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Spatiotemporal regulation of GLI target genes in the mammalian limb bud

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ABSTRACT

GLI proteins convert Sonic hedgehog (Shh) signaling into a transcriptional output in a tissue-specific fashion. The *Shh* pathway has been extensively studied in the limb bud, where it helps regulate growth through a SHH–FGF feedback loop. However, the transcriptional response is still poorly understood. We addressed this by determining the gene expression patterns of approximately 200 candidate GLI-target genes and identified three discrete SHH-responsive expression domains. GLI-target genes expressed in the three domains are predominately regulated by derepression of GLI3 but have different temporal requirements for SHH. The GLI binding regions associated with these genes harbor both distinct and common DNA motifs. Given the potential for interaction between the SHH and FGF pathways, we also measured the response of GLI-target genes to inhibition of FGF signaling and found the majority were either unaffected or upregulated. These results provide the first characterization of the spatiotemporal response of a large group of GLI-target genes and lay the foundation for a systems-level understanding of the gene regulatory networks underlying SHH-mediated limb patterning.

1. Introduction

The Hedgehog (HH) signaling pathway regulates growth and patterning in multiple tissues in a variety of metazoan embryos (reviewed in Wilson and Chuang, 2010). Secreted HH ligands can spread over several cell diameters, eliciting both short and long-range effects (Chamberlain et al., 2008; Li et al., 2006; Nahmad and Stathopoulos, 2009; Sanders et al., 2013). HH-receiving cells respond by modulating the activity of the GLI transcription factors (GLI1-3, homologs of Ci in *Drosophila*). In the absence of HH ligand, GLIs are partially degraded by the proteasome, forming a truncated protein that functions as a transcriptional repressor (GLI-R). Conversely, in the presence of HH ligand, processing of GLI proteins is inhibited, permitting the formation of GLI activators (GLI-A) (Aza-blanc et al., 1997; Méthot and Basler, 1999; Pan et al., 2006; Wang et al., 2000).

GLI proteins activate or repress transcription of their target genes by binding to a similar sequence motif within a cis-regulatory module (CRM) (Hallikas and Taipale 2006; Peterson et al., 2012). Transcriptional responses to the HH pathway can be elicited either by de-repression of GLI-R or in other cases, by transcriptional activation through GLI-A (reviewed in Falkenstein and Vokes, 2014). Recent studies suggest that additional tissue-specific factors are necessary for activating appropriate GLI target genes (Biehs et al., 2010). In the neural tube, GLI-bound CRMs are enriched for Sox binding motifs, and SOX2 and SOXB1 proteins act as neural-specific GLI co-factors (Oosterveen et al., 2012; Oosterveen et al., 2013; Peterson et al., 2012). The mechanisms underlying transcriptional specificity in other HH-mediated developmental processes remain poorly understood. In several contexts, CRMs associated with GLI-target genes that are closest to the Hh signaling source have higher affinity Gli binding sites, while genes farther away are associated with CRMs that contain lower affinity Gli binding sites (Oosterveen et al., 2012; Parker et al., 2011; Peterson et al., 2012).

In the vertebrate limb bud, Sonic hedgehog (Shh) signaling regulates digit number and growth (Chiang et al., 1996; Towers et al., 2008; Zhu et al., 2008). The timing and duration of SHH is important for establishing polarity within the limb bud (Li et al., 2014a, 2014b; Zhulyn et al., 2014), and there is some evidence suggesting that cells retain a memory of their exposure to SHH (Harfe et al., 2004). In addition, studies have suggested that a relatively brief exposure to SHH specifies digit patterning, while longer exposures are needed for subsequent growth and

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expansion (Towers et al., 2008; Zhu et al., 2008). *Shh* expression in the limb bud is maintained by FGF proteins secreted from the apical ectodermal ridge (AER). *Shh* signaling regulates the transcription of the BMP inhibitor, *Gremlin 1* (*Grem1*) (Zuniga et al., 1999, Panman et al., 2006, Zuniga et al., 2012; Li et al., 2014a, 2014b; Vokes et al., 2008). GREM1 inhibits localized BMP activity, thereby maintaining the apical ectodermal ridge (AER). Together, these interactions comprise a signaling loop between the mesoderm and the AER that regulates limb growth and digit number (Khokha et al., 2003; Laufer et al., 1994; Litingtung et al., 2002; Michos et al., 2004; Niswander et al., 1994; Te Welscher et al., 2002; Verheyden and Sun, 2008; Zuniga, et al., 1999).

Here, we determine the expression patterns of a large set of predicted GLI-target genes in the mouse limb. Using this approach we find three distinct expression domains, which have different temporal SHH signaling requirements and are predominately regulated by derepression of GLI3-R. The GLI-bound CRMs associated with genes in each domain are enriched for both unique and common DNA motifs. Finally, we show that while some of these genes are downregulated when FGF signaling is inhibited, the majority of GLI-target genes are either unaffected or are upregulated. Collectively, these results provide the first characterization of the spatiotemporal response of a large candidate group of direct GLI-target genes that mediate SHH signaling in the limb bud.

2. Material and methods

2.1. Mice and ethics statement

Experiments involving mice were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin (protocol AUP-2013-00168).

2.2. Whole-mount in situ hybridization

Antisense probes (supplementary material Table S2) were generated from plasmids using PCR templates as described previously (Yu et al., 2012). in situ hybridization was performed on a minimum of two Swiss-Webster embryos per stage at E10.5 and E11.5 using an Intavis system as described previously (Yu et al., 2012).

2.3. qRT-PCR

RNA was extracted using TRIzol (Invitrogen). 300ng of DNAse I treated RNA was used to synthesize cDNA using random hexamers and SuperScript II (Invitrogen). Primers used in qRT-PCR experiments are listed in supplementary material Table S7.

2.4. Culturing limb buds

Mouse forelimbs were cultured for 15 hours as previously described (Panman et al., 2006; Zuniga et al., 1999). To inhibit HH signaling, limb buds were cultured in 10 μM cyclopamine (Toronto Research) or in 0.125% ethanol for controls. To inhibit FGF signaling, contralateral forelimbs were cultured in 10 μM SU5402 (Tocris) or 0.125% DMSO for controls.

2.5. $Shh^{-/-}$ forelimb RNA-seq

Heterozygous Shh^{tm1amc} mice (in previous generations mated to a Cre deleter strain to generate a null allele) (Dassule et al., 2000) were crossed, and E10.25 (33–35 somites) embryos were collected and genotyped for the wild-type and null allele. Forelimbs were collected and combined from three embryos of the same genotype, and RNA was isolated using TRIzol (Invitrogen) and treated with DNase I. Two biological replicates for each genotype were sequenced. The average $Shh^{-/-}$ somite number for replicate one was 34, and replicate two was 33.3. The average wild-type somite number for replicate one was 34, and replicate two was 33.7. Library construction was performed following Illumina manufacturer suggestions, and libraries were sequenced on the Illumina HiSeq platform using paired-end sequencing. Reads were aligned to the mouse reference genome mm10 using TopHat 2.0.9 (Trapnell et al., 2009) with default parameters and the option to incorporate genome annotation (parameter "*-G*"). Aligned reads were assigned to genes by HTSeq-count (Anders et al., 2010) using the default union- counting mode. Following HTSeq-count, edgeR 3.4.2 was used to conduct differential expression analysis ('classic' edgeR) (Robinson et al., 2010). Differentially expressed genes were identified based on an FDR of 0.05 and a mean fold change of 25% (Supplementary material Table S3). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE58222.

2.6. Cyclopamine treated wild-type forelimb RNA-seq

Wild-type E10.25 (31–34 somites) forelimb pairs from seven wild-type embryos were cultured for 15 hours in 10 μM cyclopamine (Toronto Research) or in 0.125% ethanol for controls. Immediately after the incubation period, limb buds were separated from the adjacent tissue and RNA was isolated using TRIzol (Invitrogen) and treated with DNase I. Two biological replicates for each culture condition were sequenced. The average somite number for wild-type controls was 32 for replicate one, and 32.6 for replicate two. The average somite number for wild-type samples treated with cyclopamine was 32 for replicate one, and 32.8 for replicate two. Library preparations were generated following ABI manufacturer suggestions, and libraries were sequenced on an ABI SOLiD platform using paired-end sequencing. Reads were aligned to the mouse reference genome mm10 using TopHat 2.0.9 (Trapnell et al., 2009) with default parameters and the option to incorporate genome annotation (parameter "−G"). Aligned reads were assigned to genes by HTSeq-count (Anders et al., 2014) using the default union-counting mode. Following HTSeq-count, edgeR 3.4.2 was used to conduct differential expression analysis ('classic' edgeR) (Robinson et al., 2010). Differentially expressed genes were identified based on an FDR of 0.05 and a mean fold change of 25% (Supplementary material Table S4). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE58222.

2.7. de novo motif discovery and Gli motif quality analysis

DNA motifs in GLI-bound CRMs were uncovered by a de novo motif discovery method. We mapped motif PWMs to GLI-bound CRMs in each category, background sequences were modeled as a third-order Markov chain (Ji et al., 2006). Then, we compared relative enrichment levels ($r1$) of the discovered motifs in high-quality binding regions versus matched control genomic regions. We chose a motif selection procedure to select enriched motifs by simultaneously requiring $r1 \geq 2$, number of motif sites (n1B) $\geq \max(1/5*(\text{number of genes}),5)$, motif score ≥ 1. We used the TOMTOM motif comparison tool to visualize their sequence logos with their PWMs as input. The quality of Gli motifs was assessed by using a Gli motif with the highest score, and then mapped the PWM of the Gli motif to GLI- bound CRMs within each category. The Gli matrix was compared to a third-order background Markov