Visualization of Gli Activity in Craniofacial Tissues of Hedgehog-Pathway Reporter Transgenic Zebrafish

Tyler Schwend1,3*, Evyn J. Loucks3, Sara C. Ahlgren2,3*

1 Integrated Graduate Program, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 2 Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 3 Developmental Biology Program, Children’s Memorial Research Center, Chicago, Illinois, United States of America

Abstract

Background: The Hedgehog (Hh)-signaling pathway plays a crucial role in the development and maintenance of multiple vertebrate and invertebrate organ systems. Gli transcription factors are regulated by Hh-signaling and act as downstream effectors of the pathway to activate Hh-target genes. Understanding the requirements for Hh-signaling in organisms can be gained by assessing Gli activity in a spatial and temporal fashion.

Methodology/Principal Findings: We have generated a Gli-dependent (Gli-d) transgenic line, Tg(Gli-d:mCherry), that allows for rapid and simple detection of Hh-responding cell populations in both live and fixed zebrafish. This transgenic line expresses a mCherry reporter under the control of a Gli responsive promoter, which can be followed by using fluorescent microscopy and in situ hybridization. Expression of the mCherry transgene reporter during embryogenesis and early larval development faithfully replicated known expression domains of Hh-signaling in zebrafish, and abrogating Hh-signaling in transgenic fish resulted in the suppression of reporter expression. Moreover, ectopic shh expression in Tg(Gli-d:mCherry) fish led to increased transgene production. Using this transgenic line we investigated the nature of Hh-pathway response during early craniofacial development and determined that the neural crest skeletal precursors do not directly respond to Hh-signaling prior to 48 hours post fertilization, suggesting that earlier requirements for pathway activation in this population of facial skeletal precursors are indirect.

Conclusion/Significance: We have determined that early Hh-signaling requirements in craniofacial development are indirect. We further demonstrate the Tg(Gli-d:mCherry) fish are a highly useful tool for studying Hh-signaling dependent processes during embryogenesis and larval stages.

Introduction

Members of the highly conserved Hedgehog (Hh)-family of secreted proteins are essential in a variety of processes in vertebrate and invertebrate development. Hh-ligands function dually as morphogens, to pattern cells in a multicellular target field, as well as mitogens, to control cellular proliferation and survival. Understanding the many orchestrated events that transduce the Hh-signal has been of considerable interest to scientists and clinicians, as perturbations to the Hh-signaling pathway can lead to a variety of developmental disorders [1,2], while unchecked Hh-pathway regulation can develop into a variety of cancers [3,4].

Hh-signaling is initiated upon Hh-ligand binding to the large, hydrophilic extracellular loops of its cognate twelve-pass transmembrane receptor Patched (Ptc) [5–7] to relieve inhibition of a seven-pass transmembrane receptor protein Smoothened (Smo) [8,9]. Stimulation of cellular Smo leads to signal transduction events involving the activation of the Gli family of transcription factors [10,11]. Gli proteins are zinc finger transcription factors that respond to Hh-signals by regulating the transcription of Hh-target genes [12]. In zebrafish, four gli genes (gli1, gli2a, gli2b, gli3) have been identified and implicated in early development [13–19]. Hh-target gene expression may become upregulated or downregulated by Gli, as activator and repressor functions are distributed across the four zebrafish proteins, thereby creating a complex code of Hh-dependent gene transcription that influences cell proliferation and differentiation during early development. Hh-dependent gene transcription is similarly regulated among three Gli proteins (Gli1, Gli2, Gli3) in mouse [20–27] and the single bifunctional gli homolog Cubitus interruptus (c) in Drosophila [28,29]. The dual transcription activator/repressor functions are best understood for c. In the absence of Hh-signals, C is cleaved into a truncated repressor form that inhibits expression of Hh-target genes. On the other hand, in the presence of Hh-signals, C protein remains as a full-length activator version that promotes expression of Hh-target
genes [28–30]. In the mouse, both Gli2 and Gli3 can undergo post-translational proteolytic processing events that likely alter these molecules from full-length activators of Hh-signaling, to C-terminally truncated repressor forms [25,26,28,30–32]. In contrast to Gli2 and Gli3, mouse Gli1 activity is not regulated post-translationally. Instead, Gli1 levels are transcriptionally regulated in tissues responding to Hh-signaling [7,15,33–37]. Transcriptional regulation of Gli1 appears to be conserved, as studies in mouse, frog and zebrafish have all shown that Gli1 regulation is sensitive to changes in Hh-signaling [7,15,33–36]. In zebrafish, gli1 is the major activator of Hh-signaling [15,19], although gli2a/b and gli3 have roles in both activating and repressing the Hh signal, depending on the cellular context [15,17,18]. Loss of gli1 by genetic mutation, or reducing embryonic gli1 levels by antisense morpholino oligonucleotides, nearly phenocopies the ventral neural patterning defects seen in mutant zebrafish with complete Hh-signaling loss [15,38,39]. Genetic studies in the zebrafish demonstrate that combinations of gli1, gli2a/b, and gli3 are needed to precisely integrate Hh-signaling during development [13–15,18].

Gli transcription factors transduce the Hh-signal by binding to DNA in a sequence specific manner through the recognition of the DNA motif GACCACCCA, known as the Gli-consensus binding sequence (Gli-CBS) [10,11]. One widely utilized approach to show changes in Hh-responsiveness within cells has been to use a Gli-dependent (Gli-d) reporter, whereby an engineered promoter element containing eight repeating Gli-CBS drives expression of a luciferase gene [15,40]. By measuring luciferase activity in reporter construct-expressing cells, it is possible to determine the levels of cellular Gli activity. While the Gli-d luciferase reporter has been invaluable for analyzing potential agonists and antagonists of the Hh-signaling pathway in a variety of cell lines, it has not been feasible to utilize the reporter construct to determine spatial or temporal locations of Hh-target regions during animal development. As mentioned above, in the zebrafish gli1 is the major activator of Hh-signaling, and zebrafish Gli1 has been shown to activate the luciferase form of this reporter in vitro [15]. In the embryo, the presence of repressor Gli proteins refines Hh-pathway activity by suppressing Gli-mediated transcriptional activation. In this regard, a positive readout of Hh-signaling is found when Gli activation exceeds Gli repression. Therefore, probing Gli-depentent Hh-signaling is found when Gli1 expression is detected in fixed embryos analyzed by both fluorescent imaging and in situ hybridization. A 765 bp fragment of the Gli-d reporter is capable of detecting Hh-signaling in cartilage differentiation within the posterior arches [42,43].

The widespread, and dynamic, expression of Hh-ligands in the head, as well as the spectrum of phenotypes that exist depending on both the tissue disruption or the timing of the Hh-signaling deficit, suggest that the Hh-signaling mechanism that controls craniofacial development is complex and multifaceted. Although genetic mutant analysis and temporal inhibition studies have suggested roles for Hh-signaling in craniofacial and neural tissues, these studies have provided limited details concerning when specific tissue groups in the craniofacial region are receiving/responding to Hh-signals. Thus, to better understand the spatial and temporal targets of Hh-signaling in craniofacial development, we have created an in vivo reporter system for detecting Gli1 responding cells throughout development. By utilizing the previously described Gli-d reporter construct from Sasaki et al., 1997 that uses eight repeating Gli-CBS to drive expression of a mCherry reporter we have generated a novel transgenic line that can be used to dynamically visualize Hh-responding cells in zebrafish [40]. In this study we have characterized the expression of the Gli-d reporter during early zebrafish craniofacial development. Next, we determined precisely when and where Hh-signals are required during craniofacial development. Further, by performing timed inhibition studies on Tg(Gli-d:mCherry) fish, we determined that Hh-signaling controls cartilage differentiation in CNC precursors residing in the ventral arches by an indirect mechanism. Importantly, this study reveals that we now have a new and powerful tool for determining Hh-signaling targets in vivo during development.

Results

A Gli-dependent reporter is capable of detecting changes to the Hh-signaling pathway in fish

A Hh-pathway sensitive reporter construct, comprised of a Gli-d promoter element containing eight repeating Gli-CBS from the mouse FoxA2 floor plate enhancer and a minimal lens crystallin promoter, was first shown to faithfully drive luciferase reporter expression in cells activated by Hh-signaling by Sasaki et al., 1997 [40]. Since Gli activity is widely regarded as an indicator of Hh-signaling, we reasoned that a fluorescent mCherry molecule under the control of the Gli-d promoter element would allow live visualization of Hh-pathway activity in live transparent zebrafish embryos, as well as providing a unique marker for Hh-pathway activation in fixed embryos analyzed by both fluorescent imaging and in situ hybridization. A 765 bp fragment of the Gli-d

PLoS ONE | www.plosone.org 2 December 2010 | Volume 5 | Issue 12 | e14396
promoter element was PCR amplified, and subsequently cloned into a Gateway 5' entry vector and used in the Tol2 system for creating transgenic zebrafish [48]. The Gli-d promoter was cloned upstream of the mCherry open reading frame (ORF) and flanked with Tol2 transposable elements (Figure 1A).

To show that the reporter construct could be utilized to identify changes in Hh-signaling pathway activity, we created mosaic transgenic zebrafish by co-injecting 50 pg of Gli-d reporter plasmid DNA and 25 pg of Tol2 mRNA into the one-cell staged progeny of genetic mutant strains deficient in a variety of Hh-signaling components. Following the injections, the embryos were allowed to develop to 30 hpf for analysis. At this developmental stage, homozygous mutants (−/−) for Hh-signaling pathway components could be identified by the presence of U-shaped somites and a prominent ventral tail curl (i.e. Figure 1C). Heterozygotes (+/−) and homozygous wild type (+/+), siblings displayed chevron shaped somites with a straight tail (i.e. Figure 1B). These different phenotypes allowed the embryos to be sorted into mutant (−/−) or wild type (+/+,+/-) categories prior to fixation and imaging. Upon analysis, mCherry fluorescent signals were consistently visible in the ventral brain (data not shown) and within the developing somites of heterozygotes and homozygous wild-type siblings (Figure 1B,D,F). On the other hand, Gli-d reporter expression was undetectable in the homozygous mutant siblings within three separate Hh-signaling mutant lines; slow-muscle-omitted (smo) mutants, which harbor a mutation in the smo receptor (Figure 1C, [38,39]) the detour (dtr) mutant which contains a gli1 loss-of-function mutation (Figure 1E; [15]) and the you-too (yot) mutant, encoding a C-terminally truncated Gli2 protein that acts as a dominant repressor of Hh-signaling and is able to block Gli1-mediated transcriptional activation (Figure 1G; [14,15]). This data strongly suggests that mutant embryos deficient in Hh-signaling fail to express the Gli-d transgene.

The Hh-signaling pathway can be targeted by small molecule chemical compounds. Thus, we next sought to show that the

Figure 1. Gli-dependent reporter is capable of detecting modulations to the Hh-signaling pathway when expressed transiently in fish. (A) Schematic of the Gli-d mCherry reporter construct designed in this study. The construct consists of a 0.7 kb fragment encoding eight repeating Gli-CBS from the mouse FoxA2 floor plate enhancer and a minimal lens crystallin promoter (light blue and green respectively) inserted upstream of the mCherry ORF (red). The SV40 polyA signal (pink), which contains a transcriptional termination element, is directly downstream of the mCherry ORF and the entire element is flanked by Tol2 transposable elements (gray). (B–I) Lateral views, anterior to the left, of confocal stack projections of fixed trunk tissue. (B–G) Homozygous genetic mutants (−/−) for smo/smo (B,C), dtr/gli1 (D,E), yot/gli2 (F,G) were sorted from heterozygotes (+/−) and homozygous wild type (+/+) siblings based on evident characteristic Hh-signaling mutant phenotypes. Genetic mutants (−/−) for Hh-signaling pathway components failed to express mCherry protein (C,G), while strong mCherry expression was evident in trunk tissue of (+/+,+/−) siblings (B,D,F). (H,I) Hh-pathway attenuation with 100 μm Cya fully reduced mCherry protein expression in construct-expressing embryos (I) compared to vehicle-treated controls (H).

doi:10.1371/journal.pone.0014396.g001
expression of the Gli-d reporter could also be perturbed with a known small molecule inhibitor of the Hh-pathway. Cyclopamine (Cya) acts on the Hh-receptor Smo [49–51], upstream of Gli protein activation and nuclear entry, thus making it an ideal pharmacological candidate for testing the Gli-d reporter’s potential to reflect Hh-signaling changes. Using a similar strategy for visualizing reporter expression in developing trunk muscle, we saw no levels of reporter expression in wild-type embryos that had been injected as above and subsequently treated with Cya (Figure 1I). In contrast, injected siblings treated with a vehicle control (ethanol) showed robust levels of reporter expression (Figure 1H). In full, these studies clearly show that the Gli-d reporter construct, when applied to fish by transient transfection, can be used to rapidly and reliably detect changes to the Gli-d reporter construct, when applied to fish by transient transfection, can be used to rapidly and reliably detect changes to the Hh-signaling pathway. Using a vertebrate animal model to explore potential modulators of the Hh-signaling pathway, either genetic interactors or small molecules compounds, provides an alternative to performing the same studies in vitro in cell lines.

**Generation of Gli-dependent Reporter Transgenic Zebrafish**

The fact that the Gli-d reporter was sensitive to Hh-signaling when transiently expressed in fish told us that if the reporter was stably expressed it would be possible to visualize Gli activity dynamically. To generate stable transgenic lines, the construct was co-injected with in vivo transcribed Tol2 transposase mRNA [40] into one cell stage wild type embryos. The injected fish were examined for the expected mosaic expression of the transgene at 24 hpf. We identified embryos showing the strongest fluorophore expression within known regions of Hh-signaling activity, such as the forebrain, notochord and muscle fibers (Figure S1). These embryos were used to begin four independent Tg(Gli-d:mCherry) lines. Each independent line displayed identical expression patterns in the craniofacial region throughout embryonic development and during larval stages (data not shown). Incrossing of the novel lines did not reveal any gross transgene-induced defects.

To confirm that Tg(Gli-d:mCherry) progeny inherit the transgene, Tg(Gli-d:mCherry) fish were outcrossed with wild type EK or Tu adults. The resulting offspring were probed for mCherry RNA expression by RT-PCR. We detected mCherry transcripts following fertilization, in the offspring of female Tg(Gli-d:mCherry) that had been mated with male wild type fish (Figure 2). This expression declined slightly around 5 hpf, before increasing again at 7 hpf. On the other hand, mCherry transcripts did not appear until 7 hpf in the offspring of male Tg(Gli-d:mCherry) and female wild type matings (Figure 2). This indicated that the mCherry reporter was maternally deposited by Tg(Gli-d:mCherry) females and that zygotic transcription of the transgene commenced by approximately 7 hpf. When these samples were probed for gli1, it was also found to be present at fertilization and to be maintained, presumably due to maternal contributions and subsequent zygotic expression (data not shown).

We sought to determine that the Gli-d reporter is expressed in a spatially restricted fashion in Tg(Gli-d:mCherry) fish, reminiscent of the expression pattern for gli1 and ptk1, two genes which are regulated by Gli activity. Spatiotemporal expression of the reporter in Tg(Gli-d:mCherry) was analyzed throughout embryonic and early larval development by both in situ hybridization and confocal laser scanning microscopy. mCherry gene expression was evident in embryos during epiboly stages, however, at these times the signal was ubiquitous expressed at a low level in most blastomeres (Figure 3A), presumably due to maternal contributions (see above, Figure 2). mCherry gene expression first became visible in a spatially restricted fashion within the prospective anterior neural plate and axial mesoderm by the tailbud stage (11 hpf), consistent with the onset of zygote expression a few hours prior to that timepoint (Figure 3B–C*). This expression pattern is consistent with the expression of both shh and gli1 expression patterns [14,16,52]. At similar stages, we were unable to detect mCherry proteins by confocal microscopy in Tg(Gli-d:mCherry) embryos (data not shown). Failure to detect a fluorescent signal may be due to low expression levels or decreased protein stability in early-staged embryos. Despite this, by mid-somitogenesis, mCherry fluorescent protein became readily apparent in the embryonic forebrain and within the midline axis (Figure 3D), which also express shh and gli1 at these stages [15,52]. At 1 dpf (22–30 hpf), lateral and dorsal views revealed mCherry gene and
Figure 3. mCherry reporter gene and protein expression reflects known Hh-expressing domains in Tg(Gli-d:mCherry). Lateral views (A,B,D,E,G,H,J,K,L,M,N) or dorsal views (C',C',F,I,M'), anterior to the left, of Tg(Gli-d:mCherry) fish labeled with mCherry (A–F,K,L,M,N) or shh (G) riboprobes or mCherry protein which was visualized by confocal microscopy (H–J,L'). (A) mCherry is broadly expressed in blastomeres at 60% epiboly. By 11 hpf, mCherry was localized to the neural plate (np) and the midline notochord (no) tissues. (D) At 17 hpf, mCherry protein was apparent in forebrain (fb) and floor plate (fp). (E–G) At 22–24 hpf, mCherry gene (E,F) and protein (H–J) are expressed in the fb, midbrain and fp, similar to shh gene expression (G) at a comparable stage. (K–N) At later stages from 2 to 4 dpf, mCherry is shown in whole-embryo lateral views and detectable in brain, fp, myotomal adaxial cells and PA. Confocal projections of Mcherry protein corresponding to boxed region in panel L revealed expression in the adaxial cells. (M') Dorsal view of boxed region in panel M showed mCherry expression in adaxial cells and in somitic tissue neighboring the notochord in an orderly, segmental fashion. Abbreviations: di, diencephalon; fb, forebrain; fmb, forebrain-midbrain boundary; fp, floor plate; hb, hindbrain; mb, midbrain; np, neural plate; no, notochord; os, optic stalk; ov, otic vesicle; PA, pharyngeal arches; pcp, prechordal plate; te, telencephalon.

doi:10.1371/journal.pone.0014396.g003
fluorescent protein expression in the forebrain, forebrain/midbrain boundary, floor plate, axial mesoderm tissues and the otic vesicle (Figure 3E,F,H–J), consistent with shh (Figure 3G) [15].

Studying mCherry expression at later stages, from 36 hpf up to 4.5 days post fertilization (dpf), revealed dynamic expression patterns within the developing brain, pharyngeal arches (PA) and somitic myotome (Figure 3K–N). With respect to the brain, by 36 hpf mCherry expression had become restricted to ventral sites (Figure 3K). Ventral restriction in the central nervous system, after the first day of development, is similar to what has been shown for gli1 (Figure S2), and for fkh1 by other groups [15,53]. Within the developing trunk of 54 hpf embryos, lateral views reveal mCherry gene and fluorescent protein expression within the myotome (Figure 3LL'). By 72 hpf, expression persisted in neural, facial and somitic muscle tissue throughout the larvae (Figure 3M). At the same time, expression in the trunk became more spatially restricted to the population of myotome cells most proximal to the midline notochord, which secretes Hh-ligands at high levels. This reporter expression pattern in the myotome could be best visualized in dorsal views of larval trunks (Figure 3M'). Later, at 4.5 dpf, lateral views of the trunk revealed that mCherry extends along the anterior-posterior axis of the developing spinal cord and its expression is spatially restricted within each somite at the prospective sites of intervertebral discs (Figure 3N).

Changes to the Hh-signaling pathway mediates reporter gene expression in Tg(Gli-d:mCherry) fish

To confirm the Hh-sensitivity of the stable transgenic fish, we in-crossed Tg(Gli-d:mCherry) fish and then incubated the offspring with 100 μM Cya from dome stage to 30 hpf. Following Cya treatments, or similar treatments with a vehicle control (ethanol), we screened half of the embryos live to visualize mCherry protein fluorescence, while the remaining embryos were fixed and screened for mCherry RNA expression by in situ hybridization. Among vehicle-treated transgenic embryos, fluorescence was apparent in the forebrain and trunk within the majority of live anesthetized embryos (32/42 embryos displayed fluorescence) (Figure 4A,B). In contrast, fluorescence was never detected in transgenic siblings treated with Cya (0/20 embryos displayed fluorescence) (Figure 4A,B). Additionally, mCherry RNA transcripts were suppressed in a similar fashion to fluorescent mCherry protein in Cya treated embryos (0/20 embryos displayed fluorescence), while vehicle treated control siblings displayed normal gene expression patterns (26/38 embryos displayed fluorescence) (Figure 4C). Transgene expression level changes were further analyzed by RT-PCR. Using the same conditions as described above, RNA was extracted from whole embryos and Hh-sensitive (gli1) and insensitive (disp1, gapdh) genes were assessed along with mCherry. We found that mCherry expression was as sensitive to Hh-signaling inhibition as gli1 (Figure 4D).

We next sought to determine if Tg(Gli-d:mCherry) fish could respond to cellular increases in Hh-signaling. Ectopic Hh-signaling is sufficient to activate gli1 expression in mouse, chick, frog and zebrafish [7,15,33–36]. To increase the embryonic levels of Shh protein in transgenic embryos, in vitro transcribed shh mRNA was injected into one cell stage Tg(Gli-d:mCherry) fish. At 30 hpf, shh injections consistently resulted in an expansion of mCherry fluorescent protein expression, beyond the normally restricted expression patterns within the head (data not shown) and trunk regions that we have reported for Tg(Gli-d:mCherry) (Figure 4 E–H). In full, these results indicate that the Tg(Gli-d:mCherry) fish lines generated in this study express the mCherry reporter under the control of the Hh-signaling pathway.

Expression of the Gli-dependent reporter in craniofacial region of transgenic fish

The zebrafish craniofacial skeleton is comprised mostly from CNC cells. Multipotent CNC cells originate in the dorsal midbrain and hindbrain, from where they delaminate and subsequently migrate ventrally into the zebrafish head. Once they have migrated into the jaw and PA, they intricately associate with facial epithelium (both ectodermal and endodermal) which provide structural support and also secrete molecules that drive differentiation of CNC into cartilage [34,55]. Our previous studies, as well as work from others, revealed that Hh-ligands are expressed in a dynamic fashion during craniofacial development, in the brain, facial ectoderm and facial endoderm [41,42,47]. During the late pharyngula stage (32–48 hpf), Hh-ligands are secreted from at least one of these tissues and are required to induce the differentiation of postmigratory CNC cells in the posterior arches (PA 3–7) to chondrocytes [42]. Despite this knowledge, we currently have a limited understanding of which cells in the head are responding to the available Hh-signals during PA chondrogenesis. The Tg(Gli-d:mCherry) represents a useful tool for determining the spatiotemporal pattern of Gli activity within specific cellular domains of the craniofacial region during skeletal development.

As we began our studies, we speculated that the CNC mesenchyme represented the likeliest targets of Hh-signals during the late pharyngula stage. To test this hypothesis, Gli-dependent reporter activity was carefully examined in the craniofacial tissues of Tg(Gli-d:mCherry) fish, beginning with the late pharyngula stage. Concurrently, we examined the expression of each gli gene (gli1, gli2a, gli2b, gli3) within craniofacial tissues, whose collective expression patterns represent both potential activator and repressor activity on Hh-target genes. For the majority of our analysis of Gli-dependent reporter expression in craniofacial tissues, we assessed mRNA expression which likely reflects changes to the Hh-pathway more sensitively than protein as regulation of the pathway occurs at the level of transcription. Moreover, it was important to use fixed tissue in these studies as clear views of the pharyngeal region required dissection of yolk cells, and in some cases, removal of the eye.

Expression of shh, the major Hh-ligand, in craniofacial tissues during development is primarily restricted to the ventral brain from late somitogenesis up to 33 hpf at which point a new expression domain becomes apparent in the facial ectoderm and endoderm [42,47]. Similar to shh expression, mCherry and gli1 expression in the Tg(Gli-d:mCherry) fish was mainly localized to the brain prior to 33 hpf (see Figure 3E–G, Figure S2). However, at 33 hpf a new mCherry expression domain became apparent in the facial tissue (Figure 5A). To better determine the identity of the mCherry expressing cells in the face, Tg(Gli-d:mCherry) fish were outcrossed to the Fli1-GFP transgenic zebrafish line which express GFP in postmigratory CNC cells [56]. Transgenic embryos from this mating allowed us to simultaneously visualize Hh-responding domains (red, mCherry) and head CNC (green, GFP). These embryos revealed that Gli activity in the facial tissue at 33–34 hpf is spatially restricted to the oral ectoderm (oe), based on its intricate association with first arch neural crest mesenchyme (Figure 5B,C, arrow). During this time, reporter expression was also localized to the ventral brain (vb) (Figure 5A–C, asterisks denote ventral brain). Similar to mCherry reporter expression at 33 hpf, gli1 and gli2a was also expanded from neural tissue alone to facial tissues (Figure S2). Later, at 38 hpf, the reporter gene and protein remained strongly expressed in the oe, despite being expressed at reduced levels in the brain (Figure 5D–F). Interestingly, at 38 hpf a new expression pattern
emerged in the pharyngeal endoderm (pe) tissue (Figure 5D, arrowhead). Closer examination with confocal microscopy on double transgenics clearly showed reporter expression in the pharyngeal endoderm surrounding the CNC mesenchyme (Figure 5E,F). The reporter protein was localized primarily to endoderm within the third through fifth arches in the transgenic fish shown (Figure 5F). Next, at 48 hpf, the reporter gene remained expressed in the brain, facial ectoderm and endoderm (Figure 5G), similar to the expression pattern for \(\text{shh}\) at this stage [42], and also closely resembling the expression pattern for \(\text{gli1}\) (Figure S2). Consistent with reporter gene expression, \(\text{mCherry}\) protein was clearly present in the facial endoderm and facial ectoderm at these stages (Figure 5H,I, Figure S3). At this stage, we also noticed expanded gene expression in the zebrafish jaw, potentially corresponding to CNC mesenchyme in the anterior-most PA (Figure 5G). Similarly, \(\text{gli}\) transcripts, especially \(\text{gli2s}\) (\(a\) and \(b\)) but also \(\text{gli3}\), became expanded in the face at 48 hpf (Figure S2), however these genes were more broadly expressed in the jaw and other facial tissues than the reporter. Due to the balance of activator and repressor forms of \(\text{gli}\) proteins, it is likely that the expanded expression of multiple \(\text{glis}\) at this stage refines the Hh-signal, as has been shown in other developmental contexts [13,15,18,19,25,57]. In a similar vein, at this timepoint and all earlier stages, we never detected Gli-dependent reporter expression in the CNC mesenchyme within the posterior arches, strongly suggesting that the CNC mesenchyme in these arches are not actively receiving or responding to Gli activation during these stages of development, despite our previous findings that CNC mesenchyme fails to differentiate if the pathway is attenuated during this time (specifically during 32–48 hpf). Rather, expression of the Gli-dependent reporter at these developmental stages closely resembles known Hh-ligand expressing domains in the brain and facial tissues that neighbor the postmigratory CNC mesenchyme in the posterior arches.

Figure 4. Reporter expression in Tg(Gli-d:mCherry) fish is sensitive to the Hh-signaling pathway. (A–C) Lateral views, anterior to the left, of 30 hpf ethanol vehicle treated controls (left side of image) and Cya treated (right side) Tg(Gli-d:mCherry) siblings. (A,B) Live embryos were visualized using brightfield (A) and fluorescence (B) microscopy. 100 \(\mu\)m Cya treated embryos showed a significant reduction in fluorescent mCherry protein when compared to their ethanol vehicle treated siblings. (C) Fixed embryos, stained with mCherry riboprobe revealed that 100 \(\mu\)m Cya treated embryos displayed significantly reduced mCherry RNA transcripts compared to their vehicle-treated siblings. (D) RT-PCR showing reduced gli1, and mCherry expression in 30 hpf embryos treated with 100 \(\mu\)m Cya compared to their nontreated (NT) siblings. Two loading controls for disp1 and gapdh were included. (E–H) Lateral views of 30 hpf noninjected controls (E,F) or Tg(Gli-d:mcherry) siblings injected with 50 pg shh at the one cell stage (G,H). Siblings injected with shh mRNA showed expanded reporter expression throughout the trunk when compared to noninjected controls.

doi:10.1371/journal.pone.0014396.g004
While reporter expression was not detectable in posterior arch CNS mesencycle during the first two days of development, by 52–60 hpf mCherry RNA transcripts became visible in the CNC mesenchymal condensations within each PA (Figure 3J,K). By 72 hpf, mCherry was strongly expressed in nearly all ventral facial cartilage precursor cells as visualized in lateral (Figure 5L) or ventral views (Figure 5N). Consistent with reporter being in the CNC mesenchyme at this stage, the expression pattern of mcherry was nearly identical to the chondrogenic marker sox9a, a CNC marker that controls chondrification (Figure 5M,O; [58]). In addition to its expression in the CNC cells, mCherry remained detectable in the brain and pharyngeal endoderm at 72 hpf (Figure 5L, inset shows image with endoderm in focal plane [CNC cells out of focus]), similar to shh expression at this stage [42,42]. Expression of shh genes at concurrent stages of development provided further support for Hh-signaling targeting the CNC mesenchyme at later stages, as expression of gli1 and gli2a remained highly expressed in the facial tissues after the second day of development and both genes appeared strongest in the mesenchyme at 72 hpf (Figure S4). Finally, at later stages (4.5 dpf), mCherry continued to be expressed in CNC cells after their differentiation into cartilage (Figure 5P), at which point the ventral pharyngeal cartilages were completely formed (Figure 5Q).

In summary, mCherry reporter expression was dynamic in craniofacial tissues during embryonic and early larval development, first detectable in the ventral brain from 1 dpf where it remained detectable up to 4.5 dpf. Reporter expression was also detected in the facial ectoderm and endoderm from 33–38 hpf where it remained visible up to 4.5 dpf. Finally, we saw reporter expression in the CNC cell jaw precursors, from 48 hpf up to 4.5 dpf. Unlike jaw cartilage precursors, CNC that will become the cartilage elements in the posterior arches express the reporter beginning around 52–60 hpf and continue to express it up to 4.5 dpf.

**Hh-signaling controls PA chondrogenesis by its influence on the brain or facial epithelium**

Our expression analysis on Tg(Gli-d:mCherry) fish showed that posterior-arch residing CNC are not directly targeted by Hh-signaling during the late pharyngula stage (32–48 hpf); however, Hh-signaling is highly active in other craniofacial tissues during this stage, as transgene expression was visible in neural tissues and facial epithelium. Since reporter expression did become apparent in CNC mesenchyme shortly after the late pharyngula stage, we wondered if blocking Hh-signaling during the late pharyngula stage may either reduce the availability of Hh-ligands from surrounding neural or facial tissues to the postmigratory CNC mesenchyme at later stages, and/or make the CNC mesenchyme less capable of responding to Hh-signals after the late pharyngula stage.

To inhibit Hh-signaling during the late pharyngula stage, embryos were treated with Cya from 32 hpf until 48 hpf, at which time Hh-signaling was restored by washing away the Cya solution and allowing development to proceed normally. This Cya treatment protocol was chosen as it was during this developmental stage, using the same treatment protocol, that we previously identified Hh-signaling as being critical for chondrogenesis in posterior arches [42]. At 48 hpf, sixteen hours after Hh-inhibition began, Tg(Gli-d:mCherry) embryos continued to display strong transgene expression within tissues corresponding to the anterior arches, suggesting that this Cya treatment protocol did not abolish the gli-dependent response in these cells (Figure 6A,B). Reporter expression in these tissues is not likely due to persisting mRNA transcripts, produced at stages prior to inhibition, since we have found that both mcherry reporter transcripts and gli1 transcripts begin to decline at four hours of starting a Cya inhibition treatment (100 μM) and are completely reduced by six hours, as assessed by real time PCR (data not shown). Despite some reporter expression perduring in the anterior arches, transgene expression in the brain, oe, pe (Figure 6B) and trunk (data not shown) was reduced by inhibition. This indicated that late pharyngula stage inhibition reduced nearly all of the Hh-response in the brain and facial epithelium which surround cartilage precursors during this developmental stage. By 60 hpf, transgene expression had completely recovered in all tissues that normally respond to Hh-signaling at this stage. Reporter expression could be detected in neural, facial epithelium and CNC mesenchymal condensations in each arch (Figure 6C,D). Similarly, transgene expression persisted in CNC mesenchyme at 72 hpf (Figure 6E,F) and up to 4.5 dpf (data not shown), despite the fact that CNC cells in the posterior arches failed to chondrify in these larvae (Figure 6G,H). This data clearly shows that late pharyngula stage inhibition does not negatively impact the ability of CNC mesenchyme to receive Hh-signals at later stages, despite their reduced ability to differentiate properly.

We have shown previously using similar timed inhibition studies that inhibiting Hh-signaling any time after the late pharyngula stage does not impact cartilage development in the PA [42]. These findings collectively suggest that direct Hh-signaling, mediated by Gli, is not required by CNC to differentiate into cartilage. In full, this data supports a conclusion that Hh-signaling during the late pharyngula stage must be received by neural or facial tissues. Reception of the signal in these cells, which directly neighbor the cartilage precursors of the ventral head skeleton, may lead to the downstream production and secretion of an unknown, secondary signal required by the cartilage precursors to maintain expression of chondrogenic factors. More work will be required to determine the targets of Hh-signaling in the neural and facial tissues that may represent potential secondary signals in PA chondrogenesis.

**Gli activity in craniofacial tissues in the absence of Hh-signaling**

One surprising outcome of our reporter expression analysis in Tg(Gli-d:mCherry) fish was the finding that a population of cells in...
the anterior arches of 2 day old embryos continued to express the Gli reporter transgene despite abrogating the Hh-pathway with Cya in these embryos (see Figure 6B). While Hh-signaling has been shown to be necessary for normal transcription of gli genes in zebrafish and mouse [59], it has been previously reported that very weak expression of gli1 remains detectable in the absence of Hh-

Figure 6. Reduced Gli activity in craniofacial tissues following late pharyngula stage Hh-pathway inhibition. Lateral (A–F) or dorsal (G–J) views, anterior to the left, of Tg(Gli-d:mCherry) fish treated with vehicle (A,C,E,G), Cya (B,D,F,H,J) or not treated (I) and stained with mCherry riboprobe (A–F) or Alcian blue solution (G–J). (A,B) Reporter gene expression was significantly reduced in the brain and facial epithelium at 48 hpf following late pharyngula stage Cya treatment. A slight mCherry signal was detected in craniofacial tissue that likely corresponds to PA1-2 mesenchyme at 48 hpf following Cya treatment. (C–F) Reporter gene expression was fully recovered in Cya treated embryos by 60–72 hpf and is expressed in oe, pe, brain and PA mesenchyme. (M–O) Alcian blue staining to reveal cartilage deficits in Gli reporter transgenic fish following Cya treatments at 32–48 hpf (H) or 48–72 hpf (J). These deficits are consistent with our previously published findings and indicate that Cya reliably inhibited cb chondrofication upon Hh-inhibition during the late pharyngula stage (H), but did not negatively impact cartilage development when administered at later stages (J). doi:10.1371/journal.pone.0014396.g006
signaling in the zebrafish [15], at least up to 20 hpf. Since the reporter in Tg(Gli-d:mCherry) reveals the cellular sites of Gli activity, not Gli transcription outputs, we wanted to identify any persistent Gli activity in Hh-compromised fish. To achieve this, Tg(Gli-d:mCherry) fish were treated with Cya throughout development, from dome stage (4 hpf) up to the timepoint at which the fish were fixed and reporter expression was assessed at 24 hpf, 48 hpf, and 96 hpf. As shown previously in this report (Figure 4), treating Tg(Gli-d:mCherry) embryos with Cya during the first day of development completely abrogated all reporter expression in the embryo when examined at 24-28 hpf. Closer examination of head and Tg(Gli-d:mCherry) brain tissues following Cya inhibition confirms the complete reduction of Gli activity in treated embryos (Figure 7A,B). Contrary to this, when reporter expression was analyzed in Cya treated Tg(Gli-d:mCherry) that were 48 hpf (Figure 7C,D) or older (Figure 7E,F), a group of reporter expressing cells were clearly evident in the anterior arches. The persistent mCherry expression domain in the lower jaw was quite noteworthy, as the Cya treatment sufficiently eliminated reporter expression throughout all other areas of the larvae (Figure 7F). Moreover, larvae treated from 4–96 hpf displayed phenotypes characteristic of severe Hh-signaling inhibition, including U-shaped somites, a ventrally curled tail, heart edema and mild-to-severe cyclopia. A closer examination of these larvae indicated that the remaining mCherry expression domain was likely localized specifically to the second PA mesenchyme, although the transcripts were expressed in a highly diffuse fashion, at a low level, in more posterior areas of the pharyngeal region (Figure 7F’). Staining these larvae with alcian blue revealed that no cartilages formed upon complete Hh-inhibition with Cya (data not shown), similar to previous reports on smu/smo genetic mutants [39]. To be sure that Cya inhibition in these larvae was not completely eliminating both head cartilages and mCherry expressing cells in the head, we dually stained some Cya treated larvae for both mCherry

![Figure 7. Reporter expression in Tg(Gli-d:mCherry) fish following severe reductions to Hh-signaling.](image-url)

Lateral (A–F) or ventral (F’,F”) views, anterior to the right, of Tg(Gli-d:mCherry) fish treated with vehicle (A,C,E) or Cya (B,D,F,F’,F”) and stained with mCherry riboprobe (A–F’) or dually stained with mcherry and Alcian blue (F”). Vehicle or Cya treatments began at dome stage and commenced just prior to sacrificing the embryos or larvae for staining analysis. (A,B) At 24 hpf, Cya treatments suppressed all head and trunk reporter expression in transgenic embryos. (C,D) At 48 hpf, Cya treatments reduced all reporter expression in transgenic fish, with the exception of a small expressing domain in the ventral head (arrowhead). (E,F) Reporter expression was virtually absent in Cya treated 4.5 dpf transgenic larvae, with the exception of the same mCherry expressing domain in the ventral head that was seen in 48 hpf aged embryos. (F’, F”) Closer examination of the ventral head suggested that the mCherry expressing cells are within second arch mesenchyme and that no cartilage forms in this arch or any other PA in Cya treated larvae. Abbreviations: fp, floor plate; myo, myotome; vb, ventral brain.

doi:10.1371/journal.pone.0014396.g007
expression and Alcian blue expression. In these fish, we consistently noticed mcherry expression, even in the absence of any cartilages (Figure 7F).

Collectively this data suggests that a small population of cells in the ventral zebrafish head retain Gli activity in the absence of Hh-signaling. While the Gli-d reporter appears to be expressed in second arch mesenchyme, any potential Gli activity in these cells does not impact craniofacial cartilage development, consistent with our findings herein that CNC progenitors do not directly require Gli activity to differentiate to cartilage cells. While we cannot rule out the possibility that their exists low levels of reporter expression in Tg(Gli-d:mCherry) that is independent of Gli activity, our findings here are consistent with those of Karlstrom et al., 2003 where it was shown that some gliI transcripts become made in zebrafish embryos where the Hh-pathway has been fully compromised [15]. This may be unique to the fish, as GliI expression is completely absent in Smo mutant mice [59]. Moreover, reports on gliI acting independently of the Hh-signaling pathway (i.e. in a non-canonical fashion) are few in vertebrates [60] and are most often associated with tumor environments.

**Discussion**

The purpose of the experiments in this report was to create a transgenic zebrafish line that would allow for *in vivo* visualization of cells that are responding to Hh-signaling using Gli-activity as a read-out for the pathway, mCherry reporter expression in Tg(GliI-d:mCherry) embryos was spatially restricted adjacent to shh-expressing domains, most notably the ventral central nervous system, trunk myotome and craniofacial tissues. Moreover, the reporter was expressed in identical or neighboring tissues that expressed gliI genes in the craniofacial tissues, further arguing that it is responsive to Gli activity and likely reflects a balance of dual Gli promoter and repressor activity. The reporter protein could be visualized in live or fixed tissue by fluorescent microscopy without the use of antibody amplification, allowing for rapid signal detection. Being able to detect fluorescence changes in live embryos, as a result to changes to the Hh-pathway, will be beneficial in assessing in real-time the ability of a specific compound or gene product, applied topically by water-delivery or by microinjection, to alter the pathway. Furthermore, by generating an antisense RNA riboprobe for the ORF of mCherry, the reporter RNA transcripts can also be detected in a highly sensitive fashion by *in situ* hybridization. Detection of mRNA reporter transcripts in fixed tissues will allow researchers to directly visualize Hh-response in conjunction with other cell markers, as assayed by *in situ* hybridization or immunohistochemistry, as well as markers for cell proliferation or cell death.

Using Hh-signaling genetic mutants as well as treatments involving a pharmacological inhibitor for Hh-signaling, we could determine that the reporter element was sensitive to Hh-signaling inhibition *in vivo*, when stably expressed in Tg(GliI-d:mCherry) or when the reporter DNA plasmid was transiently expressed in wild type embryos by microinjection at the one cell stage. Further, the reporter element was also responsive to ectopic expression Hh signaling, as expression of Shh DNA led to expansion of mCherry expression beyond the spatially restricted domains. While reporter expression in Tg(GliI-d:mCherry) resembles known gliI expressing domains, we can not rule out the possibility that some Hh-responding domains are not represented by mCherry expression in the transgenic line. Certain cell population may display restricted transgene expression due to local chromatin effects or other epigenetic factors. It will require further examination of future reporter-expressing, transgenic lines to rule out this possibility.

Insight about the nature of Hh-signaling can be gained through analyzing the Tg(GliI-d:mCherry) under normal developmental conditions, or when the Hh-signaling pathway is experimentally modulated. In experiments reported in this study, we were able to detect changes to the Hh-signaling upon treating embryos with the pharmacological Hh-inhibitor Cya. Reductions to the Hh-signaling pathway on treated embryos could be easily detected when the reporter DNA construct was transiently expressed by microinjection into wild type embryos or stably expressed in transgenic embryos. Concurrent with our study, Yang et al., recently introduced a similar Gli-dependent transgenic line that contains a GFP reporter which is strongly expressed in the developing somites but is inhibited when the fish are ectopically treated with water soluble Hh inhibitors [61]. Our findings in this study support the use of these transgenic lines as a tool to rapidly screen chemical compounds. Additionally, we have shown here that the Tg(GliI-d:mCherry) fish represent an important line that can be utilized to report on Hh-signaling during disease and development.

The Tg(GliI-d:mCherry) zebrafish should allow Hh-signaling to be studied in a variety of embryonic or adult tissues. We designed the fish in an effort to more precisely detect Hh-responding cells in the craniofacial region during embryonic and larval development. We have previously shown that Hh-signaling controls ventral arch cartilage development by impacting CNCC patterning and differentiation [42]. It is well known that Hh-ligands are expressed in a dynamic fashion in the craniofacial tissues during development; primarily within the ventral brain up to 33 hpf, wherein *shh* expression domains expand to facial ectoderm and endoderm. Missing from this analysis was which cell populations in the brain and face are receiving the Hh-signal at synonymous timepoints. In this report, we have characterized the reporter expression in the craniofacial tissues of the Tg(GliI-d:mCherry) line throughout development to provide a detailed analysis of Hh-responding domains in these regions. To summarize the data, we found that Hh-signals are received by neural tissues, predominantly in the ventral brain, from somitogenesis stages up to 4.5 dpf. Facial ectodermal cells began expressing the reporter at 33 hpf, while reporter expression in the pharyngeal endoderm began around 36–38 hpf. At these stages, CNCC have completed their migration from the neural tube into the arches and signals from the facial epithelium are likely required to by the neural crest mesenchyme to become properly patterned. Hh-signals received by the facial epithelium cannot originate in the CNCC, as Hh-ligands are not expressed in these cells at this time. The likeliest sources of the Hh-signals to the facial epithelium are the ventral brain, or facial epithelial cells signaling in an autocrine fashion. Reporter expression could be detected in the ventral brain, facial ectoderm and endoderm up to 72 hpf.

With respect to reporter expression in the neural crest mesenchyme, we found that the first population of cells within the PA to express the reporter was post-migratory mesenchymal condensations within the lower jaw at 48 hpf. Shortly thereafter at 52 hpf, nearly all neural crest mesenchymal condensations within the PA expressed the transgene. Reporter expression could be visualized in PA mesenchymal condensations at timepoints beyond their chondrification to cartilage cells. The potential sources of the Hh-signals to the CNCC may be ventral brain or facial epithelium and more experimentation will be required to delineate the origin of the signal.

Reporter expression in the neural and facial tissues at each developmental stage examined was further supported by expression analysis of all identified zebrafish gliI genes. Collectively, these four genes represent protein products that may activate or repress Hh-target genes. Since our reporter is most likely reflects the
combination of positive and negative Gli activity functions, it was not surprising to find that its expression closely resembled the pattern for each gli gene. Overall, reporter expression could be characterized as less broad and more refined to specific tissue groups (i.e. the pharyngeal endoderm or neural crest mesenchyme) than the collective expression of gli genes. The finding that the reporter was expressed in a fine-tuned pattern at each stage of craniofacial development is in line with what one may have predicted for the readout of such a critical signaling pathway.

Upon initiation of this study, we hypothesized that Hh signals were directly required by cartilage precursors in the PA to undergo chondrogenesis into the ceratobranchial cartilage elements of the ventral zebrafish skeleton. This hypothesis was driven primarily by earlier evidence that mesenchymal condensations in the posterior arches fail to maintain expression of pro-chondrogenic transcription factors sns/aa and dx2a in con/disf mutants. Further, we had also shown that the requirement for Hh-signaling to maintain normal expression levels in the neural crest mesenchyme was during the late pharyngula stage (32–48 hpf), at which time neighboring neural and facial epithelial cells robustly express shh [42]. However, we have determined that mesenchymal condensations in the posterior arches were not positive for the Gli reporter transgene until approximately 52–60 hpf. Thus, we conclude that Hh-signaling may act by a paracrine mechanism; inducing a mediating molecule to be expressed by the brain or pharyngeal endoderm which would then be received by the CNCC residing in the posterior arches. This potential mediator signal would be sensitive to modulations in Hh-signaling and its action would promote the expression of pro-chondrogenic factors in neural crest residing in the posterior arches.

If posterior-arch residing neural crest cells do not directly require Hh-signaling to undergo chondrogenesis, why then do they robustly express the Gli-dependent reporter from the second day of development and at timepoints up to 4.5 dpf? One potential explanation is that Hh-signaling controls further aspects of facial development, such as the maturation and morphogenesis of chondrocytes and perichondrium undergoing endochondral ossification [62–65]. Hh-signaling controls chondrocyte and perichondrium ossification primarily through the expression of Indian hedgehog (Ihh) genes, which become expressed in maturing chondrocytes and provide signaling cues to both chondrocytes and the surrounding perichondral cells to influence the timing of osteoblast differentiation [65,66]. In zebrafish, shh genes become expressed in the ceratohyals by 5 dpf [67], before becoming more widely expressed in chondrocytes of the developing pharyngeal skeleton by 6 dpf [68]. Like shh ligands, shh genes exert their activity through gli genes, which are not surprisingly also expressed in chondrocytes and perichondrium at these timepoints [67]. Expression of these Hh genes is functionally relevant, as it was recently shown that increasing or deleting Hh-signaling in fish negatively influences osteoblast and osteoclast development [69]. In this study, Gli activity was mostly examined prior to shh gene expression in the head cartilages and when shh genes are believed to be the primary Hh-ligands being expressed. It is likely, however, that later reporter expression in Tg(Gli-d:mCherry) fish that are 4 days or older will allow for the visualization of Gli activity in maturing chondrocytes and perichondrium due to Ihh expression which will enhance our understanding of Hh-signaling in bone development. Additionally, reporter expression in the facial epithelium and CNC mesenchyme after timepoints required for chondrogenesis may be reflecting a requirement for Hh-signaling in other aspects of facial development, such as the development of cranial muscles. In support of this, we have found that cranial muscle development is perturbed in Hh-pathway genetic mutants (Schwend and Ahlgren, unpublished). This data has been difficult to obtain, as genetic disruptions to the Hh-pathway are often embryonic lethal and death occurs prior to developmental stages where muscles are forming (beyond 5 dpf). However, by utilizing genetic mutants where the Hh-signaling pathway is abrogated to a lesser extent (such as the con/disf1 mutant) or late-stage Cya inhibition, at timepoints after most vital organs have developed, it is possible to keep larvae alive long enough that facial development may be assessed. Currently, we are in the process of studying the role of Hh-signaling in the development of these cranial tissues. Certainly, the use of the Tg(Gli-d:mCherry) line will be highly fruitful to these continued studies.

One surprising result from our characterization of Tg(Gli-d:mCherry) line was that a domain of mCherry expressing cells in the second arch mesenchyme maintained expression despite full pathway inhibition with Cya. Gli transcription factors normally become activated in a canonical fashion, dependent on Hh-ligand presentation and Smo activation, Cya inhibits Hh-signaling at the level of the Smo receptor, and thus gli transcription should not become activated by the Hh-pathway in the presence of Cya. Thus, second PA domain may represent a group of cells in the craniofacial region that may activate gli transcription in the absence of Hh-signaling fashion. It had been previously shown that a very low level of gli transcription does occur in sma/smo mutant zebrafish, primarily in anterior tissues, however it had not been known if these sources of Gli could activate downstream targets [15]. Our data suggests that a small amount of Gli activity remains in the absence of Hh-signaling, localized primarily to the jaw after the second day of development. We cannot rule out the possibility that low levels of Hh-signaling, above the threshold for gli activation in these cells, exists in the presence of Cya. Furthermore, it remains a possibility that epigenetic factors in this cellular domain of our transgenic fish perturb the ability of the Cya to fully inhibit cells in this region, thus resulting in nonspecific expression of the transgene.

In summary, we have reported on the generation of a transgenic zebrafish line that allows for in vivo detection of cells responding to Hh-signaling. This transgenic line can be used to better understand the spatiotemporal pattern of Gli activation in a variety of organ systems in the zebrafish. Furthermore, because the fish line is sensitive to Hh-signaling, the fish can be utilized in research studies designed to test potential therapeutic agents hoping to be used as Hh-signaling agonists or antagonists. Our understanding of the Hh-signaling mechanism in development and disease will ultimately be better understood due to the generation of these transgenic lines.

Materials and Methods

Animals

Zebrafish (Danio rerio) embryos were obtained from natural crosses and staged as previously described [70]. All experimental protocols involving live animals were approved by the Animal Care and Use Committee of Children’s Memorial Research Center in Chicago, Illinois. The Tübingen (Tu) strain of fish was originally obtained from the Zebrafish International Resource Center (ZIRC) in Eugene, Oregon, and the Ekkwill strain of fish were obtained from Ekkwill Waterlife Resources (Gibsonston, Florida). detour/gh1 (dhh280), yao-too/gb2 (yol119), and slow-muscle-omitted/smu (sma3411) were previously identified in phenotypic screens for mutant phenotypes and their genetic mutations in the Hh-signaling pathway have been subsequently described [14,15,38,39,71]. Homozygous genetic mutants were identified by somite morphology (U-shaped as opposed to chevron shaped)
at 30 hpf as previously described [71]. Tg(Flk1:Gfp) transgenic zebrafish expressing the gfp-transgene under the control of the Fli1 promoter was previously characterized [56].

**Generation of Tg(Gli-d:mCherry) zebrafish**

A 765 bp basepair fragment containing eight repeating Gli-CBS from the mouse FoxA2 floor plate enhancer and a minimal lens crystallin promoter [40] were flanked by Invitrogen Multisite Gateway compatible att sites by PCR amplification using a forward PCR primer containing an attBi site and template specific sequence and a reverse PCR primer containing an attB1 site and template specific sequence. Primer sequences are available upon request. PCR products were purified using a Qiagen gel extraction kit (Qiagen, Valencia, California) and were used immediately in BP reactions with pDONR p4-p1R according to the manufacturer’s guidelines (Invitrogen Multisite Gateway system, Carlsbad, California).

The resulting plasmid was a 5’ entry clone for the Gateway system which was named p5E-8xglt-lc. The p5E-8xglt-lc entry clone, along with two MULTISITE Gateway-compatible entry vectors from the Tol2 kit [48]; a middle entry vector carrying the mCherry RFP open reading frame named pME-mCherry and a 3’ entry vector carrying a SV40 A tail from pCS2+ were incubated in the incubated in the presence of the LR Clonase II Plus Enzyme mix (Invitrogen) and the destination vector pDestTol2pA2 as previously described [48]. The resulting plasmid contained a Gli-dependent:mCherry reporter construct flanked by the minimal Tol2 transposon elements and was named Gli-d:mcherry reporter plasmid (see Figure 1A). 25–50 pg of reporter plasmid DNA was coinjected along with 30–40 pg of in vitro transcribed Tol2 transposase mRNA into wild type 1–2 cell stage embryos. The strongest expressing mCherry founders were raised to adulthood and outcrossed to wild type embryos. The F1 generation of transgenics was identified by screening for the presence of mCherry expression in progeny. Four of these fish displayed strong expression levels of mCherry and were maintained as our stable lines. Careful examination of the four stable lines failed to detect any distinguishable differences in the pattern of mCherry expression throughout embryonic development.

**mRNA synthesis**

Tol2 transposase enzyme mRNA was generated using the pCS2FA-transposase plasmid as a template [48]. Capped mRNA was transcribed from linearized DNA using RNA polymerase in vitro transcription kit, according to the manufacturer’s instructions (mMESSAGE, mMACHINE; Applied Biosystems, Foster City, CA).

**Reverse Transcriptase PCR**

Total RNA was extracted from groups of 10–20 embryos at stages of development ranging from 0.5 hpf to 30 hpf, using RNA Tissue Extraction and DNase kits (PerfectPure RNA Tissue Kit, 5 Prime). cDNA was synthesized using SuperScript III and random primers (Invitrogen) and PCR was performed using 2X PCR Supermix (Promega, Madison, Wisconsin). Additional control samples, without reverse transcriptase were performed for each sample.

The following primer sequences used were as follows: gli1 forward: 5’ CAGACGTCCTCTCGGCTTTAC3’, gli1 reverse: 5’AGTAGCCTGTCTCTGGGATGAC3’, disp1 forward: 5’GCTGTGGGTGGAGACTGAGC3’, disp1 reverse: 5’GGCCACTGTGGGTAGGAC3’, gapdh forward: 5’GAAGGTGGGAAACTGGTAGGAC3’, gapdh reverse: 5’TGCACCACCCTTTAATGTGA3’. mCherry forward: 5’ CCTGTCGCCCTTGTTCAATGTG3’, mCherry reverse: 5’ CCCTATTGTCCTTTGCTGAT3’.

**Cyclopamine treatments**

Cya (LC Laboratories, Woburn, Massachusetts) was solubilized in 95% ethanol to obtain a stock solution of 50 mM, which was further diluted in zebrafish embryo water to a working stock concentration of 100 mM. Embryos were bathed in the Cya working stock for 4–24 hours at a time. Immediately following treatment, the Cya solution was removed, embryos were washed in multiple rounds of zebrafish embryo water, and then allowed to develop further until 48–96 hpf, wherein they were analyzed by *in situ* hybridization or cartilage staining.

**Cartilage staining**

Cartilage performance was as previously described, zebrafish larvae were fixed at 96 hpf and treated with Alcian blue solution dissolved in 80% ethanol/20% glacial acetic (acid alcohol) acid for several hours or overnight. Larvae were stained in several washes of acid alcohol before being transferred to a 1% KOH: 3% hydrogen peroxide solution for further clearing of pigment cells. Larval tissue was then digested in trypsin, followed by digestion of PA cartilages which were then flat mounted as previously described [72]. Cartilage preparations were visualized on a Leica MZ16F.

**Whole mount *in situ* hybridization and Fluorescent Imaging**

Whole-mount *in situ* hybridization was conducted essentially as previously described [73], mCherry riboprobe was made by PCR amplifying a 517 basepair fragment spanning the mCherry open reading frame from the pME-mCherry using the following primers: mCherry forward 5’ CCTGTCGCCCTTGTTCAATGTG 3’ and mCherry reverse 5’ GCATGGACGAGCTGTACAAGTAAA 3’ and a Taq polymerase (Promega). PCR product were ran on an agarose gel to confirm the correct basepair size and subsequently purified using the Qiagull gel extraction kit (Qiagen) and TA-cloned into a pGemTasy (Promega) vector. Antisense digoxigenin labeled probes were generated against *hh* [47], *gli1* [15], *gli2a* [14], *gli2b* [16], *gli3* [18] and *sox9a* [58]. Embryos fixed at a stage older than 25 hpf were raised in 2 mM 1-phenyl-2-thiourea in embryo medium to prevent pigmentation. Gene expression patterns were visualized using NBT/BCIP (Roche Ltd, Basel, Switzerland). After staining completed, embryos were cleared in 80% glycerol and visualized on a Leica MZ16F microscope with camera.

Epifluorescence was visualized in live transgenic zebrafish with a Leica MZ16F microscope. For visualizing fluorescent protein in fixed embryos, whole embryos were mounted on slides in 90% glycerol and confocal stacks were generated using a ZeissMeta 510 laser scanning microscope.

**Supporting Information**

Figure S1 Expression of mCherry protein in Tg(Gli-d:mCherry) founder fish. (A–D) Lateral views, anterior to the left, of 24 hpf founder embryos visualized using brightfield (A,B) or fluorescent (C,D) microscopy. mCherry expressing cells could be clearly visualized in the forebrain (arrow in A,B) and myotome (boxed in A,B; see C,D for higher magnification) in some of the embryos injected with the Gli reporter transgene.

Found at: doi:10.1371/journal.pone.0014396.s001 (1.18 MB TIF)
Figure S2  Expression of gli genes in craniofacial region during second day of development. Lateral views, anterior to the left, of 50 hpf (A,D,G,J), 36 hpf (B,E,H,K) or 48 hpf (C,F,I,L) stained with riboprobe for gli1 (A–C), gli2a (D–F), gli2b (G–I) or gli3 (J–L). Found at: doi:10.1371/journal.pone.0014396.s002 (5.07 MB TIF)

Figure S3  mCherry protein expression in craniofacial region of double transgenics. Lateral (A,B) or ventral (C) views of confocal stack projections of 48 hpf Tg(Gli-d:mcherry) and Fli1:gfp double transgenic fish. (A) Reporter expression was visible in craniofacial structures (B,C) Closser examination within the jaw showed reporter expression in the oe (arrow) and pe (arrowhead) underlying CNC within the second arch. Abbreviations: PA, pharyngeal arch; ov, otic vesicle. Found at: doi:10.1371/journal.pone.0014396.s003 (1.43 MB TIF)

Figure S4  Expression of gli genes in craniofacial region during third day of development. Lateral views, anterior to the left, of 54 hpf (A,C,E,G) or 72 hpf (B,D,F,H) stained with riboprobe for gli1 (A–C), gli2a (D–F), gli2b (G–I) or gli3 (J–L). Second day of development. Lateral views, anterior to the left, of 54 hpf Tg(Gli-d:mcherry) and Fli1:gfp double transgenic fish. (A) Reporter expression was visible in craniofacial structures (B,C) Closser examination within the jaw showed reporter expression in the oe (arrow) and pe (arrowhead) underlying CNC within the second arch. Abbreviations: PA, pharyngeal arch; ov, otic vesicle. Found at: doi:10.1371/journal.pone.0014396.s004 (3.11 MB TIF)

References
1. Mullor JL, Sanchez P, Ruiz i Altaba A (2002) Pathways and consequences: Hedgehog signaling in human disease. Trends Cell Biol 12(12): 562–569.
2. Dellovade T, Rosner JT, Curran T, Rubin LL (2006) The hedgehog pathway and neurological disorders. Annu Rev Neurosci 29: 539–563.
3. Kasper M, Regl G, Frischaud AM, Abergier F (2006) GLI transcription factors: Mediators of oncogenic hedgehog signalling. Eur J Cancer 42(4): 437–445.
4. Taije P, Beachy PA (2001) The hedgehog and vent signalling pathways in cancer. Nature 411(6833): 349–354.
5. Fuse N, Maini T, Wang B, Porter JA, Hall TM, et al. (1999) Sonic hedgehog protein signals not as a hydrolytic enzyme but as an apparent ligand for patched. Proc Natl Acad Sci U S A 96(20): 10992–10999.
6. Hooper JE, Scott MP (1989) The drosophila patched gene encodes a putative membrane protein required for segmental patterning. Cell 59(4): 731–765.
7. Marigo V, Davey RA, Zuo Y, Cunningham JM, Tabin CJ (1999) Biochemical evidence that patched is the hedgehog receptor. Nature 384(6605): 176–179.
8. Chen Y, Struhl G (1996) Dual roles for patched in sequestering and transducing Hedgehog. Cell 87(3): 533–563.
9. Stone DM, Hynes M, Armanini M, Swanson TA, Gu Q, et al. (1996) The transcriptional reporter gene patched encodes a candidate receptor for sonic hedgehog. Nature 384(6605): 129–134.
10. Kinzler KW, Ruppert JM, Bigner SH, Vogelstein B (1988) The GLI gene is a member of the kruppel family of zinc finger proteins. Nature 332(6162): 321–324.
11. Kinzler KW, Vogelstein B (1990) The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. Mol Cell Biol 10(2): 643–649.
12. Ruppert JM, Kinzler KW, Wong AJ, Bigner SH, Kao FT, et al. (1998) The GLI-kruppel family of human genes. Mol Cell Biol 8(3): 3104–3113.
13. Devine CA, Shroga JL, Gueret B, Osgood M, Shen MC, et al. (2009) A dynamic gli code interprets hh signals to regulate induction, patterning, and endocrine cell specification in the zebrafish pituitary. Dev Biol 326(1): 143–154.
14. Karlstrom RO, Talbot WS, Schier AF (1999) Comparative syntenic cloning of hedgehog signaling pathway. Development 125(14): 2533–2543.
15. 5. Fuse N, Maiti T, Wang B, Porter JA, Hall TM, et al. (1999) Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Cell 6(1): 103–115.
16. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
17. 25. Ruiz i Altaba A (1999) Gli proteins encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
18. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
19. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
20. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
21. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
22. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
23. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
24. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
25. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
26. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
27. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
28. 22. 25. Ruiz i Altaba A (1999) Gli genes encode context-dependent positive and negative functions: Implications for development and disease. Development 126(14): 3205–3216.
59. Bai CB, Auerbach W, Lee JS, Stephen D, Joyner AL (2002) A gli9 gene required for cartilage morphogenesis. Development 129(21): 5065–5079.

60. Lauth M, Toftgard R (2007) Non-canonical activation of GLI transcription factors: Implications for targeted anti-cancer therapy. Cell Cycle 6(20): 2458–2463.

61. Yang, H., Xiang, J., Wang, N., Zhao, Y., Hyman, J. et al. (2009) Converse conformational control of smoothened activity by structurally related small molecules. J. Biol. Chem. 284(31): 20876–20884.

62. Colnot C, de la Fuente L, Huang S, Hu D, Lu C, et al. (2005) Indian hedgehog synchronizes skeletal angiogenesis and perichondrial maturation with cartilage development. Development 132(3): 1057–1067.

63. Cortes M, Baria AT, Schwartz NB (2009) Sulfation of chondroitin sulfate proteoglycans is necessary for proper indian hedgehog signaling in the developing growth plate. Development 136(10): 1697–1706.

64. Kronenberg HM (2005) Developmental regulation of the growth plate. Nature 432(6997): 332–336.

65. St-Jacques B, Hammerschmidt M, McMahon AP (1999) Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev 13(16): 2072–2086.

66. Vortkamp A, Lee K, Lanute B, Segre GV, Kronenberg HM, et al. (1996) Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273(5275): 613–622.

67. Eames BF, Singer A, Smith GA, Wood ZA, Yan YL, et al. (2010) Udp-xylose synthase 1 is required for morphogenesis and histogenesis of the craniofacial skeleton. Dev Biol 341(2): 400–415.

68. Avròn F, Hoffman L, Guay D, Akimenko MA (2006) Characterization of two new zebrafish members of the hedgehog family: Atypical expression of a zebrafish Indian hedgehog gene in skeletal elements of both endochondral and dermal origins. Dev Dyn 235(2): 478–489.

69. Hammond CL, Schulte-Merker S (2009) Two populations of endochondral osteoblasts with differential sensitivity to hedgehog signalling. Development 136(23): 3991–4000.

70. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203(3): 253–310.

71. Brand M, Heisenberg CP, Warga RM, Pelegri F, Karlstrom RO, et al. (1996) Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. Development 123: 129–142.

72. Kimmel CB, Miller CT, Krueger D, Ullmann B, Schilling TF, et al. (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203(3): 253–310.

73. Brand M, Heisenberg CP, Warga RM, Pelegri F, Karlstrom RO, et al. (1996) Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. Development 123: 129–142.

74. Kimmel CB, Miller CT, Krueger D, Ullmann B, Schilling TF, et al. (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203(3): 253–310.

75. Bhatia C, Bhatia S, Schilling TF, Poshelwait JH (1993) Structure of the zebrafish small gene and its expression in wild-type, spadetail and null mutant embryos. Development 119(4): 1203–1213.