Understanding Mechanisms of GLI-Mediated Transcription during Craniofacial Development and Disease Using the Ciliopathic Mutant, *talpid*²

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The primary cilium is a ubiquitous, microtubule-based organelle that cells utilize to transduce molecular signals. Ciliopathies are a group of diseases that are caused by a disruption in the structure or function of the primary cilium. Over 30% of all ciliopathies are primarily defined by their craniofacial phenotypes, which typically include midfacial defects, cleft lip/palate, micrognathia, aglossia, and craniosynostosis. The frequency and severity of craniofacial phenotypes in ciliopathies emphasizes the importance of the cilium during development of the craniofacial complex. Molecularly, many ciliopathic mutants, including the avian *talpid*² (*ta*²), report pathologically high levels of full-length GLI3 (*GLI3*FL), which can go on to function as an activator (*GLI*A), and reduced production of truncated GLI3 (*GLI3*T), which can go on to function as a repressor (*GLI*R). These observations suggest that the craniofacial phenotypes of ciliary mutants like *ta*² are caused either by excessive activity of the *GLI*A or reduced activity of *GLI*R. To decipher between these two scenarios, we examined GLI3 occupation at the regulatory regions of target genes and subsequent target gene expression. Using *in silico* strategies we identified consensus GLI binding regions (GBRs) in the avian genome and confirmed GLI3 binding to the regulatory regions of its targets by chromatin immunoprecipitation (ChIP). In *ta*² mutants, there was a strikingly low number of GLI3 target genes that had significantly increased expression in facial prominences compared to the control embryo and GLI3 occupancy at GBRs associated with target genes was largely reduced. In vitro DNA binding assays, further supported ChIP results, indicated that the excessive GLI3FL generated in *ta*² mutants did not bind to GBRs. In light of these results, we explored the possibility of GLI co-regulator proteins playing a role in regulatory mechanism of GLI-mediated transcription. Taken together our studies suggest that craniofacial ciliopathic phenotypes are produced via reduced GLI*T* production, allowing for target gene transcription to be mediated by the combinatorial code of GLI co-regulators.

**Keywords:** primary cilia, craniofacial, *talpid*², *c2cd3*, ciliopathies, GLI
INTRODUCTION

Primary cilia are ubiquitous organelles that serve as cellular hubs for transduction of numerous signaling pathways. Most notably, cilia have been identified as transducers of the Hedgehog (Hh) pathway. Identification of the primary cilium as a signaling hub for the Hh pathway came from seminal experiments reporting that anterograde and retrograde intraflagellar transport (IFT) proteins in the cilium are required for Sonic Hedgehog (SHH) signal propagation (Huangfu et al., 2003; Huangfu and Anderson, 2005). When SHH ligand is present, it binds to its receptor Patched (PTCH), thus allowing Smoothened (SMO) to localize and accumulate in the primary cilium (Corbit et al., 2005; Rohatgi et al., 2007). Activated, ciliary SMO, in concert with Kif7, then promotes the dissociation of GLI from Suppressor of Fused (SUFU) (Humke et al., 2010; Tukachinsky et al., 2010; Li et al., 2012) and the subsequent post-translational processing of GLI proteins necessary for their function as activators and repressors (Goetz and Anderson, 2010).

In vertebrates, there are three members of the GLI transcription factor family: GLI1, GLI2, and GLI3. GLI1 and GLI2 are considered transcriptional activators, whereas GLI3 mostly behaves as a repressor. However, there have been examples of GLI2 functioning as a repressor in the absence of GLI3, and GLI3 functioning as an activator in the absence of GLI2 (Mo et al., 1997; Theil et al., 1999; Tole et al., 2000; Bai and Joyner, 2001; Persson et al., 2002; Rallu et al., 2002; Buttitta et al., 2003; Motoyama et al., 2003; Bai et al., 2004; Lei et al., 2004; McDermott et al., 2005; Pan et al., 2009). Full-length GLI2 and GLI3 can be processed via phosphorylation and other post-translational modifications into the activator isoform (GLI\textsubscript{A}) or truncated into the repressor isoform (GLI\textsubscript{R}) (Wang et al., 2000; Pan et al., 2006). Inhibition of GLI processing prevents production of GLI\textsubscript{A} and GLI\textsubscript{R} isoforms. Thus, an essential role of primary cilia is to establish the ratio of GLI\textsubscript{A} to GLI\textsubscript{R} proteins (Haycraft et al., 2005; Liu et al., 2005), which in turn controls transcription of SHH target genes.

Three basic models have been proposed to depict the potential mechanism of how SHH target genes are activated by a gradient of GLI isoforms: (1) the ratio sensing model, (2) the threshold repression model and (3) the threshold activation model (Falkenstein and Vokes, 2014). Ratio sensing, as the name implies, is based on the ratio of GLI\textsubscript{A} and GLI\textsubscript{R} rather than concentration of either. The net balance of GLI\textsubscript{A} to GLI\textsubscript{R} then determines if, and to what extent, which target is activated or repressed. The other two models suggest threshold-specific mechanisms. The threshold activation model suggests that SHH targets are activated when GLI\textsubscript{A} reaches a threshold-specific concentration. On the other hand, in the threshold repression model (de-repression), the activation of SHH target genes is dictated by the alleviation of repression via loss of GLI\textsubscript{R}.

The talpid\textsuperscript{2} (ta\textsuperscript{2}) is a naturally occurring avian mutant that is best characterized by severe polydactyly and its oral-facial phenotype (Abbott et al., 1959, 1960; Dvorak and Fallon, 1991; Schneider et al., 1999). The face of affected ta\textsuperscript{2} embryos is characterized by a dysmorphic frontonasal prominence, facial clefting, hypoplastic maxillary prominences, incomplete fusion of the primary palate and hypoglossia (Chang et al., 2014). Our recent work determined that the ta\textsuperscript{2} mutation affected ciliogenesis via a 19 bp deletion in C2CD3 (Brugmann et al., 2010; Chang et al., 2014), a centriolar protein required for ciliogenesis (Hoover et al., 2008). Our studies also determined that the ta\textsuperscript{2} mutant genetically, biochemically and phenotypically phenocopied the human craniofacial ciliopathy, Oral-facial-digital syndrome 14 (OFD14) (Schock et al., 2015). ta\textsuperscript{2} embryos, similar to many other ciliopathies, have a significant increase in GLI3\textsubscript{A} and a reduction in the amount GLI1 (Chang et al., 2014). However, the mechanism by which this disruption in GLI isoform production affects expression of GLI targets in the developing craniofacial complex remains unknown.

Herein, we utilize a combination of several biochemical techniques to determine the impact loss of cilia has on GLI function. Specifically, we examine the expression of GLI target genes and occupation of GLI binding regions (GBRs) associated with those targets in the developing frontonasal, maxillary and mandibular prominences (FNP, MXP, and MNP, respectively) in order to uncover the mechanism by which GLI mediated transcription is being impacted in ta\textsuperscript{2} mutants. Understanding the full extent of molecular disruptions in ta\textsuperscript{2} mutants will hopefully guide future therapeutic strategies for craniofacial ciliopathies, a rapidly growing group of disorders that currently have little to no therapeutic treatment.

MATERIALS AND METHODS

Embryo Preparation
talpid\textsuperscript{2} (ta\textsuperscript{2}) heterozygous carriers were mated, eggs were collected and shipped from the UC Davis Avian Sciences Department. Embryos were incubated at 37 °C for approximately 5–7 days when embryos reached Hamburger Hamilton stage 25–31 (HH25-31).

Quantitative RT-PCR of GLI Targets
FNPs, MXP, and MNP were harvested from day 5 chick embryos. mRNA was prepared with TRIzol reagent (Thermo Fisher Scientific), and then converted to complementary DNA through reverse transcription reaction (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems™). Different amounts of cDNA (40, 20, 10 and 5 ng) was used for quantitative PCR to test PCR efficiency and a linear range of duplication (SsoAdvanced™ Universal SYBR® Green Supermix, BioRad). Expression of genes known to play a role in craniofacial development were examined with the following primer sets: ALX4 (105 bp) F: GTTACGGTAAGGAGAGCAGTTT, R: CTTTCATCTCCAGGCTCTTTC, BMP1A (100 bp) F: GTTGTGTTGGGAGCTGTTTTC, R: TGGCATTCCAACTGACATGCT, WIF1 (100 bp) F: CAACCTGGTTTCAATGGAGGAAC, R: GGCCTAATGGCAATTTGCTATTTT, OSR2 (140 bp) F: CCACGTCACACAGTCTCTACA, R: TCTCTTTGGAATTGAGTGC. The statistical significance of the data was evaluated through two-tailed Student’s t-test. p-values less than or equal to 0.05 (95% confidence level) were considered as statistically significant differences.
Western Blotting of GLI2/3 Proteins
FNPs, MXPs, or MNPs were pooled and lysed in RIPA buffer containing protease inhibitors and phosphatase inhibitors (1 mM Phenylmethylsulfonyl fluoride, 1 mM NaVO$_4$, 1X complete protease inhibitor cocktail, EDTA-free), and slightly sonicated with a microprobe to recover chromosome bound GLI2 and GLI3 proteins. BCA assay (Pierce) was used to measure protein concentration of cell lysates. Proteins were boiled with 1X Laemmli sample buffer and run on 6% SDS-PAGE for GLI2 and GLI3, or 12% SDS-PAGE for GAPDH, which later were wet-transferred to Polyvinylidene difluoride (PVDF) membrane. Anti-GLI2, anti-GLI3 (Polyclonal goat IgG 1:500, R&D systems) and anti-GAPDH (FL-335, polyclonal rabbit IgG 1:4000, Santa Cruz Biotechnology) were prepared in 1X TBST (0.1% Tween-20)/6% nonfat milk, as well as secondary antibodies (anti-goat and anti-rabbit, 1:10,000). Proteins were detected by Electrochemiluminescence assay (Amersham ECL Prime, GE Healthcare Life Science).

DNA Binding Affinity Assay
PATCHED 1 promoter oligonucleotides were designed according to the location of a Gli binding region (GBR) at −2549 from the TSS site (GGAAGAAGTGTACAGTTAAC AGTCTCAGGGTGGTGTAAGAAACCATGGCTGCCCTAC GG). 100 pmole of biotin-conjugated positive oligonucleotides and complementary oligonucleotides (Integrated DNA Technologies) were annealed in 1X TE/50 mM NaCl buffer in a PCR cycler and incubated with Dynabeads Streptavidin (Invitrogen). FNPs, MXPs, or MNPs from day 5 control or ta$^2$ embryos were pooled and processed as described for Western blotting. Cell lysates were incubated with oligonucleotides-bound Dynabeads at 4°C for 2 h. Beads were washed with 1 ml RIPA buffer three times and processed for Western Blotting analysis.

Chromatin Immunoprecipitation
FNPs, MXPs, or MNPs from day 5 control or ta$^2$ embryos were harvested, pooled and crosslinked in 1% formaldehyde. Tissue was homogenized in RIPA buffer and sonicated (Bioruptor$^®$ Pico, Diagenode) at 5 cycles of 30 s on/45 s off. Sheared DNA was distributed around 0.3 kb to 1 kb on 1% agarose gel. Cell lysates were pre-cleaned with Dynabeads protein G (ThermoFisher Scientific) and quantified by BCA assay. Dynabeads for immunoprecipitation were blocked with 20 µg/ml Glycogen, 20 µg/ml BSA, 20 µg/ml yeast RNA in RIPA buffer at 4°C for an hour. GLI3 antibody (AF3690, R&D systems) and pre-blocked Dynabeads Protein G were incubated with 90% of cell lysates at 4°C overnight. 10% of cell lysates were kept as Input. Beads were washed with IP wash buffer 1 (low salt; 50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100), IP wash buffer 2 (high salt; 50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100), IP Wash Buffer III (LiCl containing buffer; 10 mM Tris-Cl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Sodium deoxycholate, 0.5% NP-40) and TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA). ChIP samples were reverse-crosslinked by boiling with 10% Chelex-100 (BioRad), and treated with 0.2 mg/ml Proteinase K at 55°C for 30 min. Immunoprecipitated DNA samples were analyzed with quantitative real-time PCR (BioRad) with primers to GBR of target genes. Error bars in all figures represent standard error of the mean (S.E.M.) from five to seven independent experiments. The statistical significance of the data was evaluated through two-tailed Student’s t-test. p-values less than or equal to 0.05 (95% confidence level) were considered as statistically significant differences.

GBR Analysis
Sequences for GRBs from previous publications (Vokes et al., 2007, 2008) were used with a custom perl script to search for all the exact matches of the various possible sequences of the consensus GRBs in the chicken genome. Acquired positions of the motifs in the genome were run through a second custom perl script to search for genes that encompass these motif sites at a distance of 20 kb from either end. Potential GLI targets were confirmed using chromatin-immunoprecipitation (ChIP) assays.

RESULTS
The Avian Ciliopathic Mutant, talpid$^2$, has Craniofacial Anomalies Characteristic of a Ciliopathy
To understand the transcriptional networks affected in craniofacial ciliopathies we analyzed the talpid$^2$ (ta$^2$) mutant, a naturally occurring avian ciliopathic mutant that has been established as a model for the human craniofacial ciliopathy, Oral-facial-digital syndrome 14 (Chang et al., 2014; Schock et al., 2015). The ta$^2$ craniofacial phenotype characterized by facial and palatal clefting, micrognathia, and hypoglossia, is fully evident at day 7 (Figure 1). Although our previous work has characterized this phenotype (Chang et al., 2014; Schock et al., 2015), to determine the transcriptional networks that contribute, we first needed to identify when phenotypic onset occurred. At day 7 the frontonasal prominence (FNP) is shorter and wider and frequently does not fuse to adjacent prominences (Figures 1A–1B’). Two days earlier at day 5, the MXPs were medially rotated, the nasal pits were larger, thus preventing the proper juxtapositioning of the FNP with adjacent prominences (Figures 1C–1D’). Palatal views showed increased patency of the MNP, which later were confirmed using chromatin-immunoprecipitation (ChIP) assays.

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FIGURE 1 | ta² embryos have craniofacial anomalies common to craniofacial ciliopathies. (A–B') Frontal view of whole-mount day 7 control and ta² embryos. (C,D') Frontal view of whole-mount day 5 control and ta² embryos. (E–F') Palatal view of day 7 control and ta² embryo with the forming secondary palate outlined (white dotted line). (G–H’) Palatal view of day 5 control and ta² embryo. (I–J’) Dorsal view of day 7 control and ta² mandibles with tongue outlined (black dotted line). (K–L’) Dorsal view of day 5 control and ta² mandibles. Facial prominences have been pseudocolored as follows: frontonasal prominence (FNP, blue), maxillary prominence (MXP, red), and mandibular prominence (MNP, yellow). Scale bars: 1 mm (A–J’) and 650 µm (K–L’).
phenotypes we carried out our analyses in the facial prominences of day 5 embryos.

Loss of Cilia Results in Aberrant GLI Isoform Production

Our previous work determined that aberrant ciliogenesis in ta\textsuperscript{2} embryos disrupted the production of GLI proteins in such a manner that there was increased GLI\textsubscript{FL} production and decreased GLI\textsubscript{T} production (Chang et al., 2014). The GLI\textsubscript{FL} isoform typically goes on to function as an activator, whereas the GLI\textsubscript{T} isoform goes on to function as a repressor. The excessive production of GLI\textsubscript{FL} is an extraordinarily common molecular phenotype in ciliopathies, including those with craniofacial phenotypes (Huangfu and Anderson, 2005; Davey et al., 2006; Tran et al., 2008; Tabler et al., 2013). To carefully examine the differences of GLI\textsubscript{FL} and GLI\textsubscript{T} protein levels in control and ta\textsuperscript{2} mutants, we performed Western blot analysis of GLI2 and GLI3 in the three facial prominences affected in the ta\textsuperscript{2} at day 5. We detected a very low level of GLI2\textsubscript{FL} and a substantial amount of GLI2\textsubscript{T} isoforms in the control facial prominences (Figure 2A). The loss of cilia in ta\textsuperscript{2} embryos disrupted GLI processing and altered the production of GLI2 protein isoforms. In ta\textsuperscript{2} facial prominences, we detected dramatically increased levels of GLI2\textsubscript{FL}, and low levels of GLI2\textsubscript{T}, relative to control prominences. We next examined the production of GLI3 isoforms. Western blot analyses showed that, similar to GLI2\textsubscript{FL}, GLI3\textsubscript{FL} was increased in ta\textsuperscript{2} prominences relative to controls. Contrary to what was observed with the GLI2\textsubscript{T}, GLI3\textsubscript{T} was readily detectable in both control and ta\textsuperscript{2} facial prominences. Specifically, ta\textsuperscript{2} MNP had less GLI3\textsubscript{T} than control MNP. These data suggested that disrupted ciliogenesis affected the processing of GLI2 and GLI3 in distinct manners, yet the net result was an increase in GLI\textsubscript{FL} production. We did not observe a change in GLI1 protein levels between control and mutant embryos (Data not shown).

Since the GLI\textsubscript{FL} isoform typically goes on to act as an activator, these results suggest that craniofacial phenotypes in the ta\textsuperscript{2} mutant could be caused by increased GLI activator function leading to increased expression of GLI target genes. To test this hypothesis, we utilized an in silico approach to look for potential GLI target genes within the avian genome. Using previously published sequences of GBRs (Vokes et al., 2007, 2008) we scanned the chicken genome for possible GLI targets. GBR positions were run through a custom perl script to search for genes that encompass these motif sites at a distance of 100 kb from the 5' or 3' end of the gene. From this list we identified several genes known to play a role in craniofacial development (Supplemental Table 1). Confirmation of our in silico approach was carried out on selected genes using ChiP assays (Supplemental Figures 1 A–D). Through these analyses, we selected four GLI target genes known to be expressed during, and have a role in, craniofacial development: ALX4 (Beverdam et al., 2001; Mavrogiannis et al., 2001), BMPRIA (Li et al., 2011, 2013; Saito et al., 2012), OSR2 (Lan et al., 2001), and WIF1 (Hsieh et al., 1999; Darnell et al., 2007). PTCH1, a known GLI target, was used as a positive control (Supplemental Figure 1E) and IgG IP as an antibody background control. The data were all normalized to IgG IP percentage, and the genes with relative enrichment >1 were considered positive for GLI3 binding. To determine if increased production of GLI3\textsubscript{FL} in ta\textsuperscript{2} facial prominences correlated with increased expression of these target genes, we performed quantitative RT-PCR (qPCR) with mRNA from facial prominences of day 5 control and ta\textsuperscript{2} embryos (Figures 2B–E). There was not an increase in ALX4 expression in any of the facial prominences (Figure 2B); however, we detected a significant decrease in ALX4 expression in the MXP. BMPRIA expression was also not significantly increased in any of the developing prominences (Figure 2C). No significant changes in OSR2 expression were detected in the FNP or MNP; however, a significant increase in expression was observed in the MXP (Figure 2D). Finally, WIF1 expression was not changed in the FNP, yet was significantly increased in the MXP and significantly decreased in the MNP (Figure 2E). Taken together, these data do not support the idea that increased production of GLI3\textsubscript{FL} directly and uniformly results in increased expression of GLI targets throughout the facial prominences. Additionally, these data suggest that each facial prominence interprets aberrant GLI production in a unique manner.

Excess Production of GLI\textsubscript{FL} Does Not Correlate with an Increase of GLI\textsubscript{FL} Occupancy at GBRs

For GLI\textsubscript{FL} to function as an activator, it has to occupy the regulatory regions of GLI targets. We wondered if lack of uniform increases of target gene expression in ta\textsuperscript{2} embryos was due to failure of GLI\textsubscript{FL} to recognize and occupy GBRs of target genes. To test this hypothesis, we performed an in vitro DNA binding assay using the PATCHED 1 (PTCH1) promoter. We synthesized a 60 base pair biotin-labeled oligonucleotide of the PTCH1 promoter containing an endogenous GLI binding motif found at position −2549 proximally upstream of the transcription start site (TSS). Through high affinity of Biotin-Streptavidin interaction, we were able to evaluate the DNA binding ability of GLI3 isoforms by Western blot. Pre-incubation of non-labeled PTCH1 oligonucleotides depleted GLI3 protein signals, which confirmed the specificity of the GLI3-GBR interaction. Under the same exposure, the affinity based pull-down assay showed that GLI3\textsubscript{T} predominantly bound to the biotin-labeled oligonucleotides in both control and ta\textsuperscript{2} mutants. Interestingly, despite the high level of GLI3\textsubscript{FL} production in ta\textsuperscript{2} embryos, GLI3\textsubscript{FL} failed to bind to the biotin-labeled oligonucleotides (Figures 3A, B). On the contrary, comparison between control and ta\textsuperscript{2} embryos indicated that the amount of GLI3\textsubscript{T} binding to the oligonucleotides correlates with the protein concentration (Figures 3A, B). Taken together these data suggest that despite increased GLI3\textsubscript{FL} production, target gene expression is not increased via increased GLI activator function because GLI3\textsubscript{FL} does not occupy the GBRs of target genes. In addition, the predominant binding of the GLI3\textsubscript{T} repressor at GBRs supports the idea of the threshold repression model, in which GLI targets underwent de-repression due to the removal of GLI\textsubscript{T} repressor.
**FIGURE 2 | Excessive GLI_{FL} production in ta2 embryo does not correlate with increased gene activation of GLI targets.** (A) Western blot of GLI2 and GLI3 proteins from the frontonasal prominence (FNP), maxillary prominence (MXP) and mandibular prominence (MNP) of day 5 control and ta2 embryos. GAPDH was used as a loading control. (B–E) mRNA-qPCR analyses of ALX4, BMPR1A, OSR2, WiFi from FNP, MXP, and MNP of day 5 control and ta2 embryos. The data was normalized by the individual facial prominences of control and ta2 embryos. The asterisks indicate statistically significant differences and are assigned as followed: *P < 0.05, **P < 0.01. Error bars are based on the standard error of the means (S.E.M.), n = 4.
GLI3 Occupancy at GBRs within the Regulatory Regions of Target Genes Is Altered in ta^2 Mutants

In vitro DNA binding affinity assays suggested that there was not an increase of ectopic GLI3 FL at GBRs of target genes in ta^2 mutants. To determine if GLI3 binding to GBRs was altered in vivo in ta^2 mutants compared to control embryos, we next performed ChIP-qPCR (Figure 4). In ta^2 mutants, GLI3 enrichment at GBRs associated with the craniofacial genes ALX4 and OSR2 was reduced in all facial prominences, yet only significantly in the MXP and MNP (Figures 4A,B). GLI3 enrichment at the GBRs associated with BMPRIA and WIF1, was also overwhelmingly reduced in facial prominences, yet only significantly in the ta^2 FNP and MXP (Figures 4C,D). GLI3 binding in the MNP of WIF1 was below detectable levels in controls, and thus could not be evaluated. These data suggested that GLI3 occupancy at the GBR of target genes is decreased. Our in vitro data suggested that GLI3 FL did not bind at target gene GBRs (Figure 3). Thus, taken together these data indicate that the observed reduction of GLI3 binding is indicative of reduced GLI3 FL binding at the regulatory regions of target genes.

Reduced GLI3 binding at GBRs of GLI target genes appeared to be a general trend in ta^2 mutants, as several other target genes expressed in the craniofacial complex also exhibited reduced enrichment of GLI3 at associated GBRs (Supplemental Figure 2). Thus, these data suggested that altered expression of GLI targets could be caused by aberrant GLI3 FL binding in ta^2 mutants. Interestingly, there was not always a direct correlation with loss of GLI3 FL repressor binding and increased gene expression. Furthermore, different results among the three facial prominences examined pointed to context—and tissue-specific regulation of GLI targets (Supplemental Figures 1A–D). Several studies have proposed the possibility that GLI proteins work together with co-regulators to influence expression of targets genes (Brewster et al., 1998; Koyabu et al., 2001; Mizugishi et al., 2001; Lee et al., 2010; Peterson et al., 2012). To determine if target gene expression in the craniofacial complex was influenced by the action of GLI co-regulators, we next examined the proximity of GBRs to motifs for potential GLI co-regulators.

Motif Analyses Identified Sequences for Known GLI Co-Regulators Frequently Co-Localize with GBRs

GBRs have previously been identified in close proximity to binding motifs for other transcription factors, including members of the bHLH, SP, and Sox families (Vokes et al., 2008; Peterson et al., 2012; Aberger and Ruiz I Altaba, 2014). Furthermore, the co-occupancy and cooperativity of GLI with SOX transcription factors was previously shown to be essential for activating neural gene expression signatures (Peterson et al., 2012). To determine if expression of our identified GLI targets could be influenced by the presence or absence of GLI co-regulators, we first examined the genomic sequence around the GBRs of our target genes for E-box, SP, and SOX binding motifs. (Figure 5, Supplemental Figure 3). We defined the area <1 kb upstream of the transcription start site (TSS) as the promoter region, <20 kb away from the TSS as proximal upstream or downstream, and <100 kb away from the TSS as distal upstream or downstream. We analyzed the sequence surrounding GBRs in our four selected GLI target genes for sequences predictive of E-box, SP, and SOX binding motifs. (Figures 5, Supplemental Figure 3). Several other clusters containing three of the four motifs were also identified (Figures 5A–C; red box). The close proximity of these binding motifs suggested that expression of GLI targets in the developing craniofacial complex could be influenced by the cooperative function of GLI isoforms and co-regulator proteins.

GLI Co-Regulators Have a Prominence Specific Expression Pattern That Changes When Primary Cilia Are Lost

Several transcription factors synergistically cooperate with GLI proteins to influence GLI target gene expression (Aberger and Ruiz I Altaba, 2014). Their co-occupancy at the promoter of GLI targets is required for the optimal activation/repressor. ChIP-based, high-throughput analyses uncovered several transcription factor motifs located close to GBRs in the cis-regulatory modules of GLI targets. Specifically, binding sequences for Sox (Peterson et al., 2012), bHLH (Lee et al., 2010), and SP proteins (Vokes et al., 2008) have been shown to exist in very close proximity
to GBRs. Our in silico analyses confirmed these sequences exist in near GBRs in four previously identified GLI-targets. We hypothesized that differential expression of these co-regulators could contribute to the differential gene expression of GLI targets in both control and ta<sup>2</sup> mutant embryos. We first investigated the expression of genes that could bind to motifs found in close proximity to GBRs, specifically SOX8, SP3, and HAND2 (Figures 6A–C). SOX8 expression was significantly reduced in the FNP and MXP of ta<sup>2</sup> mutants, yet was not significantly altered in the MNP (Figure 6A). SP3 expression was significantly increased in ta<sup>2</sup> MNP, yet not changed in the FNP and MXP (Figure 6B). HAND2 expression was reduced in the MXP, but not significantly changed between control and ta<sup>2</sup> FPNPs and MNPs (Figure 6C). Thus, differential expression of GLI co-regulators could possibly contribute to altered target gene expression in ta<sup>2</sup> mutants and explain why changes in GLI<sub>FL</sub> and GLI<sub>T</sub> isoforms do not uniformly or directly correlate with changes in target gene expression.

Our previous analyses indicate that GLI target gene expression changed in a prominence specific manner (Chang et al., 2014). We wondered if differential expression of potential GLI co-regulators could contribute to this prominence-specific expression changes. SOX8 was robustly expressed within the FNP, with levels significantly higher than those in the MXP or MNP (Figure 6A). SP3 was more robustly expressed in the MXP and MNP, relative to the FNP (Figure 6B). HAND2 was robustly and exclusively expressed in the MNP (Figure 6C). Thus, each of these potential co-regulators has a prominences specific expression pattern that could differentially influence GLI target gene expression. Collectively, the proximity of binding motifs, coupled with the differential expression of these co-regulators could possibly contribute to altered expression of GLI targets in ta<sup>2</sup> embryos.

**DISCUSSION**

Craniofacial ciliopathies are a rapidly growing group of disorders that severely impact craniofacial growth and development. Currently, there are little to no therapeutic options for these conditions. Although the molecular mechanism behind these disorders remains nebulous, many ciliopathies have aberrant production of GLI<sub>FL</sub> and GLI<sub>T</sub> isoforms. (Huangfu and Anderson, 2005; Davey et al., 2006; Tran et al., 2008; Tabler et al., 2013; Chang et al., 2014). Herein, we attempted to identify the mechanism by which aberrant GLI protein production impacts craniofacial development. To do so we used the avian ta<sup>2</sup> model, which has recently been characterized as a bona fide model for the human craniofacial ciliopathy Orofacial-digital syndrome 14 (Schock et al., 2015). In silico and ChIP assays identified GLI target genes in the avian genome that play a role in craniofacial development. RT-qPCR of mRNA levels verified some significant
changes in the expression of these GLI targets in the developing facial prominences, yet there was not a clear, linear relationship between changes in GLI isoform production and target gene expression. Motif cluster analysis supported the hypothesis that GLI proteins work in concert with co-regulators. GBRs associated with GLI target genes were found to be situated within 1000 bp of binding motifs for several, previously identified GLI co-regulators. Differential expression within developing facial prominences of predicted co-regulators supported a hypothesis in which expression of GLI target genes is dependent upon the cooperative function of GLI isoforms and co-regulator proteins. Together, these data provide a better understanding of the complex nature of GLI-mediated transcription that occurs in normal and ciliopathic craniofacial development.

GLI Binding Regions Are Present throughout the Avian Genome in Genes That Affect Craniofacial Development

Work in other species has identified GBRs and examined the role of GLI proteins as transcription factors that affect gene expression in numerous signaling pathways (Vokes et al., 2007, 2008).
Three models currently exist to explain GLI\textsubscript{FL}/GLI\textsubscript{T} function (Falkenstein and Vokes, 2014). First the GLI\textsubscript{FL}:GLI\textsubscript{T} ratio sensing model suggest that the relative levels of GLI\textsubscript{FL} to GLI\textsubscript{T} is integrated and results in graded levels of transcription of targets. This model suggests that response to changes in ratio, rather than concentration, affect target gene expression. The threshold activation model suggests that, rather than ratio of GLI\textsubscript{FL}:GLI\textsubscript{T}, a threshold-specific concentration of GLI\textsubscript{FL} activates target gene expression (Oosterveen et al., 2012). The threshold repression model depends on the removal of the GLI\textsubscript{T} repressor from target genes, allowing for transcriptional activation by other transcription factors to initiate gene expression. From our results, mRNA expression of most target genes was not dramatically altered despite excessive GLI\textsubscript{FL} production in ta\textsuperscript{2} mutant, indicating that the ratio sensing model cannot explain our observation in ta\textsuperscript{2} mutant. Secondly, the accumulated GLI3\textsubscript{FL} in the nucleus was expected to bind and activate target gene in the threshold activation model; however, instead we found reduced occupancy of GLI3 at target genes, along with lack of detectable GLI\textsubscript{FL} binding to GBRs (Figures 3, 4), suggesting that a dysfunctional GLI\textsubscript{FL} that fails to promote GLI\textsubscript{A}-mediated activation is produced under ciliopathic conditions. Thus, these data do not support the threshold activation model for GLI\textsubscript{FL} function. Conversely, our results showed that reduced GLI\textsubscript{T} production could lead to a de-repression of target genes and potentially a gene activation, when the required transcription...
factors are available. The threshold repression model seems to be more applicable to the observation owing to less GLI3 occupancy and the failure of GLI1FL to GLI1T conversion. A more in depth understanding of GLI mechanisms of action will require tools with the ability to definitively decipher between GLI1FL and GLI1T in vivo.

**Excessive GLI1FL Production Does Not Equate to Increased GLI1A Activity in Ciliary Mutants**

A number of disorders identified as ciliopathies have craniofacial abnormalities including Oral-facial-digital syndrome, Joubert syndrome, Bardet-Biedl syndrome, Meckel-Gruber syndrome, Ellis-van Creveld syndrome (Zaghoul and Brugmann, 2011). The phenotypes for syndromes such as these, while not identical, do have several phenotypes indicative of aberrant SHH signaling including widening of the midface, cleft lip/palate, micrognathia, craniosynostosis and oral/dental anomalies (Zaghoul and Brugmann, 2011). Increased production of GLI1FL isoforms has been observed in several ciliary mutants (Huangfu and Anderson, 2005; Liu et al., 2005; Humke et al., 2010), thus a common interpretation of these data is that ciliopathic phenotypes were due to either increased activator function or skewed GLI1FL/GLI1T ratio in favor of GLI1FL. Despite these common interpretation regarding the molecular mechanism causing ciliopathic phenotypes, examination of subsequent levels of GLI processing, binding, and transcriptional function in ciliary mutants has not been performed.

Our studies are among the first to examine how and if GLI proteins function in the developing craniofacial complex of ciliary mutants; however, some questions remain regarding the mechanistic reasons as to why GLI1FL appears not to function in ciliary mutants. Prior to processing, GLI proteins associate with Suppressor of Fused (SUFU), a conserved protein known to regulate the activity of GLI proteins via modulating GLI processing, stabilization and subcellular localization (Barnfield et al., 2005; Humke et al., 2010; Tukachinsky et al., 2010; Wang et al., 2010). In the presence of a SHH signal, the SUFU-GLI1FL complex traffics through the cilium (Eggerschwiler and Anderson, 2007). Activated ciliary SMO then works through KIF7 to promote the dissociation of the inhibitory SUFU-GLI1FL complex (Humke et al., 2010; Tukachinsky et al., 2010; Li et al., 2012). Free GLI1FL is then processed into an activator and moves to the nucleus to activate downstream targets. In the absence of the SHH ligand, SMO is not translocated into the cilium and thus cannot antagonize SUFU. SUFU remains in complex with GLI1T. GLI1FL is proteolytically processed into GLI1T and the SUFU-GLI1T complex moves to the nucleus where it recruits the Sapi18-Sin3 co-repressor complex to repress GLI target genes (Ding et al., 1999; Kogerman et al., 1999; Cheng and Bishop, 2002; Paces-Fessy et al., 2004). Furthermore, our recent work with the murine ciliary mutant, Kif3β/β;Wnt1-Cre shows increased SUFU production and nuclear localization, as well as enhanced association of SUFU with GLI3 (Chang et al., in press). We hypothesize a similar mechanism is at play in ta2 mutants. Specifically, excessive GLI1FL produced in ta2 mutants cannot dissociate from SUFU because the complex cannot undergo ciliary trafficking. We further hypothesize that lack of dissociation prevents GLI1FL from occupying GBRs and directly activating target gene transcription. Our future studies will address the association of GLI1FL and SUFU in ta2 mutants and determine if their maintained association contributes to ciliopathic phenotypes.

**GLI-Binding to Target Genes Occurs in a Prominence Specific Manner**

Frequently the craniofacial complex is thought of as a singular organ system; however, the prominences that make up the face develop independently prior to their fusion. There is evidence to support that these prominences have distinct molecular profiles and develop as separate developmental fields (Brugmann et al., 2007). Our ChIP-qPCR results detected differential GLI binding to target genes in facial prominences of control embryos (Figures 2B–D). Further, the expression of co-regulators also followed a prominence specific pattern in both control and ta2 embryos (Figures 6A–C). These data supported the hypothesis that during normal development, as well as when cilia are lost, each facial prominence uses a unique mechanism to transduce a SHH-dependent GLI signal. Based on our examination of co-regulators, we hypothesize that there is a combinatorial code of GLI isoforms and co-regulators that work together to precisely regulate target gene expression. Thus, when cilia are lost and GLI production is altered, target gene expression is dictated by how the combinatorial code of remaining GLI isoforms and co-regulators function together.

In sum, our data suggest that the increased or decreased production of GLI isoforms alone is not sufficient to explain how target gene expression will be altered. To understand the molecular mechanisms responsible for ciliopathic, GLI-mediated phenotypes, future studies will have to account for tissue specificity, the presence or absence of co-regulators and the mode of GLI function (activation or de-repression) to begin to address this process.

**AUTHOR CONTRIBUTIONS**

YC designed and tested all primers, and performed and interpreted all ChIP and qPCR experiments, PC performed *in silico* GBR analysis, ES harvested and photographed embryos, SB conceived the project, analyzed data and wrote the manuscript with input from all authors.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00468

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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