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Molecular paleoecology: using gene regulatory analysis to address the origins of complex life cycles in the late Precambrian

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SUMMARY Molecular paleoecology is the application of molecular data to test hypotheses made by paleoecological scenarios. Here, we use gene regulatory analysis to test between two competing paleoecological scenarios put forth to explain the evolution of complex life cycles. The first posits that early bilaterians were holobenthic, and the evolution of macrophagous grazing drove the exploitation of the pelagos by metazoan eggs and embryos, and eventually larvae. The alternative hypothesis predicts that early bilaterians were holopelagic, and new adult stages were added on when these holopelagic forms began to feed on the benthos. The former hypothesis predicts that the larvae of protostomes and deuterostomes are not homologous, with the implication that larval-specific structures, including the apical organ, are the products of convergent evolution, whereas the latter hypothesis predicts homology of larvae, specifically homology of the apical organ. We show that in the sea urchin, *Strongylocentrotus purpuratus*, the transcription factors *NK2.1* and *HNF6* are necessary for the correct spatial expression profiles of five different cilia genes. All of these genes are expressed exclusively in the apical plate after the mesenchyme-blastula stage in cells that also express *NK2.1* and *HNF6*. In addition, abrogation of *SpNK2.1* results in embryos that lack the apical tuft. However, in the red abalone, *Haliotis rufescens*, *NK2.1* and *HNF6* are not expressed in any cells that also express these same five cilia genes. Nonetheless, like the sea urchin, the gastropod expresses both *NK2.1* and *FoxA* around the stomodeum and foregut, and *FoxA* around the proctodeum. As we detected no similarity in the development of the apical tuft between the sea urchin and the abalone, these molecular data are consistent with the hypothesis that the evolution of mobile, macrophagous metazoans drove the evolution of complex life cycles multiple times independently in the late Precambrian.

INTRODUCTION

A fascinating paleoecological problem is the origin of complex life cycles, specifically the origin(s) of marine invertebrate larvae. Given that over 70% of benthic marine invertebrates have a primary larval stage (Thorson 1950) distributed over many different animal phyla (Nielsen 1998; Peterson et al. 2000a), the origins of ciliated larvae must lie deep within the geological past (Strathmann 1985, 1993). Peterson (2005), following on the suggestions of Signor and Vermeij (1994), used both the fossil record and a molecular clock (Peterson and Butterfield 2005) to derive a paleoecological scenario for the origin of complex life cycles. Initially, ciliated larval forms evolved at least three times independently during the latest Precambrian–Early Cambrian (Fig. 1), possibly driven by the evolution of benthic grazing (Seilacher 1999), by mobile macrophagous metazoans (see also Butterfield 1997 and Peterson et al. 2005). This rise of mesozooplankton, especially crustaceans (Butterfield 1994), set the stage for the explosive evolution of epifaunal suspension feeders such as pelmatozoan echinoderms, corals, and bryozoans starting in the latest Cambrian and continuing into the Ordovician (Fig. 1). The advent of this "Paleozoic fauna" (Sepkoski 1981) then drove the evolution of larval planktotrophy by selecting for adult fecundity in at least four different clades (Fig. 1). Thus, the advent of benthic grazing drove the evolution of mesozooplankton, which in turn allowed for the evolution of epifaunal suspension feeders, which then exerted selection pressure for an increase in fecundity, and thus of larval planktotrophy.

The alternative view is that larvae, if not feeding larvae, are primitive for metazoans (Jägersten 1972). There are two modern versions of this hypothesis, the trochaea theory of Nielsen and Nørrevang (1985); Nielsen (1985, 1987, 1995, 1998, 2000), and the set-aside cell hypothesis of Davidson and colleagues (Davidson et al. 1995; Peterson et al. 1997, 2000a;

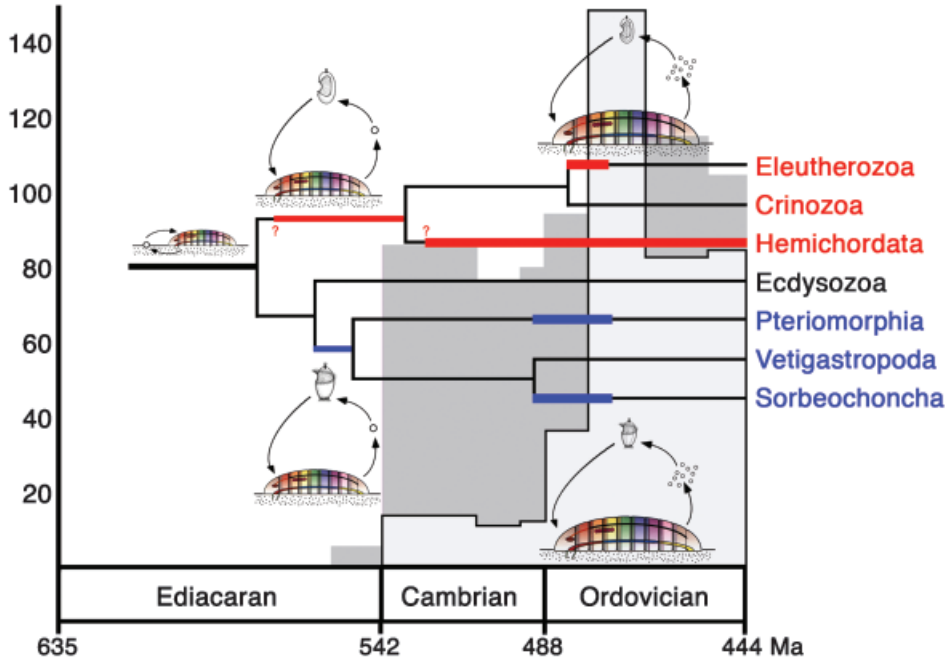

Fig. 1. Macroevolutionary interplay between benthic predators and pelagic larvae. According to the scenario of Peterson (2005; see also Signor and Vermeij 1994 and Peterson et al. 2005) basal bilaterians were small, benthic, direct-developing taxa (thick black line). These animals had a complex genetic tool kit, if not morphology, with *Hox* genes (colored bars) expressed along the A–P axis (from Carroll et al. 2005). Within the deuterostomes (red) and spiralian protostomes (blue) each evolved a ciliated larval stage, the dipleurula and trochophore, respectively. The minimum and maximum for the evolution of these larval stages are given in the thin colored bars; the "?" means that the maximum is not well constrained by the molecular clock but must be less than $\sim$ 580 Ma (see Peterson et al. 2005). Note the relative increase in adult body size, which is based on the appearance of both macroscopic trace fossils (shown in dark gray at $\times$ 1 see scale on left; Droser et al. 2005; Seilacher et al. 2005) and body fossils (Fedonkin and Waggoner 1997; Grotzinger et al. 2000). This rise in mesozooplankton, especially crustacean biomass (see Butterfield 1994) then allowed for the explosive evolution of epifaunal suspension feeders (shown in light gray at $\times$ 10 see scale on left). The continued increase in body size (Chaffe and Lindberg 1986) and the selection pressures exerted by this new mode of predation, that of tiered suspension feeders, selected for an increase in fecundity (eggs shown as small circles), and thus of planktotrophy, in multiple dipleurula- and trochophore-bearing lineages (indicated with the thick-colored lines); again the maximum for the evolution of the hemichordate tornaria larvae is not well constrained (indicated with a "?", but must be less than approximately 535 Ma, the estimated divergence between hemichordates and echinoderms). Only select taxa are shown here—see Peterson (2005) for full taxonomic analysis. Modified from Peterson (2005).

Cameron et al. 1998). Although the set-aside hypothesis was formulated independently of any ecological considerations, and made no explicit ecological predictions, the trochaea theory of Nielsen did consider some of the initial ecological drivers. Nielsen proposed that the two basic larval forms of protostomes and deuterostomes, the trochophore and the dipleurula, respectively, evolved from the trochaea, a holopelagic gastrula-like form with a blastopore surrounded by a ring of cilia used for both feeding and locomotion, and an apical organ with ganglion and tuft. These animals were able to reproduce in the plankton because they did not suffer from the same reproductive constraints that would govern post-Cambrian taxa, as detailed by Olive (1985), and thus small animals producing small numbers of small eggs could have existed during this time interval. Driving the initial exploitation of the benthos was the ability of the trochaea to begin deposit feeding using the ring of cilia surrounding the blastopore. As this new mode of feeding evolved, a benthic stage was added on to the primitive holopelagic stage, itself retained as the larval stage. Thus, holopelagic larval-like ancestors gave rise to descendants having pelago-benthic life cycles independently in at least four clades—poriferans, cnidarians, protostomes, and deuterostomes. The trochaea makes the explicit prediction that apical organs with an apical tuft are homologous across Metazoa, but that the prototrochs and neotrochs of spiralians and deuterostomes, respectively, are not.

Outside of temporal correlation and plausible mechanism, it is difficult, if not impossible, to test paleoecological scenarios such as these directly. However, correlates can be tested and refuted. The usual way to test between these two extremes is to use phylogenetics, and virtually all analyses suggest that larval feeding arose multiple times (e.g., McHugh and Rouse 1998; Rouse 1999, 2000a, b). Nonetheless, because of the phylogenetic impasse that surrounds the issue of single versus multiple origins of marine larvae (Strathmann and Eernissse 1994), and the confounding issues of evolutionary homology versus convergence with relatively simple structures like ciliary bands and apical organs (Strathmann 1993), new types of data are required to test between these two competing hypotheses. One such test between them is to ask whether the gene regulatory network underlying the development of a larval-specific structure is similar in protostomes and deuterostomes. The apical organ is the structure of choice because it

is a shared feature among a wide range of marine invertebrate larvae and is not found in benthic direct-developing taxa, nor is it part of any adult body plan as it is lost at metamorphosis (Chia and Burke 1978; Nielsen 1987, 2005; Strathmann 1993; Lacalli 1994; Marois and Carew 1997; Hay-Schmidt 2000). In both protostomes and deuterostomes, the apical organ develops within a thickened epithelium at the animal pole of the larval body, termed the apical plate, and usually consists of both a neuronal ganglion, the apical ganglion, and a group of cells bearing elongated cilia, the apical tuft. Because apical organs show several compelling structural similarities and are found in a wide range of primary marine larvae, it is the ideal structure to test hypotheses centered around the homology of primary marine larvae.

In experiments presented here, we show that in the sea urchin *Strongylocentrotus purpuratus* the transcription factor *SpNK2.1* and *SpHNF6* are necessary for the proper spatial expression profiles of five different cilia genes, all of which are expressed in the apical plate after the mesenchyme-blastula stage of development. In addition, abrogation of *SpNK2.1* results in the absence of apical tuft cilia. In the gastropod mollusc *Haliotis rufescens*, neither *HNF6* nor *NK2.1* are co-expressed with these same five cilia genes suggesting that *HNF6* and *NK2.1* can play, at best, only indirect roles into the specification of the apical tuft. But similar to the sea urchin, the gastropod expresses *NK2.1*, as well as *FoxA*, around the stomodeum and foregut, and the gastropod, like many bilaterians, expresses *FoxA* around the proctodeum as well, and the homology of both the stomodeum and proctodeum are generally well accepted. These molecular data provide strong support for the hypothesis that the larvae of protostomes and deuterostomes are the products of convergent evolution, refuting the hypothesis of a holopelagic ancestry of bilaterians, as we found no similarity in the development of the apical tufts between these two taxa.

MATERIALS AND METHODS

Injection of morpholino oligonucleotides and mRNAs

SpNK2.1 morpholino anti-sense oligonucleotides (MASO) injections, *SpDri*-MASO injections, and cadherin mRNA synthesis and injections were performed as described in Takacs et al. (2004) except that the injection concentration of LvCadherin was increased to 240 ng/μl in a 120 mM KCl solution. *SpHNF6*-MASO injections were done according to Otim et al. (2004).

cDNA subtraction

Two cDNA subtractions were performed, one for early stages (14 and 24 h), and one for later stages (approximately 42 h). For the early stages total RNA was isolated using the Clontech RNAII kit (Clontech, Mountain View, CA, USA) from normal 24-h embryos (mesenchyme blastula) and or from two stages of embryos micro-injected with synthetic E-cadherin mRNA (14 h [late cleavage] or 24 h). cDNA synthesis, PCR amplification, subtraction, and suppression PCR were carried out using the Clontech PCR-Select™ Subtraction Kit, with the following significant modifications: For relatively abundant sequences, equal quantities of tracer from 14 to 24 h E-cadherin-expressing embryos were combined with a 100-fold mass excess of driver cDNA derived from normal embryos and hybridized at 68°C to a driver Cot of approximately 20 mol sec/l (20 h). In this reaction equal quantities of each tracer that had been ligated with adaptors 1 or 2R were hybridized in the same reaction, as the 100-fold mass excess of driver effectively competes tracer self-reaction. To recover lower-abundance sequences, tracer cDNA ligated with adaptors 1 and 2R were hybridized separately with cDNA derived from normal 24-h embryos for 20 h to reduce the contribution of abundant sequences; the adaptor 1- and 2R-ligated reactions were then combined and hybridization continued for approximately 7 days to a total driver Cot of approximately 200 mol sec/l. The two hybridization mixes were then separately amplified by suppression PCR. The effectiveness of the early subtraction was monitored by using semiquantitative RT-PCR to measure the mRNA levels for *SoxB1* (expressed at 12–15 h; Kenny et al. 1999) and *NK2.1* (not expressed significantly at 14 h; Takacs et al. 2004). Candidate positive clones were identified by comparing signals produced by hybridizing selected and unselected sequences to cDNA macroarrays (Rast et al. 2000) using high probe concentrations and long hybridization times to detect low-abundance sequences. Probes for *tektin3* and *radial spoke3* were derived from the early (24 h) screens.

For the latter stage, total RNA was isolated from injected embryos using Trizol (Invitrogen, Carlsbad, CA, USA), and the cDNA was made, amplified and digested using the Super Smart PCR cDNA synthesis kit (Clontech). The digested cDNA was then used in the Clontech PCR-Select™ Subtraction Kit starting at Section IV F as described by the manufacturer (Clontech). The remainder of the protocol followed what was used for the early stages, as described above, except that the hybridization time was 44 h. After the second PCR, the DNA was ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), and sequenced using standard protocols. *Radial spoke head p63* was found with this screen.

Confirmation of gene identification involved phylogenetic analysis. Sequences were edited and aligned using MacVector 7.2.3 with minor corrections done by eye. Phylogenetic analyses of the amino acid sequences were done using PAUP v. 4.0b10 (Swofford 2002) for Macintosh. Distance analysis used minimum evolution as the optimality criterion (heuristic search with tree-bisection-reconnection [TBR]), and mean character difference as the distance measure.

These clones were transcribed into digoxygenin (DIG) labeled RNA probes and used for whole mount in situ hybridization (WMISH). Because *dynein p33* was already cloned, we amplified a fragment for a WMISH probe using the following primers (5′–3′): F—CGACATAATGATTCCACCAAACGC; R—CCGTCA CCCTACTTCTTGTTC. Finally, we isolated the 3′ UTR of *α2tubulin*, the orthologue of the apically expressed *Plα 2 tubulin*. First, degenerate primers were designed against coding sequence (F—TGYTTYGARCCNGCNAAYCA; R—CCARTGNACRA ANGCNCKYTT) and the resulting fragment, after sequence

confirmation, was labeled with ^{32}P-dCTP using the Ready-to-Go labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The radiolabeled probes were purified through sephadex G-50 mini Quick Spin DNA Columns (Roche, Indianapolis, IN, USA) before screening a 50 h lambda ZAP cDNA library as previously described (Takacs et al. 2004). Ten positive plaques were purified according to manufacturer's instructions and sequenced. Two isoforms were identified, one of which was the orthologue of *Plα 2 tubulin*. An approximately 300 bp probe derived from the 3′UTR was then amplified for use in WMISH using the following primers: F—GACAGATTATGACATTTGACAC; R—TAATCTCGTTTGACCAGA.

Microscopy and digital image analysis

To study swimming behaviors, control uninjected, control-MASO injected, and *SpNK2.1*-MASO-injected *S. purpuratus* embryos were imaged on a Nikon SMZ660 stereomicroscope equipped with a DAGE-MTI DC200 video camera fed through an ADS Technologies Pyro A/V Link analog-to-digital converter (all from Micro Video Instruments, Avon, MA, USA) using BTV Pro software (http://www.bensoftware.com/) on a Macintosh G4 computer. Pools of randomly selected embryos were placed in shallow seawater in Petri dishes on the microscope and imaged. Movies of three control embryo pools and three experimental embryo pools were captured, and the 5 sec of continuous data showing the highest number of embryos were selected for quantitation from each movie. To prevent bias in selection of embryos for quantitation, all embryos visible in a viewfield were quantified and included in the final dataset. Swimming behaviors were analyzed using Image J software with the plugins "QuickTime Movie Opener," and "Multitracker2 (MTrack2)" (available for free download from the National Institutes of Health at http://rsb.info.nih.gov/ij/).

To study cilia lengths and densities, control uninjected and MASO-injected *S. purpuratus* embryos were imaged by video-enhanced phase microscopy on a Nikon E200 microscope equipped with a Nikon 40 × /0.65 N.A. Ph2 DL objective, and Sony DFW-X700 digital video camera with Diagnostic Instruments 0.5 × C-mount (all from Micro Video Instruments) using BTV Pro software (http://www.bensoftware.com/) on a Macintosh G4 computer. All embryos were handled singly and identically by gentle mouth pipetting into viewing chambers (Morris et al. 2001) to control for shear that might remove cilia, and imaged identically to control for focal-plane depth that might influence numbers of cilia visible. Identical proportions of early, mid, and late gastrulae were imaged to control for proportions of short, medium, and long cilia on different developmental stages. Embryos were imaged from the side to view apical plate cilia and down their animal–vegetal (AV) axis to view lateral cilia. Twenty-six embryos (13 *SpNK2.1*-MASO injected and 13 controls) were imaged for an average of 640 frames each over 50 sec, and full lengths of cilia that fell entirely in the focal plane in a single image frame were traced onto transparencies, measured, and scored for motility. For quantitation purposes, a cilium was considered to originate within the apical plate if its base lay within 25 μm circumferential distance of the animal pole, itself defined as the point in the animal hemisphere most distal from the center of the blastopore. A cilium was considered "beating" if it not only originated a bend at its base but would also propagate the bend along the cilium's length (Morris and Scholey 1997), "beating weakly" if it originated a bend but did not propagate the bend, and "not beating" if it did not initiate any bends during the viewing period.

Culture of *H. rufescens* embryos and adults

Animals were obtained from the Cultured Abalone Inc. (Santa Barbara, CA, USA) and maintained at 13°C in a well-aerated aquarium. Animals were spawned by placing females and males in separate containers to which 6.6 ml of 2 M Tris was added to each liter of seawater. After 15 min 4 ml of freshly prepared 6% hydrogen peroxide was added to each liter of seawater. After 2.5 h, the animals were washed several times in fresh seawater and then checked periodically for spawning. Eggs were fertilized using standard methods (Strathmann 1987), and the cultures were kept in culture at low density at 16°C with Penn/Strep added to the culture with no stirring. Adult animals were fed after spawning.

PCR amplification and genomic library screening

A touchdown style PCR methodology (Don et al. 1991) was used to amplify highly conserved regions of nucleic acid sequence from *H. rufescens* cDNA. Primers (5′–3′), forward and reverse, respectively, for each of the genes are as follows: *NK2.1*—TTYWSNCARGCNCARGTNTAYGA and TGRAACCADATYTTNACYTGNGTNGG; *FoxA*—TNATNACNATGGCNATNCA and TARCANCCRTTYTCRAACATRTTNCC; *Hrα 2tubulin*—TGYTTYGARCCNGCNAAYCA and CCARTGNACRAANGCNCKYTT; *Radial spoke head p63*—TGYMGNTTYTGGGGNAARATH and TARTTNGCYTCRTTNCCNGGRAANGGNGG; and *Dynein p33*—GCNMGNGARACNGGNATHTGYCC and YTTNGGNGCDATDATNCCYTC. *HrHNF6* was amplified using the PCR conditions and primers previously described in Otim et al. (2004). The recovered gene specific PCR fragments were subcloned into pGEM-T Easy vectors (Promega), amplified, sequenced, and confirmed by phylogenetic analysis (not shown).

Gene-specific probes were then amplified, labeled with ^{32}P-dCTP as above, and used to screen a *H. rufescens* mRNA Lambda ZAP Express cDNA library using standard hybridization conditions (Takacs et al. 2004). Total RNA derived from embryonic stages of *H. rufescens* was combined for cDNA construction: early trochophore larvae (15 h pf), mid-trochophore larvae (18 h pf), late trochophore larvae (22 h pf), and pretorsional veliger larvae (27 h pf). The total RNA was sent to Lofstrand Labs Limited (Gaithersburg, MD, USA) for library construction. The library contained at least one million primary clones and $>10^{10}$ amplified clones. Approximately 550,000 cDNA clones were screened with each probe. Insert containing pBK-*Hr* phagemid clones were isolated by in vivo excision using ExAssist Help Phage (Stratagene) with XLOLR cells (Stratagene, La Jolla, CA, USA) as described by the manufacturer. The *HrTektin3* cDNA clone was isolated by screening the *H. rufescens* mRNA Lambda ZAP Express cDNA library with the *S. purpuratus Tektin3* ortholog isolated above. Positive pBK phagemid clones were isolated as described above.

Probe synthesis, embryo fixation and whole mount in situ hybridization

Anti-sense RNA probes for WMISH were synthesized from either linearized plasmid (pTeasy) or phagemid (pBK) DNA templates using either fluorescein-12-UTP or digoxigenin-11-UTP (DIG) RNA labeling kits (Roche). DNA templates for anti-sense RNA probe synthesis were prepared as follows. For *HrNK2.1*, a 1.8 kb fragment consisting essentially of 3′UTR was amplified by PCR from the *HrNK2.1* cDNA clone (primers: F—TGGGTCTCTGT CTCACTCTG; R—TTCCAGGGTAATCTGTGC), and then subcloned into a pGEM Teasy vector (Promega) to serve as a probe template when linearized with *Nco*I. For *Hrα 2tubulin*, a full-length clone was digested with *Eco*RV/*Xho*I removing the 3′ coding region and polyA tail. The vector-containing fragment was then self-ligated and digested with *Eco*RI to act as a template for RNA probe synthesis. For *HrFoxA*, a partially 5′ truncated clone containing most of the ORF, and 3′ UTR was used after digestion with *Sal*I. For *HrHnf6*, a partially 5′ truncated clone without a polyA tail was linearized with *Sma*I. For *HrTektin3*, a full-length clone consisting of the entire ORF, and 3′ UTR was used after being linearized with *Eco*RI. For *HrRSH* and *HrDynein* the PCR amplified fragments were used as templates for probe synthesis after linearization with *Sal*I and *Nco*I, respectively.

H. rufescens embryos were fixed and stored in 70% EtOH as described (Arenas-Mena et al. 2000). WMISH of embryos was performed as described in Arenas-Mena et al. (2000) and Takacs et al. (2004). Two-color WMISH, for the simultaneous detection in embryos hybridized with two gene-specific probes, followed the protocol of Hauptmann et al. (2001). Briefly, anti-sense RNA runoff transcripts were labeled either with fluorescein-12-UTP (Roche), used at a final concentration of 0.4 ng/μl during hybridization, or digoxigen-11-UTP (Roche), used at a final concentration of 0.2 ng/μl during hybridization. Embryos were first stained with the Fast Red Tablets (Roche) to detect the fluorescein labeled probe. Subsequently, the DIG-labeled probe was detected by staining with NBT/BCIP solutions.

RESULTS

NK2.1 and the apical tuft in the sea urchin *S. purpuratus*

To ask whether the apical tuft in a sea urchin and a gastropod mollusc are specified in a similar manner, we first explored the specification of the apical tuft in the sea urchin *S. purpuratus*. Takacs et al. (2004) showed that the *NK2* transcription factor *SpNK2.1* is expressed in the apical plate of the sea urchin *S. purpuratus*. Abrogation of SpNK2.1 function by introduction of the *SpNK2.1* MASO had no effect on the developing nervous system, including the apical ganglion, and did not appear to affect embryonic development (Takacs et al. 2004). Hence, we performed subtractive hybridization screens (see "Materials and Methods") designed to isolate downstream target genes to identify the role *NK2.1* plays in the development of *S. purpuratus*. Fragments of three cilia genes were isolated: the putative orthologue of *radial spoke 3*, *tektinA1* (one of three paralogues of *tektin3* in the sea urchin, Norrander et al. 1992), and the sea urchin *radial spoke head p63* gene (Gingras et al. 1998; see Dutcher 1995 for review of the structure of cilia). Phylogenetic analysis confirmed our gene identifications (not shown). A fourth gene, *α2tubulin*, was known to be expressed in the apical plate of the sea urchin *Paracentrotus lividus* (Gianguzza et al. 1995), and thought to be a potential downstream target of an *NK2* gene (Costa et al. 2004). We isolated the orthologue of this gene from *S. purpuratus* using PCR and cDNA library screening (see "Materials and Methods"), and found two isoforms, one of which was the clear orthologue of the *α2tubulin* as determined by comparison of the 3′ UTR sequence. Finally, a fifth gene, *dynein p33*, was already identified from *S. purpuratus* (Gingras et al. 1996), but its expression pattern during embryogenesis was unknown.

To ask where these cilia genes are expressed we performed WMISH. Transcripts of all but *radial spoke 3* were detected in maternal preparations, and all were expressed uniformly throughout the ectoderm until hatching (Fig. 2C, and data not shown). However, after hatching all five genes were expressed in the apical plate in a subregion of the *NK2.1* expression domain (Fig. 2A). We next asked whether any or all of these genes are downstream of *SpNK2.1*. Injecting a MASO directed against *SpNK2.1*, which specifically abrogates the expression of SpNK2.1 protein (Takacs et al. 2004), affected the expression of all five cilia genes. Transcripts of *α2tubulin*, *radial spoke 3*, and *tektin3* were no longer detected, whereas transcripts of *RSH p63* and *dynein p33* were detected ectopically throughout the oral ectoderm (OE) with transcripts of *dynein p33* concentrated in the ciliated band (Fig. 2B). These data suggest that *SpNK2.1* and these five cilia genes are components of a regulatory network.

To ask whether these five cilia genes behave similarly to *NK2.1* upon perturbation, we analyzed expression of all five in *SpDri*-MASO injected embryos, as well as LvCadherin-injected embryos. *SpDri*, the orthologue of *Deadringer*, is necessary for the activation and maintenance of a number of genes expressed in the OE (Amore et al. 2003) including *SpNK2.1* (Amore et al. 2003; Takacs et al. 2004). Like *SpNK2.1*, transcripts of all five cilia genes are no longer detected in embryos injected with the *SpDri* MASO after approximately 28 h of development (Fig. 2D, and data not shown) consistent with the notion that the apical plate becomes subsumed within the OE territory during gastrulation (see Takacs et al. 2004). Removal of β-catenin signaling, via injection of LvCadherin, results in embryos that lose oral–aboral (OA) polarity and expression of genes in the OE (Wikramanayake et al. 1998; Logan et al. 1999; Angerer et al. 2001; Duboc et al. 2004; reviewed in Brandhorst and Klein 2002; Angerer and Angerer 2003). Nonetheless, β-catenin

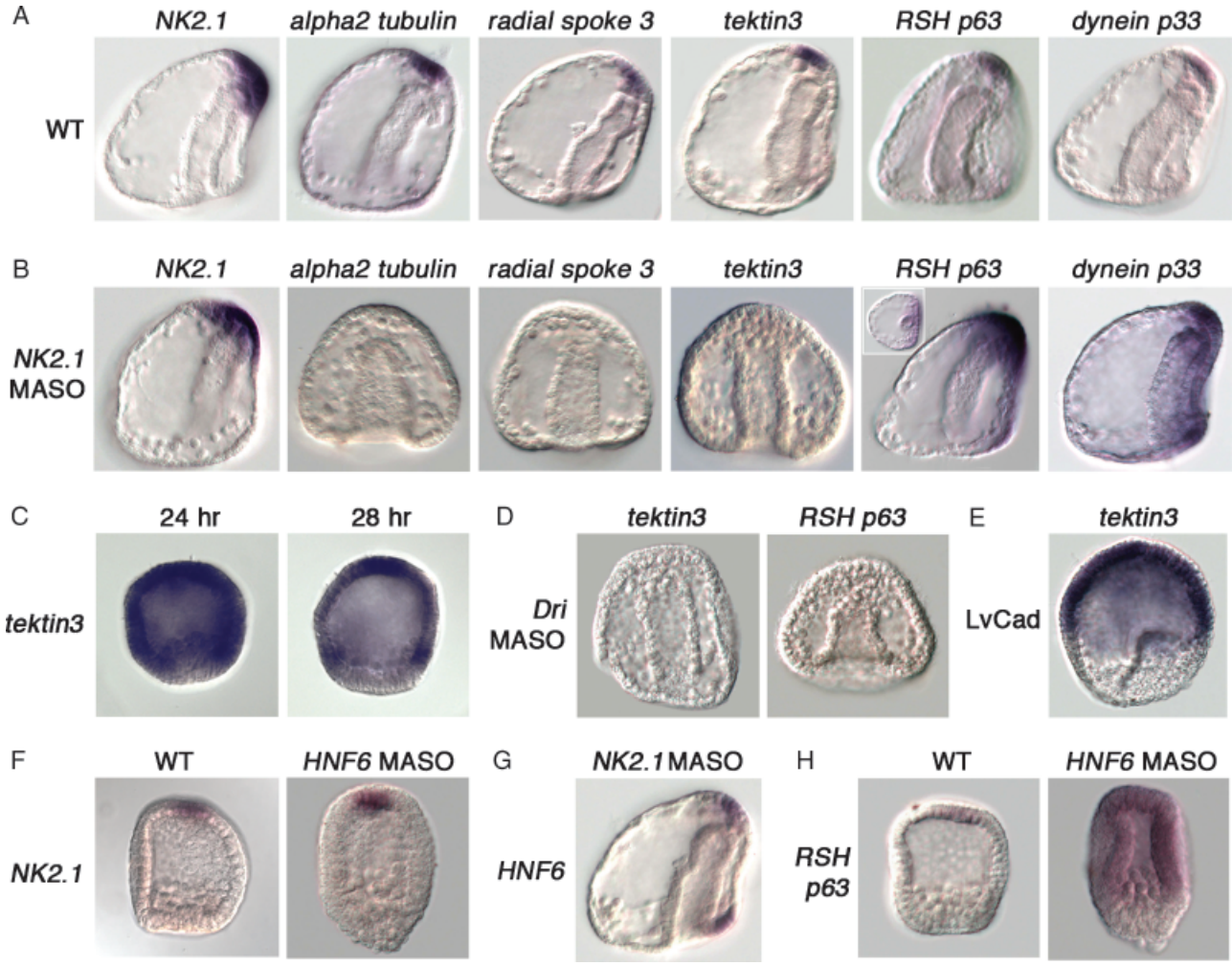

Fig. 2. Components of the gene regulatory network underlying apical tuft specification in the sea urchin *Strongylocentrotus purpuratus*. (A) Expression patterns of *SpNK2.1* and the five cilia genes examined herein at 42 h of development in wild-type (WT) embryos. All of the cilia genes have identical expression patterns that overlap the expression pattern of *SpNK2.1*. (B) Injection of a *SpNK2.1* morpholino anti-sense oligonucleotides (MASO) has no affect on transcription of *SpNK2.1*, abrogates expression of *α2tubulin*, *tektin3*, and *radial spoke* 3, but results in the ectopic expression of *radial spoke head p63* (note restriction of transcripts to the oral ectoderm, inset) and *dynein p33*. (C) Early expression of *tektin3* in WT embryos. (D) Injection of a MASO directed towards the transcription factor *SpDri* abrogates expression of all cilia genes including *tektin3* (left) and *RSH p63* (right) after approximately 28 h of development. (E) In contrast to *SpDri* MASO (D), injection of LvCadherin RNA results in the continuous expression of *tektin3* throughout much of the ectoderm. Because the effects are the same for each perturbation for all five cilia genes only data for *tektin3* are shown. (F) Injection of the *SpHNF6* MASO has no effect on the expression pattern of *SpNK2.1* (left, shown at ~24 h; compare with the WT on the right). (G) Injection of the *SpNK2.1* MASO has no affect on the expression pattern of *SpHNF6* (compare with Otim et al. 2004 and Poustka et al. 2004). (H) Injection of the *SpHNF6* MASO effects the expression pattern of the cilia genes including *RSH p63* with transcripts detected throughout the ectoderm (left, shown at ~ 24 h; compare with the WT on the right). After ~ 28 h of development though, transcripts are no longer detected, similar to what is shown in (D).

signaling is necessary for the restriction of *SpNK2.1* to the apical plate as embryos injected with LvCadherin show ectopic activation of *SpNK2.1* throughout most of the embryonic ectoderm (Takacs et al. 2004; Yaguchi et al. 2006). In LvCadherin-injected embryos, transcripts of all five cilia genes were detected throughout much of the embryo in a manner very similar to *SpNK2.1* expression (Fig. 2E, and data not shown). Thus, these data strongly suggest that the apical tuft is initially specified within a unique ectodermal territory (called the apical domain by Takacs et al. 2004), in part as a consequence of β-catenin signaling.

The phenotype resulting from the loss of SpNK2.1

Because all of these genes encode known components of cilia, we next asked whether the apical tuft developed normally in

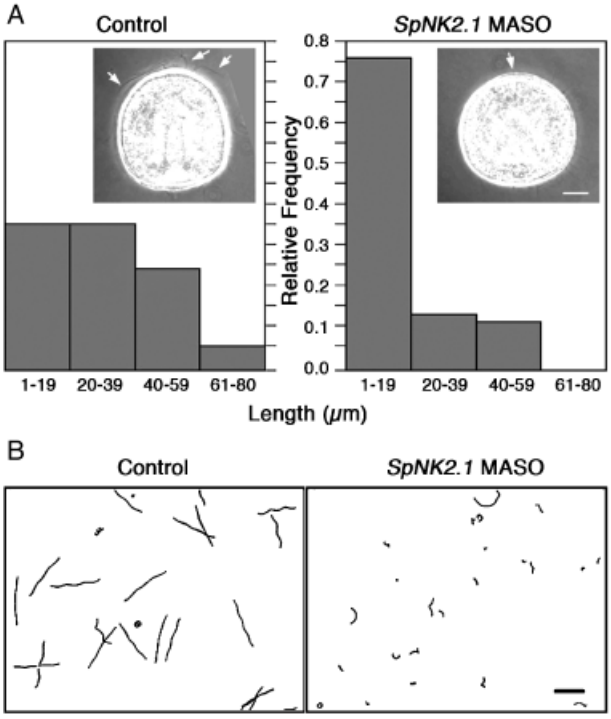

Fig. 3. Phenotype of the *SpNK2.1* morpholino anti-sense oligonucleotides (MASO) "knockdown." (A) Injection of a control MASO has no effect on the length of the apical tuft cilia—only 35% of the cilia are between 0 and 20 μm and 30% are greater than 40 μm (left; indicated with arrows on the inset), whereas 75% of the cilia are between 0 and 20 μm in the *SpNK2.1*-MASO injected embryo (indicated with the arrow on the inset) with none greater than 40 μm (right). Scale bar is 25 μm. (B) *SpNK2.1*-MASO injected embryos ($N = 24$) tracked for 120 sec swim slow and in crooked paths (right), whereas control-MASO injected embryos ($N = 24$) tracked for the same amount of time swim faster and in straighter paths (left) as compared with the *SpNK2.1*-MASO injected embryos. Scale bar is 1000 μm.

SpNK2.1 MASO-injected embryos (Fig. 3A). Wild-type embryos, and those injected with a control MASO, both showed normal morphology of the ciliary tuft with $\sim$ 5% of the cilia greater than 60 μm in length (Fig. 3A left panel, arrow), 60% of the cilia ranging in length from 20 to 60 μm, and 35% of the cilia ranging in length from 0 to 20 μm (similar to the lengths of cilia found throughout the rest of the embryo). Introduction of the *SpNK2.1* MASO eliminated all cilia greater than 60 μm in length, and reduced the number of cilia 20–60 μm in length from 60% to 24%, leaving most of the cilia in the apical plate (76%) in the 0–20 μm range (Fig. 3A, right panel, arrow). Hence, abrogation of SpNK2.1 function eliminates the apical tuft and results in an embryo with relatively uniform ciliation.

In addition to a defect to the development of the apical tuft, we noticed that the *SpNK2.1* MASO-injected embryos sat on the bottom of the Petri dish and did not swim normally in comparison with either wild-type or control-injected embryos. To quantify this apparent defect we tracked 24 control embryos for a total of 120 sec and 24 MASO-injected embryos for 120 sec (Fig. 3B). Measuring the velocity of each embryo showed that the average instantaneous velocity (i.e., speed irrespective of direction) of control embryos was 249 μm/sec and the average net velocity (i.e., distance embryos travel from their starting point) of the control embryos was 228 μm/sec. These measurements indicate that control embryos swim fast and in straight paths (Fig. 3B, left). The *SpNK2.1* MASO-injected embryos had an average instantaneous velocity of 94 μm/sec and an average net velocity of 50.3 μm/sec and thus these embryos swim slowly and in crooked paths (Fig. 3B, right). Thus, the control embryos swim faster and swim straighter than the MASO-injected embryos consistent with a defect in ciliation.

To determine whether the *SpNK2.1* MASO simply blocked cilia assembly generally and not apical tuft cilia specifically, we measured the lengths of lateral cilia away from the apical tuft region. In control embryos the average cilia length in lateral region was 19.6 μm and the standard deviation was 2.8 μm ($n = 69$ cilia on three control embryos). In MASO-injected embryos the average cilia length in lateral region was 19.1 μm and the standard deviation of cilia length in lateral region was 3.1 μm ($n = 57$ cilia on three MASO-injected embryos). Therefore, the *SpNK2.1* MASO dramatically affects assembly of the long apical tuft cilia.

NK2.1 and *HNF6* in the sea urchin *S. purpuratus*

Yaguchi et al. (2006) have shown that in the sea urchin the apical organ arises at the intersection of two transcription factors, *SpNK2.1* and *SpHNF6*. *SpHNF6* (also known as *SpOnecut*, Poustka et al. 2004) is maternally expressed and shows a complicated pattern of expression affecting several different specification pathways (Otim et al. 2004), but by gastrulation transcripts are restricted to the ciliated band (Otim et al. 2004; Poustka et al. 2004, see also Fig. 2G). Otim et al. (2004) reported that according to quantitative PCR experiments, *SpHNF6* and *SpNK2.1* appeared to play parallel roles in the apical plate given that abrogation of SpHNF6 had little or no effect upon *SpNK2.1* transcription. We confirm their findings here: *SpNK2.1* was expressed normally in *SpHNF6*-MASO injected embryos (Fig. 2F) and *SpHNF6* was expressed normally in *SpNK2.1*-MASO injected embryos (Fig. 2G). However, an interesting pattern of expression was seen for the five cilia genes—all five were expressed uniformly throughout the ectoderm (Fig. 2H, and data not shown). These data suggest that together *SpNK2.1* and *SpHNF6* are necessary for the proper spatial restriction of expression of these (and presumably other) cilia genes to the apical plate.

NK2.1, HNF6, and cilia genes in the gastropod mollusc *H. rufescens*

Having established the role that these genes play in apical tuft specification in the echinoid *S. purpuratus*, our aim was to determine the roles the orthologues of these genes play in the development of the gastropod mollusc *H. rufescens*. This constitutes our test of homology of apical tufts, and ultimately of larvae themselves, because if the mollusc shows a similar pattern whereby *HNF6* and/or *NK2.1* regulate the expression of cilia genes in the apical tuft, then homology would be indicated. Thus, we cloned fragments of the orthologues of *SpNK2.1*, *SpHNF6*, and all five cilia genes (see Fig. 2) and examined their expression patterns using WMISH on stages from early trochophore larvae (15 h pf) through pretorsional veliger larvae (27 h pf) (Fig. 4). The expression pattern of *HrNK2.1* is complex. Transcripts of *HrNK2.1* were first detected at 20 h as two dorsally located loci at the apical pole of the embryo (Fig. 4, A and B, arrow) and as a ring around the animal side of the blastopore (Fig. 4B, "b"; discussed in more detail below). Two more loci were then seen starting around 23 h again at the apical pole, but this time on the ventral side (Fig. 4, C and D, arrowhead). Then, a single, more centrally located, cell located on the apical pole expressed *HrNK2.1* (Fig. 4C, asterisk) so that for a very short time five different loci expressed *HrNK2.1* in the apical pole (Fig. 4C, inset). Then, the two dorsal loci were lost so that by 27 h only three loci were detected, the two ventral and the one central loci (Fig. 4C). Stain was also observed on the dorsal side of the larva opposite that of the blastopore (indicated with an asterisk on Fig. 4B), but this will not be discussed further.

We did not detect expression of *HrHNF6* in the prototroch, nor did we detect expression in the apical tuft (Fig. 4, M–P). Instead, starting around 20 h and continuing through 27 h of development we detected transcripts in two loci located on the dorsal side of the apical pole (Fig. 4, M–P, double arrowhead), similar to the early expression pattern of *HrNK2.1* (Fig. 4, A and B, arrow). We hypothesize that this is the anlage of the cerebral ganglion given its deep position with respect to the apical plate (Fig. 4P, double arrowhead). *HrHNF6* was also expressed in other loci, including the dorsal aspect of the larva (Fig. 4P, asterisk), but again this expression will not be discussed further.

Transcripts of all five cilia genes were detected by 20 h in two different loci, the prototroch (Fig. 4, E–N, "p"), and two apically located cells, the presumed precursor cells of the two laterally located apical tuft cilia (Page 2002a; Fig. 4, E and F, double arrow; because all five cilia genes have identical expression patterns, only *HrTektin3* is shown). These two cells come together by 27 h and appear contiguous on the ventral face of the apical dome (Fig. 4, G and H, bracket). To confirm that neither *HrNK2.1* nor *HrHNF6* are expressed in the apical tuft, we performed double-stained WMISH for each transcription factor (DIG, blue) and *HrTektin3* (fluorescein, red)—at no time did we detect *HrNK2.1* (Fig. 4, I–L) or *HrHNF6* (Fig. 4, M–P) co-expressed with *HrTektin3* anywhere in the embryo. Hence, unlike the sea urchin, these two transcription factors do not appear to belong within the same regulatory circuit as the five cilia genes, and thus cannot directly regulate the specification of the apical tuft in the gastropod mollusc.

NK2.1 and expression in the foregut

Unlike both the apical tuft and the ciliated band, similarity of expression patterns are seen between these two taxa for transcription factors known to be expressed in the gut. *NK2.1* is expressed in the pharynx/foregut of echinoids (Takacs et al. 2004; Fig. 5, A–C), hemichordates (Takacs et al. 2002), flies (Zaffran et al. 2000), and *C. elegans* (Harfe and Fire 1998), and is required for normal thyroid development in vertebrates (Kimura et al. 1996), suggesting a pan-bilaterian role in foregut development (Takacs et al. 2002). Expression in the larval foregut of *S. purpuratus* was not dependent upon expression in the apical plate as abrogation of apical expression via injection of either stabilized β-catenin (Yaguchi et al. 2006) or the *SpDri* MASO (Fig. 5C) did not affect foregut expression. *NK2.1* was also expressed in the foregut of the gastropod mollusc. At 20 h of development, transcripts of *HrNK2.1* were detected on the animal (or apical) face and the lateral edges of the blastopore (Fig. 5D, "b"), in addition to the transcripts detected on the apical pole as previously described (Fig. 4, A–D). At 27 h of development, transcripts continue to be detected in a group of cells nestled just under the prototroch, the putative stomodeum (Fig. 5E, "s"), and when viewed laterally the restriction to the future dorsal wall of the foregut is evident (Fig. 5F). The restriction of transcripts to the dorsal wall of the pharynx is obvious when compared with the expression of *HrFoxA*. Initially, *FoxA* was expressed vegetally and laterally around the blastopore (Fig. 5G, "b"), and then was restricted to the future ventral wall of the foregut in 27 h larvae (Fig. 5I). Furthermore, *HrFoxA* was also detected around the proctodeum at 27 h development (Fig. 5H, "p"). Thus, similar to other bilaterians (e.g., Kalb et al. 1998; Hinman et al. 2003a), with the interesting exception of the gastropod mollusc *Patella vulgata* where transcripts are detected throughout the endoderm as well as the ectomesoderm (Lartillot et al. 2002), *HrFoxA* is expressed in both the fore- and hindgut, but not the midgut. Hence, while *HrNK2.1* is expressed in the dorsal wall and *HrFoxA* is expressed in the ventral wall, both are expressed in the pharynx/foregut of the red abalone.

DISCUSSION

Here, we show that although both the sea urchin *S. purpuratus* and the red abalone *H. rufescens* express the transcrip-

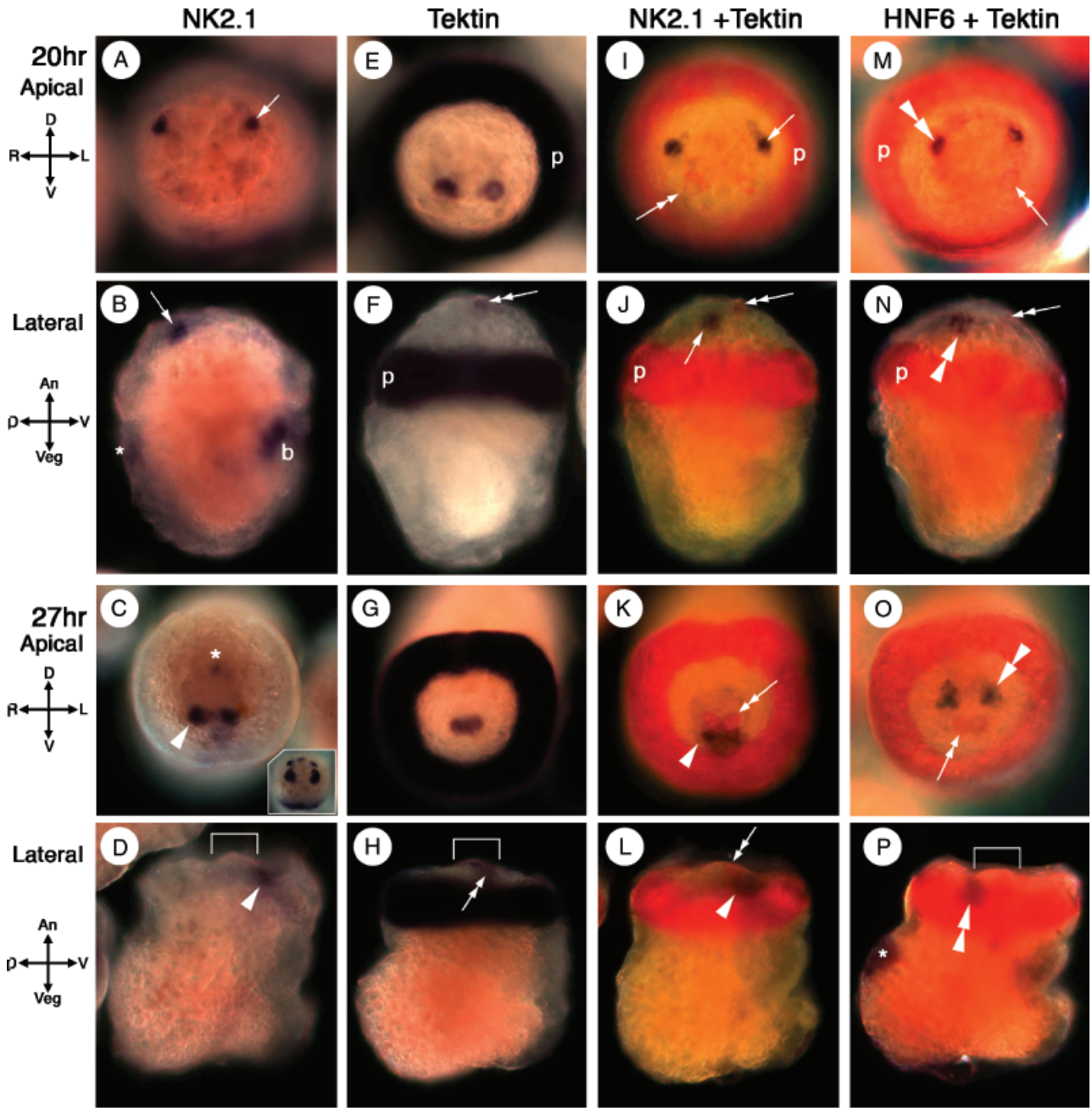

Fig. 4. Expression of *HrNK2.1*, *HrHNF6*, and *HrTektin3* in the red abalone *Haliotis rufescens*. (A, B) *HrNK2.1* is initially detected in two dorsal and apical loci in 20 h embryos (arrow) as well as around the animal aspect of the blastopore ("b"). (C–D) By 27 h of development *HrNK2.1* is now detected in a single central and apical locus (below the ∗) and two apical and ventral loci (arrowhead). For a brief period around 23 h of development *HrNK2.1* is detected in five separate apical loci (C, inset). The two ventral loci sit on the ventral surficial face of the "apical dome" (D, bracket). (E–F) Expression of *Hrtetkin3* in 20-h embryos. Stain is detected in two loci the prototroch ("p") and the two lateral apical tuft cells (double arrow). (G–H) Expression of *HrTektin3* continues in the same pattern as seen in the 20-h embryos. (I–L) Double-labeled whole mount in situ hybridization (WMISH) for *HrNK2.1* (blue) and *HrTektin3* (red)—at no time does the apical tuft express *HrNK2.1*. (M–P) Double-labeled WMISH for *HrHNF6* (blue, double arrowhead) and *HrTektin3* (red, double arrow)—at no time does the apical tuft or the prototroch ("p") express *HrHNF6*. Note that the locus of *HrHNF6* expression in 27 h embryos sits dorsal and deep (double arrowhead) with respect to the apical dome (bracket) consistent with stain restricted to the cerebral ganglia. Anatomical axes are indicated on the left (D, dorsal; V, ventral; An, animal [= apical]; Veg, vegetal). Embryos are ∼ 200 μm in length.

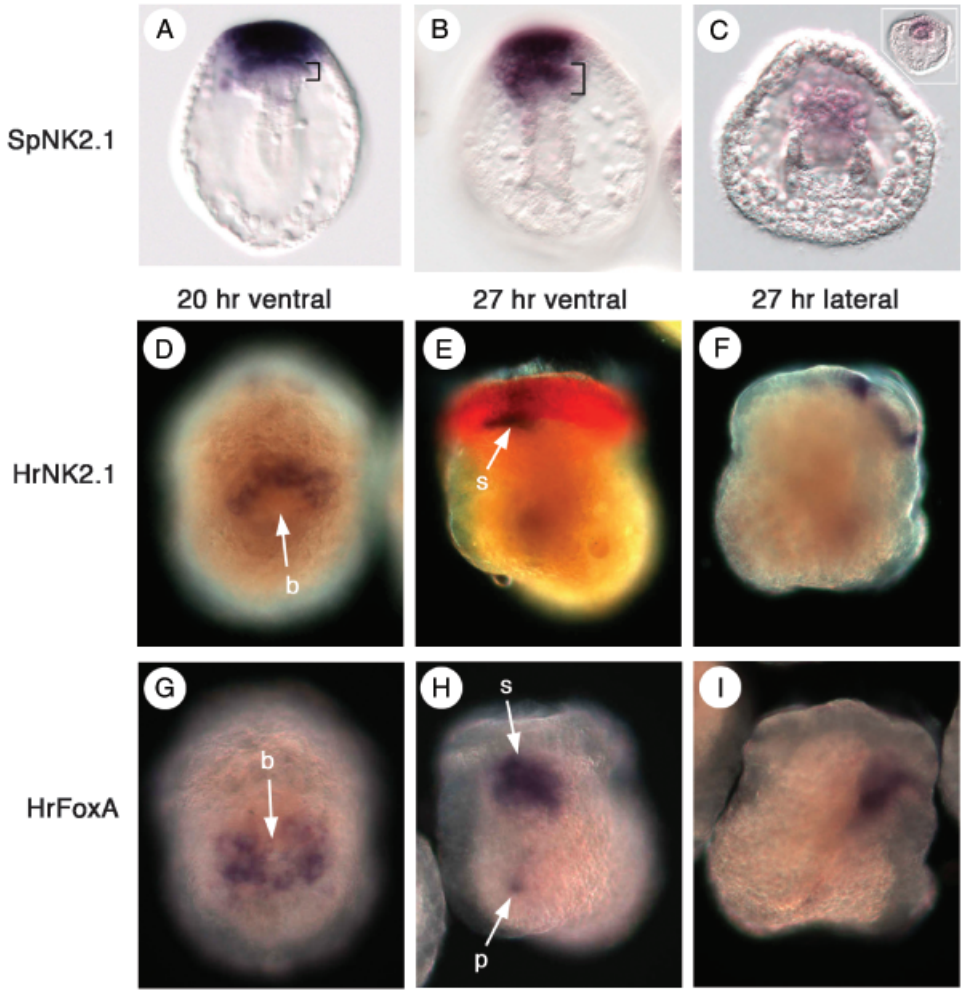

Fig. 5. Similarity of expression patterns of *NK2.1* and *FoxA* in the gut of the sea urchin and gastropod mollusc. (A, B) *SpNK2.1* expression in the sea urchin embryo photographed frontally. Bracket indicates expression in the foregut on two different embryos, one where the plane of focus is on the apical expression domain (A) and the second on the foregut expression domain (B). (C) Expression of *SpNK2.1* in a *SpDri*-MASO-injected embryo. Although expression is abrogated in the apical domain, there is no effect on *SpNK2.1* expression in the foregut domain. Inset shows expression in the gut (compare with Takacs et al. 2004 for earlier stages when *NK2.1* is restricted to the apical plate). (D–F) Expression of *HrNK2.1* in the red abalone. In addition to the apical expression domain (Fig. 4) expression is also seen on the animal face of the blastopore (D, "b") and continues to be restricted to the dorsal wall of the stomodeum (E, H "s"). (G–I) In contrast to *HrNK2.1* the expression of *HrFoxA* is restricted to the lateral and vegetal sides of the blastopore (G, "b") and is seen on the ventral wall of the foregut. Expression is also seen in the proctodeum (H, "p"), and thus like many animals transcripts of *FoxA* are restricted to the termini of the alimentary canal.

tion factors *HNF6* and *NK2.1* in the apical region of their respective embryos, these factors appear to be running different gene batteries. In the sea urchin, proper development of the apical tuft requires the input of both transcription factors. Abrogation of SpNK2.1 protein via anti-sense knockdown results in misexpression of five different genes encoding cilia proteins that are expressed in the apical plate of the embryo, as well as the loss of the long cilia normally found there. Abrogation of SpHNF6 results in the failure of restriction of the same mRNAS to the apical plate and their continued ectopic expression throughout the ectoderm. In contrast, in the gastropod mollusc these same five cilia genes are not co-expressed with either *HrHNF6* or *HrNK2.1* suggesting that, at best, they can only operate indirectly into the specification pathway for the apical tuft of *Haliotis*. These data suggest that the apical tufts of these two taxa are not homologous, and thus are consistent with the hypothesis that primary marine larvae are a convergent feature intercalated multiple times independently into an already existing direct-developing strategy.

The apical domain of metazoan embryos

Takacs et al. (2004) argued that the apical plate lies within a distinct ectodermal territory of the sea urchin embryo, the apical domain. Davidson (1989; see also Davidson et al. 1998) defined embryonic territories as polyclonal assemblages of contiguous cells whose progeny express a particular set of genes. Five distinct territories were proposed: OE (including the apical plate), aboral ectoderm, endoderm, primary mesenchyme, and the small micromeres. Although the original model has been slightly modified over the years, its essence has remained unchanged and it still constitutes a powerful explanation for how specification works in a small ciliated animal embryo (Davidson et al. 2002). Takacs et al. (2004) (see also Angerer and Angerer 2003) proposed that the apical domain of the sea urchin embryo constitutes a distinct sixth territory for the following reasons. First, the apical domain is a morphologically distinct region of the embryo fated to give rise to a specific structure, the apical organ consisting of ganglion and tuft. Second, the apical domain is

strangely refractory to genetic and chemical perturbation including perturbations to both the AV and the OA specification pathways (reviewed in Angerer and Angerer 2003; Yaguchi et al. 2006). And third, the apical domain is characterized by a unique gene expression profile: lack of accumulation or down-regulation of several mRNAs including *SoxB2* (Kenny et al. 2003), high levels of animalizing transcription factors, but very low or no nuclear β-catenin activity, and expression of the transcription factor *NK2.1*. By approximately 28 h of development though, the apical domain comes under the control of the OE specification network such that maintenance of *SpNK2.1* transcription (but not initiation) as well as maintenance of all five cilia genes reported herein (Fig. 2) depends upon the oral activator *SpDri* (Amore et al. 2003).

The fact that the apical domain exists in the sea urchin embryo as a territory both spatially and temporally distinct from the OE suggests that it could have its own separate evolutionary history as well. Whether an OE territory exists outside of the echinoderms (or possibly ambulacrarians) is unknown, but appears doubtful given that the episphere, the only potential homologue in the trochophore, does not show gene expression patterns consistent with its being a discrete embryonic territory (unlike, e.g., the prototroch or the ectomesenchyme). In other words, if the episphere were homologous to the OE of the sea urchin, one might expect oral markers such as *Goosecoid* or *Otx* to be expressed throughout the episphere rather than restricted to just the mouth/foregut and immediate area (Arendt et al. 2001). The apical domain, on the other hand, has long been recognized on morphological grounds, and now three different indirectly developing marine invertebrates (the hemichordate *P. flava* (Takacs et al. 2002), the echinoid *S. purpuratus* (Takacs et al. 2004) and the gastropod mollusc *H. rufescens* [this report]) all express *NK2.1* in the apical plate as well. These data suggest that ancestrally *NK2.1* was expressed at the apical pole of the bilaterian embryo, but was recruited into different roles in the dipleurula and trochophore larvae. Indeed, the cnidarian *Hydra vulgaris* expresses the NK2 gene *CnNK-2* at the peduncle (Grens et al. 1996), the end opposite that of the blastopore and hence homologous to the apical end of a bilaterian embryo (Nielsen 2001). However, the evolutionary relevance of this observation remains elusive given that it is expressed in the *endoderm* of the peduncle, and it is unclear if *CnNK-2* is an orthologue of *NK2.1* or *NK2.5* (Kamm and Scheirwater 2006; unpublished phylogenetic analyses).

Within the sea urchin embryo, our data suggest that NK2.1 might regulate a precise balance of expression of cilia genes. Indeed, recent studies of flagellar length control in the unicellular alga *Chlamydomonas* demonstrate that balance of cilia assembly and disassembly can determine flagellar length (Marshall et al. 2005). By such a "balance-point" model, loss of cilia proteins that promote cilia assembly such as any of the proteins essential for intraflagellar transport (Marshall and Rosenbaum 2001; Rosenbaum and Whitman 2002) might be all that is required to eliminate the apical tuft. Indeed, given the role of *tektin3* in determining the length of cilia (Norrander et al. 1995), the elimination of the apical tuft by a knockdown of SpNK2.1, an upstream activator of the *tektin* gene, may not be surprising. It will be interesting to test whether SpNK2.1 influences the expression of other essential cilia proteins as well.

Origins of primary marine larvae

Rigby and Milsom (1996, 2000) demonstrated that zooplankton have continuously invaded the pelagos through geologic time—18 of 21 planktonic groups arose on the benthos and subsequently invaded the water column, and no major clade of zooplankton evolved within the water column. The only possible exception, according to Rigby and Milsom (2000), might be the ciliated larval stages of marine invertebrates because if trochophores and dipleurula larvae are descended from a holopelagic ancestor, as hypothesized by Jägersten (1972) and Nielsen (2001), then the initial evolution of animals would have taken place in the pelagos and not on the benthos. This idea though has not received much acceptance—most authors have concluded that larval stages are products of convergent evolution intercalated into a benthic direct-developing strategy (e.g., Olive 1985; Chaffee and Lindberg 1986; Buckland-Nicks and Scheltema 1995; Haszprunar et al. 1995; Conway Morris 1998; Valentine et al. 1999; Budd and Jensen 2000; Hadfield 2000; Valentine and Collins 2000; Baguñà et al. 2001; Hadfield et al. 2001; Jondelius et al. 2002; Sly et al. 2003; Peterson et al. 2005). Indeed, ecological considerations clearly show that feeding zooplankton could not have evolved much before the Tommotian (Butterfield 1997, 2001), and Nützel et al. (2006) have recently shown that within gastropod molluscs planktotrophy did not evolve until the Cambrian–Ordovician transition around 490 Ma, as predicted by Signor and Vermeij (1994) and Peterson (2005; see Fig. 1).

Nonetheless, the only two studies designed to specifically address whether a feeding larval stage is primitive for Bilateria concluded that planktotrophy is most likely primitive for protostomes and deuterostomes. Peterson et al. (2000b) showed that, like the sea urchin (Arenas-Mena et al. 1998, 2000), *Hox* genes are not used to build the trochophore larvae of a polychaete annelid, as predicted by Davidson et al. (1995). Probe-excess titration experiments showed that the polychaete *Chaetopterus* sp. did not express four of five *Hox* genes examined during embryogenesis, but transcripts of all five were detected during adult body plan formation and were localized to what were presumed to be the teloblastic "set-aside" cells (see also Hinman et al. 2003c). However, these experiments did not actually test the primary hypothesis as they were necessarily based on the absence of expression.

The second study by Arendt et al. (2001) examined the expression patterns of *Brachyury*, *Otx*, and *Goosecoid* in the polychaete annelid *Platynereis*, and argued that because the mouth/foregut and ciliated bands of both larval types express similar genes the most likely explanation is that the last common ancestor of protostomes and deuterostomes developed through a feeding larval stage. Ignoring the ciliated bands for a moment, the common expression of genes like *Brachyury* in the larval mouth/foregut does not actually test the proposed homology of larval types because a mouth/foregut is also present in cnidarians and it too expresses *Brachyury* and *Gsc* (Broun et al. 1999; Technau and Bode 1999; Scholtz and Technau 2003). The fact that the nonfeeding larva of *H. rufescens* expresses *FoxA* (Fig. 5) in a very similar pattern with other bilaterians including cnidarians (Martinez et al. 1997, 2004; Fritzenwanker et al. 2004) suggests that the evolution of planktotrophy from lecithotrophy might just involve only relatively trivial shifts in developmental timing, especially that of gut development (Strathmann 1993; Peterson et al. 2005). Furthermore, the expression of *NK2.1* in the foregut/pharynx of most animals analyzed including both the sea urchin and gastropod (Fig. 5) suggests that the last common ancestor of protostomes and deuterostomes had a partitioned gut, a not unexpected result. Nonetheless, this does not translate to possession of a feeding larval stage—this hypothesis must be tested independently of any structure(s) found in the adult stage. Indeed, testing for homology of the mouth to infer homology of larvae is not only inappropriate, but deriving an independent test for homology of planktotrophy among larval forms outside of phylogenetic congruence (McHugh and Rouse 1998; Rouse 1999, 2000a, b) and the fossil record (e.g., Chaffe and Lindberg 1986; Nützel et al. 2006) will be difficult.

The status of the ciliated band though is another matter. Possible homology is usually discounted between the dipleurula neotroch and the trochophore prototroch because of significant structural differences between the two (Nielsen 1987). Nonetheless, ciliated bands in echinoderms (Shoguchi et al. 2000; Lowe et al. 2002; Hinman et al. 2003b), hemichordates (Harada et al. 2000), annelids (Arendt et al. 2001), and molluscs (Nederbragt et al. 2002) all express the transcription factor *Otx* suggesting to some (e.g., Nederbragt et al. 2002) that the two could be homologous. One possible explanation for the expression of *Otx* in ciliated bands is that because the ciliated band is a highly neurogenic structure (for molluscs, see review by Croll and Dickinson 2004), the expression of *Otx* simply reflects the presence of neurons, but not necessarily homology of ciliated bands. The data reported herein are consistent with the idea that the two are convergent because unlike echinoderms (Otim et al. 2004, 2005; Poustka et al. 2004), the prototroch of the gastropod *H. rufescens* does not express the transcription factor *HNF6* (Fig. 5, M–P). In addition, unlike the prototroch of *H. rufescens* (Fig. 5, E–L), the ciliated band (neotroch) of the echinoderm *S. purpuratus* does not express the five cilia genes examined herein after hatching (Fig. 2). This is a very interesting result—that in the sea urchin transcription of cilia genes spanning a range of functions is restricted only to the apical tuft after hatching and not to the ciliated band, whereas transcripts of all five genes are detected in both the apical tuft and prototroch of the gastropod. Whether this indicates a difference in function, in assembly, and/or in regulation of the echinoid ciliary band versus the other ciliated cells is as yet unresolved. Taken together, it appears that the specification of the ciliated bands in the echinoid and the gastropod are different, consistent with an argument of convergence and not homology.

Thus, deciding between the evolutionary scenarios of Peterson (2005) versus Nielsen (*op. cit.*) boils down to homology of the apical organs. Our data are consistent with the former evolutionary scenario, as we find no similarity underlying the development of the apical tufts in these two taxa. Given the constraints governing the movement of primary marine larvae through their aqueous medium (Emlet 1991), our data are consistent with the suggestion (Strathmann 1993) that both the ciliated band and apical tufts of protostome and deuterostome larvae are the products of convergent evolution. However, it is possible that apical tuft specification in one (or both) of the analyzed lineages has diverged, and the loss of the central tuft of cilia in many gastropods, including *Haliotis* (Page 2002a, b), would be consistent with this suggestion. Nonetheless, at the moment there are no data consistent with the hypothesis that these primary marine larval forms are homologous. Therefore, our molecular data support the paleoecological scenario for larval planktotrophy as proposed by Peterson (2005): the initial exploitation of the pelagos by nonfeeding larvae sometime during the late Precambrian–Early Cambrian by at least two different ciliated larval forms; and the subsequent evolution of larval planktotrophy in multiple trochophore- and dipleurula-bearing clades starting during Cambrian/Ordovician transition (Fig. 1). Now, experiments must be designed to examine the specification of apical organs in other taxa, and hopefully to test the proposed ecological interactions between benthic predators and epibenthic larvae and their macroevolutionary consequences.

Acknowledgments

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