). *A–D* Stage 14 (77 h) embryos displaying cyclical *Ph-sna1* mRNA expression in the mesoteloblasts; mRNA levels differ depending on the position of the cell cycle in a particular mesoteloblasts. *A'–D* Close up of mesoteloblasts in four embryos corresponding to bracketed area in *A*. *A'* is same embryo as *A*. *Numbers* represent position of *Ph-sna1*-expressing mesoteloblast from midline; *1* is closet to midline, *4* is furthest away. *E*, *E'* *Ph-sna1* mRNA (*E*) and corresponding nuclear DAPI stain (*E'*). *Arrowhead* in (*E*, *E'*) points to chromosomes condensing in the second mesoteloblast, indicative of prophase. *Ph-sna1* has cleared from this nucleus and is not actively being transcribed. *F*, *G* Confocal images of *Ph-sna1* expression (*red*) over nuclear DAPI stain (*blue*). *F* The first through third mesoteloblasts are in interphase and express *Ph-sna1*, while the fourth mesoteloblasts is in metaphase and does not express *Ph-sna1* (*arrowhead*). *G* The first mesoteloblast is in interphase and expresses *Ph-sna1*. The second mesoteloblasts is in either anaphase or telophase and does not express *Ph-sna1* (*arrowheads* point to condensed chromosomes of the more posterior mesoteloblast and its more anterior daughter). The third mesoteloblasts is in interphase, but does not express *Ph-sna1* (*arrow*). *H*, *I* Ph-Sna1 protein is also expressed cyclically in the mesoteloblasts. *I* Close up and false color (*red*) overlay of bracketed area in (*H*) over nuclear DAPI stain (*blue*). *Arrowhead* points to condensing chromosomes in the fourth mesoteloblast, indicative of prophase; this mesoteloblast does not express Ph-Sna1. *Arrow* points to an interphase nucleus in the first mesoteloblasts. Unlike the second and third mesoteloblasts, which are also in interphase, the first mesoteloblast does not express Ph-Sna1

mRNA or protein, as well as the DNA stain DAPI, and compared expression levels to chromosome arrangements (Table 1). We found that 24/24 mesoteloblasts expressing Ph-Sna1 protein and 103/104 mesoteloblasts expressing *Ph-sna1* mRNA were in interphase (Table 1). However, there was one mesoteloblast that expressed *Ph-sna1* mRNA that was not in interphase, but was in the beginning of prophase (Fig. 4E, E'). Unlike the other *Ph-sna1* expressing mesoteloblasts, *Ph-sna1* transcripts had cleared from this nucleus. While we observed active transcription and protein expression of *Ph-sna1* only during interphase, we also observed that 44/68 and 131/234 mesoteloblasts in interphase did not express *Ph-sna1* protein and mRNA, respectively (Table 1). In summary, *Ph-sna1* mRNA is only transcribed during a subset of interphase, and mRNA and protein are only detected during a subset of interphase and the beginning of prophase.

Table 1 *Ph-sna1* mRNA and protein expression at different stages of the cell cycle

	Protein+/Total	mRNA+/Total
Interphase	24/68	103/234
Prophase	0/7	1/9
Metaphase	0/1	0/4
Anaphase + Telophase	0/4	0/9

Three hundred thirty-six mesoteloblasts (in 42 embryos) were examined for *Ph-sna1* protein (10 embryos) or mRNA (32 embryos) expression as well as cell cycle stage (determined by chromosome state via DAPI staining). Cell cycle stages are defined as follows: interphase (uncondensed chromosomes), prophase (chromosomes condensing), metaphase (chromosomes aligned in the middle of the mesoteloblast), anaphase, and telophase (chromosomes separating/separated but still condensed). "*x*/*y*" values represent the number of mesoteloblasts examined that express *Ph-sna1* protein or mRNA out of the total number of mesoteloblasts examined for *Ph-sna1* protein or mRNA expression at that stage in the cell cycle

To further investigate the relationship between *Ph-sna1* expression and mesoteloblast behavior, we compared expression data to movies we made of mesoteloblast cell shape change and migration (Fig. 5, Supplementary Movie 2). During interphase, the mesoteloblasts produce filopodia and actively migrate along the ectoderm (Fig. 5, Supplementary Movie 1; Hannibal et al. 2012). Right before division, the mesoteloblasts stop migrating and retract their filopodia (Fig. 5, Supplementary Movie 2). The mesoteloblasts go through repeated cycles of filopodia-associated migration followed by division. As *Ph-sna1* is expressed during interphase, when the mesoteloblasts use filopodia to

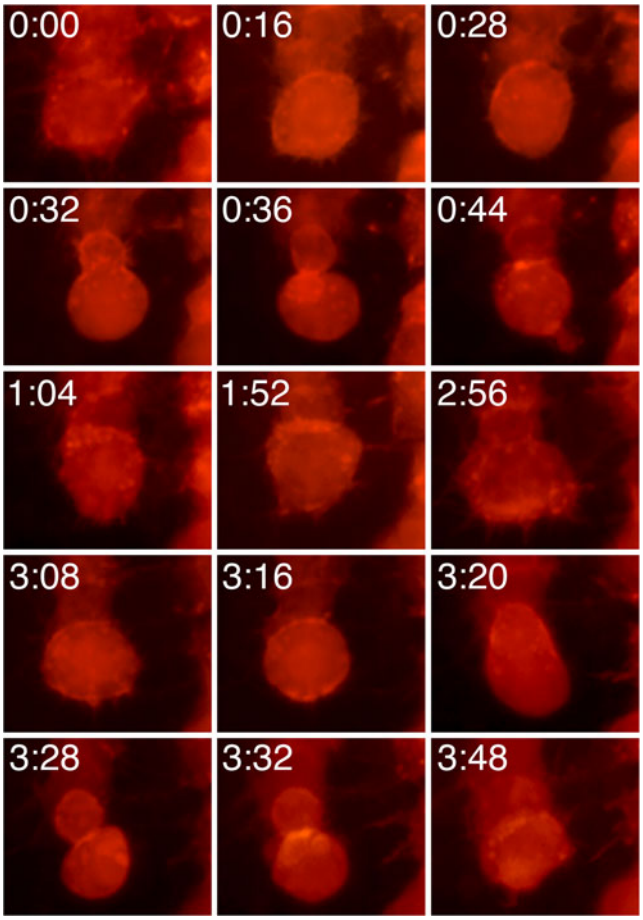

Fig. 5 Mesoteloblast display dynamic cell shape changes. Still images of the most lateral right mesoteloblast (M4), a typical mesoteloblast, from a timelapse movie (Supplementary Movie 1). The cell membranes of M4 and progeny were labeled by injecting the mesodermal progenitor cell mr with tdTomato-Moesin DNA (for details, see the "Materials and methods" section). Ventral view; anterior (the most recent daughter of M4) is to the *top*, ventral midline (and rest of mesoteloblasts and mesoblasts) is to the *right* (not shown). Time stamps (hours–minutes) are in the *upper left*. When M4 is not dividing, it produces filopodia that contact both the other mesodermal cells and the ectoderm. Before and during division, M4 retracts filopodia. This behavior is typical of all mesoteloblasts

migrate, *Ph-sna1* may be involved in mesoteloblast migration. Our data are also consistent with a role for *Ph-sna1* in mesoteloblast specification and/or mesoteloblast cell-cycle progression.

Ph-sna2 and *Ph-sna3* are expressed in neuroectoderm

Ph-sna2 and *Ph-sna3* are expressed in the neuroectoderm. *Ph-sna2* is first expressed around stage 11 (63 h; Fig. 6A, A'). It is strongly expressed in the ocular lobes and in some of the cells in the first two columns of neuroectodermal cells on either side of the midline. It is also weakly expressed in most other neuroectodermal cells, except for the midline. As development progresses, *Ph-sna2* continues to be expressed strongly in cells adjacent to the midline (Fig. 6B–D). The stronger expression of *Ph-sna2* in cells adjacent to the midline appears to be caused both by higher expression in those cells as well as a greater density of cells due to cell division. Around stage 15 (80 h), *Ph-sna2* expression clears from non-neural ectodermal cells (Fig. 6E, E'). *Ph-sna2* is still expressed in high levels in neuroectodermal cells adjacent to the midline and in the ocular lobes. Additionally, around stage 19 (96 h), *Ph-sna2* expression commences in clusters adjacent to the limb buds (Fig. 6F, F'). The position of these clusters adjacent to, but not in the limb buds, is particularly striking by stage 20 (108 h; Fig. 6G–H').

Ph-sna3 is first expressed in the ocular lobes around stage 14 (77 h; Fig. 7A, A'). As development progresses, *Ph-sna3* is also expressed in the ventral neuroectoderm of each segment, beginning just before morphological limb buds appear (Fig. 7B, B', C, C'). Expression becomes stronger in these cells, particularly in the ocular lobes, by stage 19 (96 h; Fig. 7D, D'). Late in development, around stage 20 (108 h), *Ph-sna3* is also expressed in one cell in each limb (Fig. 7E, E', F, F').

Ph-scratch is not expressed during embryogenesis

Although we performed in situ hybridizations with probes to multiple regions of *Ph-scrt*, none of them yielded an expression pattern (data not shown). *Ph-scrt* could be expressed late in embryonic development, after the formation of cuticle prevents us from detecting transcripts by in situ hybridization.

Discussion

Parhyale snail superfamily

Our phylogenetic analysis suggests that, of the four *Parhyale snail* superfamily members, three are members of the *snail* family (*Ph-sna1*, *Ph-sna2*, and *Ph-sna3*) and one is a member of the *scratch* family (*Ph-scrt*). There is strong support for the reciprocal monophyly of *snail* and *scatch* families (BPP; 1.00 each for the *snail* and *scratch* family; Fig. 1). The similarity of this phylogeny to that of other recent *snail* superfamily trees supports our classification of the *Parhyale* genes within these two families (Barrallo-Gimeno and Nieto 2009; Kerner et al. 2009). Surprisingly, we found that the three *Parhyale snail* genes group closer to *Drosophila snail* than to *Drosophila escargot, worniu*, or the *snail* gene from the crustacean *D. pulex*. This suggests that the *Parhyale* and *Drosophila snail* genes are either both more similar to the ancestral Pancrustacean

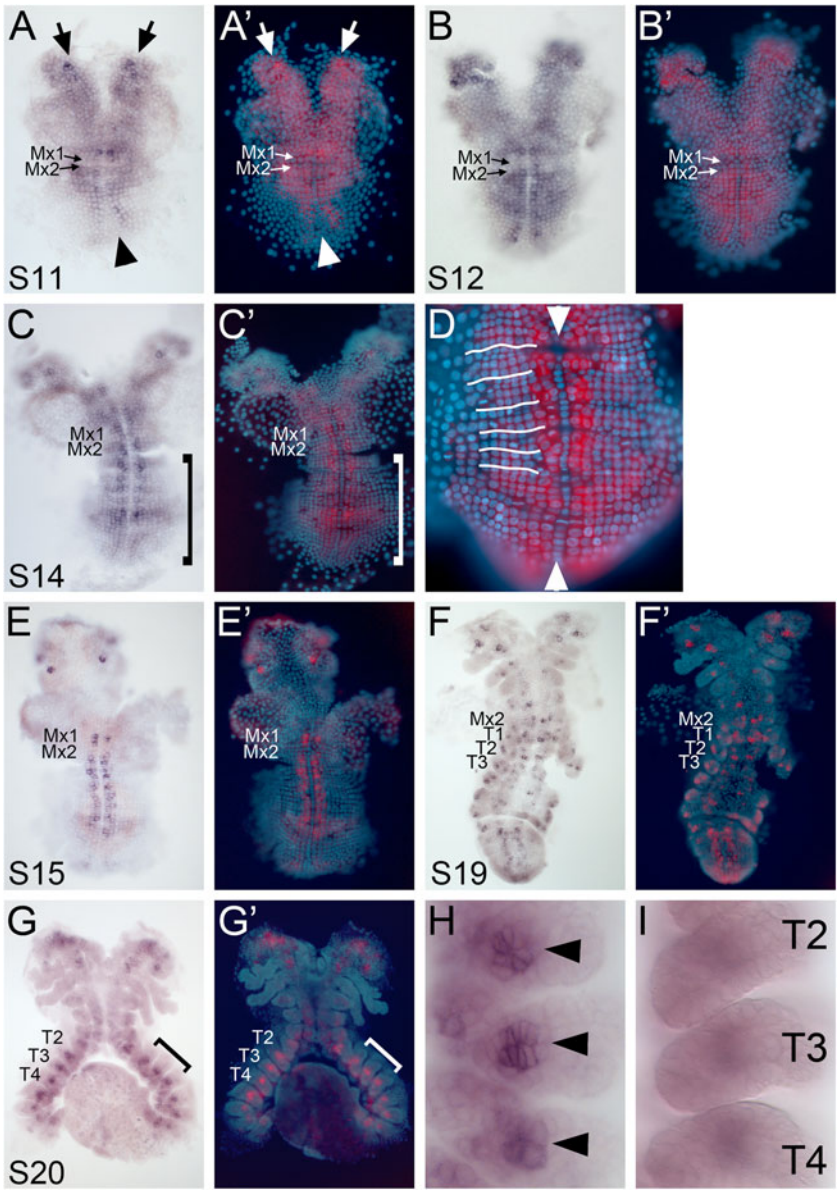

Fig. 6 *Ph-sna2* expression. Embryos stained for *Ph-sna2* mRNA. Ventral view, anterior to the top. *A'*, *B'*, *C*, *D*, *E'*, *F'*, *G'* False color overlays of *Ph-sna2* (*red*) over DAPI stained nuclei (*blue*). *A*, *A'* Onset of *Ph-sna2* expression around stage 11 (63 h). *Ph-sna2* is expressed strongly in the neuroectoderm surrounding the ventral midline (*arrowhead* points to midline) as well as in the ocular lobes (*arrows*). *Ph-sna2* is expressed weakly in the progeny of neuroectodermal parasegment precursor rows further from the midline. *Ph-sna2* is also expressed weakly in the first and second maxillary segments (Mx1 and Mx2). *B*, *B' Ph-sna2* expression at stage 12 (64 h). *C*, *C' Ph-sna2* expression at stage 14 (77 h). *D* Close up of bracketed area from (*C*, *C'*) from a similarly staged embryos (stage 14; 77 h). *Arrowheads* indicated ventral midline. *Lines* indicate parasegmental boundaries. *E*, *E'* Around stage 15 (80 h), *Ph-sna2* begins to clear from the overlying neuroectoderm but is now strongly expressed in neuroblasts and progeny in Mx1 and more posterior segments. *F*, *F' Ph-sna2* expression commences in clusters near the limbs around stage 19 (96 h). *G–H' Ph-sna2* expression in clusters of cells near the limbs in a stage 20 (108 h) embryo. *H* Close up of *Ph-sna2* expression near the base of limbs in the second through fourth thoracic segments (T2–T4; *arrowheads*) from bracketed area in (*G*, *G'*). *I* Same area as in (*H*), but now focused on a medial section through the limbs

snail or may have a conserved function that has constrained the evolution of their zinc-finger domain, which we used to build our phylogeny.

Our phylogenetic analysis also suggests that the three *Parhyale snail* genes (*Ph-sna1*, *Ph-sna2*, and *Ph-sna3*) are more closely related to each other than to copies of the *snail* gene in other species (BPP 1.00; Fig. 1). This implies that they arose from duplication events in the lineage leading to *Parhyale*, and did not arise from duplication events in the ancestral arthropod. In support of this, only one *snail* gene has been found in the crustaceans *D. magna* and *D. pulex* (Kerner et al. 2009; Ungerer et al. 2011). Independent

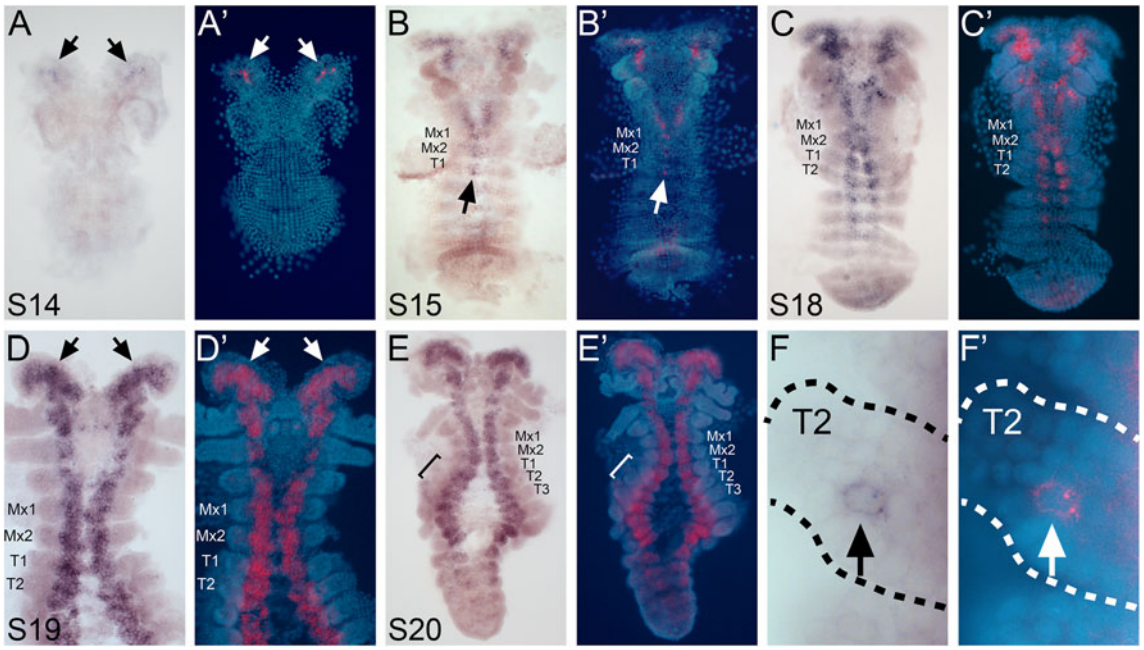

Fig. 7 *Ph-sna3* expression. Embryos stained for *Ph-sna3* mRNA. Ventral view, anterior to the top. *A'*, *B'*, *C'*, *D'*, *E'*, *F'* False color overlays of *Ph-sna3* (*red*) over DAPI stained nuclei (*blue*). *A*, *A'* Onset of *Ph-sna3* expression in the ocular lobes (*arrows*) around stage 14 (77 h). *B–C' Ph-sna3* expression at stages 15 (80 h; *B*, *B'*) and 18 (90 h; *C*, *C'*). *Ph-sna3* continues to be expressed in the ocular lobes, and begins to be expressed in the ventral neuroectoderm. *Arrow* in (*B*) points to *Ph-sna3* expression on one side of the embryo slightly before expression commences on the other side. This is due to the two sides of this particular embryo being slightly out of sync with each other. (*D*, *D'*) Close up of anterior region of stage 19 (96 h) embryo of *Ph-sna3* expression in the ocular lobes (*arrows*) and ventral neuroectoderm. *E–F'* Stage 20 (108 h) embryos. *F*, *F'* is a close-up of the second thoracic segment (T2; bracketed area in (*E*, *E'*)), illustrating *Ph-sna3* expression in a limb cell (*arrow*; limb outlined with *dashed line*)

duplications in *Parhyale* are also consistent with evidence from other *snail* superfamily trees that suggest that *snail* also independently duplicated in *Drosophila* (Barrallo-Gimeno and Nieto 2009; Kerner et al. 2009). This scenario in arthropods, where *snail* underwent independent duplications in various lineages, is different from tetrapods, where a single *snail* gene duplicated in the ancestral tetrapod, and two copies have subsequently been maintained in many modern taxa (reviewed in Barrallo-Gimeno and Nieto 2005).

Although our data are consistent with the independent duplication of *snail* in *Parhyale*, an alternative scenario is that, especially if the duplicates remained near each other after duplication, their sequences could have been homogenized through gene conversion and thus would not have evolved independently. This has been shown for the *engrailed* gene family in hexapods (Peel et al. 2006). By finding and analyzing additional *engrailed* family members in six species of hexapods, Peel et al. (2006) suggest that the *Drosophila engrailed* and *invected* genes have been in a conserved gene cassette throughout insect evolution. Therefore, *engrailed* and *invected* could have evolved together in each lineage, and thus be erroneously interpreted in phylogenetic trees that support independent duplications (Peel et al. 2006). In support of concerted evolution of the *Parhyale snail* genes, *Ph-sna1* and *Ph-sna3* are found on the same BAC, although more data are necessary to find the location of *Ph-sna2* relative to the other *snail* genes (Supplementary Fig. 2). In addition, since many arthropod *snail* genes were found via degenerate pcr, analysis of whole genomes may find more *snail* genes and therefore more evidence for/against independent duplications of *snail* in the arthropods.

Parhyale snail genes in the neuroectoderm

All of the *Parhyale snail* genes are expressed in the ventral neuroectoderm, suggesting that they play a role in neurogenesis. As *Ph-sna1* and *Ph-sna2* are expressed much earlier than *Ph-sna3*, *Ph-sna1* and *Ph-sna2* are likely expressed in the neuroblasts, the neural stem cells, while *Ph-sna3* is likely expressed in a later stage of neuron development. Furthermore, as *Ph-sna1* is expressed in more cells than *Ph-sna2*, *Ph-sna1* may be involved in neuroblast formation while *Ph-sna2* may be involved in the specification of specific neuroblasts. These patterns of expression in the ventral neuroectoderm are similar to *snail* expression in other arthropods. *Snail* homologs are expressed in the

ventral neuroectoderm during neurogenesis in the insects *Drosophila melanogaster*, the Branchiopod crustacean *D. magna*, the chelicerates *C. salei* and *A. tepidariorum*, and in the myriapod *G. marginata* (Ashraf et al. 1999; Pioro and Stollewerk 2006; Ungerer et al. 2011; Weller and Tautz 2003; Yamazaki et al. 2005). Further characterization of the specific neuronal cells that express *snail* as well as functional characterization of *snail* genes in these species will be crucial for determining how conserved the role of *snail* is in arthropod neurogenesis.

In crustaceans, *snail* homologs have been found in *Parhyale* (this study), *D. magna* and *D. pulex* (Kerner et al. 2009; Ungerer et al. 2011). As *Parhyale* and *Daphnia* belong to two different major groups of crustaceans, the Malacostracans and Branchipods, respectively, *snail* homologs could differ substantially between these groups. In *Parhyale*, we found three *snail* homologs, while only one *snail* homolog was found in each species of *Daphnia* (Kerner et al. 2009; Ungerer et al. 2011). In addition, in *D. magna*, where *snail* expression was examined, *snail* is strictly ectodermal, versus both ectodermal and mesodermal expression of *Ph-sna1*. However, *Ph-sna1* ventral neuroectermal expression is strikingly similar to *D. magna snail* (*Dam sna*) expression. Both begin to be expressed in the ventral neuroectoderm before limb bud formation and are later expressed in clusters of cells flanking the midline (Ungerer et al. 2011). Unlike *Ph-sna1*, however, *Dam sna* is also expressed in rows of ectodermal cells perpendicular to the midline coinciding with where the intersegmental furrows will form (Ungerer et al. 2011). Instead, *Ph-sna1* is expressed in a subset of the mesoderm, including in rows of mesodermal cells in each segment. It will be interesting to examine *snail* genes in crustacean species outside of Malacostracans and Branchiopods, in order to determine whether duplicated *snail* homologs and mesodermal expression exist outside of the Malacostracans.

The expression of *Ph-sna2* next to, but not in the midline, is particularly interesting since the *Drosophila snail* gene is also expressed adjacent to midline precursor cells (Kasai et al. 1992). In *Drosophila*, *snail* represses *single-minded* (*sim*), a gene expressed in the cells that will eventually form the midline. Similarly, *Parhyale single-minded* (*Ph-sim*) is expressed in the *Parhyale* midline (Vargas-Vila et al. 2010). Moreover, *Ph-sim* and *Ph-sna2* commence expression around the same time, at stage 11 (63 h; Vargas-Vila et al. 2010). Together, these data suggest that *Ph-sna2* may suppress *Ph-sim*.

Mesodermal expression of *snail*

Snail family members are expressed and function during mesoderm specification and gastrulation in insects (reviewed in Hemavathy et al. 2000; Sommer and Tautz 1994; Goltsev et al. 2007). To investigate if this expression was found only in insects, or in both insects and the crustaceans (the Pancrustacea), we examined the *snail* family in *Parhyale*. We did not find expression patterns for any *Parhyale snail* family member in the early mesoderm and/or during gastrulation, although *Ph-sna1* is expressed in mesoderm after gastrulation. One possibility is that there are yet undiscovered *Parhyale snail* family members. The discovery of *Ph-scrt*, which is divergent from the *Parhyale snail* homologs, suggests that the degenerate primers were sufficiently lenient to find all *snail* specific homologs. A more likely scenario is that the lack of *Parhayle snail* expression during early mesoderm formation and gastrulation may be related to developmental differences in *Parhyale* and insects. In *Parhyale*, mesodermal fate is restricted to two micromeres, ml and mr, at the eight-cell stage through a currently unknown mechanism (Price et al. 2010). During blastula and gastrula stages, progeny of ml and mr will become located under the ectoderm (Price and Patel 2008). After gastrulation, the progeny of ml and mr will divide to give rise to the mesoteloblasts, which will form the trunk segmental mesoderm by undergoing a series of migrations and divisions (Browne et al. 2005; Price and Patel 2008). It is only when the mesoteloblasts have formed that *Ph-sna1* is first expressed in the mesoderm. In contrast to *Parhyale*, *Drosophila* mesoderm specification and gastrulation are tightly linked (reviewed in Hemavathy et al. 2000). Ventral *snail* expression is crucial for the migration of cells into the interior of the embryo to form the mesoderm. In *Drosophila snail* mutants, ventral cells no longer invaginate (Alberga et al. 1991; Kosman et al. 1991). Moreover, these ventral cells now take on neuroectodermal fates, indicating that *snail* is also crucial for mesoderm specification (Ip et al. 1992; Kosman et al. 1991; Leptin 1991). *Snail* is also expressed in ventral mesoderm of other insects, *Tribolium* and *Anopheles*, that gastrulate similarly to *Drosophila* (Sommer and Tautz 1994; Goltsev et al. 2007).

There are two scenarios that could explain the different mesodermal *snail* expression observed between *Parhyale* and insects. First, these differences could be due to heterochrony. In the Pancrustacean ancestor, *snail* could have been expressed in the mesoderm only after gastrulation. In the lineage leading to insects, *snail* could have then become precociously expressed in the mesoderm during gastrulation. As insects display a derived form of gastrulation and mesoderm formation, it is plausible that the *snail* expression during gastrulation is also a derived character (Sander 1976). Second, mesodermal *snail* expression in *Parhyale* and insects could be due to convergence. In support of this, many *snail* genes have been co-opted to function in tissues with migratory characteristics, such as in metastasizing cancers and neural crest cells (reviewed in Barrallo-Gimeno and Nieto 2005). The formation of these tissues involves an

epithelial to mesenchymal transition, where epithelial cells detach from their neighbors and gain mesenchymal characteristics that enable them to migrate (reviewed in Barrallo-Gimeno and Nieto 2005). As both the gastrulating mesoderm in insects and the mesoteloblasts in *Parhyale* are migratory cells, it is probable that *snail* could have been co-opted in both these cases. Therefore, the ancestral arthropod *snail* could have functioned in nervous system development and later could have been independently co-opted for disparate roles in mesoderm development in different arthropod lineages.

Cycling of *Ph-sna1* in the mesodermal stem cells

We found that *Ph-sna1* cycles in the mesoteloblasts, which produce the segmental mesoderm. *Ph-sna1* mRNA and protein are expressed while the mesoteloblasts are actively migrating. *Ph-sna1* is not expressed during cell division or in the direct progeny of the mesoteloblasts. This cyclical pattern suggests that *Ph-sna1* could be important for the mesoteloblast migratory phase. Supporting this hypothesis, functional experiments have shown that *snail* drives migratory behavior of mesodermal cells in *Drosophila* (Alberga et al. 1991; Kosman et al. 1991). However, the lack of *Ph-sna1* expression during cell division could also be due to an incompatibility of cell division with *Ph-sna1* expression, as mammalian Snail has previously been described to block the cell cycle in G0/G1 phase (Vega et al. 2004). Finally, *Ph-sna1* could be important for mesoteloblast specification, and its cyclical expression could be a biproduct of a short half-life combined with a brief transcriptional block during cell division.

The close association between the cyclical pattern of *Ph-sna1* expression and the cell cycle of the mesodermal stem cells suggests that *Ph-sna1* could be important for the production of the segmental mesoderm. Interestingly, *snail* also cycles during mesoderm segmentation in vertebrates (Dale et al. 2006). *Snail1* and *Snail2* cycle in the mouse and chick presomitic mesoderm, respectively, and are important for coordinating molecular and morphological segmentation (Dale et al. 2006). However, the relationship between *snail* cycling and the production of segments differs between *Parhyale* and vertebrates. In *Parhyale*, *Ph-sna1* cyclical expression appears to be closely correlative with the cell cycle, as one segment's worth of mesoderm is formed from one row of mesoteloblast progeny. To produce one segment's worth of mesoderm, the mesoteloblasts divide once to give rise to the mesoblasts, whose progeny will populate one segment (Browne et al. 2005; Price and Patel 2008). Therefore, each mesoteloblast cell cycle will produce one segment's worth of mesoderm. In vertebrates, however, *Snail* cycling is not linked to the cell cycle, as the cell cycle is much longer than the periodicity of gene cycling (Dale et al. 2006; Palmeirim et al. 1997). In addition, in vertebrates, unlike in *Parhyale*, a region containing many cells is sectioned off to form one segment's worth of mesoderm (Pourquié 2011). In both *Parhyale* and vertebrates, there may be a general feature of *snail*, such as an autorepressive ability or a short half-life, that make it particularly amenable to cyclical behavior.

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