

Amphioxus *Gli* knockout disrupts the development of left right asymmetry but has limited impact on neural patterning

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Abstract

The Gli transcription factors are the primary mediators of Hedgehog (Hh) signaling. Vertebrate genomes contain multiple *Gli* paralogues with different functions downstream of Hh signal receipt, in part explaining the complexity of cellular responses to Hh that allow concentration-dependent target gene activation. Amphioxus is a chordate that split from the vertebrate lineage early in the evolution of chordates, before the genome duplications that occurred in early vertebrate evolution. It has a single *Gli* gene which this is alternately spliced to yield two protein isoforms called GliS and GliL. We generated two knockout mutations in amphioxus *Gli*, one that affects the whole gene and a second that only affects GliL. Both knockouts showed major morphological and molecular defects in the development of left-right asymmetry. Amphioxus *Hh* knockout also causes defects in left-right asymmetry, though there are differences between *Gli* and *Hh* knockout phenotypes. Hh signaling also patterns the amphioxus neural tube. Here, however, knockout of *GliL* only showed no identifiable phenotype, while knockout of the full gene showed only small changes to the expression of one gene family, *Olig*. Other genes that were prominently affected by Hh knockout were not altered in expression in either knockout. Reasons for the differences between *Hh* and *Gli* knockouts in the pharynx and neural tube are discussed in the context of the likely different functions of amphioxus Gli isoforms.

Key words: amphioxus; *Gli* gene; left-right asymmetry; neural tube patterning

Introduction

Hedgehog (Hh) signaling is a well-characterized intercellular signaling pathway that functions in many different capacities and contexts in the embryonic development of most animals. Canonical examples include parasegment boundary formation in *Drosophila*, and limb and spinal cord patterning in vertebrates (reviewed by (Jiang and Hui, 2008)). Hh protein is synthesized, processed and exported by *Hh* gene expressing cells, with the signal received by other cells via the receptor Patched (Ptch). Ptch interacts with another membrane protein, Smoothened (Smo), to relay the signal to the nucleus. The strength of signal received coupled with length of signal exposure can lead to different transcriptional outcomes, meaning Hh can in some contexts work as a morphogen and deliver robust patterning of cells according to their distance from a source of the signal (Briscoe and Small, 2015).

Relay of Hh signal from the Ptch-Smo complex to the nucleus is a complex process. In vertebrates this may involve translocation of proteins to the primary cilium, though the exact mechanism may differ between different lineages (Elliott and Brugmann, 2019; Huangfu and Anderson, 2006). However, a consistent feature of Hh signaling is that it feeds through the Gli transcription factors. Gli proteins have five C2H2 zinc finger domains which mediate DNA binding, as well as many other regions and sites which have been shown to be functionally significant (Hui and Angers, 2011). Importantly, the way that Hh signaling regulates the transcriptional regulatory activity of Gli and hence target gene transcription seems to be deeply conserved in animals (von Mering and Basler, 1999). Essentially, in the absence of Hh signaling full length Gli protein is modified from the C-terminus, leaving a truncated N-terminal form that still includes the zinc fingers but which acts as a transcriptional repressor (reviewed by (Hui and Angers, 2011)). When Hh signaling is relayed into a cell, C-terminal modification is inhibited leaving more full-length Gli protein, which acts as a transcriptional activator. This balance between activator and repressor forms of Gli under Hh signal control is one of the ways in which quantitative effects of signaling are realized as different transcriptional outcomes (Briscoe and Small, 2015; Sagner and Briscoe, 2019).

Most invertebrates have a single *Gli* gene, however the genome duplications that occurred in early vertebrate evolution have resulted in most vertebrate lineages having three *Gli* paralogues, *Gli1*, *Gli2* and *Gli3* (Abbasi et al., 2009). Studies of mammalian *Gli* genes show these paralogues have diverged in function. C-terminal modification of both Gli2 and Gli3 proteins is regulated by Hh signaling, however Gli3 tends to function more as a repressor and Gli2 as an activator (Hui and Angers, 2011). These characteristics help generate a dynamic network downstream of Hh that may explain some of the morphogenetic properties of the pathway. Mammalian *Gli1* functions differently: the protein is shorter than Gli2 and Gli3 and lacks some N-terminal domains, and it tends to act as an activator in a positive feedback circuit downstream of Hh (Hui and Angers, 2011; Park et al., 2000). *Gli* gene function in other vertebrate lineages has not been as well studied as it has in mammals, though it has been noted zebrafish has two *gli2* paralogues and that *gli1* and *gli2* genes may not function the same way as they do in mammals (Karlstrom et al., 2003; Ke et al., 2008).

The cephalochordate amphioxus predates the vertebrate genome duplications but shares many basic features of vertebrate development (Holland et al., 2008; Putnam et al., 2008), including a conserved pathway that regulates organismal asymmetry via the *Nodal* and *Pitx* genes (Li et al., 2017), and a dorsal neural tube with organized patterns of cell differentiation (Leung and Shimeld, 2019). Amphioxus has a Hh pathway that functions in both these processes, as established by knockout of the amphioxus *Hh* gene (Wang et al., 2015). *Hh* knockout compromises the development of left-right asymmetry, leading to animals with

symmetrical organization (Hu et al., 2017). *Hh* knockout also affects the differentiation of cell types in the central nervous system (Ren et al., 2020). Amphioxus has a single *Gli* gene like most other invertebrates (Shimeld, 2008), consistent with amphioxus not sharing the vertebrate genome duplications. Amphioxus *Gli*, however, is alternately spliced to yield transcripts encoding different isoforms: a full-length protein called GliL, and a truncated form missing part of the C terminus called GliS (Shimeld et al., 2007). It was previously considered that these resembled the full-length and C-terminus truncated forms of Gli in other species that are formed by Hh signaling regulating protein processing: this was in part tested in heterologous systems, in which amphioxus *Gli* isoforms were expressed in vertebrate and *Drosophila* embryos and the impact on target gene expression monitored (Shimeld et al., 2007). However, *Gli* function in vivo in amphioxus has not been studied.

Here we use TALEN gene knockout to examine *Gli* function in amphioxus. Two knockout lines are developed, one near the 5' end of the gene and a second that just affects the *GliL* transcript. *Gli* knockout animals show compromised development of left-right asymmetry at both morphological and molecular levels and resemble (but are not identical to) *Hh* knockouts in this regard (Hu et al., 2017). However neural patterning is only weakly affected compared to the impact of *Hh* gene knockout. Possible reasons for these differences are discussed.

Results

Generating Gli knockouts

Positioning of TALEN knockout sites is shown in Figure 1A. One TALEN was designed to the 5' region of the *Gli* gene and should result in a knockout of the whole gene. A second TALEN was designed near the 5' end of an exon that is specific to the *GliL* transcript. This should leave *GliS* functional but eliminate functions specific to *GliL*, as the mutated *GliL* transcript closely resembles *GliS*. Hereafter these two mutants are referred to as *Gli* and *GliL*, respectively (so for example as *Gli*^{-/-} and *GliL*^{-/-} when in the homozygous state). It is not possible to design a mutant specific for *GliS* as the entire coding sequence of *GliS* is included in *GliL*. Molecular characterization of knockout lines showed both genetic manipulations resulted in missense mutations (Fig. 1B).

The role of Gli in left right asymmetry

Hh knockout amphioxus show characteristic morphological defects in the pharynx related to disruption of left-right asymmetry patterning (Hu et al., 2017). In wild type animals the mouth forms on the left side, the first gill slit expands on the right side, and pharyngeal organs (preoral pit, endostyle, club-shaped gland) are asymmetrically organized. *Hh*^{-/-} animals appear symmetrical: they do not form a mouth opening, gill slits lie on the ventral midline and left-sided pharyngeal structures are duplicated on the right side while right-sided pharyngeal structures are lost (Hu et al., 2017).

Gli^{+/-} and *GliL*^{+/-} larvae showed normal asymmetric pharynx morphology as previously described in wild-type animals (Fig. 1C). Like *Hh*^{-/-} larvae, *Gli*^{-/-} larvae also showed symmetrical pharynx morphology (Fig. 1C) (Hu et al., 2017). Like *Hh*^{-/-} larvae the preoral pit was duplicated on both sides of the body, and the first gill slit was positioned on the ventral midline and did not expand up the right side as in wild-type animals. However an important aspect differed between the two phenotypes: in *Gli*^{-/-} larvae the mouth (which was absent in *Hh*^{-/-} larvae) was duplicated on both left and right sides (Fig. 1C). Similar results were found for *GliL*^{-/-} larvae, compared to *GliL*^{+/-} and *GliL*^{+/-} larvae, which appeared normal (Fig. 1C). The expression of *Ptch* was weaker in *Gli*^{-/-} and *GliL*^{-/-} embryos as compared to homozygous wild type and heterozygous embryos (Fig. 1D).

In wild-type larvae, asymmetrical pharyngeal morphology is accompanied by asymmetric expression of genes that mark the various pharyngeal territories (Hu et al., 2017). We studied the expression of such marker genes in wild type and *Gli* knockout embryos. In wild type and heterozygote embryos *Krox* expression marks the glandular region of the club-shaped gland (Knight et al., 2000), *Nkx2.1* the right part of the endostyle (Venkatesh et al., 1999), *Pou4* the mouth (Candiani et al., 2006) and *Pit* the preoral pit (Candiani et al., 2008). All four genes showed normal expression patterns in wild type and heterozygote embryos of both mutants (Fig. 2A-H). However, expression of all four genes was abnormal in *Gli*^{-/-} and *GliL*^{-/-} embryos (Fig. 2A'-H'). The asymmetrical parts of the expression domains of *Krox* and *Nkx2.1* were lost (Fig. 2A', B', E', F', compare to arrows on A, B, E, F respectively). Asymmetric expression of *Pou4* and *Pit* became symmetric with the appearance of another site of expression on the opposite side of the pharynx (Fig. 2C', D', G', H', compare arrows to C, D, G, H respectively). These data show that the development of asymmetric pharynx organs is compromised in both *Gli* knockout mutants.

Early development of asymmetry has been studied in amphioxus, revealing that *Hh* acts upstream of a conserved molecular pathway involving *Nodal*, its secreted inhibitors *Cerberus* (*Cer*) and *Lefty*, and its target the transcription factor *Pitx* (Hu et al., 2017; Li et al., 2017). To test if the same molecular pathway was being disrupted in *Gli* knockout embryos as it was in

Hh knockout embryos, we studied the expression of *Nodal*, *Cer*, *Lefty* and *Pitx* in embryos of both *Gli* mutants (Fig. 3). Expression of all four genes was normal in wild type and heterozygous embryos derived from crosses of both *Gli* knockout lines, with *Cer* predominantly expressed on the right side and the other three genes on the left side (Fig. 3A-H). However, both *Gli*^{-/-} and *GliL*^{-/-} embryos showed abnormal expression of all four genes: asymmetric expression of *Cer* was lost (Fig 3A', E'), while asymmetric expression of *Nodal*, *Lefty* and *Pitx* became symmetric with the appearance of ectopic expression of all three genes on the right side (Fig. 3B'-D', F'-H'). Note that embryos showing *Cer* expression are at an earlier developmental stage than those for the other three genes: this reflects the epistatic relationships between these genes, with *Cer* acting upstream of *Nodal*, *Lefty* and *Pitx* as we have previously described (Li et al., 2017).

The role of *Gli* in neural tube development

Hh signaling also functions in vertebrate neural tube patterning and analysis of amphioxus *Hh* mutant embryos has confirmed a developmental role in patterning the amphioxus neural tube (Ren et al., 2020). Amphioxus *Gli*^{-/-} and *GliL*^{-/-} larval neural tubes were not overtly different from those of wild type embryos in terms of gross morphology (Fig. 1C), although this is also the case in *Hh* knockout embryos. *Hh* knockout embryos do show disrupted expression of genes expressed in the ventral region of the neural tube, including *Vacht*, *Isl*, *Lhx3*, *Mnxa*, *Nkx6* and *OligA-C* genes (Ren et al., 2020). We examined the expression of these genes in *Gli* and *GliL* knockout embryos compared to wild type and heterozygote embryos. No differences were observed in the expression of the *Vacht*, *Isl*, *Lhx3* *Mnxa* and *Nkx6* genes between wild type and mutant embryos (Supplementary Figure 1). *OligA*, *OligB* and *OligC*, amphioxus specific paralogues of the *Olig* bHLH transcription factor family, were affected in *Gli*^{-/-} embryos (Fig. 4A'-C') compared to *Gli*^{+/-} and *Gli*^{+/+} embryos (Fig. 4A-C). Specifically, expression of these genes was lost from a number of cells in the neural tube in a pattern very similar to that previously observed in *Hh* knockout embryos (Ren et al., 2020). However, in *GliL*^{-/-} embryos the expression of these three genes was indistinguishable between all three genotypes (Fig. 4A''-C''), indicating that GliS can cover the necessary functions of Gli in this context.

Discussion

By designing TALENs to the 5' end of amphioxus *Gli*, and to the region specific to the *GliL* transcript, we were able to generate mutants which are likely to be a full loss of function of Gli, and loss of function of the GliL isoform only, respectively. Both mutants showed lower levels of *Ptch* expression. *Ptch* is a conserved downstream target of Hh signaling and thus we would expect disruption of Hh signaling to lead to a drop in its expression. Hh signaling is required for the correct development of asymmetry in amphioxus (Hu et al., 2017; Li et al., 2017). *Gli*^{-/-} embryos mostly phenocopy *Hh*^{-/-} embryos in this respect, showing similar disruption to pharynx development that is underlain by similar changes to genes that are normally asymmetrically expressed (Hu et al., 2017; Li et al., 2017). This is consistent with Gli playing the pivotal role as the transcription factor immediately downstream of the Hh signaling. There was one clear difference between *Hh*^{-/-} and *Gli*^{-/-} embryos, with the mouth lost in the former and duplicated in the later. The mouth is also lost in amphioxus *Smo*^{-/-} embryos, although initial asymmetry expression of the mouth marker *Pou4* is retained (Hu et al., 2021).

GliL^{-/-} embryos display the same phenotype as *Gli*^{-/-} embryos. This means that full length Gli protein is required for left-right patterning of the amphioxus pharynx by Hh signaling, while the truncated form GliS is not sufficient on its own to cover this role. Previous work in heterologous systems has shown that in these contexts GliL works as an activator, while GliS functions more as a repressor (Shimeld et al., 2007). Our analysis of *Gli*^{-/-} and *GliL*^{-/-} embryos hence indicates that the activator function of amphioxus Gli is essential for correct left-right asymmetric development.

We found clear differences between the phenotypes of *Hh* and *Gli* knockouts in neural tube patterning. *Hh* knockout embryos show significant differences in cell type specification in the neural tube (Ren et al., 2020). However, *Gli* knockout embryos only showed a difference in the expression of the *Olig* gene family, with other markers including ones disrupted in *Hh* knockout embryos showing normal expression. Overall, the impact of *Gli* knockout on neural cells was substantially less pervasive than would be expected from either the canonical view of Gli as the sole mediator of Hh signaling, or from the major phenotypes identified in the pharynx in both *Gli* and *GliL* mutant embryos. Furthermore, the neural tubes of *GliL* knockout embryos were not observably different from wild type embryos (as analyzed by the genes selected in this study), implying that perhaps only repressive functions of Gli are needed at this developmental stage.

The reasons for the difference in the strength of *Gli* knockout phenotype between the pharynx and neural tube are unknown and require further investigation, as do the differences in mouth opening between *Gli* and *Hh* knockouts. However, we know that the regulation and function of Gli are highly complex, involving feedback loops and with multiple phenotypic impacts of modified Gli proteins arising through regulated protein processing or through mutation (Falkenstein and Vokes, 2014; Hui and Angers, 2011). Furthermore, in mice both *Shh* and *Gli3* mutants have quite severe spinal cord phenotypes, while the phenotype of *Shh/Gli3* compound homozygotes is less severe than either single mutant (Litingtung and Chiang, 2000). This indicates that how different mutants feed into the gene regulatory network that turns Hh signaling into patterning via the Gli proteins can have a substantial impact on the phenotypic consequences. Resolving this in amphioxus will be a substantial challenge. However, the interbreeding of genetic knockouts to produce compound mutants has the potential to further develop our insight into this pathway.

Methods

Animal and embryo cultivation

Amphioxus (*Branchiostoma floridae*) were originally acquired from Dr. Jr-Kai Yu's lab at the Institute of Cellular and Organismic Biology, Academia Sinica, Taiwan. They were cultured and induced to spawn essentially following the protocols used for *Branchiostoma belcheri* animals (Li et al., 2013). A brief description has been included in our previous report (Yuan et al., 2020). Fertilization and subsequent culturing of the embryos were carried out essentially as described previously at 27–28 °C (Liu et al., 2013).

Mutant generation

Two TALEN pairs (BfGli-TALEN2 and BfGliL-TALEN2) targeting amphioxus *Gli* coding regions were designed and assembled according to our previous report (Li et al., 2014). Their positions and targeting sequences are shown in Fig. 1A and B. Mutation detection was conducted in a same way as a previously described (Li et al., 2017). Primers used for amplification of BfGli-TALEN2 flanking regions are BfGli-TALEN2-PCR-F1: AACGGAAAGTGGCGCTGAGT and BfGli-TALEN2-PCR-R1: CTTAGTTTCAGTGTGGGTAG, and those for BfGliL-TALEN2 are BfGliL-TALEN1-PCR-F1: GCACCGAAGCTCCCGAATA (or Bf-GliL-TALEN2-F3: CAGATGCAGTATCAGGCCAA), and BfGliL-TALEN1-PCR-R1: AAAGTTCGCGCTTTGGTCCT. As described in our previous work (Li et al., 2017; Ren et al., 2020) confirmed mutant lines were maintained as heterozygotes since homozygous mutants were not viable. To generate homozygous mutant embryos for gene expression analysis heterozygotes were crossed, yielding homozygous mutant embryos at an expected level of 25% reflecting the mendelian ratio of 3:1. Gene expression patterns were scored as wild-type (reflecting the known expression pattern for a gene) or non-wild-type. Non-wild type expression patterns were consistent between individual embryos. In each analysis the proportion of embryos in each category did not significantly deviate from the expected 3:1 ratio (chi squared test, p-values range from 0.69 to 0.93).

Probes, in situ hybridization and imaging

All probes used in the study has been described in our previous reports (Li et al., 2017; Ren et al., 2020). Embryos and larvae at required developmental stages were fixed with 4% paraformaldehyde (PFA) in MOPS buffer (pH 7.4) at 4°C overnight. In situ hybridization was then performed essentially according to a previous description (Yu and Holland, 2009). After staining, embryos were washed with phosphate buffered saline, mounted in 80% glycerol and photographed using an inverted microscope (Olympus)

Data availability

All data generated during this study are included in this published article.

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Contributions

G.L., S.S. and Y.W. designed the experiments. Q.R. generated the mutants and X.H. performed all other experiments. Q.R. and X.H. prepared the figures. All authors interpreted the data. G.L. and S.S. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Animal and human rights statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Figures and Figure Legends

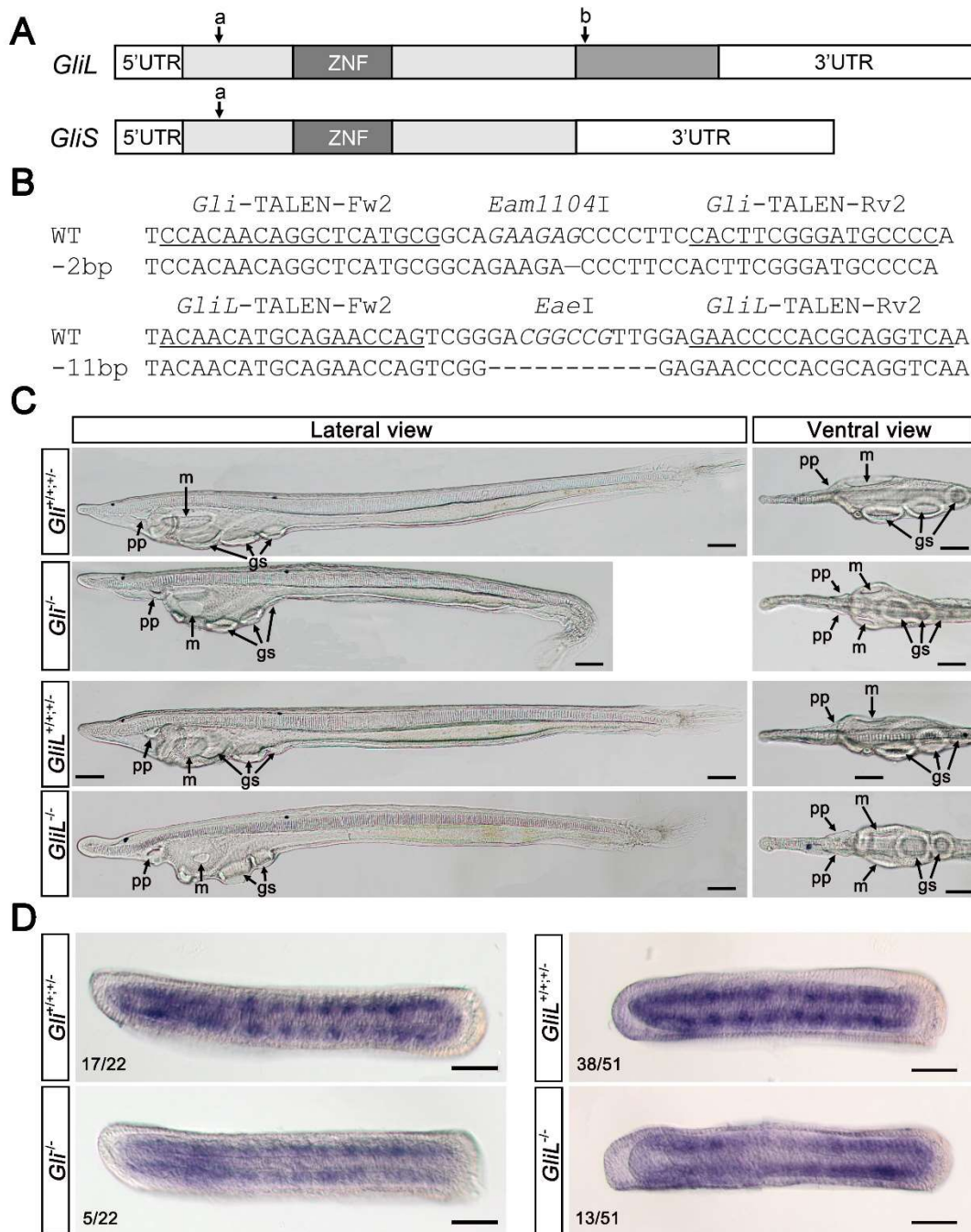


Figure 1. Generation and initial phenotyping of amphioxus *Gli* knockouts.

A. Schematic maps of the *GliL* and *GliS* transcripts showing the positions of the *Gli* TALEN (a) and the *GliL* TALEN (b). B. Sequence comparison of wild type and mutant loci for the *Gli* TALEN (top) and *GliL* TALEN (bottom). C. Morphological comparison of *Gli*^{-/-} and *GliL*^{-/-} larvae with wild type and heterozygous larvae, with lateral and ventral views showing symmetric pharynx morphology in knockout larvae. gs, gill slit. m, mouth. pp, preoral pit. D. Expression of *Ptch* in *Gli*^{-/-} and *GliL*^{-/-} embryos compared to wild type and heterozygous embryos. For the pairs of images shown in D, the numbers indicate the number of times the expression pattern was observed out of the total number of embryos analyzed for that cross. For example, for the images in the *GliL* analysis 51 embryos in total were analyzed, with 38 showing the pattern above and 13 the pattern below. All scale bars are 50 μ m.

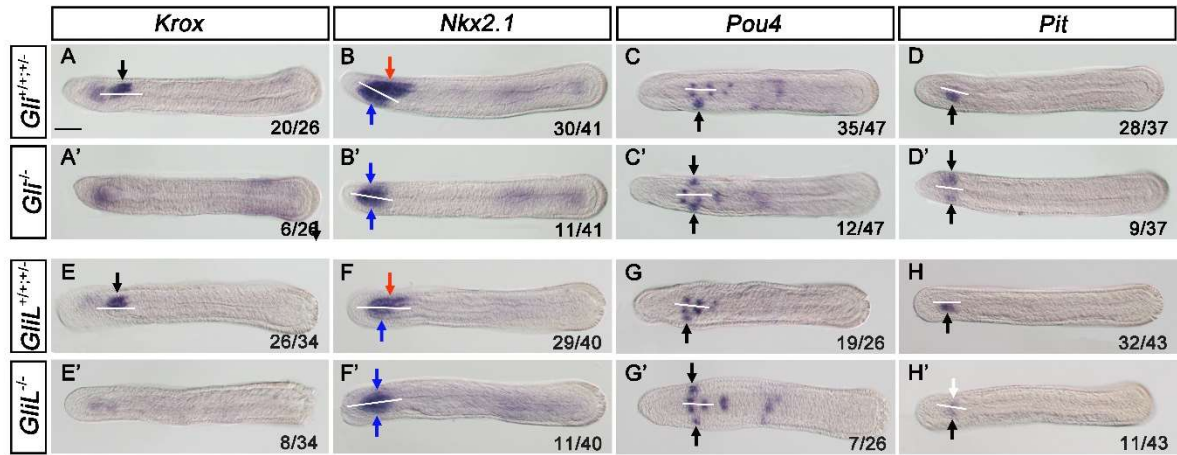


Figure 2. Expression of pharynx marker genes in amphioxus *Gli* knockout embryos.

Each pair of panels (e.g. A, A') shows expression of the gene indicated above in the wild type and heterozygous embryos (above) compared to expression in knockout embryos (below), with genotype indicated on the far left. White lines mark the separation between the left and right sides of embryos. For A and E the arrows mark asymmetric expression lost in the embryos in the panels below. For B and F blue and red arrows mark a symmetrical expression domain and right sided asymmetric expression domain, respectively. In B', F' only the asymmetric expression domain (blue arrows) is visible. On C, D, G and H the arrows mark left-sided expression domains, which are bilateral in the embryos shown in C', D', G' and H'. For the pairs of images (A, A' etc.) the numbers indicate the number of times the expression pattern was observed out of the total number of embryos analyzed for that cross. For example, for the images shown in A, A', 26 embryos in total were analyzed, with 20 showing the pattern above and 6 the pattern below. The scale bar on A is 50 μ m and applies to all panels.

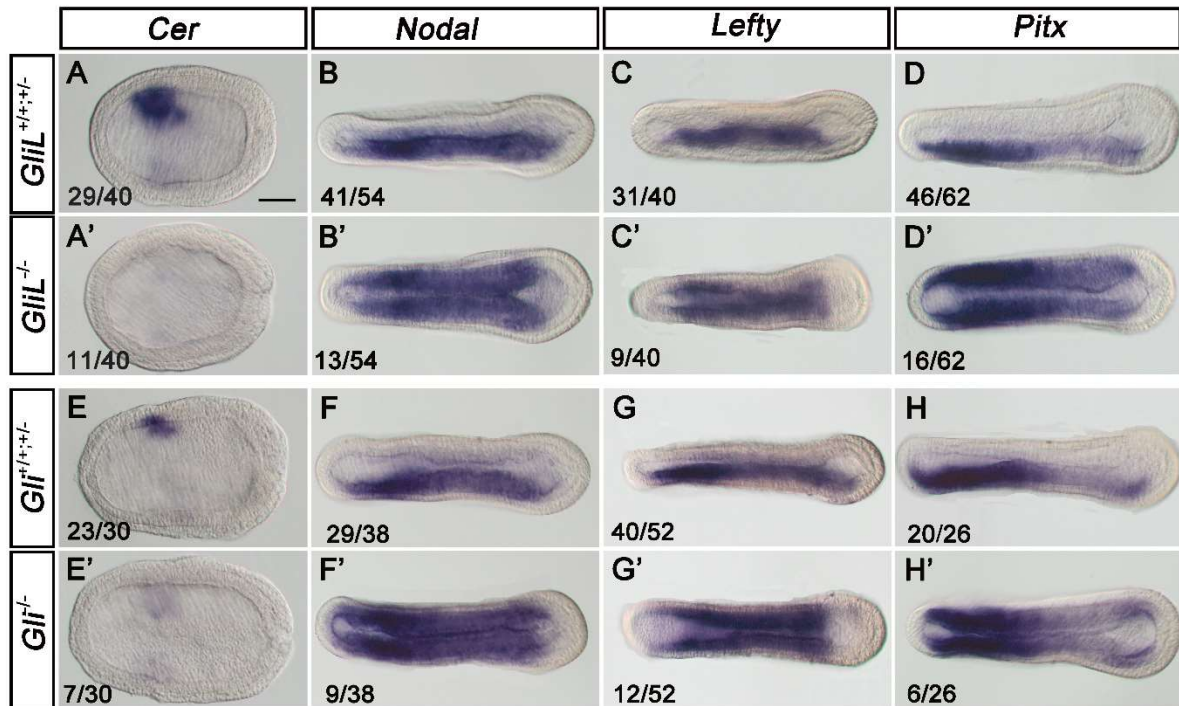


Figure 3. Expression of early left-right asymmetry genes in amphioxus *Gli* knockouts embryos.

Each pair of panels (e.g. A, A') shows expression of the gene indicated above in the wild type and heterozygous embryos (above) compared to expression in knockout embryos (below), with genotype indicated on the far left. For the pairs of images (A, A' etc.) the numbers indicate the number of times the expression pattern was observed out of the total number of embryos analyzed for that cross. For example, for the images shown in A, A', 40 embryos in total were analyzed, with 29 showing the pattern above and 11 the pattern below. The scale bar on A is 50 μ m and applies to all panels. Embryos in A, A', E, E' are at the early neurula stage, other embryos at the late neurula stage.

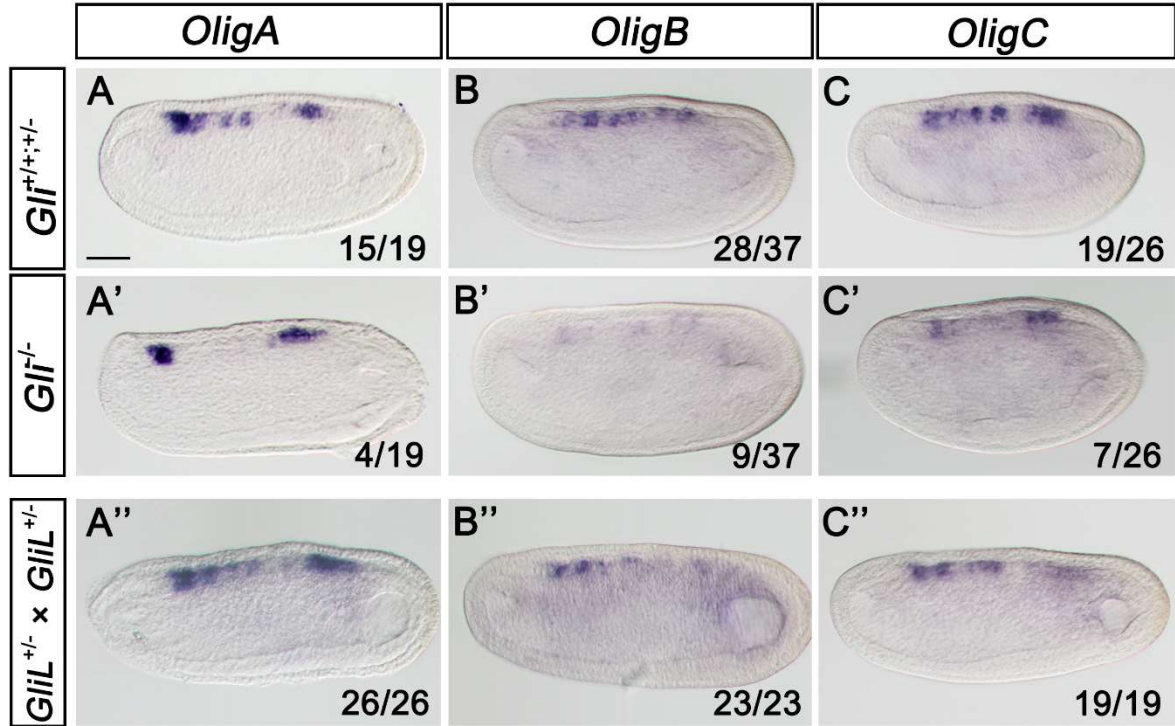
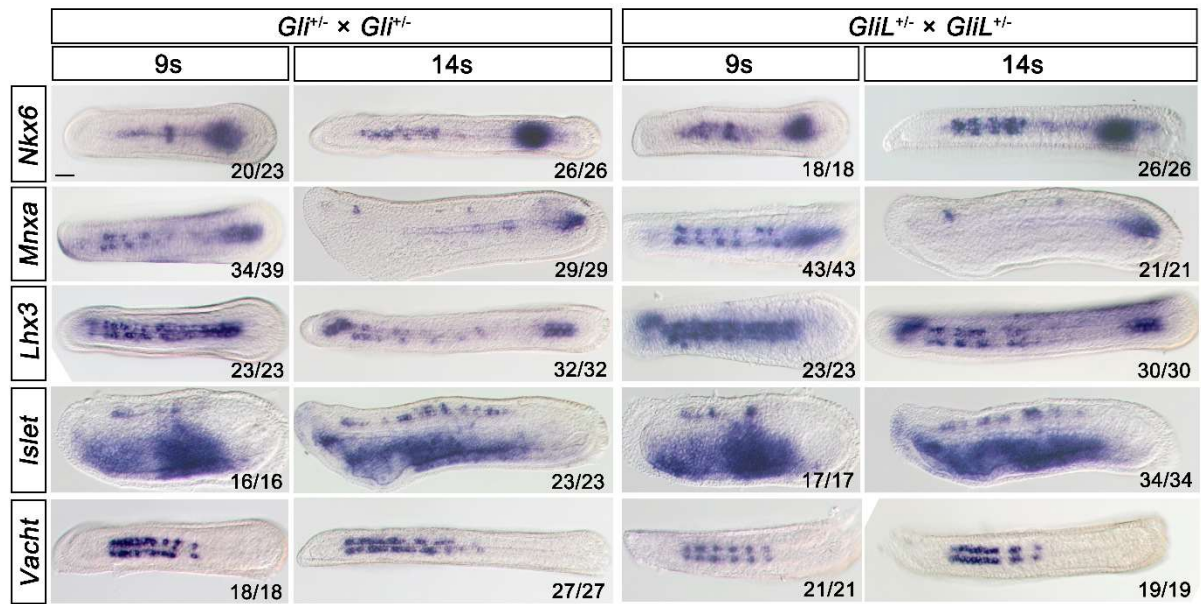


Figure 4. Expression of *Olig* family genes in amphioxus *Gli* knockouts embryos.

The expression of *OligA* (A-A''), *OligB* (B-B'') and *OligC* (C-C'') is shown in wild type, heterozygous and mutant embryos. For *Gli* knockouts, *Gli*^{-/-} embryos (A'-C') are shown compared to wild type and heterozygous embryos (A-C). For *GliL* knockouts, all three genotypes are shown combined (A''-C'') as the offspring of the *Gli*^{+/+} × *Gli*^{+/+} cross used to generate them, as there were no observed differences in gene expression. For the pairs of images (A, A' etc.) the numbers indicate the number of times the expression pattern was observed out of the total number of embryos analyzed for that cross. For example, for the images shown in A, A', 19 embryos in total were analyzed, with 15 showing the pattern above and 4 the pattern below. For A'', B'' and C'' only one pattern was observed. The scale bar on A is 50 μm and applies to all panels. Note that our experimental design means that embryos shown in A and A' were analyzed at the same time in the same tube, controlling for the between-tube variation in staining intensity sometimes encountered in in situ hybridization. The embryos represented by A'' were analyzed in the same tube as each other but in a different experiment to A and A'. Thus, while A and A' are directly comparable, neither should be compared to A'' for subtle differences in staining intensity. The same applies for B-B'' and C-C''.

Supplementary figures



sFigure 1. The expression of *Nkx6*, *Mnxa*, *Lhx3*, *Islet* and *Vacht* in embryos derived from *Gli^{+/+}* heterozygote crosses and *GliL^{+/+}* heterozygote crosses respectively, at both nine somite (9s) and 14 somite (14s) stages. No differences were observed between the genotypes. Numbers indicate the number of times the expression pattern shown was observed out of the total number of embryos analyzed for that cross. The scale bar on left top panel is 50 μ m and applies to all panels.