Coding exons function as tissue-specific enhancers of nearby genes

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Enhancers are essential gene regulatory elements whose alteration can lead to morphological differences between species, developmental abnormalities, and human disease. Current strategies to identify enhancers focus primarily on noncoding sequences and tend to exclude protein coding sequences. Here, we analyzed 25 available ChIP-seq data sets that identify enhancers in an unbiased manner (H3K4me1, H3K27ac, and EP300) for peaks that overlap exons. We find that, on average, 7% of all ChIP-seq peaks overlap coding exons (after excluding for peaks that overlap with first exons). By using mouse and zebrafish enhancer assays, we demonstrate that several of these exonic enhancers (eExons) can function as enhancers of their neighboring genes and that the exonic sequence is necessary for enhancer activity. Using ChIP, 3C, and DNA FISH, we further show that one of these exonic limb enhancers, Dync1i1 exon 15, has active enhancer marks and physically interacts with Dlx5/6 promoter regions 900 kb away. In addition, its removal by chromosomal abnormalities in humans could cause split hand and foot malformation I (SHFM1), a disorder associated with Dlx5/6. These results demonstrate that DNA sequences can have a dual function, operating as coding exons in one tissue and enhancers of nearby gene(s) in another tissue, suggesting that phenotypes resulting from coding mutations could be caused not only by protein alteration but also by disrupting the regulation of another gene.

[Supplemental material is available for this article.]

Precise temporal, spatial, and quantitative regulation of gene expression is essential for proper development. This tight transcriptional regulation is mediated in part by DNA sequences called enhancers, which regulate gene promoters. By use of comparative genomics or chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq), candidate enhancer sequences can now be identified in a relatively high-throughput manner (Heintzman and Ren 2009; Visel et al. 2009b). These sequences can then be assayed for enhancer activity using various in vitro and in vivo assays (Woolfe et al. 2005; Pennacchio et al. 2006; Heintzman et al. 2009). However, the majority of these experiments remove coding sequences from their analyses under the assumption that they do not function as enhancers, due to their protein coding role.

Previous exonic enhancers (eExons) have been reported in vertebrates (Neznanov et al. 1997; Lampe et al. 2008; Tumpel et al. 2008; Dong et al. 2010; Eichenlaub and Ettwiller 2011; Ritter et al. 2012). In addition, a recent study scanning for synonymous constraint in protein coding regions (Lin et al. 2011) found an overlap between two of these eExons (Lampe et al. 2008; Tumpel et al. 2008) and synonymous constraint elements. Here, we analyzed 25 available ChIP-seq data sets of enhancer marks (H3K4me1, H3K27ac, and EP300), also known as p300) for their overlap with coding exons. Following this analysis, we wanted to specifically determine whether eExons could regulate their neighboring genes and not the gene they reside in. This was of interest to us due to the phenotypic implications that coding mutations could have on nearby genes. For this purpose, we analyzed a specific EP300 ChIP-seq data set from mouse embryonic day 11.5 limb tissue (Visel et al. 2009a), due to its ability to identify active enhancers with high accuracy (88%) and tissue specificity (80%) in vivo. At E11.5, mouse limb development progresses along three axes: proximal-distal (P-D), anterior-posterior (A-P), and dorsal-ventral (D-V). Specific signaling centers in the limb bud create gradients and feedback loops that determine these axes (Gilbert 2000; Nissim and Tabin 2004; Zeller et al. 2009), and their alteration could lead to morphological differences. In this study, we focused on identifying limb eExons involved in the development along both the P-D and the A-P axes.
The apical ectodermal ridge (AER) is the signaling center that keeps the underlying mesenchyme in a proliferative state and allows the limb to grow, thus governing the P-D axis. In the developing mouse limb bud, the distal-less homeobox 5 and 6 (DLx5/6) genes are expressed in the AER (Fig. 1D, E), and DLx5 is also expressed in the anterior mesenchyme (Fig. 1E). Disruption of both DLx5 and DLx6 in mice leads to a split hand and foot malformation (SHFM) phenotype (Robledo et al. 2002). In humans, chromosomal aberrations in the DLX5/6 region, some of which do not encompass the coding sequences of DLX5/6, cause SHFM1 (MIM 183600) and are associated with incomplete penetrance and suggest the need to be cautious when assigning a coding mutation phenotype to protein function.

Results

Exon overlap analyses of enhancer-associated ChIP-seq data sets

To determine the genome-wide prevalence of enhancer-associated ChIP-seq peaks that overlap coding exons, we analyzed 25 available ChIP-seq data sets of enhancer marks (H3K4me1, H3K27ac, and EP300) from various human cell lines and mouse E11.5 tissues (Supplemental Table 1; see Methods). Since these enhancer marks could also identify potential promoters, we only looked for overlap with coding exons after excluding for the first exon. In all the analyses described in this study, coding exons are defined here as only those exons that are not first exons. Analysis of the individual histone marks, H3K4me1 and H3K27ac, showed that 7% and 10% of all peaks overlap coding exons, respectively (Supplemental Table 1). It is worth noting that the average peak size in the H3K4me1 and H3K27ac ChIP-seq data sets is 2441 and 3107 bp, and the average size of peaks overlapping coding exons is 3476 and 4195 bp for each mark. Analysis of the average exon size in the human genome shows that it is ~280 bp (see Methods). Compared with the average peak size of the histone marks and those that overlap exons in particular, it is quite possible that the functional entity of the peak does not constitute the exon and that the percentages above are an overestimate. Therefore, we analyzed six different EP300 ChIP-seq data sets from various human cell lines that have shorter peak sizes, the average of which was 426 bp. In these data sets, we found that on average, 4% of the peaks overlapped with coding exons (Supplemental Table 1). To get a better indication if these sequences could be functional enhancers, we examined the overlap between exonic EP300 ChIP-seq peaks and H3K4me1 and H3K27ac peaks. We used ChIP-seq data from two different cell lines, GM12878 and K562, where all three enhancer marks were available. We found that 8% and 5% of the ChIP-seq peaks that had all three enhancer marks overlapped coding exons in GM12878 and K562 cells, respectively (Supplemental Table 1). We next screened coding sequences that had all three enhancer marks for their overlap with a recently published study that scanned the genome for synonymous constraint in protein coding regions (Lin et al. 2011). We found that 9% of coding exons with all three ChIP-seq enhancer marks overlapped with synonymous constraint exons for both GM12878 and K562 cell lines (Sup...
In summary, we found that on average, 7% of the peaks in the 25 enhancer-associated ChIP-seq data sets that we analyzed overlapped coding exons after removing first exons.

**Analysis of a EP300 limb ChIP-seq data set and eExon candidate selection**

Given that several eExons were previously discovered to regulate the gene they reside in (Neznanov et al. 1997; Lampe et al. 2008; Tumpel et al. 2008; Ritter et al. 2012), we explicitly set out to search for coding eExons that do not autoregulate but rather could regulate their nearby genes. This was of interest to us due to the phenotypic consequences that coding mutations could have on their nearby genes. In order to do this, we needed a tissue-specific ChIP-seq enhancer data set. We thus focused our analysis on EP300 ChIP-seq data sets that were shown to predict functional enhancers in three different mouse E11.5 tissues (forebrain, midbrain, limb) with high accuracy and tissue specificity (Visel et al. 2009a). In this data set, we observed that 4% of EP300 ChIP-seq peaks from all three tissues overlapped with coding exons after excluding the first exon (Supplemental Table 1). These lower percentages could be due to experimental differences such as cell line versus tissue. For our functional assays, we next focused on the limb EP300 ChIP-seq data set. We scanned this data set for exonic sequences that reside in genes that are not known to be expressed in the limb but are located in the vicinity (up to 1 Mb on either side) of known limb-associated genes (see Methods). From the 252 limb EP300 ChIP-seq peaks that overlaid exons, 152 sequences overlapped coding exons and 134 were in a gene that is not expressed in the limb (Supplemental Table 3). Out of those 134 sequences, 90 had at least one limb expressed gene up to 1 Mb away on either side of the gene. We chose seven exons near important limb developmental genes (C14orf49 exon 16 [near Dicer1], CDC14B exon 13 [near Ptch1], Dync1i1 exon 15 and exon 17 [near Dlx5/6], Hdac9 exon 18 and exon 19 [near Twist1], Stx18 exons 4–5 [near Msx1]) for subsequent mouse enhancer assays (Supplemental Table 3).

**Mouse enhancer assays**

To test whether these exonic sequences function as enhancers, we tested all seven sequences for their enhancer activity in mice. The human sequences were cloned into the Hsp68-LacZ vector that contains the heat shock protein 68 minimal promoter followed by a LacZ reporter gene (Kothary et al. 1988). Transgenic mice were generated, and embryos were harvested at E11.5 and stained for LacZ. We found that four out of the seven sequences showed limb enhancer activity in mice. *Dync1i1* exon 15 drove specific LacZ expression in the limb mesenchyme and AER (Fig. 1B, B’; Supplemental Fig. 1A), and *Dync1i1* exon 17 drove specific LacZ expression in the anterior limb mesenchyme (Fig. 1C, C’; Supplemental Fig. 1C). *Hdac9* exon 18 showed enhancer activity in the anterior limb bud (Fig. 1G, G’; Supplemental Fig. 2A) and *Hdac9* exon 19 in the posterior limb bud (Fig. 1H, H’; Supplemental Fig. 2B).

**The exonic sequence is necessary for enhancer activity**

The sequences tested in the mouse enhancer assays had some intronic regions due to the ChIP-seq peak overlapping part of the intron (Supplemental Table 3). We thus wanted to assess whether the exonic sequence is necessary for enhancer activity. Since human limb and zebrafish fin development are considered highly comparable on the molecular level (Hall 2007; Iovine 2007; Mercader 2007) and since zebrafish enhancer assays are rapid and cost-efficient, we carried out a deletion series analyses using this assay. We first characterized whether our functional mouse limb enhancers were positive for fin enhancer expression in zebrafish. The four limb enhancers (Supplemental Table 3) were cloned from human genomic DNA into a zebrafish enhancer assay vector, containing an E1b minimal promoter followed by the green fluorescent protein (GFP) reporter gene (Li et al. 2009). These vectors were microinjected into one-cell-stage zebrafish embryos along with the Tol2 transposase to facilitate genomic integration. GFP expression was monitored at 48 and 72 h post-fertilization (hpf), both time points when the pectoral fin can be observed. Two of our four functional mouse limb enhancers, *Dync1i1* exon 15 and Hdac9 exon 19, were found to be functional fin enhancers in zebrafish (Supplemental Table 4). At 72 hpf, *Dync1i1* exon 15 drove GFP expression in the pectoral fin, caudal fin, and somitic muscles (Supplemental Fig. 1B), and *Hdac9* exon 19 exhibited enhancer activity in the pectoral fin and branchial arch (Supplemental Fig. 2C).

In order to determine whether the actual exonic sequences are necessary for enhancer activity, we used these two fin enhancers, *Dync1i1* exon 15 and Hdac9 exon 19, for deletion series analyses. *Dync1i1* exon 15 was divided into three segments—S’ intron, exon, and S’ intron (Fig. 2A)—and Hdac9 exon 19 was divided into the following segments: S’ distal intron, S’ proximal intron, and exon (Fig. 2C). We found that in both cases the exon and S’ intron sequence adjacent to the exon had lower enhancer activity by themselves, but when combined, their enhancer activity was substantially increased and comparable to that of the previously injected longer version of *Dync1i1* exon 15 and Hdac9 exon 19, respectively (Fig. 2B, D). These results demonstrate that the exonic sequences are necessary but not sufficient for full enhancer activity.

**Limb genes associated with eExons enhancer function**

In order to identify the limb expressed genes that could be regulated by our characterized eExons, we analyzed the RNA expression of nearby genes and carried out synteny block analysis (Ahituv et al. 2005). Whole-mount in situ hybridization of neighboring genes found that Dlx5/6 have similar limb expression patterns to *Dync1i1* eExons 15 and 17 (Fig. 2B–E; Supplemental Figs. 1, 2), and *Twist1* has a limb expression pattern that is similar to HDAC9 eExons 18 and 19 (Fig. 1G–I; Supplemental Figs. 2, 3). We also extracted RNA from E11.5 mouse limbs and adult mouse cortex and heart and performed quantitative PCR (qPCR) to validate the tissue specific expression of these genes. Dlx5, Dlx6, and *Twist1* were expressed in E11.5 limbs (Supplemental Fig. 3E, G). However, *Dync1i1* and *Hdac9* were not detected in mouse E11.5 limbs but expressed in the mouse adult cortex and heart, respectively (Supplemental Fig. 3E, G). In addition, examination of the genomic location of *Dync1i1*-DLX5/6 and HDAC9-*Twist1* in various vertebrate genomes shows that they remain adjacent to each other from human to fish (Supplemental Fig. 4). Based on a previous analysis (Ahituv et al. 2005), the human–mouse–chicken *Dync1i1*-DLX5/6 block is 1.37 Mb in size and the *HDAC9-TWIST1* is 2.52 Mb, both above the 1.02 Mb average length (N50) of a human–mouse–chicken synteny block in that study. These results further suggest that these eExons could be important for DLX5/6 and *Twist1* regulation.

**Dync1i1** eExon 15 is marked in the limb by an enhancer chromatin signature

To examine the dual role of these DNA sequences, we chose *Dync1i1* eExon 15 for further functional analysis. We analyzed this eExon
for histone modification signatures during limb development. We carried out ChIP followed by qPCR (ChIP-qPCR) on Dync1i1 eExon 15 for enhancer (H3K4me1, H3K27ac), promoter (H3K4me3), and transcribed gene (H3K36me3) chromatin signatures (Hon et al. 2009). We found that in the mouse E11.5 limb, Dync1i1 eExon 15 is marked by H3K4me1 and H3K27ac (Fig. 3B,C) but not by H3K4me3 or H3K36me3 (Fig. 3D,E). In contrast, Dync1i1 eExon 6 was not marked by H3K4me1 or H3K27ac (Fig. 3B,C) in the limb, and Dlx5/6 coding exons were marked by H3K36me3 (Fig. 3E). Thus, the chromatin status correlates with the proposed limb enhancer activity of Dync1i1 eExon 15.

3C and DNA FISH show that Dync1i1 eExon 15 physically interacts with the promoter regions of Dlx5/6

To determine whether Dync1i1 eExon 15 physically interacts with the Dlx5/6 promoter regions, we carried out 3C on mouse E11.5 heart and limb (AER enriched; see Methods) tissues. The mouse heart tissue served as a negative control, as Dlx5/6 are not expressed in the heart during that stage (Fig. 1D,E). We observed an increased interaction frequency between Dync1i1 eExon 15 and the Dlx5/6 promoters in the limb tissue compared with the heart, indicating a physical interaction between them in the limb (Fig. 4B). These results suggest that Dync1i1 eExon 15 functions as an enhancer in the AER through enhancer–promoter DNA looping.

To further analyze the chromosomal conformation around the Dlx5/6 locus during limb development, we performed DNA FISH using Dlx5/6 and Dync1i1 eExon 15 probes on mouse E11.5 limb buds and heart. After capturing images of the two fluorescent signals, the physical distance between Dync1i1 eExon 15 and the Dlx5/6 coding region was calculated (Fig. 4C–J). Frequency distribution patterns of the physical distance between Dync1i1 eExon 15 and Dlx5/6 for the AER compared with the heart were measured (Fig. 4K,L). In the AER, 35% of the Dync1i1 eExon 15 signals were in close proximity to the Dlx5/6 signals (<0.2 μm) (Fig. 4K), with a mean distance of 0.32 ± 0.06 μm. In contrast, the frequency of colocализed signals in the heart was greatly reduced (12%; P < 0.01, t-test) (Fig. 4L), and the overall frequency of separated signals was higher compared to the AER.
These results show that Dync1i1 eExon 15 is in close proximity with Dlx5/6 promoter regions in the developing AER at E11.5, supporting its proposed role as an enhancer during limb development.

Human chromosomal aberrations encompassing DYNC1I1 eExons 15 and 17 are associated with SHFM1

To test whether alterations of DYNC1I1 eExon 15 and 17 could be associated with a limb phenotype, we analyzed available individuals and previously reported cases with SHFM1 (Fig. 5). We mapped a family (GK) with SHFM1 that has a 46,XY; t(7;20)(q22;p13) translocation (Fig. 5). In addition, we mapped the inversion breakpoints of a previously published SHFM1 family (Jaekels-Horne et al. 2001) to be within chr 7: 96,219,611 and 109,486,136 (K6200 family) (Everman et al. 2005; Everman et al. 2006). In addition, we referred to two recently reported SHFM1 cases: an individual with SHFM1 who has a de novo pericentric inversion of chromosome 7: 46, XY, inv(7) (p22q21.3), with the breakpoint mapped to chr 7: 95.53–95.72 Mb (van Silfhout et al. 2009), and another individual with a split foot phenotype who has an 880-kb microdeletion of 95.39–96.27Mb (Fig. 5; Kouwenhoven et al. 2010). It is worth noting that an AER enhancer named BS1 was recently identified 300 kb centromeric to DLXS/6 (Kouwenhoven et al. 2010). However, at least two individuals with SHFM1 that are described here have chromosomal aberrations that do not include BS1 (Fig. 5), suggesting that additional limb enhancers, such as DYNC1I1 eExon 15 and 17, could lead to SHFM1. All of the chromosomal abnormalities described above overlap DYNC1I1 eExon 15 and 17 and suggest that their removal could disrupt the transcriptional regulation of DLXS/6 and be one of the causes of these human limb malformations.

Discussion

Studies aimed at discovering gene regulatory elements usually concentrate on noncoding DNA sequences as potential candidates and ignore coding sequences. However, several studies have shown that protein coding sequences may have additional encrypted information in their sequence (Chamary et al. 2006; Itzkovitz and Alon 2007; Lin et al. 2011). Here, by analyzing ChIP-seq data sets for enhancer marks from various cell lines and tissues, we found that on average 7% of peaks overlap with coding exons after excluding the first exon. With only ~1.6% of the human or mouse genomes encoding for protein, eExons could be overrepresented in these ChIP-seq enhancer-associated data sets. To test whether exons are enriched in ChIP-seq data sets, we generated a random data set from all mappable sequences that are used for any whole-genome sequencing alignment from the UCSC Genome Browser (http://moma.ki.au.dk/genome-mirror/cgi-bin/hgTrackUi?hgsid=148&c=chrX&g=wgEncodeMapability) containing an identical number of peaks as in the EP300 ChIP-seq data sets of GM12787 and K562 (51,260 and 17,883 peaks, respectively) and tested how many peaks overlap coding exons compared to the ChIP-seq data sets. We found a significantly higher percentage of peaks overlapping with coding exons (after excluding the first exon) in the EP300 ChIP-seq data sets of both GM12787 (P < 0.014; Fisher exact test) and K562 (P < 8.44 × 10−3; Fisher exact test) cell lines. In addition, using a random sampling approach, we randomly sampled 1000 peaks (from all mappable sequences) having an equal distribution to that of the two EP300 ChIP-seq data sets 1000 times and found that a significantly higher fraction of peaks overlapped coding exons in the ChIP-seq data sets versus our random samples (P < 2.2 × 10−16). Combined, these assays suggest an overrepresentation of
coding exons in ChIP-seq data sets. However, it is worth noting that the technical variability of the ChIP-seq assay due to differences in antibodies, cross-linking, pull down, sequencing depth, and others along with sequence mappability are not taken into account in these analyses.

Using a mouse transgenic enhancer assay for seven mouse E11.5 limb EP300 ChIP-seq peaks, we show that four eExons are functional limb enhancers and could regulate their neighboring genes. The observed 57% (4/7) success rate does not imply that

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mations such as lissencephaly, subcortical laminar heterotopias, and pervasive developmental disorder—not otherwise specified (PDD-NOS) (Kato and Dobyns 2003). Interestingly, an individual with PDD-NOS and SHFM1 has an inversion in the region (chr 7: 95.53–95.72 Mb) (Fig. 5), whose breakpoint has not been finely characterized (van Silfhout et al. 2009). Further analysis would be required in order to establish whether the PDD-NOS and SHFM phenotypes in this individual could be due to the disruption of both the DYNC111 gene and our characterized eExons.

Two other characterized limb enhancers, HDAC9 eExon 18 and 19, reside in the coding exons of HDAC9, a member of the histone acetyltransferase class II family. HDAC9 eExon 19 has also been shown to be an exonic remnant in zebrafish and speculated to have a cis-regulatory function (Dong et al. 2010). Both HDAC9 eExons 18 and eExon 19 appear in 3/9 HDAC9 splice isoforms (RefSeq: NM_178423.1, NM_058176.2, NM_178425.2). HDAC9 expression was shown to be more selective compared with that of other HDAC family members (de Ruijter et al. 2003). Our RNA analysis and whole-mount in situ hybridization results show that Hdad9 is not expressed in the mouse limb at E11.5 (Supplemental Fig. 3D,G). Hdad9-null mice generated by deletion of exons 4 and 5 are fertile and survive a normal life span but develop cardiac hypertrophy with age and in response to pressure overload (Zhang et al. 2002). Interestingly, despite Hdad9 not being expressed in the limb at E11.5, Hdad9 homozygous knockout mice develop polydactyly in their hindlimbs with partial penetrance (Morison and D’Mello 2008), similar to the polydactyly phenotype of Twist1 heterozygous knockout mice (Bourgeois et al. 1998). Although Hdad9 eExons 18 or 19 were not removed in these Hdad9-null mice, the regulation of Twist1 by these and other potential Twist1 enhancers could be disrupted leading to the polydactyly phenotype.

The ability of eExons to enhance the expression of their nearby genes, but not the gene they reside in, could suggest that mechanisms such as those involved in epigenetic regulation and high-order chromatin organization might control their function in each tissue. To our knowledge, the functional demonstration that DNA sequences can act as a protein coding sequence in one tissue but regulate the expression of a nearby gene/s in another tissue is novel. It raises the possibility that mutations in a certain gene, even synonymous ones, could potentially affect the regulation of a nearby gene. Therefore, careful analysis of the tissue-specific expression and function of a gene would be required in order to determine whether a phenotype is truly caused by a mutation within its coding sequence.

Methods

Computational ChIP-seq data set analyses

We identified exonic sequences in the human hg18 and mouse mm9 genome assemblies using the UCSC knownGene track (http://genome.ucsc.edu). We downloaded all exonic sequences, including 5’ UTR and 3’ UTR, using the txStrat and txEnd filter field. All exon sequence sizes were divided by the number of exons to calculate the average exon size. We downloaded coding exon sequences using the cdsStart and cdsEnd filter field. The 22 ChIP-seq data sets of human cell lines were obtained from Ernst et al. (2011), Myers et al. (2011), and Rosenbloom et al. (2012) and were downloaded from the UCSC Genome Browser, and the three EP300 ChIP-seq data sets of mouse E11.5 tissues were obtained from Visel et al. (2009a) and downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) (Supplemental Table 1 includes links for all downloaded data). In order to unify our results, human sequences with hg19 coordinates were converted to hg18 using the UCSC Genome Browser LiftOver tool. A ChIP-seq peak was considered to overlap an exon if at least 1 bp of exon sequence overlapped. BED files of all the ChIP-seq peaks that overlap exons in the various data sets can be obtained at http://bts.ucsf.edu/ahituv/resources.html. First exons for all splice isoforms of a gene were determined by the exonStarts exon Ends field in the UCSC knownGene track. To identify limb expressed genes, we used available mouse RNA in situ data from the Mouse Genome Informatics (MGI) gene expression data query form (http://www.informatics.jax.org/javawi2/servlet/WIFetch?query conforms to expressionQF and defined a limb expressing gene as one having RNA in situ expression data at either TS19 (E11.0–12.25) or TS20 (E11.5–13.0).

Transgenic enhancer assays

By use of primers designed to amplify the EP300 ChIP-seq peaks that overlap exons (Supplemental Table S5), we carried out polymerase chain reaction (PCR) on human genomic DNA (Qiagen). Primers were designed to have up to 500 bp additional sequence flanking the EP300 peak. Previous experiments have shown this to be a reliable method for obtaining positive enhancer activity when using evolutionary conserved regions (Pennacchio et al. 2006) and EP300 ChIP-seq peaks (Visel et al. 2009a). For the mouse enhancer assays, PCR products of the human genomic regions were cloned into a vector containing the Hsp68 minimal promoter followed by the LacZ reporter gene (Pennacchio et al. 2006) and sequence verified. Transgenic mice were generated by the UCSF transgenic facility and by Cyagen Biosciences using standard procedures (Nagy et al. 2002). Embryos were harvested at E11.5 and stained for LacZ expression as previously described (Pennacchio et al. 2006). For the zebrafish enhancer assays, expression and function of a gene would be required in order to determine whether a phenotype is truly caused by a mutation within its coding sequence.
the same human PCR products were cloned into the E1b-GFP-Tol2 enhancer assay vector containing an E1b minimal promoter followed by GFP (Li et al. 2009). They were injected into blastocyst stage embryos of an internal control template. Data from AER and heart were corrected by dividing the average of three PCR signals by the average signal in the control. Each data point was first corrected for PCR bias by dividing the average of three PCR signals by the average signal of a control template. Data from AER and heart were corrected by dividing the average of three PCR signals by the average signal of a control template.

Whole-mount in situ hybridization

Mouse E11.5 embryos were fixed in 4% paraformaldehyde. Clones containing mouse Dync1li (MM1013-9202215, Open Biosystems), Dlx5 (Depew et al. 1999), Dlx6 (OMMS5895-99863403 Open Biosystems), Hdac9 (EMM1052-601163 and EMM1002-6974502, Open Biosystems), and Twist1 (Chen and Behringer 1995) were used as templates for digoxigenin-labeled probes. Whole-mount in situ hybridizations were performed according to standard procedures (Hargrave et al. 2006).

DNA expression analysis

Mouse E11.5 limb and AER enriched tissues (limb buds where the AER region was carefully dissected) and adult mouse heart and cortex tissues were dissected. Total RNA was isolated using RNeasy (Qiagen) according to the manufacturer’s protocol. qPCR was performed using SsoFast EvaGreen Supermix (BioRad) and run on the Eppendorf Mastercycler ep realplex 2 thermal cycler. Samples were tested in triplicate. Specificity and absence of primer dimers was controlled by denaturation curves. β-Actin (Actb) mRNA was used for normalization. Primer sequences used for amplification are listed in Supplemental Table 5.

ChIP followed by qPCR

ChIP following standard techniques (Nelson et al. 2006) was performed on mouse E11.5 AEEnriched tissue. For each ChIP, 100–500 mg of chromatin was used. For immunoprecipitation, we used 2 μg of H3K4me1 (ab8895, Abcam), H3K4me3 (ab8580, Abcam), H3K27ac (ab4729, Abcam), and H3K36me3 (ab9050; Abcam) antibodies. qPCR was carried out using SsoFast EvaGreen Supermix (Biorad) and run on the Eppendorf Mastercycler ep realplex 2 thermal cycler. ChIP-qPCR signals were standardized to input chromatin (percentage of input). Primer sequences used for amplification are listed in Supplemental Table 5.

3C assay

3C was performed following standard procedures (Dostie and Dekker 2007). Mouse E11.5 heart and AER enriched tissues were dissected from 30 embryos, cross-linked with 1% formaldehyde, and adult mouse heart and AER enriched tissues (limb buds where the AER region was carefully dissected), and adult mouse heart and cortex tissues were dissected. Total RNA was isolated using RNeasy (Qiagen) according to the manufacturer’s protocol. qPCR was performed using SsoFast EvaGreen Supermix (BioRad) and run on the Eppendorf Mastercycler ep realplex 2 thermal cycler. Samples were tested in triplicate. Specificity and absence of primer dimers was controlled by denaturation curves. β-Actin (Actb) mRNA was used for normalization. Primer sequences used for amplification are listed in Supplemental Table 5. Each data point was first corrected for PCR bias by dividing the average of three PCR signals by the average signal of an internal control template. Data from AER and heart were normalized to a BAC library containing seven BACs obtained from the CHORI BACPAC resource center covering the SHFM1 minimal region (RP23-340G21, RP23-73K21, RP23-336F10, RP23-389M11, RP23-343G1, RP23-270A16).

DNA florescence in situ hybridization

DNA florescence in situ hybridization (ISH) was carried out as previously described (Lomvardas et al. 2006). BAC clones RP23-7703 for Dlx5/6 and RP23-430G21 for Dyncl11 were obtained from the CHORI BACPAC resource center. Probes were labeled with Digoxigenin-11-dUTP or Biotin-16-dUTP by Nick Translation (Roche). Limb or heart tissues (E11.5) were embedded without fixation, and 10 μm cryosections were collected on Superfrost Plus slides (Fisher). After drying, sections were fixed in 4% PFA for 5 min at 4°C. DNA was fragmented by incubation with 0.1 M HCl for 5 min at room temperature, and slides were treated with RNase A for 1 h at 37°C. Slides were dried by an ethanol series, denatured in a solution of 75% formamide in 2×SSC for 5 min at 85°C, rinsed immediately in ice-cold 2×SSC, and dried again by 4°C ethanol series. Pre-denatured, Cot-annealed probes were applied overnight. The probe was washed three times for 15 min in 55% formamide, 0.1% NP-40 in 2×SSC at 42°C. Probes were detected using Dylight 488 anti-digoxigenin and Dylight 549 anti-biotin (Jackson Immunoresearch). Antibody washes were carried out in a solution of PBS containing 0.1% Triton-X-100 and 8% formamide at room temperature. All images were obtained using confocal fluorescence microscopy (Nikon C1 Spectra). FISH signals were recorded in three separate RGB channels. The image stacks were reconstructed using the Velocity program (PerkinElmer), and the shortest distance between the gravity centers of the Dlx5/6 and Dyncl11 signals was calculated.

Subjects and chromosomal breakpoint mapping

The GK family consisted of a male who had ectrodactyly, micrognathia, an elongated neck, and bilateral microtia with neurosensory deafness and his female offspring who died before birth and had ectrodactyly, micrognathia, and bilateral microtia. Karyotypes of the father and his offspring demonstrated a reciprocal balanced chromosomal translocation 46,XY,t(7;20)(q22;p13) that was not found in GK’s healthy mother. By use of FISH, following standard techniques (Trask 1991), with two BACs (RP11-94N7, RP11-78B12), the breakpoint coordinates at chromosome 7 were mapped to be between 96.2 and 96.47 Mb. The K6200 family had autosomal dominant SHFM and variable sensorineural hearing loss as previously reported (Tackels-Horne et al. 2001). Subsequent studies of this family by pulse field gel electrophoresis and FISH identified a chromosome inversion with breakpoints in the SHFM1 critical region (Everman et al. 2005; Everman et al. 2006). Southern blot analysis and inverse PCR as previously described (Vervoort et al. 2002) were then used to identify the inversion breakpoints (D.B. Everman, C.T. Morgan, M.E. Laughridge, T. Moss, S. Ladd, B. DuPont, D. Toms, A. Dobson, K.D. Clarkson, F. Gurrieri, et al., unpublished). The inversion in this family was balanced, with minimal changes in the normal sequence at each breakpoint and segregated with the SHFM/hearing loss phenotype.

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