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# Hunchback-like Protein Is Expressed in Cleavage Blastomeres, Gastrula Epithelium, and Ciliary Structures in Gastropods

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**Abstract.** We report the expression of Hunchback (Hb)-like protein during embryonic and larval development in two caenogastropods, *Crepidula fornicata* and *Ilyanassa obsoleta*. During the cleavage stages of these species, Hb-like protein is uniformly expressed in micromere and macromere nuclei. At gastrulation, gastropod Hb-like protein is expressed in the surface epithelium that undergoes epiboly. During organogenesis, gastropod Hb-like protein is expressed in the developing ciliated structures associated with feeding and locomotion. We find no detectable gradient or regionalization of Hb-like protein in gastropod embryos or larvae that resembles the graded Hb pattern of expression observed in dipteran insect embryos. Rather we found that the spatiotemporal expression profile of gastropod Hb-like protein is nearly identical to the Hb-like patterns obtained from the polychaete *Capitella* sp. I and is highly similar to those reported for clitellate annelids. Based upon the comparative data collected from both ecdysozoans and lophotrochozoan lineages, our results support the hypothesis that the role of Hb in anteroposterior patterning is a derived trait specific to arthropods, and that the ancestral function of lophotrochozoan Hb-like protein played a role in the formation of the cleavage-stage blastomeres and the gastrula epithelium and in structures associated with larval feeding and locomotion.

## Introduction

One approach to understanding the basis of evolutionary change is to draw inferences from comparative sequence information, protein domain structure, gene expression studies, and functional data. As more regulatory gene products from diverse taxa are characterized at these levels, insights into the developmental mechanisms that underlie morphological change are being revealed. Hunchback (Hb) protein belongs to a subfamily of the $C_2H_2$ zinc finger family that has been characterized in three protostome phyla: annelids, arthropods, and nematodes (Fay *et al*., 1999; Patel *et al.,* 2001; Werbrock *et al.,* 2001; Schröder, 2003; Liu and Kaufman, 2004; Kontarakis *et al.*, 2006; Kerner *et al*., 2006). These comparative expression studies indicate that Hb and its protostome homologs exhibit both highly conserved and divergent pattern elements, making the Hb protein family an ideal candidate for studying the evolution of gene function and novelty.

The initial interest in characterizing *hunchback* (*hb*) gene products in protostomes arose from *Hb*'s role as a gap-class segmentation gene in anteroposterior (AP) patterning in dipteran embryos (Lehman and Nüsslein-Volhard, 1987; Tautz *et al.,* 1987; Tautz, 1988). In *Drosophila*, Hb is expressed in an anterior-to-posterior gradient and regulates the zygotic gene expression of members of the segmentation gene hierarchy and *Hox* genes (Struhl *et al.,* 1992; Yu and Small, 2008; Papatsenko and Levine, 2008; Marques-Souza *et al.,* 2008). The classic *hunchback* gap null phenotype results in a loss of several adjacent segments that normally would generate head- and thorax-specific structures (Lehman and Nüsslein-Volhard, 1987; Tautz, 1988). Recently the role of Hb in segmental patterning in insects has been refined to focus more on its overall function and less on the loss of contiguous segments (Marques-Souza *et al.,* 2008). However, the "gap" phenotype appears to be restricted to long-germ-band insects, and what appears to be shared between *Drosophila* and more basal groups is the role of Hb in specifying the boundaries of *Hox* gene expression and the interactions of the gene's expression with other gap gene

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products (Lui and Kaufman, 2004; He *et al.,* 2006; Pultz *et al.,* 2005; Mito *et al.,* 2005; Marques-Souza *et al.,* 2008).

Hb-like expression patterns obtained from other protostome lineages have suggested that the gap function may be restricted to arthropods. In annelids, Hb-like protein has been characterized in diverse lineages in terms of their life history and reproductive strategies (Werbrock *et al.,* 2001; Shimizu and Savage; 2002; Kerner *et al.,* 2006; Pinnell *et al.,* 2006). Hb-like protein is expressed maternally, in blastomeres, in the gastrula epithelium, and in the nervous system of polychaetes and clitellates (Pinnell *et al.,* 2006). It is also expressed in lineage-specific patterns such as the larval ciliary structures associated with feeding and movement in polychaetes (Werbrock *et al.,* 2001) and in epidermal structures associated with the rear sucker in leeches (Savage and Shankland, 1996). At no time are *hunchback*-like gene products restricted to an anteriorly localized gradient within the developing adult segmental tissue. The comparative data in annelids support the hypothesis that the role of *Hunchback* as an anterior organizing segmentation gene, as described in *Drosophila,* is a novel trait specific to arthropods and may be restricted further to insects in view of the expression data obtained from non-insect arthropods (Kontarakis *et al.,* 2006; Chipman and Stollewerk, 2006).

To further elucidate the evolutionary history of the *hunchback*-like gene products in metazoans, this paper provides Hb-like expression data from a key member of the lophotrochozoan super phylum, the molluscs. We used a polyclonal antibody that recognizes Hb-like protein in *Ilyanassa obsoleta* and *Crepidula fornicata*, two members of the most diverse and poorly resolved gastropod group living today, the Caenogastropoda (Bieler, 1992). The spatiotemporal expression pattern elements of Hb-like protein from early cleavage to veliger larval stages are described for these two gastropod genera. A comparison to annelid Hb-like expression patterns confirms the presence of a shared set of restricted pattern elements that include expression in cleavage-stage blastomeres, in the gastrula epithelium, and in the larval structures associated with feeding and locomotion. These data support the hypothesis that Hb-like protein has a conserved function in gastropods and annelids but does not play a role in AP pattern formation similar to that of Hb function in flies.

## Material and Methods

### *Embryos*

Adult snails were obtained from the Marine Resources Center (Marine Biological Laboratory, Woods Hole, MA) in June and July. Gravid *Crepidula fornicata* individuals were separated using an oyster knife. Embryos were released from their egg capsules with forceps in glass petri dishes filled with filtered seawater and reared at room temperature. Adults of *Ilyanassa obsoleta* were kept in running natural seawater at room temperature and fed mussel meat every 1–2 days. Egg capsules were scraped off the side of the aquarium with a razor blade and simultaneously collected using a plastic pipet. Capsules were opened using watchmaker forceps, and embryos were gently released into a glass petri dish filled with filtered seawater.

### *Molecular phylogeny*

Bayesian methods (MrBayes ver. 3.1.2; Ronquist and Huelsenbeck, 2003) were used to construct a molecular phylogenetic tree based on the comparison of 180 highly conserved amino acid residues that included the most highly conserved middle finger DNA-binding zinc finger domains. The amino acids of each zinc finger motif were aligned in CLUSTALW ver. 4 (Tamura *et al.,* 2007). The analysis was performed with two simultaneous runs with parameters set as follows: 4 chains, 4,000,000 generations, sampling every 1000 generations, and a burn-in of 40,000 generations, under a mixed model of evolution. The average standard deviation of split frequencies was used to determine when to terminate the phylogenetic analyses. The accession numbers are as follows: *Helobdella robusta* Hb (AAY43810), *Capitella* sp. I Hb (AAY43811), *Capitella* sp. I Ikaros (JGI protein ID 199858), Leech Zinc Finger 2 (*Helobdella triserialis* Hb also known as LZF2: CAA62741), *Platynereis dumerilii* Hb (AM232683), *Drosophila melanogaster* Hb (AAF54270), *Drosophila sechellia* Hb (CAA06504) *Euscelis plebejus* Hb (AAA29120), *Calliphora erythrocephala* Hb (L01591), *Clogmia albipunctata* Hb (CAA10281), *Bombyx mori* Hb (AAM34284), *Apis mellifera* Hb (XP393692), *Caenorhabditis elegans* Hbl-1 (AP063235), *Caenorhabditis elegans* Aiolos (NP001024851), *Bythnia tentaculata* Hb (P31505), *Artemia franciscana* Hb (AM055593), *Chaetopterus* sp. (AAK06713), *Lottia gigantea* Hb (JGI protein ID 167538), *Gallus gallus* Ikaros 1 (NM205088), *Gallus gallus* Aiolos (NM204607), *Gallus gallus* Helios (NP989938), *Gallus gallus* Ikaros 2 (001026766), *Xenopus tropicalis* Ikaros (AAH89243), *Ciona intestinalis* Ikaros (XP002121167), *Xenopus tropicalis* Ikaros 2 (001116930), *Homo sapien* Ikaros (NM006060), *Homo sapien* Eos (NP071910), *Homo sapien* Helios (NP057344), *Homo sapien* Aiolos (NP036613), *Danio rerio* Ikaros 2 (XP001921973), *Danio rerio* Ikaros 1 (AAL11909), and *Ilyanassa obsoleta* Hb (EST sequence provided by D. Lambert at University of Rochester). Taxonomic information and accession numbers can also be viewed in Table 1. The *Crepidula fornicata hb* sequence has not been identified and therefore is not included in the phylogenetic analysis or in the sequence comparisons.

### *Immunostaining and imaging of gastropod embryos*

Iwasa and colleagues (2000) provide a detailed description of the generation of the LZF2 polyclonal antibody that is similar to the methodology used to create the cross-

**Table 1**

*Taxonomic information and sources of amino acid sequence for Hb-like and Ikaros zinc finger protein families*

| Species/Protein | Accession Number/Source |
|---|---|
| ANNELID | |
| Polychaete | |
| *Platynereis dumerilii* Hb | AM232683 |
| *Capitella* sp I Hb | AAY43811 |
| *Chaetopterus* Hb | AAK06713 |
| *Capitella* sp I Ikaros | JGI protein ID 199858 |
| Clitellate | |
| *Helobdella triserialis* Hb (LZF2) | CAA62741 |
| *Helobdella robusta* Hb | AAY43810 |
| MOLLUSC | |
| Gastropod | |
| *Lottia gigantea* Hb | JGI protein ID 167538 |
| *Bythnia tentaculata* Hb | P31505 |
| *Ilyanassa obsoleta* Hb | Sequence provided by D. Lambert (University of Rochester) |
| ARTHROPOD | |
| Insect | |
| *Drosophila melangaster* Hb | AAF54270 |
| *Drosophila sechellia* Hb | CAA06504 |
| *Calliphora erythrocephala* Hb | L01591 |
| *Euscelis plebejus* Hb | AAA29120 |
| *Apis mellifera* Hb | XP393692 |
| *Bombyx mori* Hb | AAM34284 |
| *Clogmia albipunctata* Hb | CAA10281 |
| Crustacea | |
| *Artemia franciscana* Hb | AM055593 |
| NEMATODE | |
| *Caenorhabditis elegans* Hb | AP063235 |
| *Caenorhabditis elegans* Aiolos | NP001024851 |
| CHORDATE | |
| *Gallus gallus* Ikaros | NM205088 |
| *Gallus gallus* Ikaros 2 | 001026766 |
| *Gallus gallus* Helios | NP989938 |
| *Gallus gallus* Aiolos | NM204607 |
| *Homo sapiens* Ikaros | NM006060 |
| *Homo sapiens* Helios | NP057344 |
| *Homo sapiens* Aiolos | NP036613 |
| *Homo sapiens* Eos | NP071910 |
| *Xenopus tropicalis* Ikaros | AAH89243 |
| *Xenopus tropicalis* Ikaros 2 | 001116930 |
| *Danio rerio* Ikaros | AAL11909 |
| *Danio rerio* Ikaros 2 | XP001921973 |
| UROCHORDATE | |
| *Ciona intestinalis* Ikaros | XP002121167 |

reactive Distal-less antibody (Panganiban *et al*., 1995). In short, the strategy involved a two-step boosting regimen in which a bacterially expressed and purified 413 amino acid LZF2 peptide, which included the middle finger and the C box domains, was injected into rabbits for the first six boosts. A 128 amino acid peptide consisting entirely of the middle finger DNA-binding domain (Fig. 3) was injected into rabbits previously immunized with the larger peptide for the final boosts. The goal was to increase the titer of polyclonal antibodies that recognized epitopes that reside in the highly conserved DNA-binding domain. The specificity of the affinity-purified polyclonal has been confirmed by Western analysis in three annelid species: *Helobdella* leeches, *Capitella* sp. I polychaetes, and *Tubifex* oligochaetes (Iwasa *et al.,* 2000; Werbrock *et al.,* 2001; Shimizu and Savage, 2002). The 128 amino acid region of the leech Hb-like protein used to generate the cross-reactive antibody is shown in Figure 3.

Gastropod embryos and larvae were fixed in 4% formaldehyde in 50% HEPES-buffered saline (HBS) for 1 h and washed in HBS. Samples were incubated in 10% normal goat serum (filter sterile) for 1 h, followed by the addition of a 1:50 dilution of the primary Hb-like polyclonal antibody in HBT (HBS + 0.3% Triton) overnight at 4 °C (Iwasa *et al.,* 2000). The primary antibody was washed four times for 1 h in HBT, followed by an overnight incubation in a 1:600 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) at 4 °C. After four washes in HBT, the embryos and larvae were stained in a 1:10 dilution of diaminobenzidine (DAB) in stable peroxide substrate buffer according to manufacturer's instructions included in the Metal Enhanced DAB Substrate kit (Thermo Scientific). The DAB reaction was stopped most often after a few minutes and never longer than 2 h by transferring the samples to HBS + 1 $\mu$g/ml Hoechst 33258 nucleic acid stain. Embryos were mounted on slides in a single drop of 80% glycerol or dehydrated in an ethanol series and cleared in a 2:1 ratio of benzyl benzoate/benzyl alcohol. Specimens were imaged on a Nikon Eclipse 80i microscope with a Qimaging Retiger 2000R digital camera.

There was an absence of nonspecific staining in control embryos where primary antibody was omitted and in embryos exposed to preimmune sera or primary antibody alone. A secondary control (primary omitted) was completed with embryos from each major developmental stage (cleavage, gastrulation, and larval) for both gastropods. A secondary control from a representative stage (*I. obsoleta,* 48 h) for both gastropods is shown in Figure 6H. Hundreds of embryos and larvae were examined for Hb-like staining for each stage in *C. fornicata* and no less than 100 embryos per stage in *I. obsoleta*.

### *Preparation of* Drosophila *embryos and larvae for immunostaining*

Wild-type (*Canton-S*) adults were placed on grape juice/agar plates with yeast and left overnight. The embryos were collected and rinsed onto a nylon filter with distilled water, dechorionated in 50% commercial bleach for 3 min, and rinsed with distilled water.

The polyclonal antibody no longer recognizes Hb-like epitopes after embryos have been exposed to methanol (unpubl. obs.). This in mind, we employed two variations of

a standard fixation protocol for fly embryos (Lehmann and Tautz, 1994) that avoided organic solvents. In the first procedure, dechorionated embryos were transferred to double-stick tape, covered with ice-cold HBS, dissected out of the vitelline membrane with a 22-gauge syringe needle, transferred to a 3:1 mix of HBS and 16% methanol-free formaldehyde (Polysciences, Inc.), and fixed at room temperature for times ranging from 20 to 120 min. In the second procedure, embryos were also transferred to double-stick tape, but they were immediately covered with HBS/formaldehyde, dissected in fixative, and incubated for a similar range of times. Fixed fly embryos were processed using the immunocytochemistry technique that is described above for gastropod embryos.

We also attempted to detect misexpressed Hb in *Drosophila* larvae. *hsp70-hb (Hb476.2)/CyO* flies were obtained from the laboratory of Chris Doe (Cleary and Doe, 2006). Late third instar larvae from this stock were heat shocked for 45 min at 37 °C, then allowed to recover and accumulate Hb protein for 30 min at room temperature (Margolis *et al*., 1995). Embryos were dissected open in ice-cold HBS, and all tissue was transferred to HBS/formaldehyde and fixed for 30 min at room temperature.

To test the effectiveness of each fixation protocol, parallel samples of embryonic and larval tissues were incubated with anti-phosphoHistone H3 (Mitosis Marker) antibody (Upstate USA) at a 1:50 dilution.

## Results

### *Sequence comparison*

Hunchback-like (Hb) proteins represent a subgroup of a large and diverse family of zinc finger proteins present in eukaryotic bilaterian genomes (Lander *et al.,* 2001; Venter *et al.,* 2001; Stein *et al.,* 2003). The Hb class of $C_2H_2$ zinc finger proteins shares three distinguishing features based on sequence identity and the structural arrangement of fingers (Patel *et al*., 2001; Pinnell *et al.,* 2006). The core arrangement of zinc finger and box domains in Hb-like proteins consists of a middle finger domain (MF1-4) composed of four zinc fingers located centrally in the coding region, a C Box domain, and a C-terminal Finger domain (CF1-2) composed of two fingers. The CF2 zinc finger terminates directly into a stop codon. The MF1-4 domain has been shown to bind DNA, and the CF1-2 domain has been shown to mediate homo- and heterotypic protein binding (Tautz *et al.,* 1987; Hülskamp *et al.,* 1994; Mackay and Crossley, 1998; Kehle *et al*., 1998; McCarty *et al.,* 2003). The combination of amino acid sequence identity shared between corresponding zinc finger domains and the structural arrangement of fingers within the open reading frame shown in Figure 1 distinguish Hb-like family members from all other $C_2H_2$ zinc finger families (Patel *et al*., 2001; Pinnell *et al*., 2006)

Hb-like sequences were obtained from the gastropods *Lottia gigantia* (JGI) and *Ilyanassa obsoleta* (provided by D. Lambert; see Kingsley *et al*., 2007, for screen details). *L. gigantea* and *I. obsoleta* Hb-like sequences share similar expectation (e) values compared to the first annelid Hb-like protein discovered, called leech zinc finger 2, or LZF2 (Savage and Shankland, 1996). The MF zinc finger regions of *I. obsoleta* and *L. gigantea* Hb-like proteins received values of 7e–24 and 4e–68, respectively, when compared to the corresponding region in the LZF2 protein. The e-values obtained from sequence comparisons between two polychaete Hb-like sequences (*Capitella* sp. I 1e-56 and *Platynereis* 8e-64) to the corresponding LZF2 amino acid sequence were comparable to the molluscan scores. Therefore the e-values obtained from NCBI databases using the BLAST algorithm suggest that the *I. obsoleta* and *L. gigantea* sequences encode Hb-like homologs. A *Crepidula fornicata* Hb-like sequence is unavailable.

Comparisons of lophotrochozoan Hb-like zinc finger domains and the C Box domain reveal similar percentages of shared amino acid sequence identity across phyla (Fig. 1). For example, the polychaete (*Capitella* sp. I) and the gastropod *L. gigantea* Hb-like MF2 and MF3 zinc finger domains each share 75% and >93% amino acid identity, respectively, to the corresponding domains in the leech LZF2 protein. The MF3 domain in *I. obsoleta* shares 86% amino acid identity with the corresponding zinc finger in *Helobdella* leeches. This is, in fact, greater than the percentage (82%) of amino acid identity shared in a comparison between the *I. obsoleta* and *L. gigantea* MF3 zinc finger domains. In general, we found that gastropod zinc finger domains are more similar to their annelid counterparts then they are between themselves (not shown). The combination of shared sequence identity and the conserved structural arrangement of the motifs in the coding region supports the assignment of gastropod sequences to the Hb-like class of $C_2H_2$ zinc finger class of transcription factors (Patel *et al*., 2001; Pinnell *et al*., 2006).

### *Molecular phylogeny*

We used Bayesian methods to construct a molecular phylogenetic tree based on the alignment of up to 180 conserved amino acid residues that includes the highly conserved DNA-binding middle zinc finger domain. The Ikaros-like protein family was chosen as the outgroup on the basis of two key pieces of information. Both Hb and Ikaros protein families possess a similar structural arrangement of two zinc finger domains: a middle finger (MF1-4) and a C-terminal finger (CF1-2) domain that terminates at a stop codon (Pinnell *et al*., 2006). These structural traits distinguish Hb and Ikaros proteins from other $C_2H_2$ zinc finger proteins on the basis of genomic searches designed to identify this arrangement of zinc finger motifs (data not shown). A second defining feature of the Ikaros/Hb zinc finger

**A**

NF-1 Finger

```
LZF2   FCCHICSFVGTTEENFNSHMTQHFE
Pdhb   .F..L..Y...SKYH..A..NS... (60)
Lghb   YF..L..YA.DAKDE.DQ..SI... (44)
```

NF-2 Finger

```
LZF2   HQCPHCTYSSRTEGRLKRHIKDFHSNED
Hrohb        //..................... (100)
CapIhb .G....D.T..............E.P (82)
Pdhb   .R....D.T.........M.....DNP (75)
Lghb   YS....D.T.........I....TDDN (68)
```

MF1 Finger

```
LZF2   RKCRYCQFYTYDQVEFWVHLKKHIKPEKL
Hrohb  ............................. (100)
CapIhb F...Q.E.ASENKI...E.SRI...ED.V (46)
Pdhb   F..KQ.DYQ.EVK.D..E.SRS...E..V (45)
Lghb   YR.LQ.DYVATVKTD..Q.SRT.IKED.I (24)
```

MF2 Finger

```
LZF2   LECPHCEFVTELKHHLEYHIRVHIGSKP
Hrohb  ............................. (100)
CapIhb .Q..K.P....Y.......L.N.F.... (75)
Pdhb   .Q..K.P....Y.......L.N.F.... (75)
Lghb   .Q..R.P....Y.......L.N.F.... (75)
```

MF3 Finger

```
LZF2   FKCPKCNYSCVNKSMLNSHMKSHTNVYQ
Hrohb  ............................. (100)
CapIhb ...G....A.................... (93)
Pdhb   ...N....A.................... (93)
Lghb   ...N......................... (96)
Iohb   .........N.............KAC. (86)
Afhb   ...N..S................S.I.. (86)
```

MF4 Finger

```
LZF2   FRCRDCNYATKYSHSLKLHLIKKKHLAD
Hrohb  ............................. (100)
CapIhb Y..S..T.....C.......K.YN.KPA (64)
Pdhb   ...A..T.....C.......K.YN.KPA (68)
Lghb   Y..A..T.....C.......R.YN.KPA (64)
Iohb   ...L..PF.A.......I..K.HG.RQG (61)
Afhb   Y..A..T.....C...E...R.YN.KPA (61)
```

ExF Finger

```
LZF2   FDCSFCIEKFTTPIELKCHVEETHYRDL
Hrhohb ............................. (100)
CapIhb LN.KV.EF.AESQQT.L...LRV.AAEN (29)
Lghb   AK.NL.GFIADN.DK.NA.LMKV.SSEN (21)
Iohb   VK.EL.PFTCP.MEQ.RR.MRKV.GPEG (21)
Afhb   LK.QL.GFSTFASNQFAE.ILS-.STKE (14)
```

CF1 Finger

```
LZF2   HICRHCEMAFADQMTHRLHMGYHGYFN
Hrohb  ............................. (100)
Lghb   YE.KF.DI..R.CVMYTM.......Q. (48)
Afhb   NL.QF.DI..K.AIMYTM//
```

CF2 Finger

```
LZF2   FQCNGCGENCVDAFDFMLHLMSKAHN
Hrohb  ............................ (100)
Lghb   YK..M..NISC.KVE.F..IARA... (42)
```

C Box

```
LZF2        LADVVLNSDGSLPADGTGHFDSISKRGPPRT
Hrohb       .....................N......G.. (93)
CapIhb      KPAT...P.....T..D.I.ELV........ (64)
Pdhb        KPAT...A.....T..S.D.ELV.......G (61)
Lghb        KPAT...T.....QGLDADLSLLA......G (45)
Iohb        RQGP.........QY.L.I.GRG.GG.GGGR (45)
```

**B**

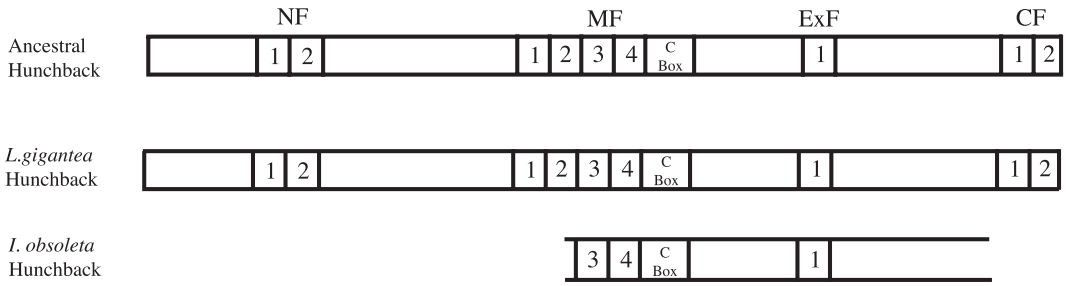


**Figure 1.** (A) Alignment of Hunchback-like zinc finger and C Box domain amino acid sequences in annelids, gastropods, and crustaceans. A dot represents an identical amino acid; a dash symbolizes a gap. The percentage of amino acid identity shared with the reference leech Hunchback known as LZF2 is in parentheses. LZF2 (*Helobdella triserialis* hb), Hrohb (*Helobdella robusta* hb), CapIhb (*Capitella sp I* hb), Pdhb (*Platynereis dumerilii* hb), Lghb (*Lottia gigantea* hb), Iohb (*Ilyanassa obsoleta* hb), and Afhb (*Artemia franciscana* hb). (B) A diagram of the conserved zinc finger and box domains in the ancestral Hb-like open reading frame (ORF) compared to the two molluscan ORFs. The organization of zinc fingers and C Box domains within the ancestral Hb-like ORF is based on comparative studies (Patel *et al*., 2001; Pinnell *et al*., 2006). The non-enclosed ends of the ORF represent partial coding sequence. The Hb-like family of zinc finger proteins possess as many as four zinc finger domains labeled NF1-2 (N-terminal finger), MF1-4 (middle finger), ExF (extrafinger), CF1-2 (C-terminal finger). The C Box domain immediately follows the middle finger domain in the Hb-like family.

proteins is that they share a homotypic dimerization zinc finger (DZF) domain located within the CF1-2 finger region (McCarty *et al*., 2003). The amino acid sequence identity shared and the biochemical mechanism by which the DZF domain mediates homodimerization are unique to this group (McCarty *et al*., 2003). Together, the data suggest that Ikaros and Hunchback proteins are members of two closely related $C_2H_2$ zinc finger families. BLAST searches com-

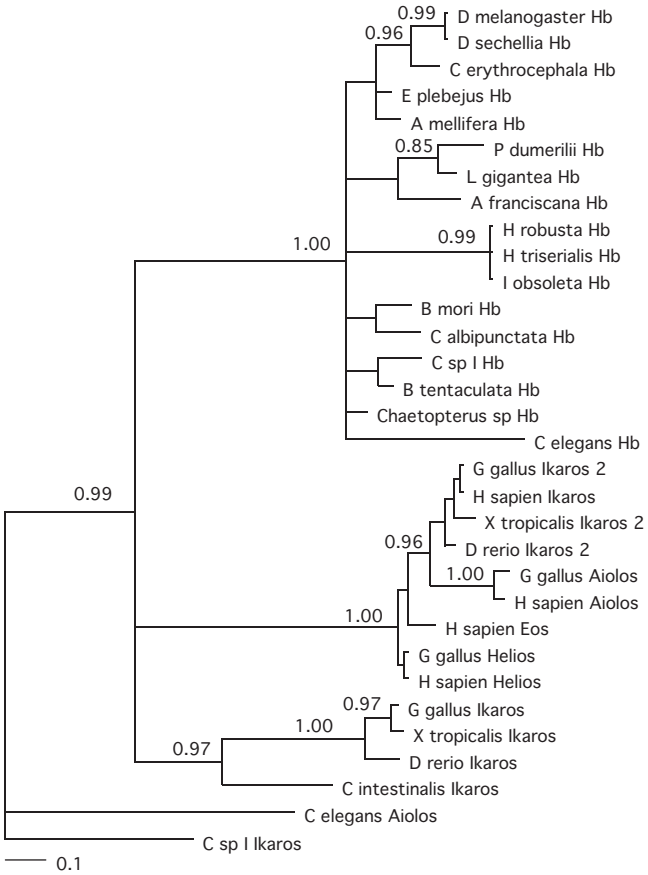


**Figure 2.** A molecular phylogenetic tree of Hunchback-like protein created using Bayesian methods and based on the alignment of up to 180 amino acid residues that compose the MF1-4 zinc finger domain. Posterior probabilities equal to or greater than 0.85 are shown, and the Ikaros protein family was used as an outgroup. The tree shows that the Hb-like protein family forms a distinct and well-supported monophyletic group separate from the Ikaros family, and the molluscan sequences reside within the Hb-like family of $C_2H_2$ zinc finger proteins.

pleted in every available deuterostome genome failed to identify an unequivocal Hb-like sequence, which confirms the findings of Kerner *et al.* (2006). The phylogeny in Figure 2 shows that the Hb-like protein family in protostomes forms a distinct and well-supported monophyletic group separate from the Ikaros zinc finger family, and the *I. obsoleta* and *L. gigantea* molluscan sequences encode members of the Hb-like $C_2H_2$ zinc finger proteins. Accession numbers and taxonomic information can be viewed in Table 1.

### *Interphyletic reactivity of the Hunchback-like antibody*

Two independent sources of data support the cross-reactivity of the polyclonal Hb-like antibody in gastropod embryos and larvae. First, high sequence identity is shared between the corresponding zinc fingers that reside in the middle finger domain, the coding region from which the polyclonal antibodies were generated (Fig. 3 and Iwasa *et al*., 2000). Gastropod *Ilyanassa obsoleta* and *Lottia gigantea* Hb-like proteins share 65% amino acid identity to the corresponding 128 amino acid region in the leech LZF2 protein (Fig. 3). This is close to the percentage (69%) obtained by comparing polychaete Hb-like proteins to LZF2. In particular, the MF3 zinc finger sequences from *Capitella* sp. I and *Platynereis dumerilii* Hb each share 93% sequence identity with the corresponding finger in LZF2 in *Helobdella triserialis*. Similarly, the MF3 finger domain from *L. gigantea* and *I. obsoleta* Hb-like proteins share 96% and 86% identity, respectively, with the corresponding finger in LZF2 in *Helobdella triserialis* (Fig. 1). Furthermore, the entire 128 amino acid middle finger region in *L. gigantea* Hb-like and *I. obsoleta* Hb-like proteins are more similar to the corresponding annelid amino acid (65%) than to each other (58%).

Second, the Hb protein expression patterns from two *Helobdella* leech species, the oligochaete *Tubifex,* and the polychaete *Capitella* sp. I (Iwasa *et al.,* 2000; Werbrock *et al.,* 2001; Shimizu and Savage, 2002) match the corresponding pattern elements in two gastropod genera, as depicted in Figures 4–6. It is also important to note that, due to high sequence conservation, the polyclonal Hb-like antibody is not likely to distinguish multiple copies of Hb in protostome genomes; therefore the molluscan proteins are referred to as Hb-like homologs.

*Drosophila* embryos and larvae were treated with the lophotrochozoan cross-reactive antibody to test the limits of cross-reactivity in a system in which the Hb expression patterns have been well described. Such information can be used to assess the cross-reactive potential of the antibody in other protostomes. *A priori* there were at least two obstacles to overcome for cross-reactivity to occur. First, the corresponding 128 amino acid MF (1-4) region in flies shares 58% identity with the corresponding domain in *Helobdella triserialis* Hb-like protein; this is lower than any known intra-lophotrochozoan comparison (Fig. 3). Second, the standard fixation protocols used for insect embryos include organic solvents that abrogate Hb-like staining in annelids (pers. obs.). Therefore, two distinct fixation protocols were developed that excluded organic solvents (see Methods). Additionally, Hb protein was ectopically expressed in larvae using the GAL4-UAS system (Cleary and Doe, 2006) to ensure the presence of high levels of Hb protein in cells. In each treatment, the annelid Hb-like antibody failed to recognize dipteran Hb protein, although positive-control antiphosphohistone H3 antibody stained dividing cells (not shown). These data suggest that a general threshold for cross-reactivity of the annelid Hb-like antibody may be at or slightly above 60% shared amino acid sequence identity. We also recognize that a change in a single residue has the potential to affect epitope binding.

```
LZF2   TYDQVEFWVHLKKHIKPEKLLECPHCEFVTELKHHLEYHIRVHIGSKPFKCPKCNYSCVNKSMLNSHMKSHTNVYQFRCR
HroHb  ................................................................................
CapIHb SENKI...E.SRI...ED.V.Q..K.P....Y.......L.N.F.......G....A...................Y..S
PdHb   .EVK.D..E.SRS...E.XV.Q..K.P....Y.......L.N.F.......N....A...................Y..A
IoHb   ----------------------------------------RKN.K.I.G.........N.............KAC....L
LgHb   ATVKTD..Q.SRT...ED.I.Q..R.P....Y.......L.N.F.......N.......................Y..A
AfHb   -------------------------------------M.N.F.......N..S................S.I..Y..A
DmHb   AITK.D..A.TRT.M..D.I.Q..K.P....F.........K.KNQ...Q.D..S.T..........R...SS...Y..A

LZF2   DCNYATKYSHSLKLHLIKKKHLADVVLNSDGSLPADGTGHFDSIS-RRG
HroHb  ..........................................N-...          (99%)
CapIHb ..T.....C.......K.YN.KPAT...P.....T..S.D.ELV.-K..          (69%)
PdHb   ..T.....C.......K.YN.KPAT...A.....T..S.D.ELV.-K..          (69%)
IoHb   ..PF.A.......I..K.HG.RQGP.........QY.L.I.GRG.-GG.          (65%)
LgHb   ..T.....C.......R.YN.KPAT...T.....Q--GLDA.LSGLSLLA         (65%)
AfHb   ..T.....C...E...R.YN.KPAM..TNE.NPAP--S-MI.LY.P...PRP       (64%)
DmHb   ..D.....C..F....R.YG.KPGM..DE..TPNP--SLVI.VYGT...          (58%)
```

**Figure 3.** Alignment of the 128 amino acid region that includes the Hb-like middle finger and C Box domains from annelids, gastropods, and arthropods. The 128 amino acid peptide was injected into rabbits previously immunized with a larger 413 amino acid that contained the MF region and adjacent 3′ sequence. The goal was to increase the titer of polyclonal antibodies that recognized the highly conserved Hb DNA-binding domain (see Methods). The percentage of amino acid identity shared with the reference leech Hb-like protein known as LZF2 is in parentheses. LZF2 (*Helobdella triserialis* hb), Hrohb (*Helobdella robusta* hb), CapIhb (*Capitella* sp. I hb), Pdhb (*Platynereis dumerilii* hb), Lghb (*Lottia gigantea* hb), Iohb (*Ilyanassa obsoleta* hb), Afhb (*Artemia franciscana* hb), and Dmhb (*Drosophila melanogaster* hb).

### *Hb-like protein expression in cleavage-stage gastropod embryos*

The spatial and temporal expression patterns of *Ilyanassa obsoleta* and *Crepidula fornicata* Hb-like protein were characterized using the cross-reactive polyclonal antibody. Within about 1 h after egg laying and continuing through the cleavage divisions, gastropod Hb-like protein accumulated in blastomere nuclei (Fig. 4A–D). *C. fornicata* and *I. obsoleta* Hb-like proteins are expressed in every micromere and macromere nucleus from one cell to at least the sixth cleavage division. One difference between *C. fornicata* and *I. obsoleta* Hb-like patterns is that the cytoplasm of 16- and 24-cell stage *I. obsoleta* embryos stains in addition to the nuclei (Fig. 4E, F). The cytoplasmic localization of this zinc finger transcription factor also occurs only in cleavage-stage blastomeres in *Helobdella* leeches (Iwasa *et al.,* 2000). The cytoplasmic staining of Hb-like protein in *I. obsoleta* gastropod and *Helobodella* leech development appears to be restricted to cleavage-stage embryos, and it has not been observed to occur at other stages.

### *Hunchback-like protein expression in the gastrula gastropod embryo*

Although there is some variation in molluscan morphogenetic movements, epiboly—the spreading of the micromere-derived epithelium over the endoderm cells in the vegetal hemisphere—is the dominant movement that characterizes gastrulation in *C. fornicata* and *I. obsoleta* (Conklin, 1897; Kumé and Dan, 1988). Prior to and during gastrulation, the epithelial cells in the animal hemisphere and the large endodermal blastomeres express Hb-like protein in both gastropods (Fig. 4C, arrow; Fig. 5A, D). As epiboly proceeds, the cells that express gastropod Hb-like protein most intensely line the leading edge of the ectoderm as shown in *I. obsoleta* (Fig. 5E, arrowheads). A similar ring-like staining pattern is observed in *C. fornicata* gastrula-stage embryos (not shown). As the blastopore closes, the immunoreactive cells are localized to the stomadeum cells that give rise to the foregut structures such as the mouth and anterior region of the esophagus, as shown in *C. fornicata* (Fig. 5B, C). It is after gastrulation that gastropod Hb-like protein transitions from a uniform to a spatially localized pattern of expression. In the equivalent embryonic stage, the 72-h *C. fornicata* and the 48-h *I. obsoleta* embryos express gastropod Hb-like protein in similar localized spatiotemporal expression patterns; there are few immunoreactive dorsal epithelial cells (Fig. 5C, inset), and the majority of DAB staining is restricted to the stomodeum cells and the cells that lie posterior to this structure (Fig. 5B, C, E, F).

### *Hb-like protein expression in gastropod veliger larvae*

The larval Hb-like staining patterns of the two gastropod species are nearly identical. The notable Hb-like expression in *C. fornicata* and *I. obsoleta* developing veliger larvae is found in ciliary structures associated with feeding and movement. The stomadeum, in addition to the developing right and left velar lobes and the foot, continues to express

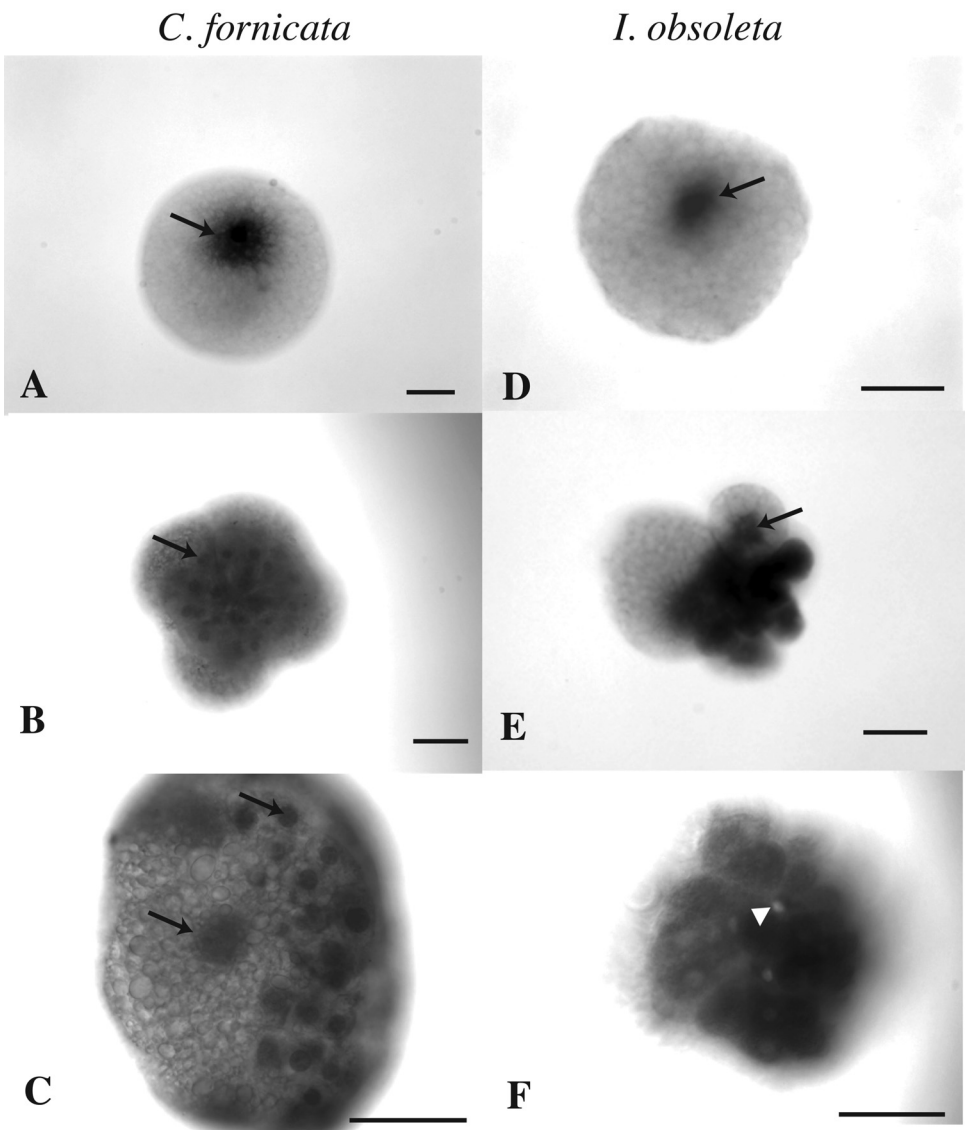


**Figure 4.** Immunolocalization of the cross-reactive Hb-like antibody during *Crepidula fornicata* and *Ilyanassa obsoleta* cleavage using HRP-conjugated secondary antibody. All blastomere nuclei show uniform nuclear staining of gastropod Hb-like protein (black arrows). Cleavage stage *I. obsoleta* embryos vary slightly in that the Hb-like protein is also found in the cytoplasm (E) and at varying levels in both the cytoplasm and nucleus (F) (white arrowhead identifies Hoechst-stained nucleus). In *C. fornicata*, Hb-like protein is localized to blastomere nuclei and is not found in the cytoplasm at this stage. Cleavage-stage embryos of *C. fornicata* in (A) 1-cell, (B) 25-cell, and (C) 36-h embryo, and of *I. obsoleta* in (D) 1-cell, (E) 16-cell, and (F) 24-cell stage. Animal hemisphere view (A, B, D, F); anterior right (C and E). Scale bar, 50 $\mu$m.

Hb-like protein (Fig. 6A, B, C, E). *C. fornicata* larvae (Fig. 6A–C) show the most intense staining in the precursor foot cells, and this staining becomes localized to its posterior rim through time (Fig. 6A, 5-day; to Fig. 6B, 7-day; to Fig. 6C, 9-day). In veliger larvae, scattered epithelial cells (see asterisk in Fig. 6B) and the precursor cells to the apical organ also express the protein in the 5-day embryo. In the 6-day *I. obsoleta* larva (Fig. 6F), the velar cells and the precursor cells that will give rise to the ciliated cells that line the intestine and esophagus express Hb-like protein. The corresponding larval stage in *C. fornicata* is not shown because it shows a similar expression pattern in the velar lobes, intestine, and esophagus. In the 9-day *I. obsoleta* veliger, the Hb-like expression is no longer present in ciliary structures that include the velum, mouth, intestine, and esophagus (Fig. 6G); the visible dark patches represent the larval eyes and pigment and are not the product of the DAB reaction. The lack of Hb-like staining observed in 9-day *I. obsoleta* and *C. fornicata* (not shown) larvae supports the specificity of the antisera.

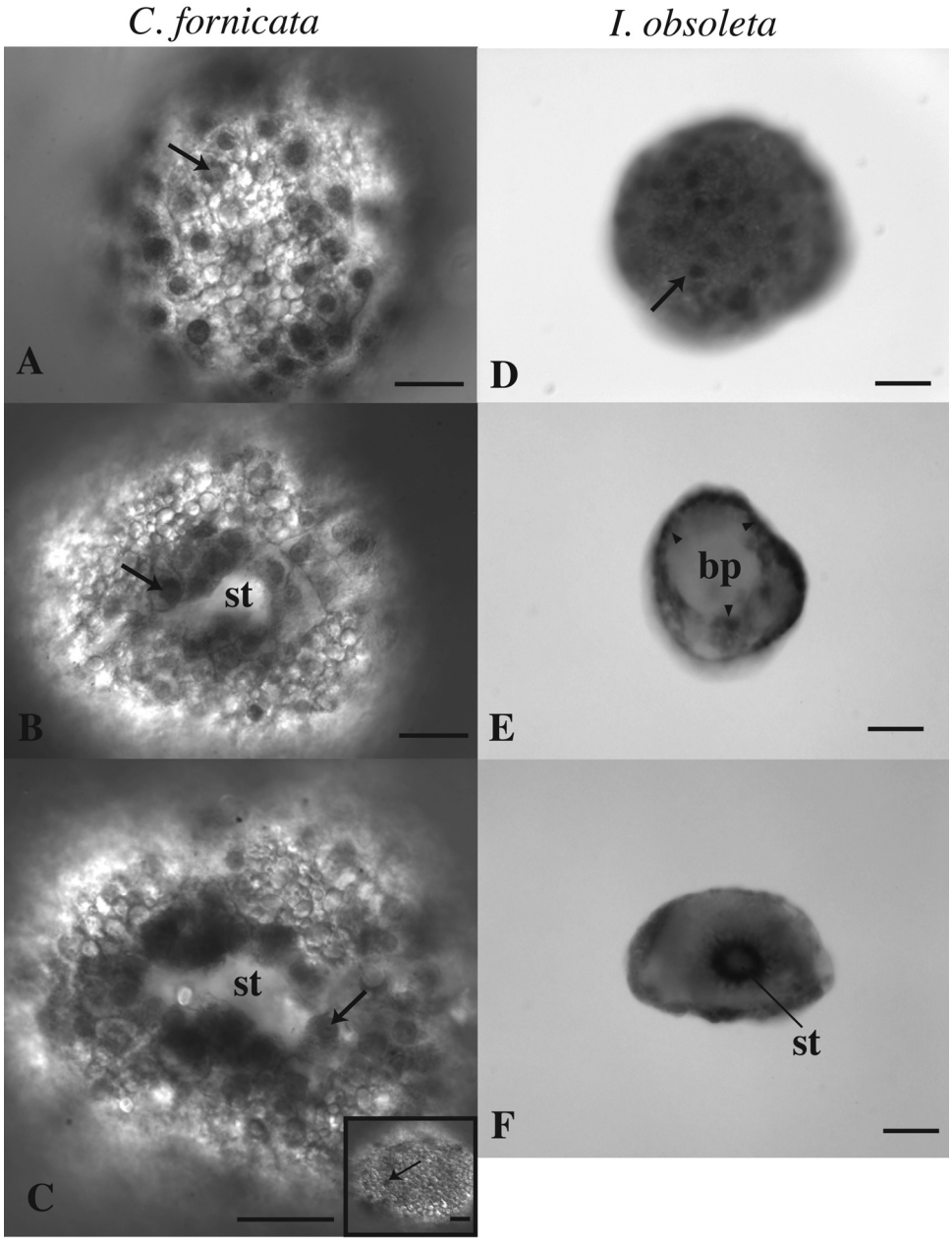


**Figure 5.** Immunolocalization of gastropod Hb-like protein during gastrulation. There is uniform nuclear staining in the epithilium prior to and during gastrulation in 2-day *Crepidula fornicata* embryo (A) and in 24-h *Ilyanassa* embryo (D). The epithelial cells that line the blastopore (bp) and the developing stomodeum (st) express Hb-like protein in the 3-day (B) and 4-day (C) *C. fornicata* embryos and the 2-day (E) and 3-day (F) *Ilyanassa* embryos. The inset in (C) indicates few immunoreactive nuclei localized in the dorsal anterior region. Filled arrows indicate immunostained nuclei, and filled arrowheads indicate Hb-like stained cells that line the leading edge of the epithelium undergoing epiboly. Animal hemisphere view (A and B); ventral and posterior right (B–F). Scale bar, 50 $\mu$m.

## Discussion

Molecular phylogenies generated over the past decade support the division of bilaterian protostomes into two distinct lineages: ecdysozoans and lophotrochozoans (Halanych *et al.,* 1995; Aguinaldo *et al.,* 1997; Dunn *et al.,* 2008; Colgan *et al.,* 2008). This revision in phylogeny impacted several leading hypotheses, including the origin of body plan segmentation. The revised phylogeny is more consistent with an independent origin of segmentation than with a shared common ancestry.

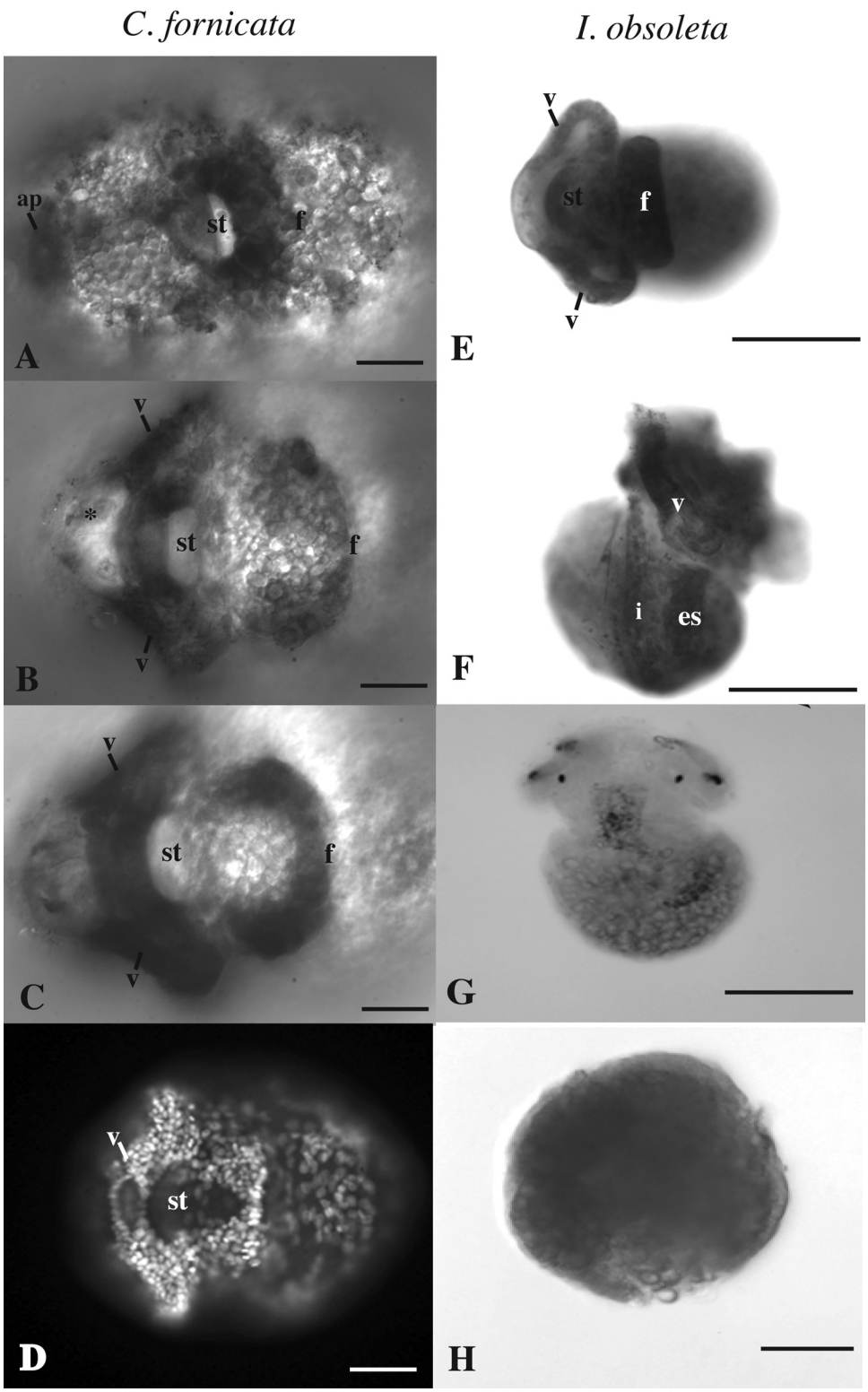


**Figure 6.** Immunolocalization of gastropod Hb-like protein during veliger organogenesis. Hb-like immunoreactivity in gastropod larvae is localized to the specialized ciliary structures associated with feeding and locomotion. In a 5-day *Crepidula fornicata* larva (A), the posterior rim of the stomodeum (st) and the apical organ (ap) region express Hb-like protein. The rim area coincides with the precursor cells that will give rise to the foot (f). In the 7-day (B) and 9-day (C) *C. fornicata* larvae, the developing velar lobes (v), the anterior region of the stomodeum, and the outer rim of the foot express gastropod Hb-like protein. The asterisk in (B) indicates

Comparative expression data from a majority of high-profile developmental regulatory proteins also support an independent origin of segmentation, but many more gene products need to be studied (Seaver and Kanishige, 2006; Pinnell *et al*., 2006). One interesting example is the characterization of the "segmentation" gene *hunchback* in protostomes. The comparative data describing Hb-like protein expression patterns in lophotrochozoans and ecdysozoans suggest that this protein has "phylogenetic signal." The signal is inferred from a combination of sources that include amino acid sequence, structural domain, and gene expression comparative analyses (Pinnell *et al*., 2006). Together, Hb-like comparative expression studies in arthropods and annelids provide strong support for the hypothesis that the anterior organizing function of Hunchback originated in insects and that this pattern element is not shared broadly among other arthropods nor in any annelid examined to date (Pinnell *et al.,* 2006; Kontarakis *et al.,* 2006; Chipman and Stollewerk, 2006; Marques-Souza *et al.,* 2008).

This study uses a cross-reactive antibody to produce a spatiotemporal expression profile of Hb-like protein in gastropod molluscs. The polyclonal Hb antibody was raised against the highly conserved middle finger DNA-binding domain (Fig. 3) in leeches, and subsequently it has been shown to be effective in every annelid tested (Iwasa *et al.,* 2000; Werbrock *et al.,* 2001; Shimizu and Savage, 2002). The combination of high sequence identity shared between middle finger domains of gastropods and annelids, the matching protein expression patterns in two gastropod genera, and the matching mRNA accumulation to Hb-like protein patterns in cleavage-stage *I. obsoleta* embryos (confirmed by D. Lambert, University of Rochester) demonstrates that the pan-annelid Hb-like antibody recognizes Hb-like protein in gastropod molluscs.

The Hb-like staining patterns in the two gastropods *Crepidula fornicata* and *Ilyanassa obsoleta* match one another throughout embryogenesis and larval development. During cleavage, gastropod Hb-like expression is uniform and labels both micromere and macromere nuclei (Fig. 4C, arrows). During gastrula stages, the epithelium undergoing epiboly, including the leading edge of the epiboly front, expresses Hb-like protein at high levels (Fig. 5E). In postgastrula larvae, Hb-like protein expression is localized to the developing ciliary structures associated with feeding and locomotion that include the stomadeum, velar lobes, foot, intestine, and esophagus (Fig. 6). In summary, there is no significant difference in Hb-like staining patterns in embryonic and larval development between the two gastropods. Hb-like expression in the veliger nervous system was not characterized in this study.

### *Conservation of Hb-like pattern elements between gastropods and annelids*

Studies in gastropod molluscs, clitellate annelids, and the polychaete *Capitella* sp. I suggest that Hb-like protein functions throughout embryonic and larval development. Our current data are consistent with the idea that the lophotrochozoan Hb-like protein has three to four distinct spatiotemporal phases of expression, depending on the presence or absence of a larval phase. In these groups, Hb-like protein is expressed in nuclei in cleavage-stage blastomeres, the gastrula epithelium that undergoes epiboly, the gut epithelium, and the ciliary structures associated with feeding and locomotion (Savage and Shankland, 1996; Iwasa *et al.,* 2000; Werbrock *et al.,* 2001; Shimizu and Savage, 2002; Pinnell *et al.,* 2006).

The Hb-like pattern elements shared between gastropod molluscs and the polychaete *Capitella* sp. I generate a number of interesting and testable hypotheses regarding the evolutionary history of *hunchback* function in lophotrochozoans. The presence of Hb-like protein in cells or tissues during the embryonic stages of cell proliferation is always followed by a downregulation in differentiated cells later in the developmental program. This occurs in the epithelium undergoing epiboly, the ciliary and locomotory structures, and the mid- and hindgut structures. These data suggest that the ancestral role of Hb-like protein may be involved in maintaining the undifferentiated state of a cell or tissue or as a general regulator of cell division. A similar role for Hb has been functionally demonstrated in studies of *Drosophila* neurogenesis (Grosskortenhaus *et al.,* 2005). Alternatively, the Hb-like protein may play a role in the specification of epithelial structures, ciliary cells, and the gut. Therefore,

immunoreactive epithelial cells that are scattered in the epithelium. A 7-day *C. fornicata* embryo (D) labeled with the Hoechst 33342 DNA stain shows that the nuclei occupy most of the volume in the cells of the velum and foot. This makes the staining appear continuous and not punctate as would be expected for a transcription factor. In the 4-day (E) and 7-day (F) *Ilyanassa obsoleta* veligers, the velar lobes, stomodeum, foot, intestine (i), and esophagus (es) are all ciliated structures that express Hb-like protein. In the 9-day (G) *I. obsoleta* larva, Hb-like expression is absent from the ciliated structures such as the velum, gut, foot, and mouth. The larval eyespots and pigmentation are visible and are not a result of DAB staining. No nonspecific staining was observed in control embryos where primary antibody was omitted from the immunostaining procedure, as shown in a 2-day *I. obsoleta* embryo (H); nor was there artifact staining in late-stage *I. obsoleta* larvae (G). Preimmune serum or primary alone treatments of gastropod embryos do not stain as well (not shown). Anterior left (A–D); anterior up (E; profile; and F, dorsal view). Scale bar, 50 $\mu$m.

functional analyses are required to determine its role or roles in lophotrochozoan development.

A second hypothesis generated from this comparative study addresses the remarkable conservation in Hb-like expression in the ciliary structures of the veliger and trochophore larvae. The stomadeum, velar lobes, and gut in gastropods compared to the trochal ciliated bands and gut in *Capitella* sp. I suggest that the structures associated with feeding and locomotion have utilized Hb-like function in some aspect of cilia patterning and thus may represent a molecular trait shared by lophotrochozoan larvae. The characterization of Hb-like expression in other indirect-developing lineages is required to determine whether this pattern element is basal to the lophotrochozoan superphylum or shared between annelids and molluscs.

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