Genomics and expression profiles of the Hedgehog and Notch signaling pathways in sea urchin development

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Abstract

The Hedgehog (Hh) and Notch signal transduction pathways control a variety of developmental processes including cell fate choice, differentiation, proliferation, patterning and boundary formation. Because many components of these pathways are conserved, it was predicted and confirmed that pathway components are largely intact in the sea urchin genome. Spatial and temporal location of these pathways in the embryo, and their function in development offer added insight into their mechanistic contributions. Accordingly, all major components of both pathways were identified and annotated in the sea urchin *Strongylocentrotus purpuratus* genome and the embryonic expression of key components was explored. Relationships of the pathway components, and modifiers predicted from the annotation of *S. purpuratus*, were compared against cnidarians, arthropods, urochordates, and vertebrates. These analyses support the prediction that the pathways are highly conserved through metazoan evolution. Further, the location of these two pathways appears to be conserved among deuterostomes, and in the case of Notch at least, display similar capacities in endomesoderm gene regulatory networks. RNA expression profiles by quantitative PCR and RNA in situ hybridization reveal that Hedgehog is produced by the endoderm beginning just prior to invagination, and signals to the secondary mesenchyme-derived tissues at least until the pluteus larva stage. RNA in situ hybridization of Notch pathway members confirms that Notch functions sequentially in the vegetal-most secondary mesenchyme cells and later in the endoderm. Functional analyses in future studies will embed these pathways into the growing knowledge of gene regulatory networks that govern early specification and morphogenesis.
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Introduction

The transducing machinery in both the Notch and the Hh signaling systems is relatively simple compared to the number of modifying components in other signal transduction pathways. Nevertheless the pathways are tightly controlled and modifiers play a clear role as reported in many animal systems. Based on published data, numerous duplications occur in vertebrates, and other duplications or losses are reported in various organisms. All of these changes tend to obscure ancestral function in development. Using a comparative approach animals such as the sea urchin, that occupy a basal position in the deuterostome clade, provide a means of gaining perspective on how these pathways operate in development. The sequencing of the sea urchin genome gives an opportunity to explore these signal transduction path-

ways in detail. The goal of this project, therefore, was to annotate all members of both signal transduction pathways and then to begin to explore where and how some of the main components of these pathways operate in the sea urchin embryo.

Fig. 1 depicts the main components of both the Hh and the Notch signaling pathways as a combination of molecules known from studies largely in *Drosophila* and vertebrates. Although differences exist between vertebrates and *Drosophila* (reviewed in Huangfu and Anderson, 2006), a generalized Hh pathway can be described as follows. Hh is a secreted protein that is enzymatically modified to make it active (Bumcrot et al., 1995; Chamoun et al., 2001; Lee et al., 1994; Pepinsky et al., 1998; Porter et al., 1995, 1996) and it binds to its cognate receptor Patched (Ptc) (Chen and Struhl, 1996; Marigo et al., 1996; Stone et al., 1996). In the absence of Hh, the receptor Ptc operates as an inhibitor by blocking the ability of Smoothened (Smo) to activate the pathway. In this case the downstream transcription factor cubitus interruptus (Ci, or Gli in vertebrates, note that
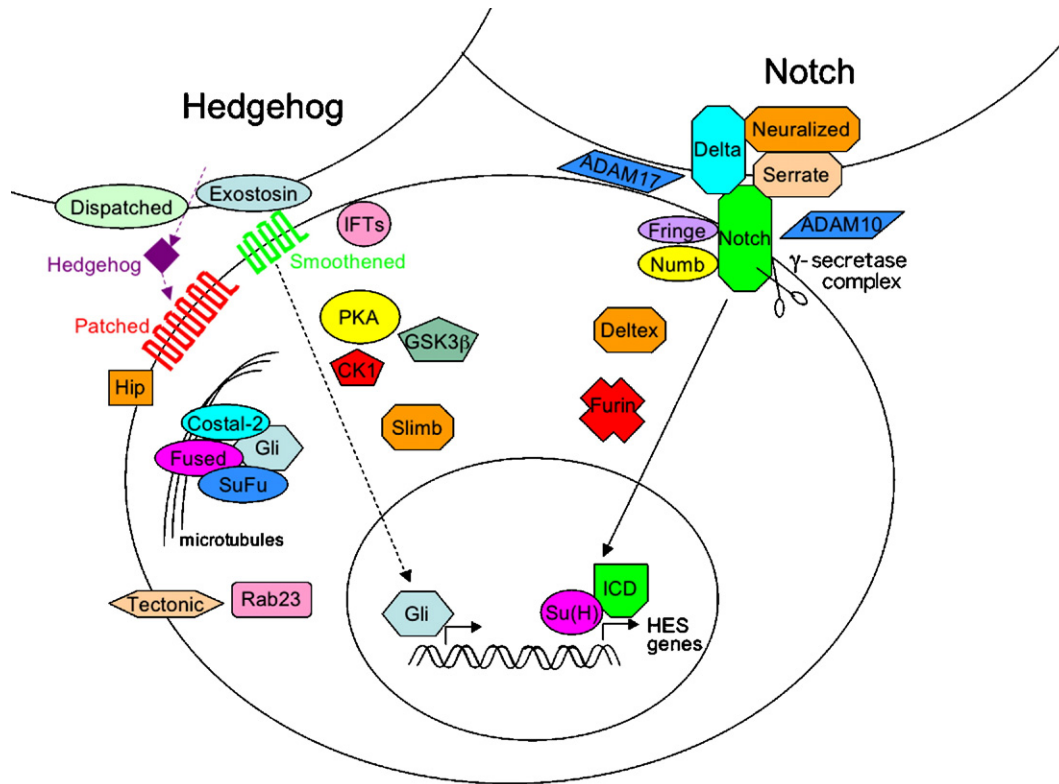
Fig. 1. Diagram of the major components and modifiers of the Hh and Notch signaling pathways that were identified in the annotation of the *S. purpuratus* genome. These components and modifiers are also listed in Supplemental Table 1 along with their other names, Glean3 model/SPU_ numbers, best human genome blast hit and relative level of embryonic expression detected by the tiling array experiment. See the text for a brief functional description of these pathways.

there are 3 forms of Gli in vertebrates: Gli1, Gli2, and Gli3) becomes phosphorylated by PKA, GSK3β and CK1, and is subsequently targeted for processing by the protease, Slimb (Chen et al., 1998; Jia et al., 2002; Jiang and Struhl, 1998; Price and Kalderon, 2002; Theodosiou et al., 1998). Slimb cleaves the full-length 155 kDa Ci to a shortened 75 kDa form and this shortened form translocates to the nucleus where it acts as a repressor (Akimaru et al., 1997; Aza-Blanc et al., 1997; Chen et al., 1999). Gli3 (and Gli2 in some contexts) is also similarly cleaved in vertebrates and it is thought that the ratio of full-length activator forms to shortened repressor forms determines the transcriptional regulatory action (von Mering and Basler, 1999). Costal-2 (Cos2) is a kinesin-like protein which is part of a complex of proteins that act to sequester Ci/Gli in the cytoplasm and promote the cleavage of the full-length Ci in *Drosophila*, however the currently identified vertebrate orthologs of Cos2 appears unable to affect subcellular localization of Glis or promote their cleavage (Chen et al., 1999; Lefers et al., 2001; Methot and Basler, 1999, 2000; Varjosalo et al., 2006; Wang et al., 2000). Suppressor of Fused (Su(Fu)) and Fused are also part of this protein complex (Preat, 1992). Su(Fu) is a weak antagonist of Hh signaling in *Drosophila*, but can act as a potent inhibitor of Hh signaling in vertebrate cells (Varjosalo et al., 2006) while Fused is a kinase thought to inactivate Su(Fu) possibly by direct phosphorylation (Lum et al., 2003; Methot and Basler, 2000).

When Hh is present, it binds to Ptc, alleviating the inhibition of Smo. Smo is then able to antagonize Cos2 activity and Slimb

protease activity is prevented. As a result the full-length form of Ci is retained and this molecule proceeds to the nucleus where it activates transcription, often of the same genes that previously were repressed by the smaller Ci protein (Methot and Basler, 2000).

In addition to the major components described above, other modifiers affect the Hh pathway including Dispatched (Disp), Exostosins (Exts), Rab23, Hedgehog-interacting protein (Hip), Intraflagellar transport proteins (IFTs), Tectonic (Tect), SIL, Talpid3 and FKBP8. Disp is required for Hh secretion from Hh releasing cells (Burke et al., 1999). The *Drosophila tout-velu* (*ttv*) genes, which are homologs of the vertebrate *ext* genes, are critical to the movement of the Hh signal between cells, and are required in the receiving cells (Bellaiche et al., 1998; The et al., 1999). Rab23 is a negative regulator of vertebrate Hh signaling and is a member of the small GTP-activated proteins, which are associated with membrane trafficking (Eggenschwiler et al., 2001). Its function appears to be in localizing some factor in Hh signaling that acts between Ptc and Smo and the downstream transcription factor, Gli (Eggenschwiler et al., 2006). Hip, a Hh-binding protein previously thought to be vertebrate specific, is upregulated by Hh signaling and has a negative effect on that signal, thereby providing a feedback mechanism (Chuang and McMahon, 1999). IFTs are required for assembling cilia and flagella (Rosenbaum and Witman, 2002) and are necessary for Gli activity in response to Hh signaling in vertebrates at a step between Smo and Gli (Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005). This requirement for IFTs in Hh

signaling appears not to be conserved in *Drosophila* since IFT knock out flies do not have the phenotypic patterning defects of Hh pathway mutations (Avidor-Reiss et al., 2004; Han et al., 2003; Ray et al., 1999). Tect, SIL, Talpid3, and FKBP8 are some relatively new additions to the vertebrate pathway. Tect appears to function in the Hh pathway somewhere downstream of Smo and Rab23 (Reiter and Skarnes, 2006). It is a transmembrane protein and is required for full activation of Hh signaling and specification of the ventral most cell types in the neural tube and may also play a separate role in repressing Hh activity. SIL is a cytosolic protein required in mouse for left–right axis development and Shh signaling (Izraeli et al., 1999, 2001). Talpid3 is another cytoplasmic protein required for the function of Gli repressor and Gli activator in chickens (Davey et al., 2006). Finally, FKBP8 is a member of the FK506-binding protein family and is an antagonist of Shh signaling in the development of the central nervous system (Bulgakov et al., 2004).

In contrast to the Hh pathway, activation of The Notch pathway (Fig. 1) involves direct cell contact. Ligands of the Notch pathway are transmembrane proteins of the DSL family known as Delta and Serrate (Fehon et al., 1990; Rebay et al., 1991) while the receptor Notch is also a transmembrane protein. Prior to contact between the ligand and receptor, several modifiers are involved. In the cells displaying Delta or Serrate, the neuralized and the mind bomb genes encode ubiquitin-3 ligases that are thought to interact with the ligands and stimulate their endocytosis and signaling activity (Deblandre et al., 2001; Fleming et al., 1997; Lai et al., 2001; Panin et al., 1997; Pavlopoulos et al., 2001; Pitsouli and Delidakis, 2005). On the other hand in the cells expressing Notch, the Notch receptor has to be processed before its arrival at the membrane. The Notch protein is made as a long precursor molecule with 36 EGF repeats on the external chain (35 in the sea urchin (Sherwood and McClay, 1997)), a single pass transmembrane region and a long cytoplasmic domain containing ankyrin repeats (Artavanis-Tsakonas et al., 1999). Prior to its arrival at the cell surface Notch is cleaved on the future extracellular surface by the furin protease and the two fragments are linked by disulfide bonds (Logeat et al., 1998). Some Notch-bearing cells express a Fringe protein (Fleming et al., 1997; Peterson and McClay, 2005), a glycosyl transferase, that modifies the Notch receptors making them receptive to the Delta signal. Absence of Fringe-catalyzed glycosylation makes the Notch-bearing cell selectively receptive to the Serrate signal (Fleming et al., 1997). After the binding of either Delta or Serrate, Notch receptors undergo two further specific cleavages required for the activation and transduction of the signal. The second cleavage (after the first furin-dependent cleavage) follows ligand binding and is mediated by an ADAM metalloprotease while the third is based on γ-secretase activity and releases the Notch Intracellular Domain (NICD) (Annaert and De Strooper, 1999; Brou et al., 2000; Lieber et al., 2002; Mumm et al., 2000; Sotillos et al., 1997; Weinmaster, 2000). When the NICD goes to the nucleus it binds to a transcription factor of the CSL family, such as Suppressor of Hairless (Su (H)), where it converts the CSL factor's regulation from repression to activation (Kopan, 2002). Targets of Notch signaling are quite varied throughout the animal kingdom, but a common target is *hairy* (Davidson et al., 2002; Ransick and Davidson, 2006).

Additional modifiers of Notch pathway are found in the Notch-bearing cell as shown in Fig. 1. Nicastrin is a membrane protein that is part of the Presenilin complex in vertebrates. Functionally, it enables the γ-secretase cut of Notch and therefore enables activation of the transcription of Notch target genes. Numb is a cytosolic protein that is localized close to the membrane of the Notch containing cell and that acts in many animal systems as an antagonizer of Notch signaling possibly through a role in the endocytosis of a positive modifier of the signaling pathway (Cayouette and Raff, 2002). Deltex is another cytosolic protein that regulates Notch signaling in some contexts. It is an E3 ubiquitin ligase that binds to the ankyrin repeats of Notch and promotes Notch endocytic trafficking to the late endosome and possibly activates Su(H), although it appears not to be essential during development in *Drosophila* (Diederich et al., 1994; Fuwa et al., 2006; Matsuno et al., 1995).

Homologs for all of the genes in the Hh and Notch signaling pathways listed in Supplemental Tables 1 and 2 have been identified within the sea urchin genome. Functional roles for all of the Hh pathway components as well as for many of the Notch pathway components have not yet been determined in detail in the sea urchin embryo but an early picture has emerged. Oligonucleotide tiling microarrays covering the genome were explored *in silico* and temporal and spatial patterns of expression for some of the main components of these pathways were examined to determine when and where the pathways operate. Here, we begin to address Hh signaling function and add to a growing knowledge about Notch signaling function in the sea urchin during development.

Materials and methods

Identification and naming of sea urchin homologs

Strongylocentrotus purpuratus homologs were identified by blasting (tblastn)(Altschul et al., 1997) protein sequences from other species against the *S. purpuratus* Glean3 predicted model database released July 18, 2005 (http://www.hgsc.bcm.tmc.edu/blast/blast.cgi?organism=Spurpuratus). Matches were confirmed by blasting (blastp) the predicted protein sequence of the Glean3 model against the National Center for Biotechnology Information (NCBI) GenBank protein database. Glean3 models were named according to the best blast matches identified from GenBank and confirmed by bootstrap and neighbor-joining phylogenetic tree analysis against human, *Ciona intestinalis*, *Drosophila melanogaster*, and *Nematostella vectensis*. Sequences for human and *Drosophila* were obtained from GenBank and Ensembl. *Ciona* sequences were obtained from GenBank and the *Ciona* database ANISEED or Ascidian Network for In Situ Expression and Embryological Data (http://crfb.univ-mrs.fr/aniseed/index.php). *Nematostella* sequences were identified by domain searches and blast searches in the *N. vectensis* genomics database StellaBase (http://www.stellabase.org/). Neighbor-joining and bootstrap phylogenetic tree analyses were performed in PAUP from nexus files prepared with Clustal-X. 1000 repetitions were used for the bootstrap analyses.

High-density oligonucleotide tiling microarrays

High-density oligonucleotide tiling microarrays were performed as described in Samanta et al. (in press). Briefly, 50 oligonucleotide lengths at a time were arrayed on glass slides and hybridized with labeled RNA pooled from equal quantities of egg, early blastula, gastrula and prism RNA. Where labeled

RNA hybridized with the arrayed oligonucleotides, levels of hybridization were optically detected. Hybridization levels for each of the oligonucleotides were then coordinately matched with their corresponding location on the genomic scaffolds of sequence so that the levels of hybridization could be viewed as they appear along the sequence scaffold. This information is available for review using Genboree (http://www.genboree.org/java-bin/login.jsp). Each Glean model listed in the Supplementary tables was examined in Genboree. The resulting transcript levels for each area were considered against the exon predictions for each Glean model and relative levels of transcript were assigned as described in the figure legends.

Cloning of sea urchin genes

Primers were designed within highly conserved regions of the predicted *S. purpuratus* Glean3 models to amplify partial sequences of those genes by PCR. Primers were also used in some cases with *L. variegatus* cDNA at lower annealing temperatures. PCR products were ligated into pGEMT Easy vector and electroporated into XL-1 blue competent cells. Plasmids were extracted using Qiagen miniprep and maxiprep kits. Clones were sequenced to confirm their identity and orientation within the vector.

Quantitative PCR

RNA was extracted from embryos at various stages of development using Trizol reagent followed by ethanol washes and resuspension in nuclease free water. 2 μg of total RNA was treated with DNaseI and used as a template for reverse transcription with the TaqMan cDNA synthesis kit (Roche Cat #N808-0234). 0.5 μl of cDNA was then used in a quantitative PCR reaction using the Roche LightCycler Fast Start DNA Master SYBR Green I kit (Roche Cat #12 239 264 001). Primers were designed using the Probe Design program for the Roche Light Cycler. Primer sets were as follows: *Sp/Lvhh*, F: GACACATTTGGTGC-CAGTGG, R: GTCTTTGCATCGCTGTGTC; *LvPtc*, F: GATCCTTCAGACC-GGC, R: TGACTTACTTGTGACATCG; *Lv*Smo, F: GCTCAGTGGGAGAAGG, R: TCCGCTTCCATATAGCC; *LvGli*, F: TCTGTCGATGGCGTGA, R: GGTA-CATCCGGCGTGA; *LvNotch*, F: TCCCGGCTTAGTCCCGT, R: AGGTTGTC-GCGCTG; *LvDelta*, F: TTCGGCGGACCCAACTG, R: CCCGGATGTTAGGCCGT.

Primer sets were validated using plasmid-cloned templates for each gene for a positive control or water as a negative control. Threshold crossing cycle determined by the Light Cycler program was averaged for at least two runs per set of developmentally staged cDNAs. Within each data set, the threshold crossing cycle was also determined for ubiquitin. The number of transcripts per embryo was calculated assuming 87,000 transcripts for ubiquitin per embryo at each stage of development and a QPCR amplification rate of 1.9-fold per cycle (Nemer et al., 1991; Ransick et al., 2002). Transcripts per embryo at each stage of development were plotted using a logarithmic scale to allow the timecourses to be evaluated on the same scale. Dashed lines at 150 and 350 transcripts were included to indicate the minimum and maximum range of significance for biological activity of a gene transcript.

RNA in situ hybridization

Embryos were fixed in 4% PFA/ASW with 10 mM EPPS for 1 h at room temperature. Then they were washed with ASW and stored in methanol at −20°C. Stored embryos were rehydrated and prehybridized with 50% formamide, 25% 20× SSC pH 5.0, 0.001% of 50 mg/ml of heparin, 0.001% of 50 mg/ml of yeast tRNA, and 0.002% of 50% Tween 20. Digoxigenin-labeled probes were hybridized overnight at 65°C at 1 ng/ml and washed through a series of hybridization solution, and SSCT from 2× to 0.1× at 65°C. Embryos were then blocked in 0.5% BSA and 2% heat-inactivated goat serum in 1× TBST for 1 h and then incubated in anti-digoxigenin antibody at 1:2000 in blocking solution for 2 h. Embryos were then washed several times in 1× TBST. Color reactions were performed with NBT/BCIP for 2 h at room temperature or overnight at 4°C. Images were taken at 200× magnification using a Zeiss Axioplan2 microscope with a Zeiss AxioCam HRc camera and AxioVision Rel.4.4 software. Processing of the images was performed in Adobe Photoshop to orient and crop the images.

Results and discussion

The sea urchin Hh pathway

To date the composition of the Hh pathway has mainly been studied in two evolutionary groups, vertebrates representing deuterostome lineages and arthropods for the protostome lineages. The evolutionary position of the sea urchin as a basal deuterostome provides an opportunity to examine basal deuterostome components, thus is an organism that could answer some questions about the evolution of this pathway. The vertebrate and *Drosophila* Hh pathways contain 30 and 18 genes respectively (excluding newly identified modifiers of the pathway). The sea urchin genome was examined to find orthologs for each pathway component and to augment the study we also included in our survey Hh pathway orthologs in the urochordate genome of *C. intestinalis* and *Nematostella*, a Cnidarian, to include an animal basal to bilaterians. We identified 25 genes (30 including the newly identified modifiers of the pathway) belonging to the Hh pathway in the sea urchin genome (Supplemental Table 1). All of the Hh pathway genes in *Drosophila* and most of the vertebrate genes are present in *S. purpuratus*, although in some cases fewer paralogs of the genes were found, probably due to the later genome duplication in the vertebrate line. Below we describe some of the more intriguing results from the annotation as well as the expression patterns of some of the main components in the Hh pathway.

In vertebrates three distinct Hh ligands are present, *Ciona* has two, and *Drosophila* and sea urchins each have only one. Despite the numbers of paralogs, *S. purpuratus* Hh, *Nematostella* Hh and *Drosophila* Hh were equally similar to the human Hhs. Surprisingly, all were more similar to the human Hhs than *Ciona* Hhs (Supplemental Fig. 1A) suggesting that the *Ciona hh* gene diverged from the ancestral, and more conserved gene.

Vertebrates also have more *ptc* paralogs (two *ptc* genes) than *Ciona*, *Drosophila*, or *S. purpuratus*, each of which have only one *ptc* gene. *S. purpuratus* Ptc is similar to other deuterostome Ptc proteins as its predicted protein clades with bootstrap values of 92 to *Ciona* Ptc and to human Ptc 1 and Ptc 2. *Drosophila* Ptc demonstrates an earlier divergence (Supplemental Fig. 1B). One *disp* and two *dispatched-like* genes were also identified as homologs to human and *Ciona disp* along with two *Nematostella* predicted proteins.

A single *smo* gene was found in the *S. purpuratus* genome which agrees with the number found in all other organisms sequenced to date. The sea urchin Smo protein is most similar to human Smo and the most different from a predicted *Nematostella* protein and the *Drosophila* Smo (Supplemental Fig. 1C). This correlates with an apparent divergence of the cytoplasmic domain in *Drosophila* relative to deuterostome Smos. The cytoplasmic portion of the Smo protein was shown to convey the Hh signal (Hooper, 2003; Jia et al., 2003; Nakano et al., 2004), yet there is a great difference in the length of these tails between *Drosophila* and vertebrates and differences in the role that phosphorylation of this domain may play in Hh signaling as well differences in its interaction with Cos2 (Huangfu and Anderson, 2006; Jia et al., 2003; Varjosalo et al., 2006). Protein alignment

of human, *Drosophila* and *S. purpuratus* Smo reveals that *S. purpuratus* Smo is 43% similar to human Smo and 30% similar to *Drosophila* Smo while an alignment of the C-terminal portions (CTD) of these Smo proteins shows that this portion of *S. purpuratus* Smo is similar in length to the vertebrate CTD and is 31% similar to human and only 18% similar to *Drosophila* Smo. The similarity of the sea urchin Smo in CTD length to vertebrates may suggest that the cytoplasmic function of Smo may be more similar to that of vertebrates than *Drosophila*.

Sea urchin and *Ciona* each have a single ortholog of *gli* that is related to the three vertebrate *gli* transcription factors. Sea urchin GliA is most similar to the *Nematostella* Gli and is more similar to human Gli2 and Gli3 than it is to human Gli1 by neighbor-joining phylogenetic analysis (Supplemental Fig. 1D). *Drosophila* Ci and vertebrate Gli2 and Gli3 are cleaved to produce a shortened repressor form while Gli1 is not cleaved. Phosphorylation of Ci/Gli by PKA, CKI and GSK3-β is required for proteolysis to the shortened form. There are 3 PKA, 3 CKI, and 2 GSK3-β sites occurring in 3 clusters that are conserved between Ci and the Gli proteins (Lefers and Holmgren, 2002). Alignment of *S. purpuratus* GliA with human and mouse Gli1, 2 and 3 and *Drosophila* Ci revealed conservation of these sites (Fig. 2), indicating the likelihood that *S. purpuratus* GliA is cleaved similarly to a shortened form. Additionally, there are two sea urchin *gli-similar* genes (*glisB* and *glisC*) that were predicted as compared to the three in vertebrates. *S. purpuratus* GlisC is most similar to human Gli2 and *S. purpuratus* GlisB is more similar to human Gli3 and Gli1. The *S. purpuratus* Glis appear similar in length to the shortened form of Ci when aligned with Ci and the Gli proteins, suggesting that these genes may represent repressor forms of Gli-similar proteins in the sea urchin.

Hedgehog-interacting protein (hip), previously thought to be vertebrate specific, was also identified in the sea urchin extending its history to all deuterostomes. Phylogenetic analysis revealed that sea urchin Hip is an ortholog of a *Ciona* Hip with a bootstrap value of 100. Sea urchin and *Ciona* Hip have a weaker but significant similarity to the human protein (Supplemental Fig. 1E).

Several other components of the Hh signaling pathway were also annotated and are represented in the schematic of the signaling pathway in Fig. 1. In summary, the main Hh pathway components are all present in the sea urchin genome. The function of some of the vertebrate-specific components of the pathway (Hip, IFTs, SIL, Rab23, FKBP8, Tect, and Talpid3), remains to be investigated in detail. In examining the 30 genes in the Hh pathway family it is clear that a number of patterns of evolutionary dispersion are found

indicating that the pathway was not treated as a unit by evolutionary selection.

Embryonic expression of Hh genes in sea urchin

To assess when and where genes present in the Hh pathway function in the developing embryo, we first surveyed the high-density oligonucleotide tiling microarray hybridizations performed by Samanta et al. for embryonic expression of the genes (Samanta et al., in press; accessed through Genboree at http://www.genboree.org/java-bin/login.jsp). These data reveal that all genes in the Hh pathway are expressed embryonically (Supplemental Table 1) with the exception of *su(fu)*, *disp*, *tect* and *hip*. For those genes not showing embryonic expression on the high-density oligonucleotide tiling microarray hybridizations, we searched in silico for transcripts of these genes by Blast searching of an embryonic EST database (accessed at http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=7668). These searches revealed ESTs for *su(fu)* and *disp*. These were respectively: CD306952.1 and CD291291.1. EST CD306952.1 is from a 2- to 3-week larva stage library. Using the sequence of EST CD291291.1 in a tblastn comparison against the GenBank protein database reveals that this EST is most similar to *su(fu)*. EST CD291291.1 is from a primary mesenchyme cell library. When the sequence of EST CD306952.1 is compared to the GenBank protein database by tblastn, it is most similar to *ptc*. No ESTs were discovered by blast comparison of the predicted proteins for the annotated *tect* and *hip* genes. EST data were not available for many of these genes since most of the Hh signaling pathway components seem to be expressed at low levels at the early stages of development when most of the EST libraries are currently available.

Temporal expression patterns of key Hh pathway components

Since most pathway components are present in the embryo, we next cloned several Hh pathway components in *L. variegatus* and *S. purpuratus* to confirm annotation predictions and analyze in situ patterns for pathway localization. The predicted Hh sequence matched well with the GenBank database sequences for *S. purpuratus*, *L. variegatus*, and *H. pulcherrimus* (gi60593030, gi3089555, gi60677667). The timing of expression of several of the key components of the pathway was examined by Quantitative PCR. *Lytechinus variegatus hh* mRNA begins to accumulate during the late blastula to mesenchyme blastula stages and accumulates throughout gastrulation

Fig. 2. Protein alignment of *Drosophila* Ci, Human Gli1, 2 and 3 and *S. purpuratus* GliA showing conservation of PKA, CKI, and GSK3β phosphorylation sites thought to be important in Slimb cleavage of these proteins (modified from Lefers and Holmgren, 2002).

and into the prism and pluteus stages (Fig. 3A). *L. variegatus ptc* mRNA is expressed in a highly varying pattern with an overall increase in expression from the blastula stages through gastrulation (Fig. 3B). *L. variegatus smo* mRNA is expressed in a pattern similar to that of *L. variegatus hh*. There is a small peak at 16-cell stage with an overall increasing trend from the hatched blastula stage through the gastrula stages and a large increase at the late pluteus stage (Fig. 3C). *L. variegatus gliA* appears to have maternal transcripts with zygotic mRNA beginning to be expressed during the hatched blastula stage and increases throughout the later blastula stages and gastrulation (Fig. 3D). Much of the specification for endoderm and mesoderm occurs prior to the gastrula stage of development, and this is followed by a later refinement of specification and patterning of these tissues in the gastrula stage. The pattern of accumulation of *hh* pathway members during gastrulation and into the pluteus stage suggests that Hh signaling is a relatively late event during the development of the endoderm and mesoderm of the sea urchin. Specification of SMC cell types appears to occur in 3 phases during the separation of the endomesodermal compartment (A. Ransick, personal commu-

nication). During phases 1 and 2 initial specification of endoderm and mesoderm occurs and specification of the pigment and blastocoelar cell SMC subtypes takes place. Little is known about the signals involved in the third phase when the late specification of muscle and coelomic pouch SMC cell types occurs, except that more endomesoderm is committed toward these late SMC subtypes.

RNA localization patterns of key Hh pathway components

RNA in situ hybridization shows a localization pattern for *L. variegatus hh* in the endoderm of the embryo during the gastrula stages, which continues through the prism and early pluteus stages (Figs. 4A–D). At a later stage of pluteus larva, *L. variegatus hh* expression is confined to the esophagus with higher expression at the sphincters than elsewhere along the archenteron (Fig. 4E). Our in situ analysis was unable to repeat previously published patterns of Hh expression in *S. purpuratus* and *Hemicentrotus pulcherrimus* (Egana and Ernst, 2004; Hara and Katow, 2005). Egana and Ernst (2004) used what were purported to be cross-reactive antibodies to localize *S.*
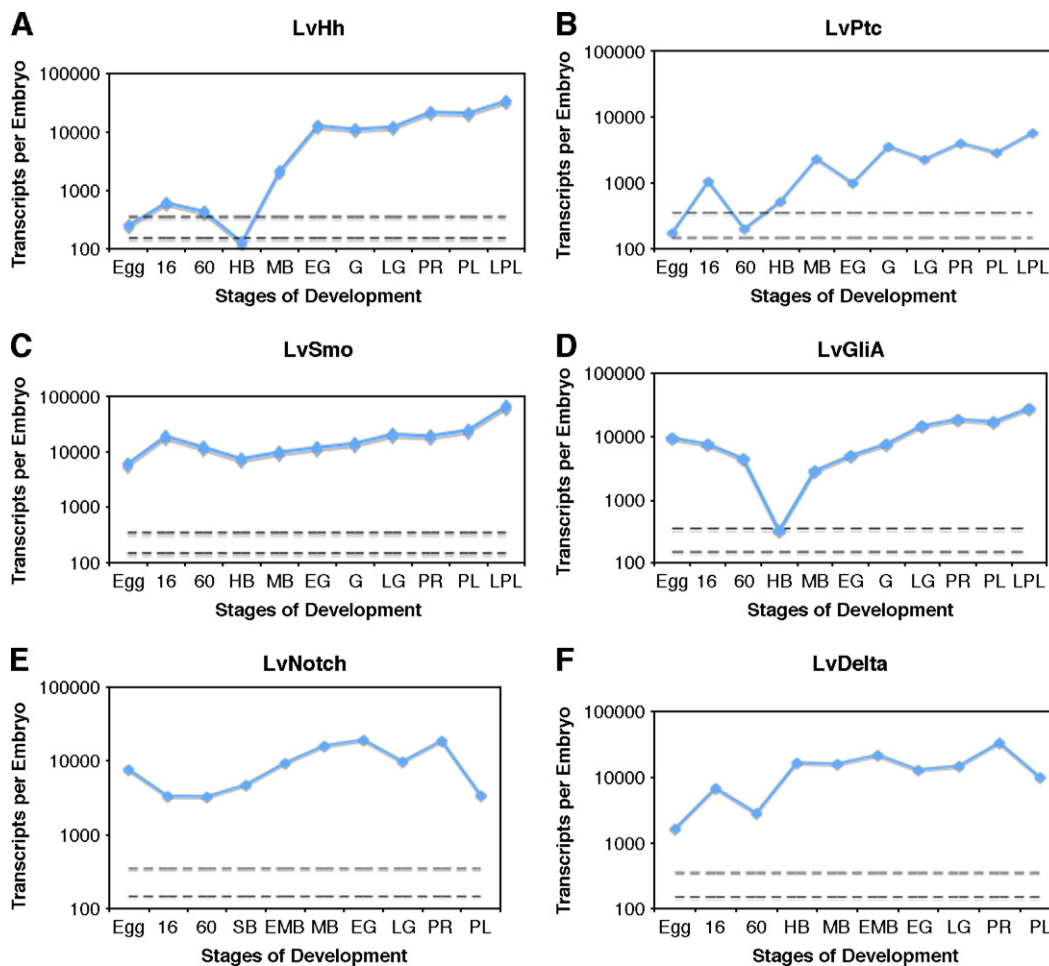

Fig. 3. Quantitative PCR for major components of the Hh and Notch signaling pathways. (A) *hh mRNA*; (B) *ptc mRNA*; (C) *smo mRNA*; (D) *GliA* mRNA; (E) *Notch* mRNA; (F) *Delta* mRNA begins to accumulate during the blastula and gastrula stages. Stages are: egg, 16-cell, 60-cell, hatched blastula, early mesenchyme blastula, mesenchyme blastula, early gastrula, mid-gastrula, late gastrula, prism, pluteus, and late pluteus. Data are shown on logarithmic scales as the number of transcripts per embryo at a given stage of development. Dashed lines at 150 and 350 transcripts were included to indicate the minimum and maximum range of significance for biological activity of a gene transcript.
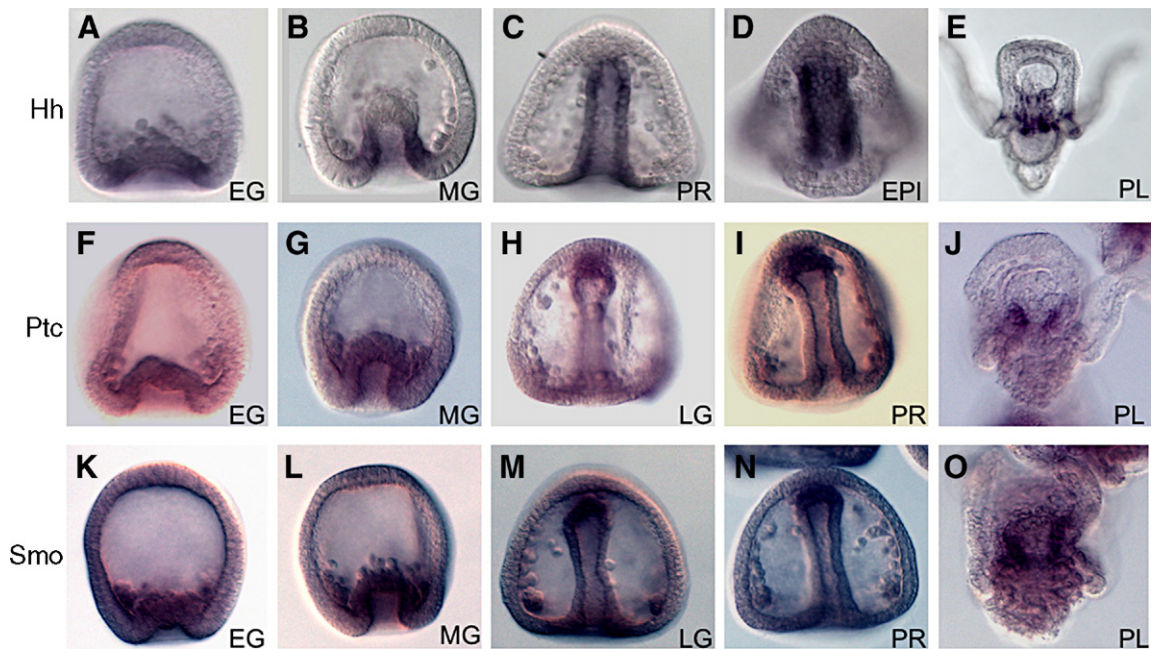
Fig. 4. RNA in situ localization pattern for main components of the Hh signaling pathway. (A–E) *hh*; (F–J) *ptc*; (K–O) *smo*. Stages are: EG, early gastrula; MG, mid-gastrula; LG, late gastrula; PR, prism; EPl, early pluteus; PL, 2-day pluteus. All views are lateral.

purpuratus Hh in the secondary mesenchyme cells, however the in situ pattern of expression occurs in the endoderm. The RNA localization of *H. pulcherrimus hh* to the small micromeres by Hara and Katow (2005) also was not repeated and that pattern fails to correlate either with the expression pattern for *S. purpuratus hh* RNA published in Egana and Ernst (2004), nor with the QPCR pattern for *L. variegatus hh* RNA shown here in Fig. 3A. The localization of *hh* to the endoderm in *L. variegatus,* shown here (Figs. 4A–E) is in agreement with the QPCR data in terms of timing and is by a strong RNA in situ hybridization signal that is highly repeatable. Further, one might predict that the *hh* expression profile is adjacent to the tissue expressing *ptc* and *smo*, and the data on expression of these genes support that prediction, reported here for the first time in the sea urchin. Further, expression of the Hh pathway in the sea urchin matches a well-described comparative function of endoderm signaling to mesoderm in both *Drosophila* and vertebrates (see below), RNA in situ analysis shows that *L. variegatus ptc* is expressed in the secondary mesenchyme cells throughout gastrulation and continues to be expressed in SMC-derived tissues in the 2-day-old pluteus (Figs. 4F–J). RNA in situ analysis shows that *L. variegatus smo* is expressed in the same pattern as *L. variegatus ptc* with localization in the secondary mesenchyme cells throughout gastrulation with continued expression in SMC-derived tissues (Figs. 4K–O).

These data on all Hh pathway members suggest that the pathway functions most strongly during late embryonic development. A functional role for Hh signaling has not yet been described in the sea urchin, however the patterns seen here by RNA in situ hybridization for *L. variegatus hh*, *L. variegatus ptc*, and *L. variegatus smo* suggest that the Hedgehog signal is produced by the endodermal tissues of the archenteron and function in formation of the mesodermal tissues.

Role of Hh in germ layer specification across the animal kingdom

Hh signaling is used in a wide variety of contexts during embryonic development in other model systems. One such role for Hh signaling is in the development of the gut where pathway components are expressed in a variety of animals during development including: chicken (Sukegawa et al., 2000), leech (Kang et al., 2003), *Amphioxus* (Shimeld, 1999), mouse (Bitgood and McMahon, 1995; Echelard et al., 1993), *Drosophila* (Mohler and Vani, 1992), zebrafish (Strahle et al., 1996), and *Xenopus* (Ekker et al., 1995). Hh signaling is essential for patterning the gut in these animals since mutations or interference with other components of the Hh signaling pathway result in a variety of gut malformations (reviewed in Lees et al., 2005). Communication between endoderm and the overlying mesoderm is essential to specification and patterning of the gut. Sonic hedgehog is one molecule that is essential to this communication since it has been shown to be expressed in the epithelium and signals to Ptc and Gli that are expressed in the neighboring mesenchyme cells (Ramalho-Santos et al., 2000). Based on expression patterns of *hh* and *ptc* plus *smo*, a similar function in sea urchins may occur as the embryonic coelomic pouches are specified in preparation for metamorphosis. Functional studies based on the molecules described herein will further our understanding of how these two pathways are involved first in specification of germ layers, and later in modification of those germ layers to generate functional larval structures.

An overview of the Notch signaling pathway

The Notch pathway in sea urchins. The Notch pathway in vertebrates, arthropods, and nematodes is well studied, with

much less information available about the Notch pathway from other phyla. Analysis of the Notch signaling system in the sea urchin (as a basal deuterostome) provides important information about the evolution and comparative function of this pathway. Similar to the Hh signaling pathway, the Notch pathway in the sea urchin has an intermediate global number of genes as compared to *Drosophila* and vertebrates (23 sea urchin genes compared to 36 in humans and 20 in *Drosophila*, excluding Notch-ligands besides Delta and Serrate/Jagged). Orthologs for all major components of the Notch pathway were identified with thirty-one members of the identified in the *S. purpuratus* genome (including Notch-like ligands). A complete list of genes that were examined is in Supplemental Table 2. Several other main components were also identified and are represented in the schematic of the signaling pathway in Fig. 1. Below we describe the most informative of our results from the annotation as well as the expression patterns of *notch* and *delta* in the sea urchin.

Notch Pathway components present in the sea urchin genome

Relative to vertebrates, the sea urchin genome contains fewer paralogs in some gene families. For example humans have 4 *notch* genes while *Drosophila* has one *notch* gene and sea urchins have one notch gene plus two notch-like genes. In humans there are seven *hairy* gene homologs (hairy and enhancer of split) while sea urchin appears to have two and *Drosophila* just one. Most other genes had equal numbers in all three genome groups examined with the exception of *fringe*, *su(h)* and *deltex*, each of which was expanded, presumably by duplications, in vertebrates.

The *notch* receptor, as well as its ligands *delta* and *serrate/ jagged*, were identified and subjected to phylogenetic analysis for comparison. The computationally predicted gene sequences for *notch, delta* and *serrate* matched strongly to previously published sequences in *L. variegatus* and *S. purpuratus* (GenBank gi2570351, gi18535661, gi18535657). Neighbor-joining phylogenetic tree analysis shows that sea urchin Notch is most closely related to *D. melanogaster* Notch with a bootstrap confidence level of 68 (Supplemental Fig. 2A). *Drosophila* and sea urchin form a clade with human and *Ciona* Notch with a bootstrap value of 99. *L. variegatus delta* was cloned and previously described (Sweet et al., 2002). Phylogenetic analysis showed that sea urchin Deltas (*H. pulcherrimus*, *S. purpuratus* and *L. variegatus*) join in a clade with Delta from other species and are most closely related to *Ciona* Delta with a bootstrap value of 70 (Supplemental Fig. 2C). Finally, *S. purpuratus* Nicastrin was analyzed and is most similar to human Nicastrin (bootstrap value of 96) suggesting that the *nicastrin* gene is conserved in deuterostomes (Supplemental Fig. 2B). Taken together, these data suggest that the Notch pathway did not evolve as a unit, but rather each component changed subject to independent evolutionary selection.

Much more is known about the function of the Notch pathway in the sea urchin as compared to the Hh pathway. Notch is known to play a key role in the segregation of mesoderm from endoderm, the specification of the SMC-derived pigment and blastocoelar cells, and in setting the boundary between endoderm and ectoderm (Sherwood and McClay, 1997, 1999, 2001).

Complementary to the Notch experiments, Delta expressed in the micromeres is the ligand that activates the Notch receptor triggering pigment and blastocoelar cell specification, while Delta expressed by veg2 progeny signals to specify blastocoelar cells, coelomic pouches and muscle cells (Sweet et al., 2002). *Gcm* is a target of Notch signaling in the sea urchin and is essential for pigment cell specification (Ransick and Davidson, 2006). Fringe modification of Notch is also required for Notch signaling in the sea urchin to specify SMCs and some endodermal genes (Peterson and McClay, 2005).

Expression of Notch genes in the sea urchin embryo

Data from the high-density oligonucleotide tiling microarray performed by Samanta et al. were examined for Notch pathway components (Samanta et al., in press). These data reveal that all pathway genes are expressed embryonically with the exception of *serrate* (Supplemental Table 2). We were unable to amplify *serrate* from embryonic cDNA. An in silico search for sea urchin serrate transcripts by Blast comparison of the Glean3 predicted sea urchin Serrate protein against the sea urchin EST database did identify several possible *serrate* ESTs (http://www. ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi? taxid=7668). However, these same ESTs matched with higher identity to *delta* than to *serrate*. Additionally, blast comparison of the ESTs against the GenBank protein database matched the *delta* sequence of other species. Several other Notch ligands were also identified as possible DSL family members since they have EGF repeats and a DSL domain, however they did not form a clade with Delta or Serrate/Jagged in the phylogenetic analyses. Thus, by some criteria, *serrate* is present, but the embryo does not express this gene, and it does not form a clade distinct from Delta-related ligands.

Temporal RNA expression patterns of sea urchin delta and notch

Three sea urchin Notch pathway genes were cloned. *Notch* and *delta* were cloned in *L. variegatus* and have previously been described (Sherwood and McClay, 1997, 1999, 2001; Sweet et al., 2002). The RNA expression patterns of *notch* and *delta* were examined by Quantitative PCR. *Notch* is expressed maternally, decreases during the first cleavages and then begins to increase again at the blastula stages (Fig. 3F). This pattern agrees with the previously published northern analysis data (Sherwood and McClay, 1997). *Delta* begins to accumulate during the blastula and gastrula stages (Fig. 3G). This pattern agrees with the previously published northern analysis (Sweet et al., 2002) with the exception of a continued expression pattern at later stages not seen in the published northern analysis.

RNA localization patterns for delta and notch

The *delta* RNA in situ expression pattern observed matches the previously published pattern (Sweet et al., 2002), in which *delta* first localizes to micromeres. Then it localizes to a ring of cells at the vegetal pole at the hatched blastula stage and is
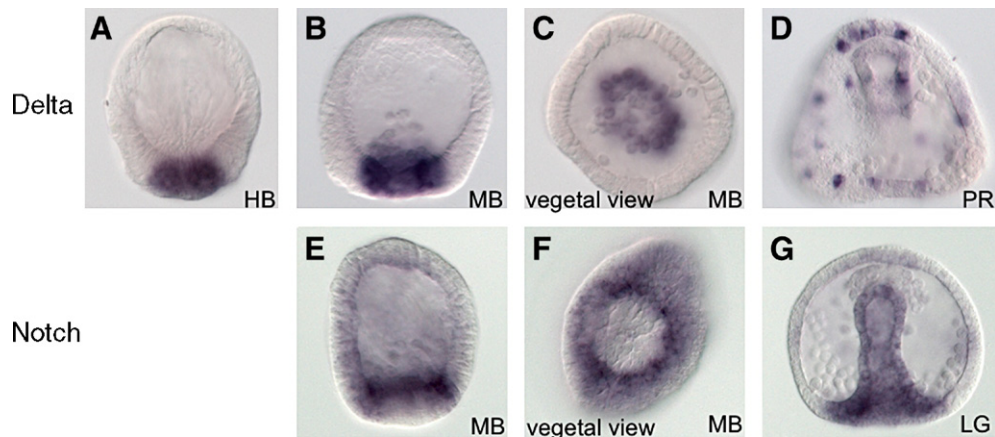
Fig. 5. RNA in situ localization pattern for main components of the Notch signaling pathway. (A–D) *delta*; (E–G) *notch*. Stages are: HB, hatched blastula; MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; PR, prism. All views are lateral except panels C, F and J which are indicated as vegetal views.

expressed in the vegetal plate cells with the exception of the primary mesenchyme cells at the mesenchyme blastula stage (Figs. 5B and C). Expression is confined to the secondary mesenchyme cells at the prism stage (Fig. 5D) with the possible exception being expression in presumptive neuroblasts at the apical region (see the paper by Burke et al., in this issue).

Zygotic *notch* mRNA is confined to ring of cells in the presumptive endoderm at the mesenchyme blastula stage (Figs. 5E and F). That ring of *notch* expression is predictably outside the ring of *delta* expression at the same stage (Fig. 5C). Earlier in development Notch protein was shown with antibodies initially to be expressed on all cells of the embryo, and the early maternally expressed Notch on presumptive SMCs is activated by Delta from micromeres as a necessary step in SMC specification (Sherwood and McClay, 1997, 1999, 2001). *Notch* continues to be expressed in the endoderm throughout gastrulation where it plays an essential role in defining the fate of endoderm, just as it earlier had an important role in separation of SMCs and endoderm from early endomesoderm (Sherwood and McClay, 1999). Notch also appears to have a later role in determining the border between endoderm and ectoderm (Sherwood and McClay, 2001). A further role in patterning of the endoderm itself is suggested by the interaction of Notch with other molecules within the Endomesodermal Gene Regulatory Network (McClay, unpublished data), however further investigation is needed to define this endodermal patterning role.

Role of Notch in germ layer specification across the animal kingdom

Notch is expressed in the gut of *amphioxus* (Holland et al., 2001) and developing mouse embryos (Schroder and Gossler, 2002) and is essential to gut development. Notch has also been shown to play a role in endoderm development in *Xenopus* (Contakos et al., 2005), chicken (Matsuda et al., 2005), zebrafish (Kikuchi et al., 2004), and *Drosophila* (Fusse and Hoch, 2002). Thus, in addition to its well-characterized role in neural specification, the Notch pathway is involved broadly in the specification of other tissues in the embryo. A later pattern of expression in the sea urchin suggests that Delta-Notch could be used for neural specification (Fig. 5D), but functional data testing this hypothesis are lacking at present.

Summary

Thirty Hh pathway genes and twenty-three Notch pathway genes (excluding Notch-ligands besides Delta and Serrate) have been identified from the sea urchin genome. Analysis of these genes revealed that for the most part all main components and modifiers were present in the sea urchin and that the number of copies of genes was consistent with its place in evolutionary history. In addition, the differences in relationships of the proteins produced from these genes relative to humans, *Ciona*, *Drosophila* and *Nematostella* suggest that the pathways did not evolve as a unit, but rather each component was subjected to its own evolutionary selection. Finally, temporal and spatial RNA expression patterns for these genes suggest their importance in gut development is conserved in the sea urchin relative to similar known functions in other species.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.064.

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