Introduction

The zinc-finger transcription factor GLI3 (a member of the GLI family) acts as an antagonist or mediator for the Sonic hedgehog (Shh) signaling cascade in a context-dependent combinatorial fashion. GLI3 participates in the patterning and growth of many organs, including the central nervous system (CNS) and limbs. Previously, we reported a subset of human intronic cis-regulators controlling many known aspects of endogenous Gli3 expression in mouse and zebrafish. Here we demonstrate in a transgenic zebrafish assay the potential of two novel tetrapod-teleost conserved non-coding elements (CNEs) docking within GLI3 intronic intervals (intron 3 and 4) to induce reporter gene expression at known sites of endogenous Gli3 transcription in embryonic domains such as the central nervous system (CNS) and limbs. Interestingly, the cell culture based assays reveal harmony with the context dependent dual nature of intra-GLI3 conserved elements. Furthermore, a transgenic zebrafish assay of previously reported limb-specific GLI3 transcriptional enhancers (previously tested in mice and chicken limb buds) induced reporter gene expression in zebrafish blood precursor cells and notochord instead of fin. These results demonstrate that the appendage-specific activity of a subset of GLI3-associated enhancers might be a tetrapod innovation. Taken together with our recent data, these results suggest that during the course of vertebrate evolution Gli3 expression control acquired a complex cis-regulatory landscape for spatiotemporal patterning of CNS and limbs. Comparative data from fish and mice suggest that the functional aspects of a subset of these cis-regulators have diverged significantly between these two lineages.

Key words: Cis-regulators, conserved non-coding elements, enhancer, fin, GLI3.
Congenital abnormalities associated with human GLI3 are listed under the term “GLI3 morphopathies”, including Greig cephalopolysyndactyly syndrome (GCP), non-syndromic polydactyly, Pallister Hall syndrome (PHS), acrocallosal syndrome, preaxial polydactyly type IV (PPD-IV) and postaxial polydactyly type A (PAPA) (Vortkamp et al. 1991; Kang et al. 1997; Radhakrishna et al. 1997, 1999; Elson et al. 2002). Moreover, GLI3 is also associated with oral-facial-digital syndrome (OFDS) and Opitz syndrome (OS) (Liu et al. 2001; Johnston et al. 2010). A dominant developmental syndrome, GCP with polydactyly and craniofacial abnormalities, is linked with large deletions, developmental syndrome, GCPS with polydactyly and are listed under the term “GLI3 morphopathies”, GLI3 (Liu 2002). Moreover, 1997; Radhakrishna et al. type A (PAPA) (Vortkamp polydactyly type IV (PPD-IV) and postaxial polydactyly syndrome (PHS), acrocallosal syndrome, preaxial (GCPS), non-syndromic polydactyly, Pallister Hall including Greig cephalopolysyndactyly syndrome (Pohl et al. 1990; Schimmang et al. 1992, 1994; Hui & Joyner 1993). The limbs of Gli3−/− mutant mouse embryos show severe polydactyly characterized by many un-patterned digits, a loss of A-P polarity, and the absence of apoptosis in inter-digital regions (Welscher et al. 2002b).

The pleiotropy of GLI3 is indicative of a highly complex and sophisticated cis-acting regulatory network governing GLI3 expression in the correct spatiotemporal manner during embryonic development and postnatally. Previously, a set of 12 human-fugu ancient gene regulatory elements were identified that participate in the spatio-temporal expression of GLI3 (Abbasi et al. 2007, 2010, 2013). Eleven of these act as enhancers or repressors in a cell-type dependent manner in cultured cells (Abbasi et al. 2007; Paparidis et al. 2007). GLI3-CNEs, having regulatory potential in human cell lines, were also tested in zebrafish and mouse embryos (Abbasi et al. 2010). Reporter gene expression in transgenic animal models is observed in many un-patterned digits, a loss of A-P polarity, and the absence of apoptosis in inter-digital regions (Welscher et al. 2002b).

In the present study, we identify two novel tetrapod-teleost conserved intronic regions at the GLI3 locus using comparative genomics. Our results obtained through Tol2-based transgenesis in zebrafish demonstrate that these novel intronic CNEs act as tissue-specific enhancers and regulate reporter gene expression in zebrafish hindbrain and pectoral fin. Interestingly, in vitro studies (luciferase reporter assays) of these novel regulators reveal the distinct activities of intra-GLI3 conserved genomic intervals. Furthermore, we test the regulatory potential of previously reported GLI3 associated limb specific cis-regulators, CNE6 and CNE11, in zebrafish (Abbasi et al. 2010). Taken together our studies, based on comparative functional data from fish and mice, suggest that appendage-specific activity of a subset of GLI3-associated cis-regulators might be a tetrapod innovation.

**Materials and methods**

**Identification of conserved non-coding elements at the GLI3 locus**

The human GLI3 genomic sequence was obtained from the ENSEMBL genome browser along with orthologous sequences from mouse (NCBIM37), chicken (GaGa4), lizard (AnoCar2.0), fugu (Fugu4) and zebrafish (Zv9). Multi-species sequence comparisons were performed using the Shuffle LAGAN (SLAGAN) alignment tool kit (Brudno et al. 2003). The human sequence was used as a baseline and annotated by exon/intron information available at ENSEMBL genome browser. The SLAGAN alignment was visualized using VISTA (Mayor et al. 2000). The conservation was measured using a 50 bp window and a cutoff score of 50% identity.

In silico mapping of conserved transcription factor binding sites (TFBSs)

To identify conserved TFBSs for each CNE, the orthologous sequences of terrestrial and non-terrestrial vertebrates were retrieved from the Ensembl genome database. Each of the CNEs with orthologous sequences were analyzed using the MEME motif discovery algorithm (Bailey et al. 2009). MEME is a position weight matrixes (PWM) based algorithm that identifies over-represented motifs in the query data et. The criteria for minimum length was set from 6 to 12 bp. The identified motifs of each CNE were further characterized using the STAMP tool (Mahony & Benos 2007) to determine known transcription factors against TRANSFAC (v11.3) library (Matys et al. 2003). Each of the specified transcription factors were then chosen for endogenous gene expression (RNA in-situ hybridization) studies using the Mouse Genome Informatics database (http://www.informatics.jax.org/).

**Luciferase reporter assay**

For the luciferase reporter assay, CNEs were cloned into pGL3 with minimum TK promoter, and confirmed by nucleotide sequencing. The NIH3T3 cell line is maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 units/mL penicillin/streptomycin. Cells
were plated at 3 × 10^4/well into a 48 well plate, and luciferase reporter constructs (100 ng) and pRL-TK (5 ng) were transfected using Fugene 6 (Promega), according to the manufacturer’s instructions. Cells were harvested 40–44 h after transfection and luciferase activities measured using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions. Experiments were done in triplicate, and the results represented shown as average standard +/− deviation. Statistical significance was evaluated by t-test.

Zebrafish transgenic assays

We used two approaches, a co-injection assay and a Tol2 transposon-based assay to test activities of CNEs in vivo using zebrafish embryos. Zebrafish were bred and raised according to standard protocols (Kimmel et al. 1995). The co-injection assays were performed, as described previously (Woolfe et al. 2006; Minhas et al. 2015). For the preparation of DNA and micro-injection, CNEs were polymerase chain reaction (PCR)-amplified from human genomic DNA. The reporter expression cassette consisting of EGFP under the control of a minimal promoter from the mouse β-globin gene was amplified from plasmid vector by PCR (Thermo Scientific DNA Taq), and purified using the PureLink PCR purification kit, according to manufacturer’s instructions (Life Technologies). PCR purified product of CNEs (30 ng/µL) and β-globin-GFP promoter-reporter cassette (15 ng/µL) were combined, and 0.5% phenol red (Sigma) was used as a tracer dye. The injected embryos were raised at 28.5°C in 1x embryo medium containing 0.003% PTU to prevent pigmentmentation. The zebrafish embryos were dechorionated manually by fine forceps at day 2 and anaesthetized by Tricaine.

The Tol2 system is based on a transposon system, which allows for efficient transgene integration (Kawakami 2007). To test CNEs using a Tol2 GFP system (Fisher et al. 2006), the CNEs were first amplified with a final 10–30 min extension step. The freshly amplified PCR (~500 ng/µL) products were cloned into pCR8/GW/TOPO vector (Life Technologies) to make entry clones (Pauls et al. 2012; Chen et al. 2014). Orientation screening to determine the sense strand was followed by a LR (attL and attR) recombination reaction between Topo entry clone (~100 ng/µL) and destination vector pGW_cfosEGFP (~100 ng/µL); Gateway LR Clonase II enzyme (Life Technologies) was used. The destination clones consisting of CNEs and a minimal c-fos promoter were sequenced for confirmation of a positive orientation into the transposon construct. The purified transposon construct (25 ng/µL), 0.5 µL transposase RNA (175 ng/µL), and 0.5 µL phenol red stock, were injected into one-cell stage zebrafish embryos.

Images and screening

The transgenic embryos were screened after approximately 24 and 48 hpf for GFP signals using an Olympus IX71 inverted fluorescence microscope. Photographs were taken with an Olympus DP72 camera.

Results

Identification of CNEs at the GLI3 locus by comparative sequence analysis

Previously, we identified 12 anciently conserved genomic elements in the intronic intervals of human GLI3 through human–fish comparative sequence analysis (Abbasi et al. 2007). Cis-regulatory potential of 11 of these GLI3 intronic regions was elucidated by using in vitro and in vivo assays (Fig. S1) (Abbasi et al. 2007, 2010, 2013; Paparidis et al. 2007). In the present study, we extended our previous work and analyzed the sequence alignments more extensively to capture any remaining anciently conserved GLI3 intronic intervals (Fig. S1). This careful comparative sequence analysis pinpointed two novel conserved sequences within intronic intervals of human GLI3, thereby named CNE13 (intron-4) and CNE14 (intron-3). Taken together with our previous investigations, human GLI3 intronic intervals thus harbor 14 ancient conserved CNEs in total with at least 50% identity over a 50 bp window (Fig. 1 and Fig. S1).

Functional assays of GLI3-associated CNEs in transgenic zebrafish embryos

The regulatory potential of a selected subset of novel CNEs identified in the present study (CNE8, 13 and 14) was tested independently in zebrafish embryos by using two independent strategies: exploiting a co-injection assay (β-globin promoter) and directly cloning into a Tol2 vector (c-fos promoter) (Woolfe et al. 2006; Fisher et al. 2006). In contrast, previously identified limb-specific CNE6 and 11 (which have shown transcriptional activity in developing limb bud of mice and chicken) (Abbasi et al. 2010) were only tested using a Tol2 vector with a c-fos minimal promoter in zebrafish transgenic assays. The details of CNEs selected for functional analyses are depicted in Figure 1 and also listed in Table 1. The results from both assays are highly reproducible, while GFP expression is generally stronger using the Tol2 strategy due to efficient inte-
CNE13 drives expression predominantly in the hindbrain, notochord and pectoral fin bud

This novel element (CNE13) is deeply conserved (human-fish) and resides within intron-4 of human GLI3. The human genomic segment of 624 bp spanning the human-fish conserved core sequence of 88 bp was co-injected with a GFP reporter into zebrafish and then monitored for enhancer activity at set time points. From this assay it appears that CNE13 directs reporter gene expression prominently in the hindbrain after approximately 48–56 hpf (Fig. 2A and Fig. S2). To further confirm results obtained with the co-injection experiment, the Tol2 based assay was also used on CNE13. In the Tol2 assay, the major activity domain for CNE13 was again hindbrain territory (Fig. 2B). Reproducible GFP expression in the hindbrain with two independent assays using independent promoter systems strongly suggests that CNE13 plays a role in...
neuronal development. In the Tol2 assay, notable reporter gene expression was also detected in the notochord, muscle cells and pectoral fin (Fig. 2C–E). The CNE13 induced GFP signal in the pectoral fin, which appeared after 48 hpf, was robust and reproducible. It is notable that with the co-injection assay, CNE13 was unable to upregulate GFP expression in the developing pectoral fin, possibly due to high levels of mosaicism associated with this strategy (Woolfe et al. 2005; Abbasi et al. 2007; Kawakami 2007; Minhas et al. 2015).

**CNE14 drives GFP expression exclusively in the pectoral fin**

The second novel evolutionarily conserved intronic interval identified through comparative sequence analysis in the present study was named CNE14. This element resides within intron-3 of GLI3 and is highly conserved among diverse lineages of tetrapods. When tested in a co-injection experiment, a genomic segment of approximately 2.0 Kilobase pair (kbp) was unable to induce reporter gene expression in fish embryos at day 2 or day 3 of development. However, when tested using the Tol2 strategy, CNE14 was able to drive robust and reproducible GFP expression in the pectoral fin after 48 hpf (Fig. 3). Interestingly, CNE14...
induced reporter expression was limited to the pectoral fin only, whereas no significant expression was observed in any of the other developmental domains of zebrafish embryos at day 2 or day 3 of development. Failure to detect reporter expression with co-injection assay again illuminates the fact that this strategy is not suitable for detecting the activity of limb-specific regulators.

**CNE6 and CNE11 limb-specific enhancers did not induce reporter gene expression in the zebrafish fin**

Previously, we have shown in chicken and mouse transgenic embryos that the human-fish evolutionarily conserved GLI3-intronic enhancers named CNE6 and CNE11 autonomously control individual aspects of GLI3 expression in the developing limb skeletal structures. For instance, the prominent activity domain of CNE6 was autopod-specific, whereas CNE11 appeared to be a stylopod/zeugopod specific cis-regulator (Abbasi et al. 2010).

In this study we sought to evaluate the regulatory potential of CNE6 and CNE11 in the pectoral fin of the zebrafish to investigate whether the appendage specific activity of these two enhancers is conserved across bony vertebrates. For this purpose, we used the Tol2 vector based strategy and amplified CNE6 and CNE11, which are 862 bp and 1185 bp, respectively (Abbasi et al. 2010). Interestingly, neither CNE6 nor CNE11 could induce reporter gene expression in the developing pectoral fin of the fish at day 2 or day 3 of development (Fig. 4B,D). Instead, the Tol2 assay indicated the prominent expression domain for CNE6 to be blood precursor cells, and for CNE11 the notochord (Fig. 4A,C). Therefore, our data reflect that GLI3-associated cis-regulators underwent both genetic and developmental alteration during vertebrate evolution, acquiring prominently divergent roles in tetrapod and bony fish lineages.

**Human-fish conserved CNE8 is unable to upregulate reporter expression in transgenic zebrafish assays**

The human-fish conserved intronic interval CNE8 was identified in our previously reported comparative analyses of the GLI3 locus. However, the functionality of this element was not tested previously (Abbasi et al. 2007). In this study we sought to investigate the enhancer potential of this human intronic patch of 574 bp sequence located in intron-10 of the GLI3 gene. This human interval spans a human-fish conserved core sequence of 144 bp. Approximately 200 embryos were screened for GFP reporter activity at set time points for both the co-injection and Tol2 based transgenic assays. However, both assays indicate that CNE8 is unable to activate reporter gene expression.

**Luciferase reporter assays of GLI3-associated CNEs**

Our in vivo data demonstrate that both CNE13 and CNE14 can function for tissue-specific expression of GFP reporters. In order to further examine activities of these CNEs and compare them with activities of other previously-identified CNEs, we set up in vitro luciferase reporter assays. We used the NIH3T3 cell line, a mouse mesenchyme cell line, which can respond to Hedgehog signaling, a major regulator of GLI function (Kim et al. 2010).

We observed upregulation of reporter activities by CNE14, which is consistent with GFP reporter activation in the fin bud in an in vivo assay. Contrary to this,

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**Fig. 4.** Limb-specific enhancers cannot upregulate reporter gene expression in the pectoral fin with Tol2 assay. Images of live zebrafish embryos at day 2, lateral views, anterior to left, dorsal to top. Arrowheads indicate green fluorescent protein (GFP) expressing cells. Conserved non-coding element 6 (CNE6) (A) induced GFP expression in blood precursor cells and CNE11 (C) in developing notochord. CNE6 and CNE11 do not induce reporter gene expression in pectoral fin (B and D). b, blood precursor cells; nc, notochord; y, yolk.
In the present study we identified two novel ancient non-coding intervals in intron-3 and 4 of GLI3 by employing comparative sequence analysis of the GLI3 containing locus of humans, mice, chickens, lizards, fugu, and zebrafish (Fig. 1 and Fig. S1). The in vivo regulatory potential of these two novel CNEs (named CNE13 and CNE14) was tested via a zebrafish co-injection assay (Woolfe et al. 2005) and Tol2-based transgenesis in zebrafish (Fisher et al. 2006). CNE13-controlled GFP expression was strongly expressed in the hindbrain, a finding consistent with the known roles and expression pattern of GLI3 during CNS development (Table 2). Therefore, taking into consideration our previously reported GLI3 enhancers (CNE1, 2, 6, 9 and 11), we identified six GLI3-intronic enhancer regions in total that control reporter gene expression in the developing neural tissues of mouse and zebrafish embryos (Fig. 1). With multiple independent enhancers controlling early CNS patterning, GLI3 resembles other key developmental genes that feature a high level of complexity in their genetic regulatory mechanisms governing CNS patterning (Nobrega et al. 2003; Kimura-Yoshida et al. 2004).

Within the somites, Gli3 expression is widespread and known to play a vital role in epaxial and hypaxial myotome formation (McDermott et al. 2005). Consistent with such roles, CNE13 upregulates reporter expression predominantly within the muscles of developing zebrafish (Table 2 and Fig. S2). Together with our previously reported GLI3-associated enhancers, it thus appears that at least three independent enhancer regions (CNE9, 11 and 13) govern reported GLI3 expression and function during muscle formation (Fig. 1).

Based on the Tol2 transgenesis methodology, in the present study we report two novel appendage-specific enhancers residing within GLI3 intronic intervals. In addition to the hindbrain and muscles another prominent activity domain of CNE13 in zebrafish was the pectoral fin. CNE13-induced robust and reproducible reporter expression in the pectoral fin at 48 hpf (Fig. 2E). Similarly, the second novel element (CNE14), which resides within intron-3 of GLI3, induced widespread reporter expression in the pectoral fin bud after 48 hpf (Fig. 3). Interestingly, CNE14-induced enhancer activity was explicitly restricted to the pectoral fin and a careful examination revealed no significant reporter expression in any of the other developmental compart-

**Fig. 5.** Luciferase reporter assays of GLI3-associated conserved non-coding elements (CNEs) in the NIH3T3 cell line. (A) Schematic drawings of the luciferase constructs. CNEs were cloned into a pGL3 luciferase vector with the TK minimum promoter (TK min). (B) Luciferase reporter activities without CNE and with indicated CNEs in the NIH3T3 cell line. Shown is average ± standard deviation. *P < 0.05.
During early embryonic development of the tetrapod limb, GLI3 plays multiple roles: Shh-independent polarization of nascent limb bud (Welscher et al. 2002a; Osterwalder et al. 2014) and regulating anteroposterior patterning of the autopod by counteracting Shh signaling (Wang et al. 2000; Littingtung et al. 2002; Welscher et al. 2002b). GLI3 also regulates specification of skeletal precursors for development of specific limb skeletal elements (Barna et al. 2005; Robert & Lallemand 2006). These distinct roles of GLI3 during limb development suggest that a complex cis-regulatory landscape might be instrumental in deploying GLI3 product at different time/domains of limb development. Accordingly, CNE13 and CNE14 exhibited distinct activities in the in vitro luciferase reporter assays, supporting the notion of complex and cellular context-dependent regulation of GLI3 expression. The exact molecular mechanisms that define the different activities between CNE13 and CNE14 in the developing fin remains to be elucidated. It is conceivable that endogenous factors to drive CNE13 in the fin bud and hindbrain would not be present in NIH3T3 cells, while CNE14 could regulate reporter expression both in NIH3T3 cells and developing fin buds. Similarly, CNE-1, 2, 6, 9, and 11, which also exhibited activities to drive reporter gene expression in neural tissue in previous studies, were also not activated in NIH3T3-based luciferase reporter assay. Taken together with our previously reported data, we defined four independent GLI3-intronic intervals (CNE6, 11, 13 and 14) regulating reporter expression in the developing limb/fin bud (Fig. 1) (Abbasi et al. 2010).

Here, we propose that the spatial and temporal activity of novel enhancers reported in the present study (CNE13 and CNE14) must also be investigated thoroughly in mice or chicken. Experimental data from such tetrapod model animals would further define the spatiotemporal aspects of CNE13 and CNE14 activity during anterior-posterior polarity of the limb and patterning of the CNS.
Conclusions

Taken together with our previous reports, the identification of two novel cis-regions at the GLI3 locus reflects that GLI3 harbors multiple cis-acting regulatory modules that participate in an overlapping fashion during development of vertebrate neural tube, limb, and muscles. A subset of these cis-regulators is dual in nature and demonstrated context-dependent regulation of GLI3 expression. These findings suggest that even though GLI3 in tetrapod and teleost shared multiple evolutionarily conserved cis-acting regulators, the target site specificity of some of these elements has diverged significantly between these two lineages. This sort of functional differentiation might have been achieved either through changes in the overall span of enhancers or through the turnover of transcriptional factor binding site inputs. Furthermore, this complex catalogue of GLI3-associated cis-regulators will help in understanding the genetic basis of those potential human birth defects that cannot be attributed to a mutation in coding sequence of GLI3. In such cases, these cis-regulatory modules can be investigated among those mutations that can potentially affect the space and time availability of the GLI3 transcript during embryogenesis.

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Author contributions

A.A.A. and S.S.A. conceived the project. A.A.A., G.E. and Y.K designed the experiments. Computational analysis were performed by S.A., S.S.A., and R.M. The in vivo experiments were performed by S.A. and R.M. In vitro assays were performed by Y.K and N.L. The manuscript was written by A.A.A., R.M., S.A., and Y.K

Conflict of interests

The authors declare that they have no competing interests.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Conservation of the non-coding elements across the GLI3 locus.

Fig. S2. Schematic representation of GFP expression induced by GLI3 associated CNE13 in zebrafish embryos at day 2 (~24 hpf) and day 3 (~48 hpf).