An Amphioxus Gli Gene Reveals Conservation of Midline Patterning and the Evolution of Hedgehog Signalling Diversity in Chordates

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Background. Hedgehog signalling, interpreted in receiving cells by Gli transcription factors, plays a central role in the development of vertebrate and Drosophila embryos. Many aspects of the signalling pathway are conserved between these lineages, however vertebrates have diverged in at least one key aspect: they have evolved multiple Gli genes encoding functionally-distinct proteins, increasing the complexity of the hedgehog-dependent transcriptional response. Amphioxus is one of the closest living relatives of the vertebrates, having split from the vertebrate lineage prior to the widespread gene duplication prominent in early vertebrate evolution. Principal Findings. We show that amphioxus has a single Gli gene, which is deployed in tissues adjacent to sources of hedgehog signalling derived from the midline and anterior endoderm. This shows the duplication and divergence of the Gli gene family, and hence the origin of vertebrate Gli functional diversity, was specific to the vertebrate lineage. However we also show that the single amphioxus Gli gene produces two distinct transcripts encoding different proteins. We utilise three tests of Gli function to examine the transcription regulatory capacities of these different proteins, demonstrating one has activating activity similar to Gli2, while the other acts as a weak repressor, similar to Gli3. Conclusions. These data show that vertebrates and amphioxus have evolved functionally-similar repertoires of Gli proteins using parallel molecular routes; vertebrates via gene duplication and divergence, and amphioxus via alternate splicing of a single gene. Our results demonstrate that similar functional complexity of intercellular signalling can be achieved via different evolutionary pathways.

INTRODUCTION

A key challenge faced by embryos with large cell numbers is to regulate the patterning of cell fields. While short-range intercellular signals can play a role in such processes, morphogen gradients provide a conceptually attractive alternative and key examples include nodal signalling in early vertebrate gastrulation and hedgehog signalling in vertebrate limb and neural tube development [1]. A critical component of a morphogen-based patterning system is the signal reception and transduction pathway that senses morphogen concentration and activates appropriate target gene expression. From an evolutionary perspective such components can be informative for study, as one possible route to evolving complexity in patterning is increasing the fidelity of gradient sensing, and hence the complexity of concentration-dependent transcriptional responses.

The hedgehog signalling pathway has been extensively studied in Drosophila and vertebrates, and significant similarities in genes and mechanisms are observed in these two lineages. There are important differences too, for example the roles of Drosophila Cad2 and Suppressor of FusedSu(fu) appear to differ from those of their mammalian orthologs [2,3] (though see also [4]). Vertebrates and Drosophila also differ in the number of genes encoding many pathway components. For example, a single hedgehog gene (hh) has been described from Drosophila, while vertebrates have multiple hedgehog genes falling into three distinct groups, related to Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Studies on the protostomes Branchiostoma flordae (amphioxus) and Ciona intestinalis have shown these extra vertebrate hedgehog genes evolved via gene duplications specific to the vertebrate lineage [5,6].

Receipt of hedgehog signalling by target cells requires the cell membrane proteins patched and smoothened, which then relay the signal intracellularly to a conserved family of transcription factors encoded by the Gli genes in vertebrates and Cubitus interruptus (Ci) in Drosophila (hereafter referred to collectively as the Gli gene family). Evidence from Drosophila suggests all hedgehog signalling is transduced via Ci protein [7]. The regulation of Ci/Gli protein activity levels by hedgehog is, however, a complex affair [reviewed in [8]]. Briefly, in Drosophila cytoplasmic Ci protein is cleaved in the absence of hedgehog signalling to yield an N-terminal form with potent repressor activity. Hedgehog signalling blocks this cleavage, increasing the concentration of full length protein and hence activator activity. Cleavage of Ci requires phosphorylation on specific sites by PKA and additional serine/threonine kinases. These phosphorylation events also appear to result in differently-active protein forms, presenting an additional opportunity for regulation by hedgehog signalling. Thus the single Drosophila Ci gene can produce varying concentrations of activator and repressor protein under the regulation of hedgehog signalling.

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As with the hedgehog genes, in vertebrates there are more Gli genes than in Drosophila. In mammals, three distinct genes, Gli1, Gli2 and Gli3 have been described [9,10]. Like Ci, hedgehog-dependent cleavage and phosphorylation plays a role in the post-translational regulation of the vertebrate Gli proteins [11,12], showing this to be an ancient aspect of hedgehog signal interpretation. Furthermore, recent studies suggest that graded hedgehog signalling results in graded levels of activation of Gli protein, and hence concentration-dependent target gene activation [13]. Consistent with the central role of Gli proteins in hedgehog signalling experiments in which the three vertebrate Gli genes were expressed in Drosophila imaginal discs showed that, at the subcellular level, the combination of activator and repressor activities displayed by all three proteins could be accounted for by Gli alone [14,15]. Importantly, however, these activities are not distributed evenly between the three vertebrate paralogs, and Gli1, Gli2 and Gli3 have been demonstrated to have distinct activator and repressor functions in a variety of embryonic contexts [11,16–18]; reviewed by [19]. Gli1 and Gli2 appear to mainly provide positive transcriptional activity while Gli3, although harboring latent positive transcriptional activity appears to act predominantly as a transcriptional inhibitor. Furthermore, the Gli1, Gli2 and Gli3 genes are differentially expressed during development, for example in the mouse neural tube Gli1 is expressed ventrally, while Gli2 and Gli3 are more dorsally expressed [9]. This differential expression is at least partially regulated by hedgehog signalling [16], and hence itself by levels of Gli protein activation [13].

Thus, while aspects of Gli family function are similar in Drosophila and vertebrates, there are also important differences. From an evolutionary perspective, the duplication and divergence of Gli genes in vertebrates has increased the complexity of the mechanism receiving hedgehog signalling. In this context it is intriguing to note that hedgehog signalling is deployed in the developing face [22]. To gain insight into when and how Gli gene duplication and divergence occurred, we have characterised a Gli ortholog from the cephalochordate amphioxus. Amphioxus develops a classic chordate body plan, but split from the vertebrate lineage prior to the widespread gene duplications [23]. Hence we predicted it would have only one Gli ortholog. Accordingly, we demonstrate the presence of a single Gli ortholog in amphioxus, and show it is expressed in developing embryos and larvae in a pattern consistent with a role in hedgehog signalling. We also find the amphioxus Gli mRNA is differentially spliced to yield two transcripts encoding different open reading frames and with spatially-distinct expression patterns. We utilise tests of Gli protein function to show the proteins encoded by these transcripts differ in function. Together, these results demonstrate that the duplication and divergence of Gli genes is specific to vertebrates, but also show that amphioxus has evolved an increased diversity of Gli proteins through a separate mechanism, that of alternate splicing.

MATERIALS AND METHODS

Sequence alignments, phylogenetic analyses and database comparisons

Sequence alignments were constructed using ClustalX [24,25], and molecular phylogenetic analyses carried out using the embedded Neighbor Joining function of ClustalX and using TREEPUZZLE [26]. Evidence of alternate splicing of Gli genes was gathered as follows: For C. intestinalis; via the JGI Ciona version 2 website (http://genome.jgi-psf.org/Cion2/Cion2. home.html). For mouse (version NCBI m36) and Human (version NCBI 36); via the Ensembl Alternative Splicing Database (http://www.ensembl.org/index.html).

Expression of amphioxus Gli proteins in NIH-3T3 cells and chick embryos

Full length cDNAs encoding AmphiGli-S or AmphiGli-L were excised with Xba1 and Xho1, and cloned into the plasmid pCAGGS IRES-NLS-GFP [13,27] that had been linearised with Xho1 and Nhe1. Construct integrity was verified by sequencing. Transcriptional activity in NIH-3T3 cell culture was assayed as previously described using a multimeric Gli Binding Site-Luciferase reporter [13]. In ovo electroperoration was used to transfet the neural tube of Hamburger and Hamilton (HH) stage 10–12 chick embryos [28]. Electroporated embryos were incubated for 24–48h, then fixed and processed for the immunohistochemical localization of proteins as described [29]. The antibodies used have been described previously [13,30,31].

Expression of amphioxus Gli proteins in Drosophila imaginal discs

Full length cDNAs encoding AmphiGli-S or AmphiGli-L were excised with EcoR1 and cloned into the plasmid pUAST [32]. Construct integrity was verified by sequencing. Transgenic lines were generated as described [33]; several independent insertions of each construct were obtained and localised to their respective chromosomes. Lines were kept over balancer chromosomes medium.

To generate cells expressing these constructs in the fly, the lines were mated to the 30A GAL4 driver line. This line drives expression of the UAS transgene in a ring surrounding the future blade area of the wing imaginal disc [32]. To assess activation of the hedgehog pathway a transgene that has the regulatory sequences important for expression of the decapentaplegic (dpp) gene, a recognised hedgehog target, coupled to a marker gene (LacZ), was added to this genetic background. To generate random cells expressing the transgenes the lines were mated to FLP-out lines as described [34].

To detect effects on the hedgehog signalling pathway, imaginal discs were isolated. For LacZ detection discs were fixed with 0.5% glutaraldehyde. They were washed in PBS and stained for β-
Galactosidase activity using standard assays. They were washed in PBS, mounted in 75% glycerol/PBS and imaged using DIC optics on a Zeiss AxioFluor. For immuno-stainings, the tissues were fixed in 4% paraformaldehyde, washed (PBS+0.1% saponin) and blocked using 3% Normal donkey serum. The discs were incubated with primary antibodies in PNT (PBS+0.1% saponin+3% normal donkey serum) overnight at 4°C, washed the next day five times for five minutes with PNT and incubated with appropriate secondary antibodies (Jackson Immunoresearch) for two hours in PNT. Washes were done as before and wing discs were mounted in Vectashield (Vector Laboratories) and imaged using a Zeiss Meta501 Confocal Laser Scanning Microscope.

RESULTS

Cloning and characterisation of an amphioxus Gli gene

A 327bp fragment encoding part of the zinc finger region of AmphiiGli was amplified from an amphioxus embryo cDNA library [35] using primers described previously [36]. Five independent clones contained the same fragment. This fragment was then used to screen the same cDNA library, and 11 cDNA clones isolated and sequenced. Sequence comparison revealed the clones derived from the same gene which encoded five conserved Gli-type C2H2 zinc finger domains (Fig. 1A, E), but represented two splice variants, with one type (represented by 5 clones) including an extra 1128 bp inserted 12 bp from the 3' end of the open reading frame. This insert contains an alternate stop codon, and results in the insertion of an additional 130 amino acids. Thus the two splice forms encode open reading frames that differ at the carboxy terminus (Fig. 1B, C). Specifically, AmphiGli-Long (AmphiGli-L) includes a conserved carboxy terminus domain found in Gli proteins in insects and vertebrates (Fig. 1B-D), while in AmphiGli-Short (AmphiGli-S) this domain is absent.

Next we sought to assess Gli gene number in amphioxus. Considerable evidence suggests gene duplication via genome duplication marked early vertebrate evolution, as exemplified by the Hox clusters [37]. The linkage of GLI1 to HOXC and GLI3 to HOXA on human chromosomes 12q15 and 17p13-14 respectively suggests the Gli genes were duplicated at the same time as the Hox clusters, that is before the divergence of jawed vertebrates. We also examined the draft assembly of the Branchiostoma floridae genome (http://genome.jgi-psf.org/Brafl1/Brafl1.home.html) for Gli, Glis and Zic related genes. This identified only one Gli ortholog in the current assembly.

Expression of Amphioxus Gli transcripts

To examine the combined expression of both transcripts, we used a riboprobe derived from the entire cDNA encoding AmphiGli-S. For comparison, we also examined the expression of the amphioxus hedgehog gene AmphiHh [6]. AmphiGli expression was first detected in late gastrulae, in a broad dorsal domain in both expressing cells located in AmphiGli expression was gradually down regulated in the neural tube and paraxial mesoderm until becoming undetectable in these tissues in larvae (Fig. 2E, H, I-K). Expression was maintained in the cerebral vesicle and head cavity, and activated in the developing gill slits (Fig. 2E, H). At this time, AmphiHh is expressed in the anterior extension of the notochord that underlies the cerebral vesicle, and in endoderm

Figure 1. Sequence and structure of the AmphiGli transcripts. A: Alignment of the zinc finger region of AmphiGli with the equivalent region of a selection of vertebrate Gli proteins, Drosophila Ci, AmphiZic and mouse Zic1. The 5 zinc finger domains are indicated, and the paired cysteine and histidine residues of each finger are in bold. B: Schematic comparison of conserved domains in AmphiGli with vertebrate and Drosophila Gli proteins. The arrows indicate the approximate position of cleavage of Drosophila Ci. NR: N terminal regulatory domain . ZNF: zinc finger region. CT: C terminal transactivation domain that is absent from AmphiGli. Numbers indicate protein length in amino acids (aa). C: Inferred differential splicing that yields the two AmphiGli transcripts. D: Sequence alignment of the C terminal transactivation region found in AmphiGli-L with a selection of other Gli proteins, E: Molecular phylogenetic tree of amphioxus, human and Drosophila Gli zinc finger sequences, rooted with the related Zic and Glis sequences. Previous identified gene groups have been boxed [38], and AmphiGli groups robustly within the Gli group. Accession numbers of sequences used are indicated. Numbers next to nodes indicate percentage puzzling support values, and for clarity values lower than 80 have been omitted. Species codes are: Mm, Mus musculus. Hs, Homo sapiens. Xl, Xenopus laevis. Gg, Gallus gallus. Dr, Danio rerio. Ci, Ciona intestinalis. Dm, Drosophila melanogaster.

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associated with the anterior pharynx and forming gill slit (Fig. 2N; [6]). This pattern essentially persisted in larvae, with transcripts detected in the cerebral vesicle, pre-oral pit, club-shaped gland and gill slits (Fig. 2J, K).

To determine whether the two AmphiGli splice variants are differently expressed, we sought to develop transcript-specific probes. This is not possible for AmphiGli-S, as the entire mRNA sequence is included in that of AmphiGli-L. We were however able...
to develop a specific probe for the extra sequence included in AmphiGli-L, and hence were able to examine the localisation of this transcript. The AmphiGli-L transcript was not detected in gastrulae, in embryos that had finished neurulation or in larvae (Fig. 2R and data not shown). In neurulating embryos, AmphiGli-L expression was detected in the somitic mesoderm and in the neural plate (Fig. 2P, Q), but was not detected in the left head cavity, which was intensely stained by the full AmphiGli probe (Fig. 2D, E). These data demonstrate that AmphiGli expression is typically found adjacent to cells expressing AmphiHh, and that the two AmphiGli transcripts are differently expressed during development. Specifically, we were able to show some sites of expression were specific to AmphiGli-S, as they did not label with the AmphiGli-L-specific probe. However due to the overlap between the transcripts, we were not able to determine if sites labelled by both probes expressed both AmphiGli-L and AmphiGli-S, or just expressed AmphiGli-L.

AmphiGli-L and AmphiGli-S encode functionally distinct proteins

Since AmphiGli-L and AmphiGli-S encode different ORFs, we hypothesised that the distinct proteins they produce may have different functions. To test this, we exploited three different methods for assessing Gli protein activity. First, we used a cell culture assay, in which Gli protein regulates the expression of a reporter regulated by eight multimeric Gli binding sites [17]. This showed that AmphiGli-L is a potent transcriptional activator with similar or greater activity in these assays to vertebrate Gli2. In contrast, AmphiGli-S, like vertebrate Gli3, did not affect reporter activity and thus does not act as a transcriptional activator in these assays (Fig. 3).

Second, we assessed the activity of the two AmphiGli variants in the vertebrate spinal cord, which provides a sensitive in vivo assay for the strength of Gli activity. In response to different levels of Gli activity, established by a gradient of Shh signalling, distinct neuronal subtypes emerge in a characteristic spatial order from progenitors arrayed along the dorsal ventral axis of the neural tube. Thus the expression of a set of markers of different progenitor domains and neuronal subtypes can be used as a reliable indicator of Gli activity (Fig. 4A). To take advantage of this system we cloned each full length AmphiGli transcript into a bi-cistronic vector in which both AmphiGli and GFP were driven from the same promoter, facilitating identification of transfected cells by their expression of GFP. Each construct was then ectopically expressed in the chick neural tube using in ovo electroporation, and 24–48 h post-transfection the expression of neural progenitor domain markers assessed. In this assay, AmphiGli-L induced expression of markers of the somatic motor neuron domain (as detected by MNR2/HB9 expression) and p0–p2 interneuron progenitor domain (as detected by Cad7), while it repressed the dorsal marker Pax7 (Fig. 4B–J). AmphiGli-L was however unable to induce ectopic Nkx2.2 expression, a marker of progenitors ventral to the motor neurons that requires high levels of Shh signalling for its induction (Fig. 4K–M). These data are consistent with the in vitro assay and indicate AmphiGli-L induces responses characteristic of moderate levels of Shh signalling, and is approximately equivalent in activity to full length Gli2 [13]. In contrast, AmphiGli-S did not alter the expression Pax7, Cad7 or MNR2/HB9, however AmphiGli-S repressed the expression of Nkx2.2, (Fig. 4N–V). This suggests AmphiGli-S acts as a weak repressor of Shh signalling in the vertebrate neural tube.

Third, we utilised a Drosophila imaginal disc assay previously used to assess vertebrate Gli activity [14,15]. We cloned each full length Gli transcript into the vector pUAST. Transgenic lines containing these constructs were made. By crossing these lines to driver lines, expression of the genes can be directed spatially and temporarily [32]. The expression of these genes in a defined region or random expression in small groups of cells in imaginal discs was...
used to assay the alterations to the hedgehog pathway, which we visualised using markers and pathway components. Ectopic AmphiGli-L activated a Dpp-LacZ reporter that responds to hedgehog pathway activity, while ectopic AmphiGli-S did not seem to affect this reporter (Fig. 5). To assay if either of the Gli isoforms has activity as a repressor, we looked at another transcriptional target of hedgehog signalling in the imaginal discs, Engrailed. Engrailed expression is only induced and maintained at highest levels of hedgehog signalling. Figure 6 shows that the AmphiGli-S form abolishes Engrailed expression from cells in which it is expressed.

In summary three independent assays, utilising cell culture, the chick neural tube and Drosophila imaginal discs, demonstrate that the two AmphiGli proteins differ functionally. Specifically they show that the AmphiGli-L form, which includes the conserved C terminal domain, acts as an activator, while the AmphiGli-S form in which this domain is absent can act as a repressor.

DISCUSSION

Amphioxus has one Gli ortholog, and this is alternately spliced

Our data confirm that amphioxus has a single Gli ortholog. The urochordates Ciona intestinalis and Ciona savignyi and the sea urchin Strongylocentrotus purpuratus also have single Gli orthologs ([38,39]; our unpublished observations), and hence conclude that the gene duplication that yielded vertebrate Gli1, Gli2 and Gli3 occurred in the vertebrate lineage, after amphioxus and urochordates had diverged. Therefore, the complexity of Gli genes and resulting protein forms seen in vertebrates is a vertebrate innovation.

We also show the AmphiGli yields two splice variants encoding proteins with different transcriptional activities (see below). There are few descriptions of alternate splicing of Gli genes in the literature. An exception is human GLI2, for which alternate splicing in gonad tissue and relating to viral function has been reported [40,41]. However the splicing reported in these studies involves differential usage of exons encoding the N terminal regions of GLI2, and hence differs from the splicing in amphioxus, which affects the C terminal. Other vertebrate Gli genes, Ci in Drosophila and Ci-Gli in C. intestinalis have not been reported to be to be alternately spliced. Examination of chordate Gli loci and associated EST data also do not indicate splicing that would alter the C termini (as seen in amphioxus), although there is evidence that other vertebrate Gli genes may also be spliced in a similar way to human GLI2 such that the N terminus is affected (SMS, unpublished observations). Hence we conclude that, while alternate splicing affecting the N terminus may occur for vertebrate Gli genes, the alternate splicing yielding different C termini observed in amphioxus is specific to the amphioxus lineage.

AmphiGli expression correlates with AmphiHh expression, and indicates conserved mechanisms operate in chordate midline patterning

AmphiGli is dorsally-expressed in both neurectoderm and mesoderm during neurulation. In the neurectoderm, expression is excluded from the floor plate but found throughout the rest of the neural plate. During the same period, AmphiHh is expressed by notochord and floor plate cells [6]. In vertebrate embryos, midline-derived signalling by Shh plays a critical role in establishing the DV pattern of neuronal differentiation in the
In mouse and chick embryos, the expression of genes involved in dorsal cell-type determination, such as Msx, Pax7, Irx3 and Pax6, is repressed by Shh, while expression of ventral genes, such as Nkx6.1, Nkx2.2 and Dlx2, is induced by Shh [42]. A number of additional transcription factor genes are also activated in specific types of differentiating neuron, including Mox, En1, Eex and Ibx1, as well as several Islet and Lim genes. Knowledge of neural DV patterning outside of the amniotes is less advanced, however studies on zebrafish embryos suggest a high degree of similarity within the vertebrates [43].

Orthologs of many of these vertebrate genes, including Pax3/7, Mox, Eex, Nkx2.1, Mox, Islet and Ibx3, have been isolated from amphioxus and shown to be expressed in the developing neural plate and/or specific populations of cells in the neural tube [44–50]. Our data show that AmphiHh and AmphiGli are expressed at the right time and place to be involved in regulating the expression of these genes in amphioxus, while the functional analysis of AmphiGli (discussed below) demonstrates that AmphiGli is able to act as an effector of hedgehog signalling. These data suggest conservation of neural DV patterning between amphioxus and vertebrates, with an ancient role for Gli proteins in transducing midline-derived hedgehog signals and hence in generating the bilateral organisation of the central nervous system.

Similar to its expression in the neurectoderm, AmphiGli mesodermal expression is excluded from the midline (i.e. the notochord), but found through the majority of each mesodermal segment. Amphioxus mesodermal segments are often referred to as somites. However these differ from vertebrate somites, as in vertebrates the ventral mesoderm remains unsegmented, while in amphioxus all mesoderm (other than the notochord) is segmented. Hence each amphioxus somite includes cells fated to form the myotome (also derived from the somite in vertebrates), plus cells fated to form the mesoderm that surrounds the gut (derived from the unsegmented lateral plate mesoderm in vertebrates) and an additional population of dorsolateral cells of distinct but uncertain fate (with no clear counterpart in vertebrates) [51]. These populations of cells express different combinations of genes suggestive of homology with vertebrate mesoderm compartments; for example Mox and Zic in the dorsal somite [47,52], and FoxF

![Figure 5. Expression of a Decapentaplegic-LacZ (Dpp-LacZ) reporter gene in Drosophila wing imaginal discs. All discs are shown with the anterior compartment to the right. A. Control Dpp-LacZ disc. B. Dpp-LacZ 30A GAL4 UAS AmphiGliS. C. and D. Dpp-LacZ 30A GAL4 UAS AmphiGliL. The arrows in C and D point to an expansion of LacZ expression into the anterior compartment, indicating that the AmphiGliS construct is inducing the Dpp transgene. doi:10.1371/journal.pone.0000864.g005](image)

![Figure 6. Expression of Engrailed in Drosophila wing imaginal discs. All discs are shown with anterior compartment to the right. The cell specific marker that indicates the cells that have the FLP’ed construct, i.e. are expressing the AmphiGli-S (A–D) or the AmphiGli-L (E–H) constructs is in green (A, D, G and H). The Ci protein is labelled in red (B, D, F and H). Ci is expressed only in the anterior compartment and its expression delimits the anterior compartment. The expression of Engrailed is in blue (C, D, G and H). Engrailed is expressed throughout the posterior compartment, overlapping with Hh expression. Engrailed also becomes expressed in a small group of cells just within the anterior compartment (and thus overlapping with Ci expression). This expression is dependent on Hedgehog and is thought to represent the highest level Hedgehog signalling target [73]. Expressing AmphiGli-S in small clones of cells leads to a complete down-regulation of Engrailed expression in the anterior compartment domain of expression (arrows, A–D). A similar clone of cells expressing AmphiGli-L shows some but weaker down regulation of Engrailed (arrows, E–H). doi:10.1371/journal.pone.0000864.g006](image)
and Vent in ventral, gut-associated mesoderm [33,34]. Notochord-derived hedgehog signalling regulates somite subdivision in vertebrates (see for example; [55–60]). Single Hh and Gli genes are predicted to have been present in the common ancestor of the Deuterostomes. In the Ambulacaria (represented by the echinoderm Lytechinus variegatus and the hemichordate Saccoglossus kowalevskii), these genes do not appear to have duplicated, though hemichordate Gli has yet to be isolated. The spatial expression of Gli genes is unreported in Ambulacaria, however Hh expression has been reported as localised to the apical tip in S. kowalevskii and to the endoderm in L. variegatus. In the Chordata, midline Hh expression is observed in all three lineages, with corresponding Gli expression in adjacent tissues (though note neural restriction of Hh and Gli in C. intestinalis, indicating the loss of notochord Hh and associated mesodermal Gli expression from this lineage). Adjacent to the B. floridana and M. musculus diagrams are simplified representations of the diversity of activator and repressor forms of Gli present in each lineage. Superscript numbers adjacent to genes indicate the following references from which the data were taken: 1 [74]. 2 [39]. 3 [75]. 4 [6]. 5 [5]. 6 [38].

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Hedgehog signalling in chordate pharyngeal development

We also detected AmphiiGli transcripts in the developing left head cavity towards the end of neurulation, and in the developing cerebral vesicle, pre-oral pit (which forms in part from the left head cavity) and endoderm associated with the developing club-shaped gland and gill slits. These sites correlate with expression of AmphiiHh in adjacent tissues, specifically the anterior notochord, central pre-oral pit and pharynx [6]. The pre-oral pit of amphioxus has been suggested to be homologous to the vertebrate pituitary on the basis of embryological and gene expression data, and due to its post-metamorphic fate, when it establishes a connection to the ventral cerebral vesicle [64–67]. Hh/Gli
signalling has been reported to be required for vertebrate pituitary development [66,69], and hence the expression of AmphiHh and AmphiGli in the developing pre-oral pit may reflect a conserved role. Similarly, Shh is expressed by vertebrate anterior endoderm cells and plays a role in patterning the developing pharynx [70]. The expression of AmphiHh and AmphiGli in the amphioxus pharynx may indicate that the regulation of pharyngeal development by hedgehog/Gli signalling is a primitive chordate character.

**AmphiGli transcripts encode functionally distinct proteins**

Three different tests of Gli protein activity demonstrate that the proteins encoded by AmphiGli-L and AmphiGli-S are functionally distinct. All three tests show AmphiGli-L is an activator of the hedgehog pathway, and in the chick neural tube assay it has similar activating ability as full length vertebrate Gli2 [13]. In contrast AmphiGli-S was not able to activate reporter expression in vitro, and in both chick neural tube and Drosophila imaginal disc assays it functioned as a repressor of the hedgehog pathway. This is similar to the activity of Gli3 in these assays. A similar effect on the transcriptional activity of vertebrate Gli2 was achieved by deleting the last 93 amino acids of the protein [71], an engineered change that effectively reproduces the structure of AmphiGli-S. Comparison of the sequence of Gli proteins from vertebrates, amphioxus and Drosophila reveals a high level of conservation in the C terminal region (Fig 1D). This raises the possibility that the C terminal region of Gli family proteins are involved in activating transcription through a common mechanism, and removal of this region provides one mechanism for modulating the transcriptional activity of a Gli protein.

**Conservation and diversity of hedgehog-Gli signalling in the chordates**

Our data suggest a model for the diversification of hedgehog signalling in the chordates (Fig. 7). The characterisation of the amphioxus Gli gene demonstrates that two different chordate lineages have used different genetic routes to evolve an increased complexity of transcription factor capability downstream of hedgehog signalling. In vertebrates, Gli genes have duplicated then diverged both in the function of the encoded proteins and in spatiotemporal expression. The amphioxus lineage originated before these duplications, and amphioxus has not independently duplicated the Gli gene. However, diversification of protein function and regulation of expression has evolved via alternate splicing. Perhaps most interestingly, in vertebrates Gli3 usually acts as a strong repressor, with a major role of hedgehog signalling being to alleviate this repression. This is illustrated by Shh+/−/Gli3−/− mouse embryos, which show partial rescue of the phenotype seen in Shh+/− embryos [72]. Our data show this division of Gli function between duplicated genes is a vertebrate innovation. However AmphiGli-S has similar transcriptional properties to Gli3 and is expressed without AmphiGli-L in several sites, indicating that alleviating repression is also likely to be an important mechanism in amphioxus hedgehog signalling. We conclude that functionally similar end-points in hedgehog signalling complexity can be reached by different molecular routes, as different solutions evolve to solve similar evolutionary-developmental problems.

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**Author Contributions**

Conceived and designed the experiments: JB SS Mv. Performed the experiments: JB SS Mv RD. Analyzed the data: JB SS Mv. Contributed reagents/materials/analysis tools: SS. Wrote the paper: JB SS Mv.

**REFERENCES**

1. Ashe HL, Briscoe J (2006) The interpretation of morphogen gradients. Development 133: 385–394.
2. Svard J, Henricson KH, Persson-Lek M, Rozell B, Lauth M, et al. (2006) Genetic elimination of suppressor of fused reveals an essential repressor function in the mammalian hedgehog signaling pathway. Dev Cell 10: 187–197.
3. Varjosalo M, Li SP, Taipale J (2006) Divergence of hedgehog signal transduction mechanism between drosophila and mammals. Dev Cell 10: 177–186.
4. Tay SY, Ingham PW, Roy S (2005) A homologue of the Drosophila kinesin-like protein Costal2 regulates Hedgehog signal transduction in the vertebrate embryo. Development 132: 625–634.
5. Takatori N, Satou Y, Satoh N (2002) Expression of hedgehog genes in Ciona intestinalis embryos. Mech Dev 116: 235–239.
6. Shimeld SM (1999) The evolution of the hedgehog gene family in chordates: Inaugis from amphioxus hedgehog. Genes Dev 2009: 40–47.
7. Methot N, Basler K (2001) An absolute requirement for Cubitus interruptus in Hedgehog signaling. Development 128: 733–742.
8. Ruiz i Altaba A (1999) The works of GLI and the power of hedgehog. Nat Cell Biol 2: E147–148.
9. Hui CC, Shuarski D, Platt KA, Holmgren R, Joyner AL (1994) Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggest multiple roles during postimplantation development. Dev Biol 162: 402–413.
10. Ruppert JM, Kanzier KW, Wong AJ, Bigner SH, Kao FT, et al. (1988) The Gli-Kruppel family of human genes. Mol Cell Biol 8: 3104–3113.
11. Wang B, Fallon JF, Beachy PA (2000) Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 100: 423–434.
12. Pan Y, Bai CB, Joyner AL, Wang B (2006) Sonic hedgehog signalling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Mol Cell Biol 26: 3363–3377.
13. Stamatakis D, Ulhoa F, Tseni SV, Mynett A, Briscoe J (2005) A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube. Genes Dev 19: 626–641.
14. Aza-Blanc P, Lin HY, Ruiz i Altaba A, Kornberg T (2000) Expression of vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities. Development 127: 4293–4301.
15. Mering Cv, Basler K (1999) Distinct and regulated activities of human Gli proteins in Drosophila. Current Biol 9: 1319–1322.
16. Ruiz i Altaba A (1996) Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. Development 125: 2203–2212.
17. Sasaki H, Hui C, Nakaoka M, Kondoh H (1997) A binding site for Gli proteins is essential for HNF3beta floor plate enhancer activity in transgens and can respond to Shh in vitro. Development 124: 1313–1322.
18. Lee J, Platt KA, Censullo P, Ruiz i Altaba A (1997) Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. Development 124: 2537–2552.
19. Ingham PW, McMahon AP (2001) Hedgehog signaling in animal development: paradigms and principles. Genes Dev 15: 3059–3087.
20. Riddle RD, Johnson RJ, Laufer E, Tabin C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75: 1401–1416.
21. Vorckamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, et al. (1996) Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273: 613–622.
22. Harrickson Z, Ho R, Hui CC, Sharpie PT (1998) The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants. Development 125: 2803–2811.
23. Panopoulos G, Herrig S, Groth D, Krause A, Poustka AJ, et al. (2003) New evidence for genome-wide duplications at the origin of vertebrates using an amphioxus gene set and completed animal genomes. Genome Res 13: 1056–1066.
24. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence
weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 22: 4673–4680.
25. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality control tools. Nucl Acids Res 25: 4876–4882.
26. Strimmer K, von Haeseler S (1996) Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Mol Biol Evol 13: 964–969.
27. Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants of Escherichia coli: novel esakaryotic vector. Genes Dev 5: 193–198.
28. Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. J Morphol 88: 49–92.
29. Briscoe J, Piersani A, Jessel TM, Ericson J (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101: 435–445.
30. Nakagawa S, Takeichi M (1995) Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. Development 121: 557–568.
31. Ericson J, Rashbash P, Schoeder, A, Newsome M, Sakasakaa N, et al. (1997) Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signalling. Cell 90: 169–180.
32. Brandt AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401–415.
33. Rubin GM, Spradling AC (1982) Genetic transformation of Drosophila with transposable element vectors. Science 218: 348–353.
34. Basler K, Struhl G (1994) Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. Nature 368: 208–214.
35. Langeland JA, Torna KA, Jackman WR, Kimmel CB (1998) An amphioxus snail gene: Expression in paraxial mesoderm and neural plate suggests a conserved role in patterning the vertebrate embryo. Dev Genes Evol 208: 569–577.
36. Holland PW, Williams NA, Lanza JF (1991) Cloning of segment polarity gene homologues from the unsegmented brachipod Terebratalia retusa (Linnaeus). FEBS Lett 291: 211–215.
37. Dehail P, Boore JL (2005) Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biol 3: e314.
38. Yamada L, Koyashki K, Degnan B, Satoh N, Satou Y (2003) A genomewide expression survey of developmentally relevant genes in Ciona intestinalis. IV. Genes for HMG transcriptional regulators, bZIP and GATA/Zic/Snail. Dev Genes Evol 213: 245–253.
39. Materna SC, Howard-Ashby M, Gray RF, Davidson EH (2006) The CHZ2 zinc finger genes of Strongylocentrotus purpuratus and their expression in embryonic development. Dev Biol 300: 108–120.
40. Tanimura A, Dan S, Yoshida M (1998) Cloning of novel isoforms of the human Gli2 oncogene and their expression in embryonic and neural tissues. J Neurosci Res 55: 543–551.
41. Ferrier DE, Minguillon C, Cebrian C, Garcia-Fernandez J (2001) Amphioxus AMH transcriptional regulators, bZIP and GATA/Zic/Snail. Dev Genes Evol 210: 193–199.