

Published in final edited form as:

Dev Biol. 2014 July 15; 391(2): 133–146. doi:10.1016/j.ydbio.2014.04.016.

A Gene Expression Atlas of Early Craniofacial Development

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Abstract

We present a gene expression atlas of early mouse craniofacial development. Laser capture microdissection (LCM) was used to isolate cells from the principal critical micro-regions, whose development, differentiation and signaling interactions are responsible for the construction of the mammalian face. At E8.5, as migrating neural crest cells begin to exit the neural fold/epidermal ectoderm boundary, we examined the cranial mesenchyme, composed of mixed neural crest and paraxial mesoderm cells, as well as cells from adjacent neuroepithelium. At E9.5 cells from the cranial mesenchyme, overlying olfactory placode/epidermal ectoderm, and underlying neuroepithelium, as well as the emerging mandibular and maxillary arches were sampled. At E10.5, as the facial prominences form, cells from the medial and lateral prominences, the olfactory pit, multiple discrete regions of underlying neuroepithelium, the mandibular and maxillary arches, including both their mesenchymal and ectodermal components, as well as Rathke's pouch, were similarly sampled and profiled using both microarray and RNA-seq technologies. Further, we performed single cell studies to better define the gene expression states of the early E8.5 pioneer neural crest cells and paraxial mesoderm. Taken together, and analyzable by a variety of biological network approaches, these data provide a complementing and cross-validating resource capable of fueling discovery of novel compartment specific markers and signatures whose combinatorial interactions of transcription factors and growth factors/receptors are responsible for providing the master genetic blueprint for craniofacial development.

Keywords

mammalian craniofacial development; gene expression atlas; RNA-seq; microarrays

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Introduction

An atlas of gene expression profiles can provide a valuable resource for the research community. A prime example is the Allen Brain Atlas, which was initiated in 2003 to create a comprehensive expression dataset to advance fundamental discovery into brain function (Lein et al., 2007). Hundreds of thousands of *in situ* hybridizations were carried out to define gene expression patterns in the developing and adult mouse brain, human brain, and mouse spinal cord.

The GenitoUrinary Molecular Anatomy Project (GUDMAP.ORG) provides another example of a gene expression atlas (Harding et al., 2011; McMahon et al., 2008). A few thousand *in situ* hybridizations were carried out. In addition, however, the diverse compartments of the kidney were gene expression profiled using a combination of laser capture microdissection (LCM) and microarrays (Brunskill et al., 2008) as well as RNA-seq (Brunskill et al., 2011a; Brunskill et al., 2011b; Brunskill and Potter, 2010; Brunskill et al., 2011c). The results define the changing waves of gene expression as the kidney progenitor cells progress through the different stages of nephrogenesis.

The FACEBASE consortium was established by NIH to provide a resource for the craniofacial research community (Hochheiser et al., 2011). One purpose of this consortium is to generate a gene expression atlas of mouse craniofacial development. In this report we describe the results of an extensive LCM/microarray/RNA-seq analysis of the gene expression patterns of early mouse craniofacial development, during E8.5, E9.5 and E10.5. At each developmental stage the multiple craniofacial compartments were isolated by LCM and gene expression patterns characterized by microarray and RNA-seq. The results define the gene expression blueprint of early craniofacial development. All growth factor, receptor and transcription factor domains of expression are defined. Novel compartment specific molecular markers are identified. In addition the RNA-seq data defines RNA splice patterns and provides a comprehensive catalog of noncoding transcripts, including those derived from enhancers. In summary, this is an extensive gene expression compendium meant to augment craniofacial research.

Materials and Methods

LCM protocols

In brief, embryos were rapidly collected from CD1 outbred mice (Charles River) with the day of vaginal plug designated E0.5. Embryos were flash frozen in O.C.T (Sakura Finetek) with liquid nitrogen cooled isopentane. Cryostat sections were made and processed, and LCM was carried out with an Arcturus Veritas machine, with membrane slides and using a combination of UV cutting and IR capture lasers as previously described (Potter and Brunskill, 2012). For a typical sample approximately 10–30 LCM collected tissue sections were pooled for analysis. Detailed protocols, with representative LCM images, are available at <https://www.facebase.org/node/154>.

RNA Purifications and Amplifications

RNA was purified using the ZR RNA MicroPrep kit (Zymo). Nugen RiboSpia Ovation Pico WTA System V2 was used for target amplification for RNA-seq. For microarrays we used the SCAMP method previously described (Brunskill et al., 2011c). RNA-seq was carried out using 50b single end reads on the Illumina Hi-Seq 2500 machine according to Illumina protocols, with read depths > 40 million. For microarrays we examined a minimum of three biological replicates, and commonly four to six. Over one hundred microarrays in total were used. Exact replicate numbers are shown in heatmaps and at FaceBase.Org. For RNA-seq single samples were analyzed.

Single Cell

Single, neural-crest cells were isolated from the cranial mesenchyme using *WNT1CRE-Rosa26GFP* reporter mice. The cranial mesenchyme and overlying epidermal ectoderm were separated from the neural fold, transferred to an eppendorf tube, digested with 0.05% trypsin for 5 min and the digestion stopped with ice-cold 1% FBS/PBS. Under a fluorescent microscope GFP-positive cells neural crest cells, representing neural crest, and GFP-negative paraxial mesoderm and epidermal ectoderm cells, were isolated using pulled glass pipettes. Each cell was serially transferred through several petri dishes to confirm that only a single-cell was isolated. The cell was then transferred to an eppendorf tube containing lysis buffer and quick-frozen on dry-ice for later SCAMP amplification and microarray analysis.

Data Analysis

Microarray and RNA-seq data were analyzed by a combination of Bowtie, Tophat, and GeneSpring versions 7.3.1 and 12.6 software. A standard workflow for RNA-seq analysis included removal of probesets that did not map uniquely to the mm9 ENSEMBL genome or ENSEMBL-annotated genes. For the microarray samples, probesets were selected whose RMA-estimated expression level was at least 6.2 in at least one sample. Differentially expressed genes were identified using both unpaired t-tests for pairwise comparisons and one way ANOVA for multiple compartment comparisons ($FDR < 0.05$), with subsequent fold-change of 2–5 fold depending on the comparisons so as to optimize the identification of expression signatures for each compartment. RNA-seq BAM files generated using the Bowtie-Tophat2 pipeline were analyzed for the expression of known and unknown genes/transcripts using both Cufflinks2 and GeneSpring 12.6. Workflows included filtering to remove duplicate reads, and those with post-aligned read metrics mapping quality below 40, as well as the exclusion of samples that exhibited strong outlier 3'/5' read distribution ratios. Transcript/isoform and gene summarized expression tables were filtered to identify entities whose expression was at least 3 FPKM (Cufflinks) or 3 nRPKM (GeneSpring) in at least one sample. Differentially expressed gene signatures were identified using Audic Claverie tests ($P < 0.05$) and Welch t-tests ($FDR < 0.05$) followed by a two to five fold change requirements. Gene Ontology and other enrichment and biological network analysis was carried out with GeneSpring, ToppGene (<http://toppgene.cchmc.org>), and ToppCluster (<http://toppcluster.cchmc.org/>) yielding both similar and complementary results.

Biological replicate microarray Pearson correlation coefficients were generally in the range of 0.93 to 0.99. For example, at E10.5, we observed for the lateral nasal prominence (0.944,

0.937, 0.962), medial nasal prominence (0.950, 0.950, 0.943), olfactory pit (0.966, 0.956, 0.955), mandibular arch (0.943, 0.947, 0.978), and maxillary arch (0.976, 0.992, 0.972).

Data is available on the FaceBase.Org website and in GEO under superset GSE55967, which includes individual data series GSE55964, GSE55965, and GSE55966 for ST1.0 single cell samples, LCM microarray, and LCM RNA-seq samples, respectively.

Generating a Global Facebase Datamine

In order to establish integrated, comprehensive, mineable, and community-useful tables and maps of gene expression patterns across the series of cell types, regions and developmental stages that were profiled in this project, we followed a strategy that allowed us to generate two large normalized data matrix files (one being RNA-seq, the other Affymetrix GeneChip ST1.0) of all the measured values in each sample in the study. Prior to “baselining”, these normalized “raw” values correspond to the estimated expression level of each gene, transcript, or probeset as measured using either technology. The RNA-seq values are FPKM values, and the microarray are based on the RMA normalization approach. These absolute expression value tables were then baselined to a global median expression value.

The RMA microarray data algorithm provides a log₂ based relative intensity value for each probeset for each microarray. Our baseline referencing approach for these RMA values was to convert these into relative ratios per sample using the transform of $\text{Intensity} = 2^{\text{RMA}}$, and then to define the ratio of each gene/probeset’s expression relative to its median expression value across all samples in the dataset. Thus, the relative expression profile for a given gene is relative to this baseline. The data submitted to GEO described above includes the global normalized value data matrices that we used in these analyses. For the FPKM-based RNA-seq data, we carried out a similar strategy, but used a denominator for relative expression that was the ratio of FPKM per sample versus the global median of (FPKM+1) so as to define the lowest level of log₂FPKM = 0. Thus, the final normalized and baselined expression values represent a ratio of expression in each sample relative to the median of that gene or transcript across the sample series.

To define compartment-specific gene lists, we subjected the two expression tables to a “shredding protocol” in which a series of expression signatures per sample type were generated using relative expression rank cutoffs of 100, 250, 500 and 1000 transcripts per compartment. Additional sub-signatures from these parent lists were then generated by subjecting each gene list to K-means clustering using 5 Pearson-correlation-based K-groups, thus providing gene sets that are both highly ranked for a given compartment, and similarly expressed across the other samples in the datasets when they belong to the same K-means cluster (note that different K-means clusters have different numbers of genes per cluster). For each list of mouse genes, Toppgene only lists those that have an NCBI Homologene-mapped human gene ortholog. This generates a reference signature database composed of the series of genelists that were then placed into the ToppGene (<http://toppgene.cchmc.org>) and ToppCluster (<http://toppcluster.cchmc.org>) web site data analysis resources. Each of the genelists from this “shredding procedure” are named according to a scheme that generates descriptors such as “Facebase RNA-seq E10.5 Mandibular Arch 500”, or “Facebase ST1 E10.5 ColumnEpith Mandib 500 #4”, where the first number refers to the number of genes

in the relative expression-ranked list, and the second number, if present, would refer to the K-means cluster to which it belonged. In this way, any single gene of interest can be queried through Toppgene to see if appears in one of these top-ranked gene lists. After a user inspects the names of the genelists that contain it, one or more of those lists can be downloaded or directly re-analyzed for its functional associations and enrichments with respect to all Gene Ontologies, pathways, protein-protein interactions, and known mouse and human gene knockout phenotypes as per normal analysis protocols of ToppGene and ToppCluster3 (Chen et al., 2009; Kaimal et al., 2010). By our registering the data from the present studies into Toppgene data tables, we thereby enable the analysis of a list of genes submitted by user as a query to be compared to the Facebase datasets themselves. The goal of our enabling this approach is to provide a flexible and focused analysis resource for the craniofacial research community to detect compartments and study a group of genesets that have a high likelihood of being coexpressed in specific developing craniofacial compartments. And then by using connections provided by all of the other Toppgene data features (such as mouse knockout phenotypes, gene ontology terms, protein-protein interactions, etc.) Facebase gene expression signatures can be analyzed for their relationships to known or putative functional, mechanistic, and interactions-based features, allowing for new hypotheses to be formulated.

Results and Discussion

E8.5 gene expression profiles

The neural crest cells are a multipotent and migratory cell population that will give rise to several lineages of the developing face, including melanocytes, cartilage, bone, smooth muscle and neurons (Bush and Jiang, 2012; Chai and Maxson, 2006; Kulesa et al., 2010; Szabo-Rogers et al., 2010). At around E8.5 the first neural crest cells depart the neural fold/epidermal ectoderm boundary and migrate as streams between the outer epidermal ectoderm, and the neural fold. The neural crest cells then join with resident paraxial mesoderm cells. To better understand this initial stage of craniofacial development we defined gene expression profiles of several compartments, including the mesenchyme (neural crest and paraxial mesoderm), dorsal neural fold, ventral neural fold (including the floor plate region), as well as the total caudal neural fold region (Fig. 1). A total of 18 samples were analyzed using Affymetrix mouse Gene 1.0 ST arrays, providing four or five biological replicates per compartment.

The mesenchyme cells are of particular interest, as they construct the major components of the face, while the other compartments examined are primarily of interest because of their possible function in signaling to the flanking neural crest and paraxial mesoderm cells. The mesenchyme gene expression state at E8.5 was first compared to the dorsal neural fold, yielding a list of 331 upregulated genes (Table S1). These include genes up-regulated as cells from the dorsal neural fold undergo epithelial to mesenchymal transformation and generate the neural crest. Gene ontologies analysis of these 331 genes identified the top ranked molecular function as sequence specific DNA binding, with 28 transcription factor genes, including *Snai1*, *Snai2*, *Foxc1*, *Foxc2*, *Foxf2*, *Foxd3*, *Alx1*, *Prrx1*, *Prrx2*, *Bcl2*, *Irx3*, *Irx5*, *Msx1*, *Msx2*, *Sp5*, *Etv2*, *Hhex*, *Six1*, *Six2*, *Nr2f1*, *Lmo2*, *Sox10*, *Nfil3*, *Atf3*, *Nkx6-1* and

Pitx2. Many of these represent previously identified neural crest markers. The data suggest that these encoded transcription factors combine to determine the early mesenchyme gene expression state. Key biological processes identified included vascular development (57 associated genes), mesenchyme development (23 associated genes), and neural crest development (11 genes) (Table S2).

Of the 331 genes called by microarrays to be more abundantly expressed in mesenchyme, 312 were annotated genes with assigned gene symbols. 92% of these (all but 26) were validated as significantly ($P < 0.05$) changed in expression by independent RNA-seq analysis (Table S1). All but 40 of these also showed greater than two-fold change in expression with RNA-seq. These two independent technologies therefore showed quite good agreement for the genes called differently expressed.

Of interest, however, RNA-Seq called many more genes differentially expressed between mesenchyme and dorsal neural fold, with 1849 showing more than two fold change. It has been commonly observed that RNA-seq gives more genes called differently expressed than microarrays, and this is often attributed to the lower background with RNA-seq, which improves the signal to noise ratio (Wang et al., 2009). Arrays give a resulting fold change compression compared to RNA-seq. It has been observed that RNA-seq with a four fold change cutoff can identify as many differentially expressed genes as microarrays with a much less stringent two fold change (Raghavachari et al., 2012). A large scale comparison of RNA-seq and microarray technologies, however, found that in general they show excellent concordance, producing comparable results, consistent with what we observed (Guo et al., 2013).

Several of the genes showing the greatest fold change by RNA-seq, however, were not even called differently expressed by microarrays. For example the *Ddr2* gene gave 2,542 raw reads for mesenchyme, and only one read for dorsal neural fold, with a fold change of 707 called by RNA-seq, while this gene was not called differently expressed by array. Similarly, *Pear1* (nRPKM of 10.6 vs zero), *Fzd10* (nRPKM of 13.8 versus zero) and *Ebf2* (nRPKM of 22.6 vs 0.06), were not detected as differently expressed by microarray. This failure could reflect the different amplification technologies used (SCAMP for microarray versus Nugen RiboSpia for RNA-seq), and/or imperfections in microarray design.

Therefore, the independent dual technology analyses used in this study, with both microarray and RNA-seq, does provide a high throughput cross validation, with the RNA-seq independent technology typically confirming about 90% of the genes called differently expressed by arrays. The RNA-seq dataset, however, provides more accurate fold change calls, identifies a greater number of differently expressed genes, examines gene expression in an unbiased manner that does not depend on array design, and defines RNA splicing patterns (Wang et al., 2009).

The RNA-seq data showed an interesting co-expression of 5' flanking non-spliced sequences for a number of genes, including *Six1* (Fig. 2). At E8.5 these *Six1* 5' flanking transcripts were restricted to the mesenchymal neural crest/paraxial mesoderm, but at both E9.5 and E10.5 the *Six1* gene and its 5' transcripts were widely expressed, in all compartments

assayed. These 5' transcripts represent candidate enhancer RNAs, since it is now established that many enhancers are transcribed, giving rise to short transcripts (Core et al., 2008; Hah et al., 2011; Kim et al., 2010; Wang et al., 2011). Further, recent results indicate that these enhancer transcripts can be functional (Lam et al., 2013; Li et al., 2013; Melo et al., 2013). Because we carried out the RNA-seq using a combined dT plus random primer reverse transcription reaction we detected nonpolyadenylated transcripts from “non-canonical” regions, which do not correspond to exons of the reference genome. It is interesting to note that *Six1/Eya1* have been shown to play an important role in craniofacial development as upstream regulators of FGF8 signaling (Guo et al., 2011).

As mentioned, the cranial neural crest cells first migrate in close apposition to the dorsal neural fold, which may secrete important growth factor signals. Interesting signaling candidates identified included *Fgf8*, and *Wnt8b*, as well as *Bmp5*, which was made by both the flanking neuroepithelium and the mesenchyme cells themselves, suggesting possible paracrine and autocrine functions.

The transcription factor expression code of the dorsal neuroepithelium at E8.5 is of particular interest, as this includes the region of origin of neural crest cells. We identified 70 transcription factors with elevated expression in comparison to the ventral region of the neuroepithelium (Table S3). These included a number of genes with previously established expression in this zone, including *Sp8* and *Dlx5*, providing positive control historic validation of the dataset.

As the neural crest cells continue to migrate they pass by the ventral region of the neuroepithelium, and again are exposed to flanking growth factors, in this case including SHH, BMP2 and BMP4, FGFs 7,11, 14, 15, and 18, as well as TGF β 1 and 2. In addition the RNA-seq data defined a transcription factor code of the ventral floor plate region, with 144 transcription factors expressed at significantly higher levels (>2FC) compared to the more dorsal neuroepithelium (Table S4).

The E8.5 mesenchymal RNA-seq gene signature was then analyzed using a screen that required a minimum 2 FC enrichment of transcripts against all other E8.5 compartments profiled, yielding a list of 704 genes (Table S5). This more stringent analysis of the RNA-seq dataset identified a total of 83 transcription factors defining the E8.5 mesenchyme (neural crest plus paraxial mesoderm) transcription factor code (Table S6), a number significantly larger than the 28 mentioned previously, defined with microarrays and using a less stringent screen. This transcription factor code for E8.5 mesenchyme included *Alx1,4*, *Ebf1,2*, the *Fox* genes *c1, c2, d2, d3, f2, p1, p2*, *Gata2*, *Lhx6*, *Msx1*, *Myocd*, *Nkx3-1*, *Pax9*, *Pitx2*, *Six1*, *Snai1*, *Sox7,9,10,17,18*, *Stat3,6*, *Twist1,2*, and many others.

E9.5 expression data

At E9.5 we used LCM to isolate six separate spatial compartments. These included the cranial mesenchyme (CM), as well as the underlying neuroepithelium (NE) and overlying epidermal ectoderm (EE) (Fig. 1). The mandibular and maxillary arches, as well as otic vesicles were also captured and gene expression profiling was carried out with Affymetrix

mouse gene 1.0 ST arrays. In addition we gene expression profiled the cranial mesenchyme, overlying ectoderm, mandibular arch, and maxillary arch using RNA-seq.

We first analyzed the microarray data, removing probesets with only low expression levels, performing oneway ANOVA ($P < 0.05$), and requiring $FC > 2$ compared to other regions sampled. For example, when the cranial mesenchyme gene expression pattern was compared to mandibular arch, maxillary arch, neuroepithelium, overlying ectoderm and otic vesicle, 26 cranial mesenchyme compartment enriched genes were identified (Table S7). It is interesting to note that two of these comparison compartments, mandibular arch and maxillary arch, are also primarily made up of neural crest cells. The similarities in cell type likely partially explain the relative paucity of genes with restricted expression. This is also in part the result of microarray technology, with more differences found by RNA-seq, as described later. The list of cranial mesenchyme enriched transcription factors included *Alx1*, *Alx3*, *Batf3*, *Six2* and *Sox8* (Table S7).

The overlying ectoderm, including primarily but not exclusively the olfactory placode, showed elevated expression of 68 genes, when compared to all other E9.5 compartments, excepting otic vesicle. These cells signal to the underlying cranial mesenchyme, and subsequently invaginate to form the olfactory pit. The enriched gene transcripts encoded ALDH1a3, thought to play an important role in establishing retinoic acid gradients, as well as the growth factors BMP4, PDGFA and PDGFC. Enriched transcription factor genes included *Isl1*, *Pax6*, *Pitx1*, and *Six3*. In addition there were a number of claudins (3,4,6,7,9), keratins (8,14,18,19), and Epcam, all associated with the epithelial nature of these cells. For a complete list see (Table S8).

The mandibular arch, when compared to cranial mesenchyme, overlying ectoderm, neuroepithelium and otic vesicle, gave 232 genes with enriched transcripts. A series graph of these enriched genes shows that the expression patterns for the mandibular and maxillary arches were quite similar, with genes showing elevated expression in one generally also showing elevated expression in the other (Fig. 3). Five genes gave particularly robust elevated expression in these two compartments, *Barx1*, *Emcn*, *Lhx8*, *Mab2111* and *Kcne3*. *Barx1* and *Lhx8* encode homeobox transcription factors. Endomucin (*Emcn*) interferes with the assembly of focal adhesion complexes and inhibits interaction between cells and the extracellular matrix (Kinoshita et al., 2001). *Mab2111* is closely related to the *mab21* gene of *C. elegans*, which has been shown to play a critical role in multiple cell fate decisions, with mutants showing posterior to anterior homeotic transformations (Chow et al., 1995). *Kcne3* encodes a voltage gated ion channel. For a complete list of mandibular/maxillary arch enriched gene transcripts see (Table S9).

The underlying neuroepithelium notably expressed *Fgf15*, *Fgf17*, *Fgf18*, and *Wnt1*, as well as the chemokine *Ccl19*, which could all provide important signals to the flanking cranial mesenchyme (Table S10).

A heatmap of the E9.5 compartment specific gene lists is shown in Fig. 4. This was generated with GeneSpring software, using Pearson's centered distance metric and Ward's linkage rule. The mandibular arch and maxillary arch profiles cluster together, reflecting

their very similar gene expression profiles. The otic vesicle and olfactory placode/overlying ectoderm also show strongly overlapping gene expression patterns, as might be expected given their common epidermal ectodermal origin. In contrast the cranial mesenchyme and underlying neuroepithelium produced lists of genes with more distinct expression patterns. The reproducibility was generally excellent, with some expected variability due to the sampling differences resulting from random sets of LCM sections through the compartments of interest.

E9.5 RNA-seq

As noted previously, E9.5 craniofacial gene expression patterns were also examined with RNA-seq. Cranial mesenchyme, mandibular arch, maxillary arch, and overlying ectoderm, including the olfactory placode, were profiled. Although representing a more limited set than examined with microarray, these are the compartments that directly construct the face.

Once again, as for E8.5, the RNA-seq analysis provided an independent high throughput technology for validation of the microarray data. For example, in comparing the microarray and RNA-seq cranial mesenchyme data, 13/26 genes called enriched by arrays were similarly called differently expressed by RNA-Seq. Another 5/26 genes were also called differently expressed by RNA-seq, but with low expression levels ($nRPKM < 3$), therefore not making our normal expression threshold cutoff. Other genes showed the same directional change, but did not quite make the two fold change cutoff. In the end only three of the 26 genes showed a complete failure of validation, with either no transcripts detected by RNA-seq (one gene), or no corresponding directional change in expression observed (two genes). So, as for the E8.5 gene expression data, about 90% of genes called differently expressed by microarray were validated in significant measure by the RNA-seq data.

The RNA-seq data again produced lists with more differently expressed genes, and with generally greater fold changes than seen with microarrays. For example 1,009 genes were called cranial mesenchyme enriched (greater than two fold change compared to other compartments)(Table S11). A Gene Ontologies analysis using ToppGene gave a very strong protein synthesis signature, with many of the top molecular functions and biological processes relating to ribosomes, translation, and protein targeting to the ER (Table S12). Growth factors expressed included midkine, neurturin, and colony stimulating factor1. The list of cranial mesenchyme enriched transcription factors was expanded compared to that seen with arrays, now including *Alx1*, *Alx3*, *Alx4*, *Btf3*, *Rarb*, *Shox2*, *Six2*, *Sox7* and *Sox8*. For the complete list see (Table S11).

A similar RNA-seq analysis was carried out to find genes with enriched expression in the overlying ectoderm, including the olfactory placode. A list of 1270 genes emerged (Table S13). The gene with the greatest fold transcript enrichment was *Sp8*, which encodes a zinc finger transcription factor, previously shown to be highly expressed in the olfactory placode (Zembrzycki et al., 2007), and previously shown to be a key regulator of craniofacial development (Kasberg et al., 2013), providing historic validation of the dataset. The epidermal ectoderm was clearly a rich source of growth factors, including BMPs 2,4,5,7,8b, FGFs 8,9,11,12,16,17,18, as well as WNTs 4 and 5b. A gene ontologies analysis confirmed this, with the top molecular function ($P = 5 \times 10^{-5}$) being growth factor activity. The top

biological process was neuron differentiation ($P = 6 \times 10^{-10}$), which likely reflects the beginning stages of the generation of the olfactory neuroepithelium.

The RNA-seq data further defined distinct transcription patterns for certain genes. For example, consider the homeobox transcription factor gene *Dlx6* (Fig. 5). The cranial mesenchyme showed very low expression, with almost no reads (top panel). The region of the *Dlx6* gene is shown at the bottom, with introns in light blue. In contrast the epidermal ectoderm/olfactory placode showed robust expression, with standard RNA splicing as shown by the lines that span the sequences of the introns, which are deleted in the RNA-seq reads of the cDNA. Also note the abundant reads from the 5' promoter region of *Dlx6*, representing candidate enhancer transcripts. These transcripts did not show evidence of RNA splicing since none of the RNA-seq reads spanned introns. *Dlx6* was also strongly expressed in the maxillary arch, but in this case there were abundant spliced antisense transcripts in the region 5' of *Dlx6*, with introns again represented by lines. In the mandibular arch the expression pattern was yet more complex, with the presence of two species of spliced antisense transcripts, with one spanning the entire *Dlx6* gene region, representing the 3' Opposite Strand (3' OS) transcript shown at the bottom of Fig. 5. Both the 3' and 5' antisense transcripts spliced to an exon located further 5' and not included in Fig. 5. In summary, each of the four compartments examined showed a distinct transcription/RNA processing pattern in the region of *Dlx6*.

The RNA-seq data also revealed novel transcripts and unusual RNA splicing patterns. One example is a novel transcript flanking the *Rps29* gene. This transcript has two exons separated by a very large single intron, of approximately 196 Kb. More than six genes reside within this intron, including *Klhdc1*, *Klhdc2*, *Nemf*, *Pde2*, *Mgat* and *Lrr1*. The two exons of this novel transcript showed multiple splice site alternatives, including one noncanonical AT-AC junction sequence (data not shown). This RNA was robustly expressed, with raw reads of over one thousand in all compartments examined. Of interest, the splicing pattern observed in the cranial mesenchyme for this transcript was unique to that compartment. In summary, the RNA-seq dataset provides a useful resource for the analysis of the RNA-splicing patterns for all genes expressed in these compartments at E9.5.

E10.5 Microarray analysis

At E10.5 craniofacial development has progressed to form the lateral nasal process, medial nasal process, and olfactory pit. (Fig. 1). We used LCM to capture samples from these compartments for microarray analysis. We also isolated the maxillary arch, mandibular arch, and the neuroepithelium underlying the Lateral and Medial Nasal Processes, as well as the most medial neuroepithelium, which is a known growth factor signaling center. In addition we LCM purified Rathke's pouch, the epidermal ectodermal layers of the maxillary and mandibular arches, as well as neuroepithelium that did not underly the facial processes as a control. A minimum of three biological replicates were examined for each compartment, with a total of 47 microarrays used to examine gene expression in the E10.5 developing face.

This dataset was first examined by performing ANOVA ($P < 0.05$), minimum fold change of at least five for any pairwise comparison, giving 1516 differentially expressed probesets

(Table S14). Cluster analysis showed excellent reproducibility, with some variation expected as a result of the LCM sampling of distinct subregions of compartments (Fig. 6). Although all of the compartments were predominantly ectodermal in origin there were similarities in the heatmap that reflected their distinct lineages. Each of the neuroepithelial (NE) compartments, for example, shared a large number of active genes distinct from the other regions. The epidermal ectodermal cells of the mandibular and maxillary arches also showed similar gene expression patterns, which partially overlapped the nasal pit and Rathke's pouch, which are derived from the invagination of epidermal ectoderm. Moving to the far right of the heatmap of Fig. 6 are genes expressed predominantly by neural crest rich compartments, including the lateral nasal prominence, medial nasal prominence, mandibular and maxillary arches.

Relatively few genes showed compartment specific expression, as might be expected given their strongly overlapping cellular compositions. It is not surprising therefore that transcripts for only 24 probesets, including 17 annotated genes, were identified, for example, as lateral nasal prominence enriched (Table S15). This screen required two fold enrichment compared to all other compartments, excepting the medial nasal prominence, which showed strongly overlapping gene expression with the lateral prominence. The lateral nasal prominence enriched list of genes encoded the transcription factors GSC and ALX1, as well as the vacuolar sorting protein VPS13d, the chemokine PF4. Enriched genes also included *Bicc1*, which encodes an RNA that regulates protein translation during development. The further requirement for two fold enrichment in the lateral nasal prominence versus the medial nasal prominence reduced the list to ten annotated genes (*Bicc1*, *Gsc*, *Nfe2*, *Snhg1*, *S100a13*, *Wdr65*, *Vps13d*, *Inmt*, *Tyrobp* and *Hpgd*).

A similar analysis of the olfactory pit identified the growth factor FGF17, the transcription factor neurogenin1, likely involved in differentiation of the olfactory system, *Aldh1a3*, again involved in establishing retinoic acid gradients, and the ephrin gene *efna3*.

The neuroepithelium underlying the lateral nasal prominence showed elevated expression for several transcription factors, but little evidence of FGF, BMP or SHH signaling. In contrast the more medial neuroepithelium showed expression of the *Noggin*, *Fgf8*, *Fgf15*, and *Fgf17* genes, which likely play important roles in the development of the nearby neural crest cells of the medial nasal prominence.

The epithelia surrounding the mandibular arches expressed *Igf2*, *Pdgfa* (very high expression), *Pdgfc*, *Wnt4* and *Wnt6*, which likely signal the underlying neural crest/paraxial mesoderm.

The gene expression patterns of the E10.5 craniofacial compartments were also examined by RNA-seq. Once again, this provides an independent high throughput global strategy for validation of the microarray data, as well as lending the many advantages of RNA-seq.

RNA-seq analysis of the olfactory pit confirmed and extended the microarray results. The data once again showed highly elevated levels of the *Efna3*, *Aldh1a3*, and *Fgf17*, as seen with microarrays. The RNA-seq further defined the signaling center function of the nasal pit, showing for example higher expression of multiple FGFs, including FGF 8, 9,12, 15,16, 17

and 18, compared to the other compartments. The olfactory pit is clearly a major FGF signaling source, with seven FGF genes showing striking elevated expression, and with FGFs 8, 9, 17 and 18 showing robust nRPKM expression levels above nine. In addition the olfactory pit expressed WNT4 (nRPKM 7), and connective tissue growth factor CTGF (nRPKM 15), which plays a role in driving chondrocyte proliferation and differentiation. Other growth factor genes expressed included inhibin alpha (*Inha*: nRPKM 6), *Pdgfa* (a very high nRPKM of 144) and *Pdgfc* (nRPKM 50), *Tgfa* (nRPKM 14), *Nrg1* (nRPKM 15), and *Notch1* (nRPKM 24).

The RNA-seq dataset can be screened in a rich variety of manners to yield different insights. Compartment specific growth factors, transcription factors and receptors can be defined. For example, an analysis of the mandibular arch elevated genes, compared to all other compartments excepting the maxillary arch, which shows a strongly overlapping gene expression pattern, identified the strongly expressed transcription factor genes *Hand1*, *Hand2*, *Osr1*, *Twist2*, *Lhx6*, *Lhx8*, *Barx1*, and *Dlx1*. Because of their restricted and robust expression these genes are therefore strong candidates for representing the transcription factor code that defines the mandibular arch. Of interest, all of these gene have been previously implicated in craniofacial development (Barbosa et al., 2007; Denaxa et al., 2009; Jeong et al., 2008; Liu et al., 2013; Nichols et al., 2013; Tukul et al., 2010; Zhao et al., 1999). It is also interesting to note that one of the genes with the highest expression level in the mandibular arch encodes the growth factor IGF2. In the different compartments *Igf2* showed nRPKM values of: olfactory pit, 108; medial eminence 137; lateral eminence 94; maxillary arch 139, and mandibular arch 310. These high *Igf2* expression levels likely help drive the rapid growth of these compartments. Of interest, altered expression of *Igf2* has been associated with Russell-Silver syndrome, which includes a distinctive craniofacial phenotype (Chopra et al., 2010).

***In situ* hybridizations**

The use of two independent high throughput gene expression profiling technologies, microarrays and RNA-seq, provided the primary method of data validation for this study. In addition, however, we carried out *in situ* hybridizations for a group of genes predicted to have elevated expression levels in restricted sets of compartments. The results were quite consistent with predictions, as illustrated in Fig. 7. At E8.5 *Pou3f2* and *Hes3* were expressed in the caudal brain neuroepithelium. At E9.5 *Epcam*, *Aldh1a3* and *Pitx2* were expressed in the epidermal ectoderm, *Alx1*, *Alx3*, *Lum* and *Six2* were expressed in the cranial mesenchyme, and *Rap2c* and *Pth1r* were expressed in the mandibular arch. At E10.5 *Barx1* was expressed in the mandibular arch, and also at a somewhat lower level in the maxillary arch. *Cited 1* showed elevated expression in the mandibular arch, while *Epcam*, *Fezf1* and *Cldn3* showed elevated expression in the nasal pits. For *Barx1*, *Rap2c*, *Lum*, *Aldh1a3*, *Alx1* and *Alx3* we generated section *in situ* hybridization data to better localize expression (Fig. S2). Together these results provide significant data validation, although for a more restricted gene set than possible with the RNA-seq/microarray comparison.

Single Cell Analyses

To better define cell-type specific gene expression profiles that correlate with individual cell types within the early tissue layers responsible for craniofacial development, we compared a series of E8.5 cells sampled from three compartments critical for craniofacial development; epidermal ectoderm, paraxial mesoderm and neural crest. Although as previously described we had used LCM to isolate and profile populations of cells that are present within each of these compartments at this stage, these profiles are complicated by the admixture of different cell types within these compartments, for example the presence of both neural crest and paraxial mesoderm cells in cranial mesenchyme. This complicates the issues of identifying cell type-specific transcriptional programs and leads to results that provide ensemble averages of the mixed populations of cells, here for example based on the ratio of neural crest and paraxial mesoderm cells that are present as well as the distribution of varying degrees of cellular maturation. The separation of these programs requires the use of single cell based profiling methodologies.

The neural crest cells were GFP labeled by crossing the *Wnt1-Cre* and floxed stop *Rosa26-GFP* reporter transgenic mice. We used manual microdissection coupled with mild trypsin treatment to isolate GFP positive single cells from the E8.5 cranial mesenchyme, flanking the neural folds, representing neural crest, as well as GFP negative cells, representing paraxial mesoderm and overlying epidermal ectoderm. In total eleven cells were purified, microscopically confirmed to be single cell, rinsed repeatedly in PBS, and then used for SCAMP amplification as previously described (Brunskill et al., 2011a) and hybridized to Affymetrix Mouse Gene 1.0 ST arrays, CEL files from which were quantitated using the RMA method.

Probesets were filtered for those that exhibited RMA –estimated expression level of at least 6.0 in at least one sample, and were then subjected to ANOVA, fold-difference, and clustering based analyses to identify probesets that exhibited differential compartment specific expression with $p < 0.05$, without FDR to allow identification of genes that were not necessarily expressed by all of the individual cells of the distinct sampled compartments. The resulting 377 probesets with compartment specificity of expression, were then subjected to hierarchical clustering using median baseline referenced relative expression values (Fig 8). We identified 90 candidate epidermal ectoderm genes, 217 candidate neural crest genes, and 70 candidate paraxial mesoderm genes (Table S16). The significant degree of variability among the individual cells is likely in part due to the pulsatile nature of individual cell-level gene expression (Chubb et al., 2006; Losick and Desplan, 2008; Raj et al., 2006), which can cause the gene expression profile of even a single cell to fluctuate significantly as a function of time. Regional heterogeneities of the single cells sampled will further contribute to the observed differences in gene expression. In addition the low number of transcripts per gene in a cell results in technical noise, as it is impossible to capture each transcript for analysis with 100% efficiency.

Topgene analysis for shared properties and connections among the genes in each genelist revealed both known/expected associations as well as unknown genes and gene functions. These included known neural crest genes, *Gng3*, *Lmo4*, *Myc*, *Snai1*, *Sox9*, *Sox10*, and

Twist1, as well as significant numbers of genes associated with transcriptional control, signal transduction, cell cycle regulation, and mRNA splicing. Many of the neural crest genes play key roles in mesenchymal development and the determination of multiple lineages including neuronal (*Gemin2*, *Sox9*, *Sox10*, *Ddx20*, *Wrap53*), and craniofacial bone development (*Itgb1bp1*, *Lmo4*, *Myc1*, *Nkd1*, *Plekha1*, *Snai1*, *Sox9*, *Twist1*). Other genes were known to be critical to prevent facial or palatal clefting, but were not previously known to be associated with neural crest-specific/enhanced expression (*Arhgap29*, *B3gat3*, *G6pc3*, *Gsk3a*, *Lasp1*, *Orc1*, *Pex7*, *Phf6*, *Polr1d*, *Pqbp1*, *Ugdh*). Interesting early candidate neural crest markers to emerge in the analysis included *Pnp2*, *Lxn*, *Ddx47*, *Riok3*, *Tgds*, *Zfp105* and *Zfp259*. These results provide, to our knowledge, the first single cell resolution global analysis of the gene expression profiles of these early neural crest cells and provide a resource through which we can improve our understanding of the genomic basis of their function.

Data Mining the Integrated Craniofacial Gene Expression Atlas

In order to enable flexible use of the data from this project for data mining and signature comparisons across the entire series of cell types, regions, and developmental stages that we have profiled, we combined, normalized, and baseline referenced all of the data into a single data matrix per technology (RNA-seq or Affymetrix GeneChip ST1.0). To illustrate the range of patterns manifested in these data, Fig 9 illustrates gene expression clusters in the RNA-seq dataset of 511 genes that are known from mouse knockout phenotypes to result in abnormal craniofacial morphology or development. For this map we used the relatively high cutoff of 5 FPKM in order to identify the most robust clusters and patterns among these genes with known effects on craniofacial development. The heatmap is formed by hierarchical clustering by genes, and by samples, of the relative gene expression values as measured in each sample (Table S17). This map also provides a framework for considering which samples and genes are similar to each other and what types of patterns represent major and minor differences. For example, note the strong similarity of E8.5 MES, E9.5 CM, and the E9.5 Ma-AR and Mx-AR samples in the portion of the heatmap that includes *Twist1*, *Prrx1*, etc. Outside of this area, however, the similarities of these samples are less prominent. Note also that this map is only showing the patterning of known craniofacial genes, and that a much larger number of other genes that are not yet known to be essential for craniofacial development follow similar types of patterns and constitute a potential discovery resource for important genes and their interactions.

To divide the complete datasets, both ST1.0 and RNA-seq, into specific geneset modules, which allows for both analyses of these modules themselves or comparisons of a given list of genes in relation to these, we subjected the normalized data matrices to a shredding protocol as described in Methods in which sample-type-specific signatures were computationally generated and then placed into the Toppgene/Topfun database for further use. For example, querying Toppgene for *Twist1*, (<http://toppgene.cchmc.org/enrichment.jsp>), (for single gene queries, do not use FDR statistical correction), returns a list of genesets that each contain *Twist1*, including a list of 226 genes titled “Facebase ST1 E10.5 MaxilArch 500”, and another a list of 207 genes titled “Facebase_ST1_E8.5_ParaxMesoderm_250”. The lists can include fewer than the stated

500/250 genes because they represent non-redundant genes that map to human orthologs and there are also sublists that were formed from these parent lists based on K-means clustering (eg cluster#1). Clicking through the listed numbers of genes in Toppgene allows for detailed view of the genes in each list and allows for immediate analysis of the enriched functions, pathways, and properties that are shared among the genes in each list. For these two particular gene lists above, only 14 genes overlap, indicating a potentially interesting shift in the genes that *Twist1* interacts with in the two developmental stages and compartments. To test this hypothesis using the independent samples and platform offered by the FaceBase RNA-seq dataset, we used Pearson correlation to first identify genes most tightly correlated with *Twist1*'s pattern of expression and then subjected the 193 top-correlated genes to hierarchical gene clustering as shown in Fig. 10. The heatmap illustrates the difference in the E8.5 and E.10.5 patterns of expression for these *Twist1* related genes. We subdivided these 193 genes into three clusters based the hierarchical tree structure (not shown), giving genes showing similar levels of expression in both stages, or predominantly only E8.5 or E10.5 (Fig 10, Table S17). Using Toppgene to carry out comparative enrichment analysis of these sets is extremely revealing about each of those two compartments, and strongly suggests context-specific roles for *Twist1*. In the E8.5 cranial mesenchyme (including paraxial mesoderm) gene list are 16 genes that are known to regulate mesenchyme development ($P < 10^{-9}$; *Bmp7*, *Cyp26A1*, *Dab2*, *Efna1*, *Eng*, *Foxc1*, *Foxc2*, *Foxd1*, *Foxf2*, *Frzb*, *Pitx2*, *Six1*, *Snai1*, *Snai2*, *Sox10*, *Twist1*), 31+ genes associated with vascular development, 18+ genes associated with craniofacial development, 6+ genes associated with palate bone morphology ($P < 2E-4$; 5 from above plus *Pdss2*) and 18+ genes with additional roles in craniofacial development (including *Dkk1*, *Prrx1*, *Hesx1*, *Hapln1*, *Wls*, etc). 25 of these genes in the E8.5 module are sequence specific transcription factors. Overall the group of genes that *Twist1* is correlated with in the E8.5 mesenchyme is richly involved in the morphogenesis and vascularization of extracellular matrix that shapes the morphology of a broad range of craniofacial structure. In contrast, the group of genes associated with the E10.5 maxillary arch module that contains *Twist1* is also rich in craniofacial phenotype determining genes, but has little enrichment in extracellular matrix biology or vascular development and rather is richly involved in bony growth and development with upwards of 15 genes associated with cleft palate in mice or humans ($P < 4E-6$; *Alx1*, *Barx1*, *Chd7*, *Dlx1*, *Fgf10*, *Gsc*, *Lhx8*, *Msx1*, *Prrx1*, *Tbx2*, *Tbx3*, *Tmem107*, *Twist1*). Whereas this cluster of genes is also enriched in sequence specific transcription factors (26 genes, $P < 4E-9$) only 6 of these are in common with the E8.5 Paraxial Mesoderm geneset. To demonstrate how these data can be further mined and used to develop new hypotheses, Fig. 11 demonstrates the combined use of ToppCluster and Cytoscape to compare the properties and functions of the *Twist1*-correlated genesets shown in Fig 10 (Table S17). As shown in the Fig. 10 heatmap, these signatures overlap for one group of genes as well as have their own additional genes and thus can be used to form three genelists that are then functionally compared using ToppCluster, which similar to ToppGene carries out GO and other functional analyses. The top-ranked features from a variety of functional categories can be selected within ToppCluster and then exported as an XGML document that can then be analyzed by Cytoscape network-based algorithms and visualization [Smoot, 2011 #44]. In contrast to the single gene list queries that are discussed above, network-based representation approaches allow functional co-relationships to be demonstrated such that the

connections of one set of specific molecular or functional pathways can be linked to others by shared genes, protein-protein interactions, and other properties including specific phenotypes shared by multiple genes per cluster. These connections can thus provide highly instructive relationships and implications for the existence of larger scale gene networks that are responsible for specific aspects of craniofacial development.

Twist1-correlated genes shown in Fig. 10 were divided into three clusters corresponding to expression in E8.5 cranial mesenchyme, E10.5 maxillary arch, or both. These three patterns are represented as the clusters of genes (hexagons) that are on the left (E10.5), center (E10.5+E8.5), or the right (E8.5) (Fig. 11). These three sets of genes are further divided into those previously associated with abnormal craniofacial or vascular development, shown above, and those not yet known to be associated with those phenotypes, shown below (mouse phenotypes are lower case, human phenotypes are capitalized). Distinct classes of gene-associated properties are shown as different colored squares around the outside of the diagram. All E8.5-specific cranio/vascular functionally known genes are selected and appear as yellow hexagons, and the functions and properties that they are connected to are indicated by the red edges. The gray edges that go into those connected concepts or the concepts that have no red edges, are thus not linked to the core set of early genes. For example the facial bony abnormalities that are in the upper left are not connected to any of the E8.5 specific genes. Similarly, only 5 of the genes associated with integrin function are known to be craniofacial phenotype associated, but 9 additional genes are also associated with integrin signaling and function. This implicates a more significant role for integrin signaling and function in craniofacial mesenchyme development than might have been anticipated. The specific modules and connectivities of genes in these networks provide powerful suggestions about the gene interactions and overarching biological processes that drive craniofacial development. The importance of these additional gene function categories is suggested by the number known craniofacial connections that they have (red edges) or the number of unknown craniofacial genes that they are connected to (gray edges).

In summary, in this report we describe a murine craniofacial atlas of gene expression for the E8.5, E9.5 and E10.5 developmental time points. We used LCM to capture the multiple compartments, which were then used for gene expression profiling with both microarrays and RNA-seq. The use of two independent global gene expression technologies provided a high throughput cross validation of the resulting dataset. The RNA-seq data confirmed approximately 90% of genes found differentially expressed by microarray. As might be expected, however, the RNA-seq data identified more differentially expressed genes, with greater fold changes. In addition the RNA-seq data provides a global view of gene expression that extends to non-polyadenylated RNAs, including long intergenic noncoding RNAs and enhancer transcripts, as well as defining RNA-splicing patterns.

The results create a comprehensive atlas of gene expression during the early stages of craniofacial development. The changing waves of gene expression that occur during this process are defined. The transcription patterns of genes encoding all transcription factors, growth factors and receptors are characterized in a sensitive and quantitative manner. The results identify novel compartment specific markers, guide the construction of new transgenic mouse tools and globally characterize potential inter compartmental cross talk.

The gene expression blueprints of the elements of craniofacial construction are delineated. This atlas component resource of the FACEBASE Consortium is intended to facilitate further discovery by the craniofacial research community (FACEBASE.ORG).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant UO1 DE020049. We thank Paul Trainor for insightful advice.

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Highlights

A gene expression atlas resource of craniofacial development is presented

Laser capture microdissection isolation of craniofacial compartments

Gene expression profiling by both microarray and RNA-seq

Defining expression patterns of all growth factors and transcription factors

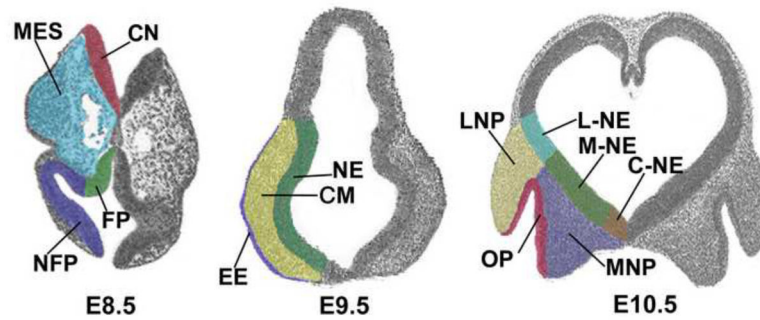


Fig. 1. Compartments isolated by LCM

Some of the compartments isolated by LCM are illustrated. At E8.5 the cranial mesenchyme (MES), with neural crest and paraxial mesoderm, as well as the flanking neuroepithelium, divided into floorplate (FP) and more dorsal non-floor plate (NFP), as well as more caudal neuroepithelium (CN). At E9.5 LCM was used to purify the cranial mesenchyme (MES), the flanking neuroepithelium (NE), the epidermal ectoderm, including the olfactory placode (EE), as well as the otic vesicle, mandibular and maxillary arches (not shown). At E10.5 we collected the lateral nasal prominence (LNP), medial nasal prominence (MNP), flanking neuroepithelia (L-NE, M-NE, C-NE), olfactory pit (OP), and, Rathke's pouch, both mandibular and maxillary arches, including both mesenchyme and epidermal ectoderm compartments (not shown).

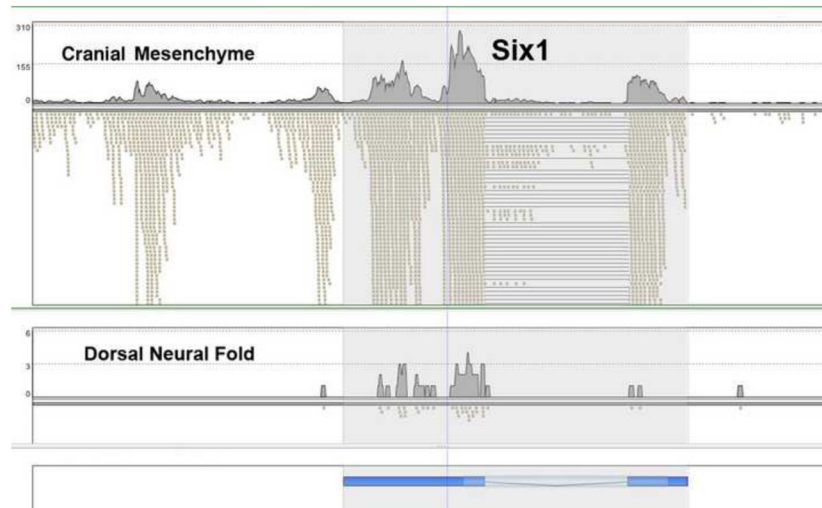


Fig. 2. RNA-seq analysis of *Six1* expression

The positions of the *Six1* exons are shown at the bottom in blue. The lightest shade is intron, intermediate shade is coding, and darker blue represents 5' and 3' UTR. The cranial mesenchyme, top panel, shows strong expression with many RNA-seq reads, marked by small tan rectangles. The lines indicate positions of introns, where single cDNA RNA-seq reads align to the two flanking exons. Note the many reads from the 5' region of *Six1* in the top panel, coincident with high expression of the *Six1* one gene itself. In the dorsal neural fold, bottom panel, there is very low expression of both the *Six1* gene and the 5' flanking region.

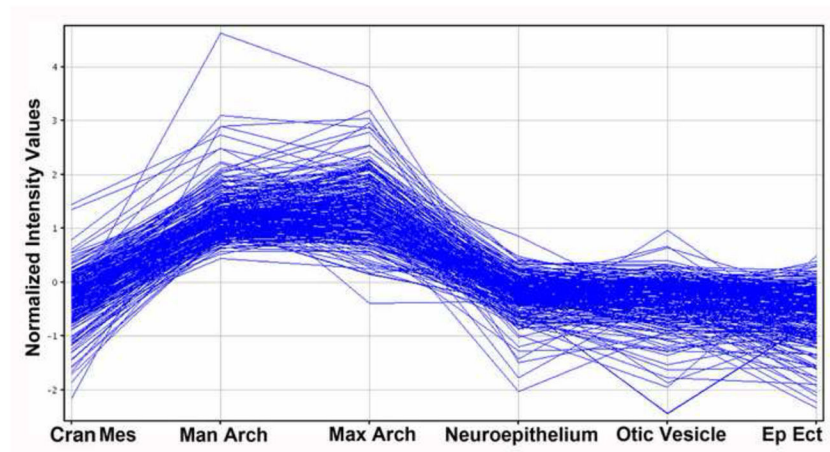


Fig. 3. Series graph of genes with elevated expression in the E9.5 mandibular arch

Genes with elevated expression in the mandibular arch were identified by comparing its microarray defined gene expression profile with those of all other E9.5 compartments examined, excepting the maxillary arch. As shown, genes with elevated expression in the mandibular arch are, with rare exception, also elevated in expression in the maxillary arch. It is interesting to note that the cranial mesenchyme, mandibular arch and maxillary arch are all composed primarily of neural crest cells. Ep Ect; epidermal ectoderm, including olfactory placode. Affymetrix Gene 1.0 ST microarray data.

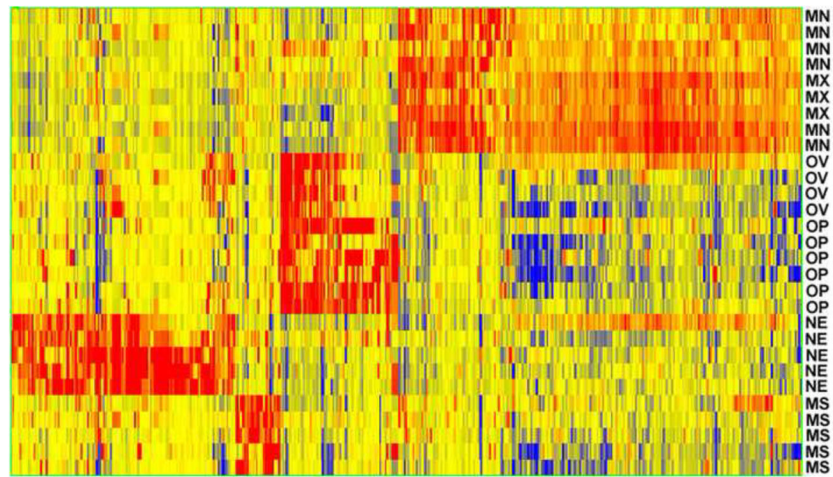


Fig. 4. Heatmap of genes with compartment elevated expression at E9.5

The mandibular (MN) and maxillary (MX) arches cluster together, as do the otic vesicle (OV) and olfactory placode (OP), which are both derived from the epidermal ectoderm. The neuroepithelium (NE) shows a very distinct gene expression signature, and there are a limited number of genes that distinguish the cranial mesenchyme (MS). Based on microarray data. See Fig. 1 for compartment locations. Red indicates high expression and blue indicates low expression. Based on Affymetrix Gene 1.0 ST microarray data.

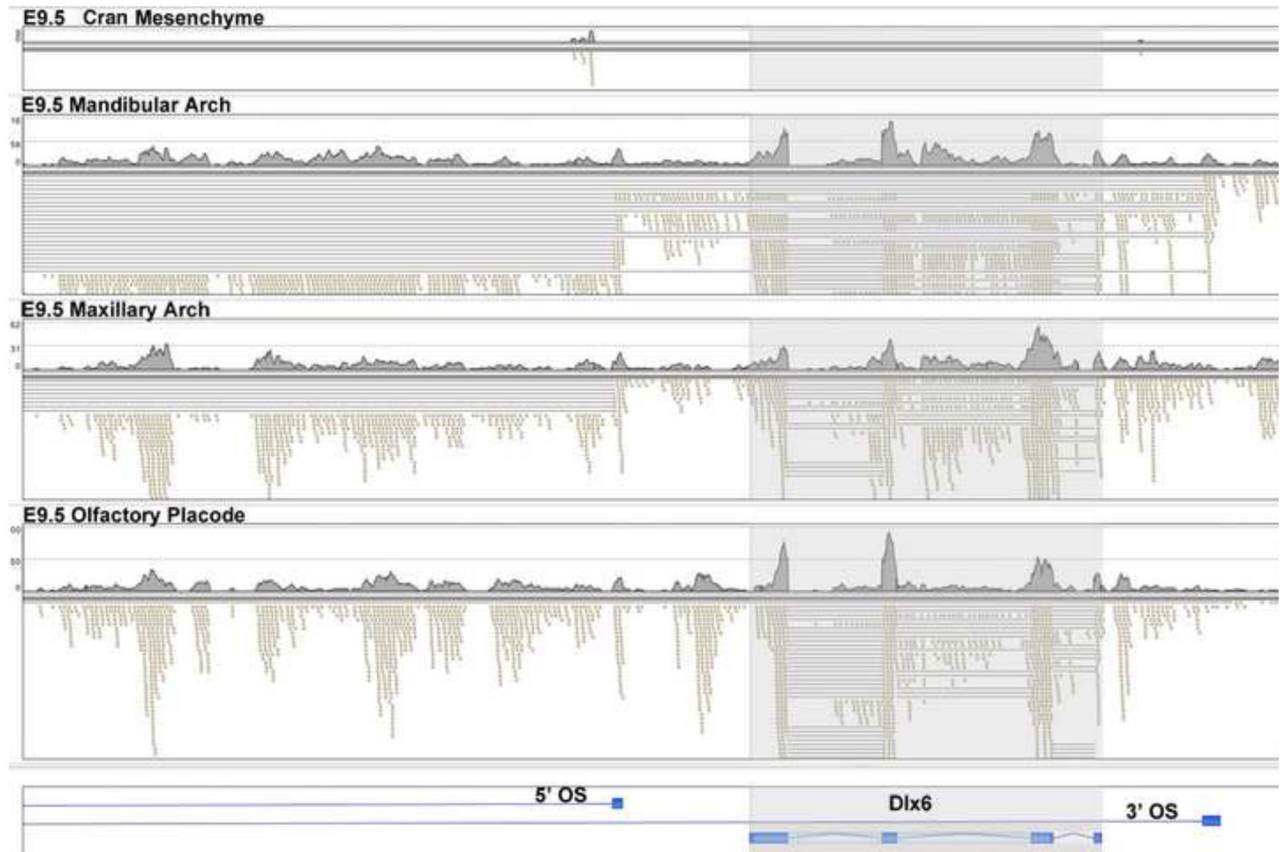


Fig. 5. Compartment specific expression patterns of the *Dlx6* gene at E9.5

The position of the *Dlx6* gene is shaded, with exons shown at the bottom of the figure in darker blue and introns in light blue. The E9.5 cranial mesenchyme (top) shows very low expression, with few RNA-seq reads (small rectangles). The olfactory placode, bottom, shows strong *Dlx6* expression, with normal splicing. The lines show RNA-seq reads that spanned introns. In addition there are multiple RNA-seq reads from the 5' flanking region of the gene (to the left), that did not showing splicing (no lines). The maxillary arch showed strong expression of *Dlx6*, as well as strong expression of a spliced antisense noncoding transcript in the 5' promoter region, indicated by the lines marking the opposite strand (5' OS) intron. In the mandibular arch there is strong expression of the *Dlx6* gene, the 5' antisense spliced transcript, and also an antisense transcript that initiates 3' of the *Dlx6* gene (3' OS), with both antisense transcripts splicing to exons further 5' and not shown in this figure.

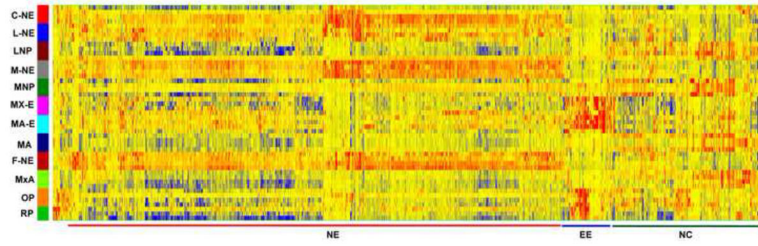


Fig. 6. Heatmap of genes with compartment elevated expression at E10.5

Compartments with neuroepithelial cells show closely related gene expression signatures, as marked at the bottom with NE (red line). Similarly, the compartments made of, or derived from, epidermal ectoderm show very closely related gene expression patterns, as marked with EE at the bottom (blue line). The NC denotes genes with elevated expression in a compartment made up primarily of neural crest cells (green line). See Fig. 1 for positions of most compartments. C-NE, central neuroepithelia; L-NE, neuroepithelia flanking the lateral nasal prominence; M-NE, neuroepithelia flanking the medial nasal prominence; MNP, medial nasal prominence; LNP, lateral nasal prominence; MxA, maxillary arch; MA Mandibular arch; OP, olfactory pit; RP, Rathke's pouch; MX-E, epidermal ectoderm of maxillary arch; MA-E, epidermal ectoderm of mandibular arch; F-NE, control neuroepithelium from dorsal region of brain. Microarray data. Red indicates high expression and blue indicates low expression.

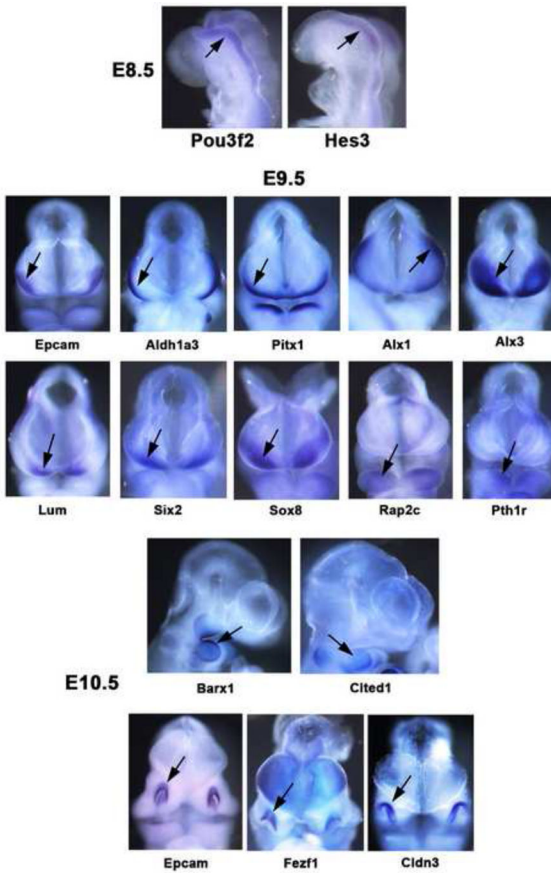


Fig. 7. *In situ* hybridizations showing elevated gene expression in select craniofacial compartments

Whole mount *in situ* hybridizations at E8.5, E9.5 and E10.5. At E8.5 *Pou3f2* and *Hes3* show elevated expression in the caudal brain neuroepithelium. At E9.5 *Epcam*, *Aldh1a3* and *Pitx1* show elevated expression in the olfactory placode/epidermal ectoderm, *Alx1*, *Alx3*, *Lum*, *Six2* and *Sox8* show elevated expression in the cranial mesenchyme, while *Rap2c* and *Pth1r* show elevated expression in the mandibular arch. At E10.5 *Barx1* and *Cited1* show elevated expression in the mandibular arch, while *Epcam*, *Fezf1* and *Cldn3* show elevated expression in the olfactory pits.

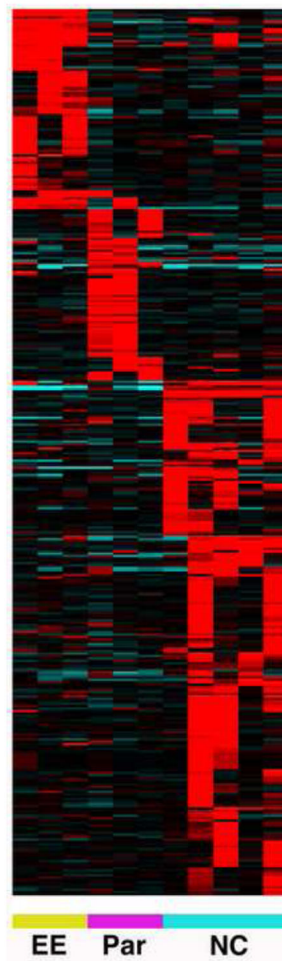


Fig. 8. Heatmap of gene expression patterns of single cells at E8.5

We performed single cell gene expression studies to begin to define the gene expression profiles of the early neural crest (NC), paraxial mesoderm (PM), and flanking epidermal ectoderm (EE). The data defines the gene expression programs of the early neural crest cells. Red is high expression, blue is low, and black is intermediate. Affymetrix Gene 1.0 ST microarray data.

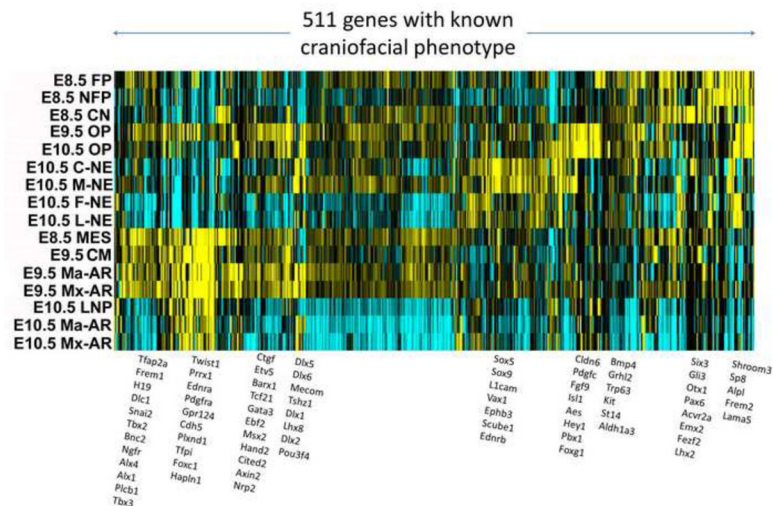


Fig. 9. Heatmap of gene expression patterns revealed by known craniofacial genes as measured in the normalized RNA-seq dataset

Hierarchically clustered gene expression patterns in the RNA-seq dataset by genes that are already known to result in abnormal craniofacial morphology or development suggest that the shared membership of genes within a given pattern may be highly informative as to specific modules of expression associated with different cell types and stages of developing craniofacial structures. Representative genes within each cluster are shown to the right of the heatmap. Yellow indicates high expression and blue indicates low expression.

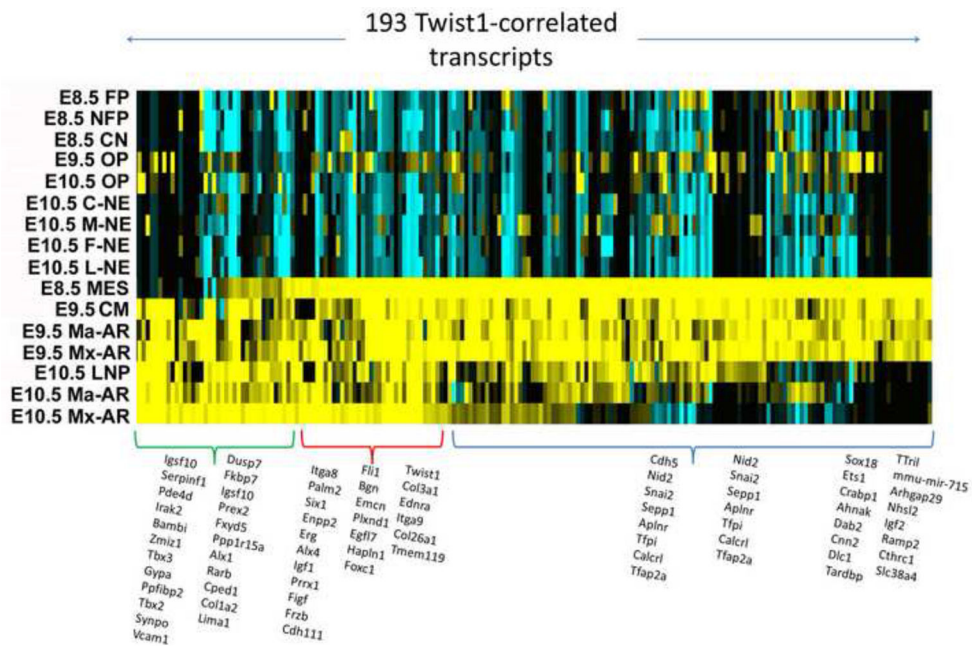


Figure 10. Heatmap of the top 193 genes whose expression correlates with *Twist1* in the RNA-seq dataset

Hierarchically clustered heatmap reveals three different patterns that correspond to genes with strongest expression in relevant samples from E8.5, E10.5, or both. The E8.5 cranial mesenchyme-prominent geneset is at the top (blue), and E10.5 maxillary arch-prominent set is at the bottom (green). *Twist1* itself is strong in both, red group). In the heatmap, yellow indicates high expression and blue indicates low expression.

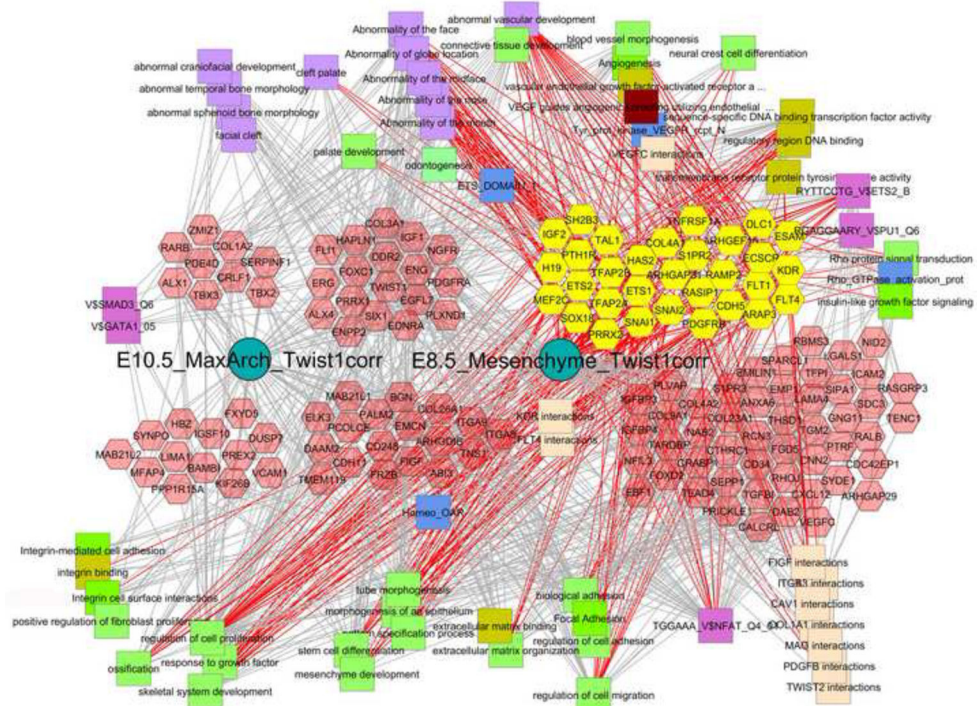


Fig. 11. Cytoscape-ToppCluster-based network analysis of genes that differentially co-express with *Twist1* expression at E8.5, E10.5 or at both stages
Twist1-correlated genes shown in Fig. 10 were divided into three clusters corresponding to expression in E8.5 cranial mesenchyme, E10.5 maxillary arch, or both. These three patterns are represented as the clusters of genes (hexagons) that are on the left (E10.5), center (E10.5+E8.5), or the right (E8.5). These three sets of genes are further divided into those previously associated with abnormal craniofacial or vascular development, shown above, and those not yet known to be associated with those phenotypes, shown below (mouse phenotypes are lower case, human phenotypes are capitalized). Distinct classes of gene-associated properties are shown as different colored squares around the outside of the diagram. All E8.5-specific cranio/vascular functionally known genes are selected and appear as yellow hexagons, and the functions and properties that they are connected to are indicated by the red edges. The gray edges that go into those connected concepts or the concepts that have no red edges, are thus not linked to the core set of early genes. For example the facial bony abnormalities that are in the upper left are not connected to any of the E8.5 specific genes. Similarly, only 5 of the genes associated with integrin function are known to be craniofacial phenotype associated, but 9 additional genes are also associated with integrin signaling and function. This implicates a more significant role for integrin signaling and function in craniofacial mesenchyme development than might have been anticipated. The specific modules and connectivities of genes in these networks provide powerful suggestions about the gene interactions and overarching biological processes that drive craniofacial development. The importance of these additional gene function categories is suggested by the number known craniofacial connections that they have (red edges) or the number of unknown craniofacial genes that they are connected to (gray edges).