ABSTRACT

The limb is widely used as a model developmental system and changes to gene expression patterns in its signaling centers, notably the zone of polarizing activity (ZPA) and the apical ectodermal ridge (AER), are known to cause limb malformations and evolutionary differences in limb morphology. Although several genes that define these limb signaling centers have been described, the identification of regulatory elements that are active within these centers has been limited. By dissecting mouse E11.5 limbs that fluorescently mark the ZPA or AER, followed by fluorescence-activated cell sorting and low-cell H3K27ac ChIP-seq, we identified thousands of specific signaling-center enhancers. Our ChIP-seq datasets show strong correlation with ZPA- and AER-expressed genes, previously characterized functional ZPA and AER enhancers and enrichment for relevant biological terms related to limb development and malformation for the neighboring genes. Using transgenic assays, we show that several of these sequences function as ZPA and AER enhancers. Our results identify novel ZPA and AER enhancers that could be important regulators of genes involved in the establishment of these specialized regions and the patterning of tetrapod limbs.

KEY WORDS: Enhancer, AER, ZPA, Limb, Mouse

INTRODUCTION

The tetrapod limb has long been used as a model of embryonic development in three dimensions. The limb bud begins as a small protrusion of cells from the flank of the embryo. As it develops, the limb bud must establish polarity along three axes: anterior-posterior (AP), proximal-distal (PD) and dorsal-ventral (DV). The AP axis is controlled by a small region in the posterior limb bud called the zone of polarizing activity (ZPA). These cells express sonic hedgehog (Shh), which acts as a morphogen to determine the AP axis. When Shh is expressed ectopically in anterior parts of the limb bud, the pattern of the AP axis is disrupted. The most common phenotype caused by anterior ‘ZPA activity’ is the formation of extra digits at the anterior of the limb or the adoption of posterior identity by anterior digits (reviewed by Anderson et al. (2012)). The PD axis is maintained by the apical ectodermal ridge (AER), which develops from the ectoderm at the distal edge of the limb bud. Without a functioning AER, the growth of the limb is severely stunted. There is significant crosstalk between the ZPA and AER, with each signaling center required for the maintenance of the other [reviewed by Zeller et al. (2009)].

Gene expression changes in signaling centers are known to affect limb morphology. As the genes involved in the ZPA and AER are also expressed in other tissues, mutations in coding regions usually cause additional phenotypes. Mutations in enhancers that are specific to these limb-signaling centers, however, only cause limb phenotypes. For example, mutations in the ZPA regulatory sequence (ZRS) enhancer that controls expression of Shh in the ZPA are known to cause isolated limb malformations in humans, mice, cats and dogs, without any other phenotypes caused by coding disruptions to Shh [reviewed by VanderMeer and Ahituv (2011)]. Another study showed that replacing the mouse Prxl limb enhancer with the bat homologous enhancer leads to longer bones in the mature limb, but no other phenotypes (Creteskos et al., 2008).

The identification of enhancers in the ZPA and AER is challenging. Cells in these signaling centers form only ~4% of the developing limb. Whereas previous studies have used chromatin immunoprecipitation followed by sequencing (ChIP-seq) on histone marks (Cotney et al., 2012, 2013) or E1A binding protein p300 (EP300/p300) (Visel et al., 2009), these address the limb as a single tissue. Because the ZPA and AER have functions distinct from the larger limb mesenchyme, it is likely that they have distinct epigenetic signatures that would be diluted in such an experiment. Tellingly, not a single one of the twenty p300 ChIP-seq peaks that were positive for limb enhancer activity (Visel et al., 2009) were expressed specifically in the ZPA or AER.

To identify active enhancers specific to signaling centers, it is necessary to study these tissues independently. We therefore bred mouse lines that endogenously express GFP in either the ZPA or AER to isolate the cells of each signaling center using fluorescence-activated cell sorting (FACS). We then used ChIP-seq to identify regions with the epigenetic signature H3K27ac, which is associated with active enhancers (Creighton et al., 2012, 2013). Subsequent mouse transgenic enhancer assays on selected ZPA and AER ChIP-seq peaks found them to be active in these signaling centers. Through this work we have identified two novel sets of signaling center-specific enhancers which can play important roles in limb development and morphology.

RESULTS

We bred transgenic mouse lines that express GFP in the ZPA or AER. For the ZPA, we used Shh-GFP-cre mice (Harfe et al., 2004) that have a cassette containing an in-frame fusion between GFP and cre inserted at the ATG of the mouse Shh gene, leading to specific GFP expression in the ZPA (supplementary material Fig. S1A). For the AER, we crossed Msx2-cre mice that expresses cre specifically in the AER under the control of the Msx2 promoter (Sun et al., 2000) to GNZ (ROSAS26-nGFP) mice that contain aloxP-flanked STOP cassette followed by a GFP/β-galactosidase fusion protein sequence with an SV40 nuclear localization signal (Stoller et al., 2008), thus
allowing for AER-specific GFP expression (supplementary material Fig. S1B). Embryos were harvested at E11.5, and ZPA or AER cells were isolated from dissected limbs by FACS. Similar to previous measurements (Rock et al., 2007), the ZPA and AER cells each comprised 2-4% of the total cells of the limb. These cells were subjected to ChIP-seq with an antibody for H3K27ac (Fig. 1A).

In the ZPA, we identified 14,571 H3K27ac peaks (supplementary material Table S1). Of these, 45% were at promoters [defined as \(-2500 \text{ and } +500 \text{ bp from the transcription start site (TSS) based on UCSC known gene annotations}]), 4% in exons (excluding exons that overlap the promoter region) and 51% in intergenic or intronic regions (Fig. 1B). In the AER, we identified 4936 peaks (supplementary material Table S1), 57% of which were located at gene promoters, 8% in exons and 35% in intergenic or intronic regions (Fig. 1B). We next compared these peaks with 208 functionally validated limb enhancers from the VISTA Enhancer Browser (Visel et al., 2007). As a negative control, we used a set of 281 forebrain enhancers with no limb expression. We observed a significant overlap of our peaks with the VISTA limb set versus the forebrain (\(P<0.0001\); Fisher’s exact test, one-tailed) for both the ZPA and AER (Fig. 1C). Of the 208 VISTA limb enhancers, we identified 11 with ZPA/posterior expression and 18 with AER/ectoderm expression. Our ZPA and AER ChIP-seq peaks overlap more with the validated enhancers in their respective tissue, but the number of enhancers was too low for statistical significance.

We used GREAT (McLean et al., 2010) to identify biological functions associated with the ZPA and AER peaks. Numerous limb-associated genes reside near our ChIP-seq peaks (supplementary material Table S1).
material Table S2). In both datasets, we observed a significant enrichment for terms related to limb development and malformation phenotypes (Fig. 1D; supplementary material Table S2). The ZPA includes mouse and human phenotypic terms related to polydactyly and abnormal digit patterning, which are common effects of ZPA disruptions. The AER terms include ‘abnormal limb bud morphology’, ‘aplasia of the nails’ and ‘brachydactyly’, all of which are phenotypes associated with AER defects. Limb development and malformation phenotype terms are also enriched when using only non-TSS peaks (supplementary material Fig. S2; Tables S1 and S2), showing that this enrichment is not due to promoter binding. We also examined gene loci known to be highly expressed in the ZPA or AER and observed a correlation with H3K27ac peak presence. As examples, we observed a specific H3K27ac peak for Shh and its enhancer ZRS in the ZPA and for engrailed homeobox 1 (En1) in the AER, whereas Alx4, a gene expressed specifically in the anterior mesenchyme, did not have an H3K27ac signal in either tissue (Fig. 2).

To identify ZPA and AER-specific enhancers, we selected peaks that did not overlap with the promoters of known genes or with previously published E11.5 whole-limb H3K27ac and p300 ChIP-seq datasets (Cotney et al., 2012; Visel et al., 2009). The resulting lists contain 1233 ZPA-specific and 715 AER-specific peaks (Fig. 3A). To functionally validate these peaks, we tested five candidates from each signaling center for enhancer activity in mice, prioritizing enhancers near genes involved in limb development. These genes include transcription factors, signal receptors and genes involved in cilia transport that are required for evolutionary changes in limb morphology and human congenital limb malformations. We have previously demonstrated that replacing the mouse Prx1 limb enhancer with the bat homologous enhancer can lead to longer bones (Cretekos et al., 2008), and our dataset of ZPA and AER enhancers can provide a unique resource to identify additional limb enhancers that could have led to limb morphological differences. Human limb malformations are the second-most common form of birth defect, with a frequency of nearly 1 in 500 live births (Moore and Persaud, 1998). Some of these occur as part of a syndrome; however, limb defects are also seen in isolation as the only phenotype in an individual and have been found to be caused by enhancer mutations (Dathe et al., 2009; Kurth et al., 2009; Lettice et al., 2003). An enhancer that regulates the ZPA or AER could be a site where mutations result in limb defects. Our ZPA and AER ChIP-seq peaks thus provide specific candidate sequences in the genome that, when mutated, can lead to morphological differences between species and limb malformations and can be used to guide mutation analyses between organisms and in individuals with isolated limb malformations.

Previous whole-limb approaches have failed to identify enhancers that are active in these important limb development signaling centers, as seen by in vivo results (Visel et al., 2009), possibly because the cells of these regions comprise only a small proportion of the limb tissue. By investigating these specific tissues, our study provides a novel resource of putative ZPA and AER enhancers. The rate of in vivo enhancer validation for this dataset was low, particularly in the ZPA. This could be due to the stringency of our peak selection. By eliminating peaks in our dataset that had any overlap to a region called as a peak in the whole-limb set, we probably eliminated enhancers that are biologically specific to the ZPA or AER, but produced a signal that was identifiable in the whole-limb set. These appear to include some of the strongest enhancers because we find that the distribution of peak heights in the signaling center-specific sets is lower than the distribution for any overlap to a region called as a peak in the whole-limb set, we probably eliminated enhancers that are biologically specific to the ZPA or AER, but produced a signal that was identifiable in the whole-limb set. These appear to include some of the strongest enhancers because we find that the distribution of peak heights in the signaling center-specific sets is lower than the distribution for...
ZPA-specific expression of *Shh*, is called as a peak in the whole-limb H3K27ac dataset under some parameters (Cotney et al., 2012).

In summary, using FACS followed by low-cell ChIP-seq on ZPA and AER cells, we identified thousands of potential limb signaling center peaks. We observed a strong correlation between known ZPA- and AER-specific gene loci and H3K27ac peak prevalence. We also observed a strong correlation with fitting biological terms and limb-associated genes using GREAT (McLean et al., 2010) (supplementary material Table S2). These ZPA- and AER-specific regions represent a set of enhancers active in regions that determine limb growth and patterning and can help inform our understanding of tetrapod limb development, evolutionary differences in limb morphology and the genetic etiology of human limb malformations.

**MATERIALS AND METHODS**

**Tissue collection**

All mouse work was approved by the UCSF institutional animal care and use committee.

To isolate cells from the ZPA and AER, transgenic mouse lines were established that express GFP in one of these regions. For the ZPA, we used the *Shh*tm1(EFPU-cre)F5 line (Harfe et al., 2004). Heterozygous males were mated to CD-1 females and GFP-positive embryos were used for tissue collection. For the AER, homozygous male tg(Msx2-cre)5Rem mice (Sun et al., 2000) were mated to females homozygous for a floxed reporter B6.129-GT(ROSA)26Sortm1joe (Stoller et al., 2008). All mice were backcrossed onto a CD-1 strain to address any variation of embryo size between lines.

Embryos were collected at E11.5 from timed matings. For the ZPA, embryos were examined briefly with a dissecting fluorescent microscope to select those carrying the GFP allele. Both forelimbs and hindlimbs were collected and crosslinked with 1% formaldehyde for 10 min at room temperature, followed by quenching with glycine and rinsing with cold PBS. Tissue was homogenized with a Dounce tissue grinder (Kontes) to a single-cell suspension for FACS. Cells were kept on ice in 0.5 µM EDTA and 0.05% BSA in PBS for sorting as previously described (Rock et al., 2007) and sorted on a FACSAria II (BD Biosciences). GFP-positive cells represented ~2-4% of the limb tissue for both ZPA and AER. Cells were collected in the sorting buffer, pelleted by centrifugation and flash-frozen to ~80°C.

**ChIP-seq**

ChIP was performed using the LowCell#ChIP kit (Diagenode) with modified wash conditions (see methods in the supplementary material). Cells from dissected limbs from an estimated 20–35 embryos were pooled to give ~70,000 cells per IP; they were sonicated with a Covaris sonicator (S220 Focused-ultrasonicator, Covaris), and 30 µl of sheared chromatin was used for each ChIP. The antibody anti-acetyl histone H3 (Lys27) clone (S220 Focused-ultrasonicator, Covaris), and 30 µl of sheared chromatin was used for each ChIP. The antibody anti-acetyl histone H3 (Lys27) clone (S220 Focused-ultrasonicator, Covaris), and 30 µl of sheared chromatin was used for each ChIP.

ChIP-seq data from this study is available in SRA (http://www.ncbi.nlm.nih.gov/sra) (SRA experiment: SRX597164; AER: SRR1393727; ZPA: SRR1393728).

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

J.E.V. and N.A. designed experiments. J.E.V. and S.J. performed experiments. J.E.V. and R.P.S. analyzed data. J.E.V. wrote the manuscript with contributions from N.A., S.J. and R.P.S.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110965/-/DC1

**References**


