Supplemental Experimental Procedures

IPSC culture

hESCs (H9 line), human iPSCs (WT-33 and ADRC-40) and chimp iPSCs (1209, 0818) were expanded in feeder-free, serum-free medium mTESR-1 from StemCell technologies. Cells were passaged ~1:6 every 5–6 days by incubation with accutase (Invitrogen) and the resulting small cell clusters (50–200 cells) were subsequently re-plated on tissue culture dishes coated overnight with growth-factor-reduced matrigel (BD Biosciences).

CNCC derivation and culture

Pluripotent lines were differentiated into CNCC as previously described (Rada-Iglesias et al., 2012). Briefly, hESCs/iPSCs were incubated with 2mg/ml collagenase. Once detached, clusters of 100-200 cells were plated in CNCC differentiation medium: 1:1 Neurobasal medium/D-MEM F-12 medium (Invitrogen), 0.5× B-27 supplement with Vitamin A (50× stock, Invitrogen), 0.5× N-2 supplement (100× stock, Invitrogen), 20 ng/ml bFGF (Peprotech), 20 ng/ml EGF (Sigma-Aldrich), 5 µg/ml bovine insulin (Sigma-Aldrich) and 1× Glutamax-I supplement (100× stock, Invitrogen). Medium was changed every other day. After seven days of differentiation, neuroepithelial spheres attached to the dish and gave rise to migratory CNCC. Three-four days after the appearance of the first CNCC, cells were dissociated with accutase until single cells and passaged onto fibronectin-coated plates. CNCCs were then transitioned to CNCC early maintenance media: 1:1 Neurobasal medium/D-MEM F-12 medium (Invitrogen), 0.5× B-27 supplement with Vitamin A (50× stock, Invitrogen), 0.5× N-2 supplement (100× stock, Invitrogen), 20 ng/ml bFGF (Peprotech), 20 ng/ml EGF (Sigma-Aldrich), 1 mg/ml bovine serum albumin, serum replacement grade (Gemini Bio-Products # 700-104P) and 1× Glutamax-I supplement (100× stock, Invitrogen). CNCCs were passaged onto fibronectin-coated plates 1:3 every three days, and after 1-2 passages, transitioned to CNCC long term maintenance media, which is composed of CNCC early maintenance media plus 3uM ChIRON

99021 (Selleck, CHIR-99021) and 50ng/ml BMP2 (Peprotech). Cells were maintained on fibronectin with passaging every ~ 3days, and collected at passage 4 for all ChIPs and downstream assays. For directed differentiation to later lineages, cells were cultured in media that promoted differentiation to smooth muscle (D-MEM F-12 + 10% FBS), or neurons/glia (D-MEM F12 + B27 + 2mM glutamine + 50ng/ml BMP2 + 50ng/ml LIF + 1% heat-inactivated serum).

ChIP-seq antibodies

All antibodies used were previously reported as suitable for ChIP and/or ChIP-seq: p300 (sc-585, Santa Cruz Biotechnology), H3K4me1 (ab8895, Abcam), H3K27ac (39133, Active Motif), H3K4me3 (39159, Active Motif), H3K27me3 (39536, Active Motif), NR2F1 (PP-H8132-00, Perseus Proteomics), and TFAP2A (sc-184, Santa Cruz Biotechnology).

Immunocytochemistry

Cells were fixed in fresh 4% paraformaldehyde. For stainings with antibodies recognizing intracellular epitopes, phosphate buffered saline with 0.5 mg/ml BSA and 0.1% Triton X-100 was used for blocking and permeabilization. For cell surface stainings (e.g., p75) TritonX-100 was eliminated from the blocking buffer and cells were additionally methanol-fixed for 10 minutes. Appropriate Alexa 488, or Alexa 594, labeled secondary antibodies and/or DAPI counterstaining was used for visualization on a confocal microscope (Leica TSC SP2).

Xenotransplantation of human and chimp CNCCs into chick embryos

Chick embryos were cultured in a humidified 37°–38°C incubator until HH st. 8-11, then embryos were transferred to weigh trays for injections. Human and chimp CNCCs were transiently transfected with pMAX-GFP, cultured overnight, washed with PBS and injected via mouth pipette under a fluorescent microscope into the dorsal-most portion of the chick neural tube anterior to rhombomere 5. Embryos were then treated with antibiotics and returned to the incubator for 48 hours before dissection to remove extraembryonic tissues and yolk followed by

fluorescence imaging. Samples were then frozen and sectioned on a Cryostat at 12uM thickness.

Surface marker identification and analysis

In preliminary work for this study, we observed a level of variation in the transcriptome between different derivations of CNCCs which was obscuring true interspecies differences, which we deemed unacceptable. Scarcity of suitable flow cytometry markers for human CNCCs prompted us to identify in an unbiased fashion a panel of 5 “cluster of differentiation” (CD) antibodies to standardize the culture conditions to promote reproducibility and homogeneity of CNCCs. First, we identified the subsets of significantly expressed genes with highest variance across multiple conditions (high vs low stress, early vs late neural crest) in our RNAseq data, and identified 17 candidate surface marker genes among this group. Due to antibody availability and some redundancies based on gene cluster identities, we further reduced this set to 13 candidate markers and tested each in flow cytometry (AriaII SORP and BD Fortessa) for surface expression and clear dynamic changes during the course of our CNCC derivation from the iPSC/ESC state. Finally we settled on a non-redundant subset of 5 antibodies consisting of CD10 (MME), CD99, CD105 (ENG), CD266 (TNFRSF12A) and CD271 (NGFR, p75) (Miltenyi Biotec) that trace differentiation progress *in vitro*. Of note p75/CD271 is a known marker of neural crest cells and was re-identified in the process. All 5 antibodies were cross-reactive with both species, and were used to assess homogeneity of derived CNCCs.

Analysis of chimpanzee and human enhancer activity in a lacZ reporter transgenic mouse assay

Divergent regulatory regions for CNTNAP2 and PAPPA were chosen for *in vivo* testing in the mouse embryo in a lacZ reporter transgenic assay, as previously described (Ferretti et al., 2011; Vitobello et al., 2011). Briefly, enhancer candidate regions consisting corresponding to chr7: 145,843,942-145,844,366 (hg19) for the CNTNAP2 enhancer and chr9:118163085-118163446 (hg19) for the PAPPA

enhancer and their orthologous chimp sequences respectively were PCR-amplified from genomic DNA using the same primer sets for both species. Three concatenated copies of each fragment were cloned into the hsp68-basal promoter-lacZ reporter vector (DiLeone et al., 1998). Transgenic mouse embryos were generated by pronuclear injection of the relative construct (by Cyagen Biosciences, Santa Clara, CA) (Attanasio et al., 2013; Ferretti et al., 2011; Vitobello et al., 2011) . F0 embryos were collected at E11.5, a time-point that allows evaluation of most developing craniofacial structures and is consistent with other transgenesis analyses of craniofacial enhancers (Attanasio et al., 2013). Embryos were PCR-screened for the presence of the transgene, using oligoprimers designed within the lacZ gene, and stained for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal). Only patterns of craniofacial expression that were observed in at least three different embryos resulting from independent transgenic integration events of the same construct were considered reproducible (Visel et al., 2009). Whole-mount images of all lacZ-positive embryos and close-up images of the heads were taken and expression patterns were annotated according to X-Gal staining of defined anatomical regions.

ATAC-seq

ATAC-seq libraries were performed starting from 50,000 CNCCs from each population, according to published protocols (Buenrostro et al., 2013). Libraries were multiplexed 4 samples per lane and sequenced with 2x50bp paired-end reads.

RNA-seq

Total RNA was extracted from >$1x10^6$ CNCCs at p4 in Trizol (Invitrogen). 5 µg of total RNA were subjected to two rounds of oligo-dT purification using Dynal oligo-dT beads (Invitrogen), then fragmented with 10× fragmentation buffer (Ambion). Fragmented RNA was used for first-strand cDNA synthesis, using random hexamer primers (Invitrogen) and SuperScript II enzyme (Invitrogen). Second

strand cDNA was obtained by adding RNaseH (Invitrogen) and DNA Pol I (New England Biolabs) to the first strand cDNA mix. The resulting double-stranded cDNA was used for Illumina library preparation as described for ChIP-seq experiments, and sequenced with 2x100bp paired-end reads.

Luciferase reporters

The pGL3-control vector (Promega) was modified to remove the SV40 enhancer. Human and chimp enhancers were amplified from genomic DNA and cloned between the BglII and XhoI sites. Reporter vectors were co-transfected with FuGENE 6 (Promega) into p4 CNCC at a 1:200 ratio with pRL-SV40 renilla luciferase (Promega), and luminescence was measured using the Dual-Luciferase® Reporter Assay System after 24 hours post-transfection. In all cases, the same primers were used to amplify orthologous human and chimp enhancers. Luminescence measurements were repeated for all reporters in three independent CNCC differentiations of each species. For testing the Coordinator motif activity, four versions of the motif were used: V1 = natural sequence with best fit to consensus PWM, 4X: CCCATCTGGTTCCCATTAA; V2 = consensus PWM, 4X: CACATCTGTTTTTAATTAA; V3 = mix of 4 strong versions, V4 = version from strongest H3K27ac-marked enhancer, 4X: GCCTTCTGGTTTTAATAAC; empty = empty pGL3 vector.

ChIP-seq analysis and identification of modal peak positions

All sequencing reads were aligned to both reference genomes (hg19 and panTro3) using default settings with bowtie2.2.4, regardless of species of origin. ATAC-seq was paired-end sequenced, Nextera adapter sequence was trimmed away and each mate was treated as independent transposition events for downstream analysis. Wig files for genome browser visualization were generated with QuEST2.4. Peak calls were performed using default settings on MACS2. To generate the list of candidate genomic regions with robust coordinates in both assemblies, we applied the following strategy: for each reference genome,

summit positions from p300, AP2A, NR2F1 ChIPs and ATAC-seq were assigned unique names and combined in one file. LiftOver (-minMatch=0.1 -multiple) was used to map each peak to the reciprocal reference genome. Peak positions in each genome assembly (both original and remapped) where then combined into a single file, sorted on unique names and combined in one table by full outer join. Peaks that could not be mapped by liftOver received a “chr0” placeholder chromosome with numerical coordinates of the original genome. Next we used a mean shift algorithm in two dimensions with 300bp bandwidth and applied a Gaussian kernel to cluster the peaks into candidate regulatory regions (hence incorporating evidence from both genome coordinates simultaneously). Of note, at our depth of sequencing (~50M reads per sample), using ATAC-seq data only would miss 45% of the putative enhancer sites, while incorporating ATAC-seq to the p300 and TF data increases the number of discovered sites by 20%. For motif analysis we restricted the region set further by identifying regions with bijective (1-to-1) orthology down to the single-base level by repeating liftOver on the ±100bp regions relative to the mean-shift modal peak position, excluding elements whose peripheral (±100bp) coordinates did not remap to the expected coordinates in both species.

Overlaps of genomic intervals with annotated genomic features such as HARs, repetitive elements and VISTA enhancers were calculated using Bedtools and statistical analysis was performed with Fisher’s exact test followed by q-value calculation (using a Storey-Tibshirani procedure). HAR coordinates were found in Hubisz and Pollard, 2014, and repeat coordinates and classifications were extracted from the UCSC genome browser repeat masker track. VISTA enhancer coordinates are available from the VISTA database

RNA-seq analysis and enhancer association

RNA-seq were aligned to hg19 and panTro3 reference genomes with tophat and quantified against human ENSEMBL 78 (GRCh37) gene models using htseq-count. Differential expression analysis was performed with DESeq2. To assign most likely target genes for the regions identified in ChIP-seq we applied default

GREAT association rules (McLean et al., 2010). To calculate the effect of multiple enhancers, all enhancers from either species were scored as 0 (invariant, p > .0001), +1 (human-biased p-value < .0001) or -1 (chimp biased p-value < .0001) in hg19. Genes with divergent gene expression (padj <0.1) are then binned by the cumulative count of all enhancers within 250kb of the TSS and violin plots were generated in Matlab for the log(fold change human/chimp) of expression according to DESeq2.

Conservation plots

Conservation plots were generated using the Conservation Plot (version 1.0.0) tool available through Galaxy/Cistrome.

Variance-edit distance

To estimate the effect of genetic distance on divergence of epigenetic marks we binned the chromatin regions based on Levenshtein distance of the 200bp orthologous regions and calculated variance of pairwise difference in the ChIP signal for each alignment within each Levenshtein distance bin. For plotting purpose the variances were normalized to that of distance=0 by subtracting the variance at zero distance.

Similarity with other datasets

To assess similarity with other human cell types, we downloaded over 50 H3K27ac public ChIP-seq datasets from a representative set of cell types. The kernel density estimate was calculated at approximately 50,000 genomic regions based on the superset of ENCODE transcription factor ChIP-seq data, DNase hypersensitivity and our own datasets. 1-Spearman correlation coefficient was used as metric for clustering. Since no epigenomic data from *in vivo* human CNCCs are available, we calculated correlation coefficients for the portion of sites defined above that were remappable to the chick genome with H3K27ac ChiP-seq data from neural crest from HH stage 20 chick facial prominence (SRX148743, Rada Iglesias et al.).

Pleiotropic Versus NCC specific motif enrichment

NCC chromatin regions were classified as increasingly pleiotropic/promiscuous based on activity detected in additional tissue types and fraction of the long motif was plotted for each class.

Motif discovery

Enriched and *de novo* motif discovery was performed using SeqPos tool in Cistrome with the top 1904 active enhancers as well as with top 3499 CNCC-specific enhancers. To analyze motif usage in the annotated set of genomic regions, we considered approximately 200,000 x 200bp intervals (centered at the summits) with bijective orthology at the base resolution (as defined above). Fasta files were then generated from hg19 and panTro3 reference genomes corresponding to the set of genomic regions, and scanned for motifs with FIMO using combined MEME Jolma2013 and Jaspar core 2014 vertebrates databases plus de novo motifs at cutoff of $p<0.0001$. To detect motif instances that have changed between species, the full outer join of the fimo outputs was performed with missing matches assigned conservatively a p-value of 0.005. The log ratio of the p-values for orthologous motifs was calculated and used in downstream analyses. In addition, the odds ratio and p-value for enrichment at biased sites was calculated with Fisher's exact test.

To determine which motifs might have functional input into chromatin modifications in CNCCs, we calculated a correlation coefficient per motif between the log ratio p-value for its PWM and log ratio of chromatin feature signal at all sites containing the motif with discoverable species bias genome-wide. For visualization, the resulting matrix was bi-clustered with heatmap.2 and fastcluster functions in R.

For selected motifs we used the experimental information on the strength of the allele (H3K27ac enrichment) to further resolve which particular nucleotide mutations within the motif are favored at the stronger allele/ortholog. For each motif we tabulated mutations present in the strong allele and the resulting PWM

was visualized with SeqLogo. As a control, the same analysis was performed for the weak allele/ortholog.

Sequence comparison with ancestral outgroups

To identify directionality of evolutionary changes at Coordinator sites we calculated p-value scores with FIMO at orthologous human, chimp and human-chimp ancestor sequence inferred by the ENSEMBL EPO pipeline. To visualize changes we plotted -log10 p-values for each of three reference genomes and for subsets of enhancers as orthographic projections along space diagonal. The sequences of Neanderthal (Altai) and Denisova individual were obtained from Max Planck Institute for Evolutionary Anthropology server. (Meyer et al.2012; Prüfer et al., 2014).

Calculation of divergence score and identification of clusters of regulatory divergence

All enhancers with 1-to-1 orthology were assigned a p-value corresponding to species divergence of H3K27ac enrichment (see details above). To calculate a divergence score, enhancers with human bias (p-value<0.1) and, separately, enhancers with chimp bias (p-value <.1) were sorted by chromosomal position in hg19. For each sorted enhancer, the –log10(p-value) of the closest 8 enhancers (with q<0.1 bias toward the same species, in either chromosomal direction) was summed, and divided by the distance (bp) between the centers of the two furthest (±8) enhancers. To estimate background distributions, the analysis was repeated after p-values of species bias were randomly reassigned across all enhancers. To consolidate into distinct clusters and distinguish the boundaries of the regions, the window of integration (from the start of the -8 enhancer to the end of the +8 enhancer) for all enhancers with a divergence score over $2.5x10^{-4}$ were merged using Bedtools. Association with genes was done using GREAT default settings. All plots were generated in Matlab.

Supplemental References

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