

1 Supplementary Information

2 KEGG pathway sign test

3 KEGG pathway release 92.0 was downloaded using KEGGREST R package. We used the sign
4 test to test for independent *cis* changes showing deviations from neutrality, thus pointing to
5 lineage-specific selection^{9,49}. If any pathway deviates significantly from the random expectation,
6 then the null hypothesis of neutrality can be rejected in favor of polygenic selection. We
7 discarded pseudogenes and used pathways with a minimum of 50 genes linked to them, with no
8 threshold on ASE significance. *Drug metabolism - other enzymes*, *Steroid hormone biosynthesis*,
9 *Drug metabolism - cytochrome P450*, and *Metabolism of xenobiotics by cytochrome P450*
10 pathways were removed from the analysis as genes associated with them are found mainly in
11 clusters and therefore their expression changes cannot be treated as independent events. Disease
12 and genetic information processing pathways (e.g., *Transcription*) were not tested. Chromosome
13 20 and chromosome X were removed from the analyses as well due to potential aneuploidy¹² and
14 non-random deactivation that could shift results. We also examined how various FDR and fold-
15 change thresholds affect the test and found that the down:up ratio among Hh genes increases as
16 more stringent thresholds are used (Extended Data Fig. 3a).

17 Similarly, we tested protein translation rates⁵². This dataset provided information for 34 out of
18 the 50 Hh proteins annotated in KEGG. Differential translation rates data (33 of the proteins) and
19 detectable/non-detectable translation levels data (one protein) were combined for the sign test
20 analysis.

21

22 EVC2 expression

23 To pick a top candidate for further analyses, we took all genes that are expressed in both hybrid
24 cell types (mean FPKM ≥ 1), show significant ASE in both, and are linked to at least one HPO
25 phenotype with human-chimpanzee divergence information (see above). Out of 268 such genes,
26 EVC2 was the gene farthest from the origin of axes (Fig. 3b). For its phenotypic analyses,
27 phenotypes were extracted from HPO build 1268 and from a review of the literature
28 (Supplementary Tables 21-23).

29 EVC2 acts in a complex with EVC, whose loss-of-function phenotypes are indistinguishable
30 from EVC2. We detect up-regulation of *EVC* human alleles compared to chimpanzees in the
31 hybrid CNCCs (2.25x, FDR = 2.52×10^{-19}), but not parental samples, suggesting counteracting
32 *cis*- and *trans*-acting changes. As the abundance of the EVC/EVC2 complex is determined by the
33 less abundant protein⁹¹, and the expression levels of *EVC* are 7-8x higher than those of *EVC2* in
34 parental and hybrid CNCCs, it is likely that the down-regulation of EVC2 reduces the overall
35 abundance of the complex.

36

37 DPSCs

38 **Human DPSCs:** Human DPSCs were ordered from Lonza (catalog #PT-5025). Cells were
39 grown in DPSC medium catalog #: PT-3005 (AXOL).

40 **Chimpanzee DPSCs:** An incisor and a canine tooth were recovered from a forty-seven year old
41 female chimpanzee euthanized for clinical reasons at the Yerkes National Primate Research
42 Center. The teeth surfaces were cleaned and dissected at the cementum-enamel junction to reveal

43 the pulp chamber. The dental pulp was recovered followed by digestion in 3 mg/ml collagenase
44 type I (Invitrogen, Inc.) and 4 mg/ml of dispase (Invitrogen, Inc) for one hour at 37°C. The cell
45 suspension was filtered through a 70 µm cell strainer (Falcon, Inc) to remove cell debris and
46 undigested tissues. The single-cell suspensions of the dental pulp were recovered and cultured in
47 α-MEM (Invitrogen, Inc.) media supplemented with 20% fetal bovine serum (Hyclone, Inc), 100
48 µM L-ascorbic acid-2-phosphate (Sigma, Inc), 2 mM glutamine, 100 units/ml penicillin and 100
49 µg /ml streptomycin (Invitrogen, Inc.), and incubated at 37°C with 5% CO₂. Medium was
50 replaced every three to four days and cells were passaged at 80% confluence. Cells were grown
51 in DPSC medium catalog #: PT-3005 (AXOL).

52

53 *Candidate regions underlying EVC2 differential expression*

54 CNCC ATAC-seq, and NR2F1 and TFAP2A ChIP-seq reads were downloaded from GEO
55 accession number GSE70751. Reads were aligned to the human hg38 and chimpanzee panTro5
56 genomes using Bowtie2 [⁹²] and the parameters --very-sensitive --dovetail --maxins 2000. Peaks
57 were called using MACS2 v2.1.1.20160309 [⁹³] with the parameters -g hs -B -q 0.05. First, we
58 searched for any region within the gene body or promoter (up to 5 kb upstream) of *EVC2* for loci
59 with an ATAC-seq peak in all individuals of one species, but none of the individuals of the other
60 species (two chimpanzees and three humans, with two replicates each, except for human 1).
61 Three such loci were found (hereinafter, intron 6, intron 9 and intron 19, Extended Data Fig. 4a-
62 c). Next, we picked the boundaries for the sequences used for the reporter assay (see next) based
63 on the center of TFAP2A or NR2F1 binding (the stronger of the two transcription factors per
64 each locus), up to 10 kb away from the ATAC-seq peak. Given its regulatory role, the promoter
65 of *EVC2* was cloned as well. We also cloned a locus within intron 1 which was previously

66 reported to bear chimpanzee-biased H3K27ac marks⁴³ (but did not show any species-specific
67 chromatin accessibility).

68

69 To construct reporter plasmids containing chimpanzee and human *EVC2* genomic loci, DNA
70 sequences were amplified from genomic DNA samples using primers listed in Supplementary
71 Table 24. The amplified products were then inserted into the pGL4.11b (Promega) vector for the
72 promoter and pGL4.23 (Promega) vector for candidate enhancers. Cloned DNA sequences were
73 validated by Sanger sequencing. For the chimpanzee sequence within intron 6, we were unable to
74 amplify the full region denoted by the primers from genomic DNA due to repetitive regions.
75 Instead, we were able to amplify a 626 bp sequence that included the beginning and end of the
76 region, without the repeats in its center (Supplementary Table 25). We confirmed that the
77 sequence was still specific to *EVC2* intron 6 via the UCSC Genome Browser BLAT tool. This
78 626 bp is in fact more homologous to the human sequence than the entire length, as the repeats in
79 the center of the chimpanzee sequence do not exist in the human sequence. For the intron 19
80 locus, repetitive sequences in the primer regions did not allow us to clone sequences with
81 identical boundaries: the resulting chimpanzee sequence had additional 16 bp in its 5' end, and
82 45 bp in its 3' end, while the rest of the 1.4 kb was homologous. Reporter plasmids and the
83 Renilla luciferase plasmid pGL4.73 (Promega) were co-transfected into human DPSCs in each
84 well of a 48-well plate using X-tremeGENE HP DNA Transfection Reagent (Sigma-Aldrich)
85 according to the manufacturers' protocol. To correct for transfection efficiency, 240 ng of
86 reporter plasmid and 60 ng of pGL4.73 were transfected. 48 hours post-transfection, cells were
87 harvested and reporter activity was measured using the Dual-luciferase reporter assay system
88 (Promega) according to the manufacturers' protocol. Reporter activity was quantified using the

89 Glomax 96-well plate luminometer (Promega). Relative firefly/Renilla luciferase values were
90 determined in two independent experiments of quadruplet measurements ($n = 8$).

91 The intron 6 and 19 loci showed increased expression of the chimpanzee allele (3.6-fold and $P =$
92 8.2×10^{-4} for intron 6, and 4.1-fold and $P = 9.4 \times 10^{-4}$ for intron 19, one-tailed t -test, Extended Data
93 Fig. 4d,e). The promoter and intron 9 loci did not show significant differential expression ($P =$
94 0.07 for the promoter, and $P = 0.08$ for intron 9, one-tailed t -test), while the intron 1 locus
95 showed human-biased expression ($P = 4.7 \times 10^{-9}$). Based on the ATAC-seq data, intron 1 is not
96 accessible in either species. Therefore, it remains to be determined whether this region plays an
97 active regulatory role *in vivo* (possibly attenuating the effect of other loci), or alternatively, has
98 the potential to drive differential expression, but is not accessible in its endogenous context. The
99 intron 6 and intron 19 loci each have over ten substitutions between humans and chimpanzees,
100 making the investigation of specific expression-altering sequence changes a challenging task.

101 The intron 19 locus also has two indels (7 bp and 24 bp long), and the intron 6 locus has a 1.2 kb
102 long indel. Given its length, the 1.2 kb indel serves as a promising candidate to underlie the
103 expression difference we observed. Comparing this locus with the gorilla genome (gorGor5), we
104 found that this sequence does not exist in gorillas, suggesting that the insertion likely emerged
105 along the chimpanzee lineage. At first glance, this does not fit with the observed differential
106 expression of *EVC2*, which likely emerged along the human lineage. However, although humans
107 show lower *EVC2* expression compared to gorillas (Extended Data Fig. 3e), the human-gorilla
108 ratio is lower than the human-chimpanzee ratio, suggesting there might have been an additional
109 event along the chimpanzee lineage increasing *EVC2* expression. Importantly, the chimpanzee
110 region amplified for the reporter assay did not include most of this insertion (due to its
111 repetitiveness), and therefore serves as a more closely matched sequence to the human sequence.

112 Regardless, the comparative ATAC-seq, TFAP2A and NR2F1 analyses described above
113 identified sites outside this insertion showing chimpanzee-biased patterns. This suggests that
114 intron 6 might have experienced a more complex regulatory evolution, with changes affecting
115 *EVC2* expression in both directions.

116 To identify potential sequence changes that might underlie these expression changes, we scanned
117 the intron 6 and intron 19 loci for predicted transcription factor binding sites that differ between
118 human and chimpanzees. We downloaded the 4,351 *directly determined* human transcription
119 factor binding motifs from the Catalogue of Inferred Sequence Binding Preferences (CIS-BP)
120 database (<http://cisbp.ccb.utoronto.ca/>), and used FIMO⁹⁴ to map each motif to the human and
121 chimpanzee sequences. For each predicted binding site, we required that at least one species had
122 a binding *q*-value ≤ 0.05 . Then, we searched for instances where the predicted binding score of
123 the motif differed between the species. In the intron 6 locus, we found 1,091 predicted binding
124 differences, all of which are in the repetitive regions of the intron, and 51 of those are within the
125 homologous repetitive region that is outside the chimpanzee insertion. In the intron 19 locus, we
126 found 31 predicted binding differences, and two additional predicted binding motifs outside the
127 region that was amplified in the human sequence (Supplementary Tables 26-27). Next, we
128 analyzed ChIP-seq data from ENCODE⁹⁵ to determine if any of the predicted binding sites are
129 indeed bound by the predicted transcription factor in embryonic stem cells (as CNCC ChIP-seq
130 data for these factors were not available). We found that four of the transcription factors (TCF12,
131 RXRA, SP1, and SRF) were mapped in the ENCODE project. For one of them (SRF) there are
132 reported peaks (chr4:5573026-5573222 and chr4:5573079-5573309) overlapping the predicted
133 motif in intron 19 (chr4:5573215-5573228). SRF is expressed in CNCCs (TPM = 39.2),
134 suggesting this binding site may be occupied by this factor in CNCCs as well. Notably, none of

135 the positions of the substitutions in intron 6 nor intron 19 are particularly conserved among
 136 vertebrates (PhyloP score range: -3.54 to -0.13 for intron 6, and -3.42 to 0.07 for intron 19).
 137 Thus, further work is required to determine whether the expression changes we report were
 138 driven by these divergent sequences, and if so, to tease out the individual contribution of each of
 139 these substitutions and indels to the overall expression change.

140

141 **Supplementary Table 24:** Genomic coordinates, primers and vectors used in the construction of chimpanzee (Ch)
 142 and human (Hu) *EVC2* reporter plasmids.

Reporter Plasmid	Genomic Coordinates	Genomic DNA Primers	pGL vector
<i>EVC2</i> promoter	panTro5=chr4:6171370-6174138	Fwd: CCAGCTTGTTTCTAGTTTGTT TCATCATTTCTCATGGC Rev: TGGGCCAGACCATTGACC	pGL4.11b
	hg38=chr4:5707417-5710191	Fwd: CCAGCTTGTTTCTAGTTTGTTTCATCATTTCTCATGGC Rev: TGGGCCAGACCATTGGCC	pGL4.11b
<i>EVC2</i> intron 1	panTro5=chr4:6164402-6166648	Fwd: GCAGACCATTCTACTGGAAGTTG Rev: AAATACACCCATGTGGTCTCTGA	pGL4.23
	hg38=chr4:5700284-5702536	Fwd: GCAGACCATTCTACTGGAAGTTG Rev: CTTAACAATAACATCCATGTGGTC	pGL4.23
<i>EVC2</i> intron 6	panTro5=chr4:6146850-6148511	Fwd: CAGAACTCTGAATGAAATGAAATG Rev: GGGGTTCTAAGGGGTAGAG	pGL4.23
	hg38=chr4:5683604-5684029	Fwd: CAGAACTCTGAATGAAATGAAATG Rev: GGGGTTCTAAGGGGTAGAG	pGL4.23
<i>EVC2</i> intron 9	panTro5=chr4:6106169-6107052	Fwd: GATCAGTGGGGTGGCTATC Rev: TTGATCAACTGCGGTCTTTATTC	pGL4.23
	hg38=chr4:5647354-5648240	Fwd: GATCAGTGGGGTGGCTATC Rev: TTGATCAACTGCGGTCTTTATTC	pGL4.23

<i>EVC2</i> intron 19	panTro5=chr4:6031719-6033215	Fwd: AGAGGATGCTCAATCAGTGCTAA Rev: ATAACAGGCATGGCAGGTGTT	pGL4.23
	hg38=chr4:5573074-5574468	Fwd: CCAGTCGATGTCTTTTCTGTGAT Rev: GTG TTCAGGAAGTACCAGCTCCT	pGL4.23

143

144

145 **Supplementary Table 25:** Chimpanzee *EVC2* intron 6 locus amplified from genomic DNA sample.

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GGGGTTCCTAAGGGGTAGAGTCAATATCACAGACAGGAACCAAACAAAGTAACAGGCGGGACTAT
TTCAGGGTGGGGCAGGGGAAGCCACGAGGAAGGTCCTCCCGGAGCATGCAGGGAGCCAGGCTCTG
CGGAACCACAAACCTCCCCCTGCTCAAGCCAGCCCAGCCCAGGAACACACACAGCTGCCAGGAAC
ACACACAGCTGGCCAGGAACACACACAGCTGCCAGGAACACACACAGCTGCCGGGAACATACA
CACAGCTGCCGGGAACACACACAGCTGCCGGGAACACACACAGCTGCCGGGAACACACACAC
AGCTGCCGGGAACACACACAGCTGCCGGGAATACGCACAGCTGCCGGAAACACACACAGCTG
CCCAGGAACACACACAGCTGCCAGGAACACACAGAGCTGCCGGGAACATACACACAGCTGCC
GGGAACACACACAGCTGCCAGGAACACACACAGCTGCCAGGAACACACAGAGCTGCCGGGAA
CACACACAGCTGCCGGGAACACACACAGCTGCCGGGGCTTCAGCTCCTGAGGCTCCTGCCTGAG
GACATGGAAGCTTCCATTTTCATTTTCATTCAGAGTTTCTG

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146

147 See Supplementary Figure for BLAST⁹⁶ alignments.

Species	Seq ID	Sequence	Position
Chimp	1	GGGTTCTTAAGGGTAGACTCAATATCACAGCAGGAACCAACAAAGTAACAGGCGGG	60
Human	1T.....T.....T.....C.....	60
Chimp	61	ACTATTTTCAGGCTGGGCAGGGGAAGCCAGAGGAGGTCTCCCGAGCATGCAGGGAG	120
Human	61A.....	120
Chimp	121	CCAGGCTCTGCGGAACCACAACTCCCCCTGCTCAagccagccagcccaggaacacac	180
Human	121G.....	180
Chimp	181	acagctgccaggaacacacacagctgcccaggaacacacacagctgcccagga-----	236
Human	181G.....ACAC..	240
Chimp	237	acacacagctgccgggaacatacacacagctgccgggaacacacacagctgccggga	296
Human	241A.....G.....A.A.....	298
Chimp	297	acacacacagctgccgggaacacacacacagctgcccgggaacacacacacacacacac	356
Human	299G.....	358
Chimp	357	G 357	
Human	359	. 359	
Chimp	371	gcccgggaacacacacagctgccgggaacacacacagctgccgggaacacacacagCT	430
Human	167A.G.....G.....G.....C.....	226
Chimp	431	GCCC-GGGA-acatacacacacagctgccgggaacacacacagctgccgggaacacacac	488
Human	227A.A.C..C.....A.....G.....	286
Chimp	489	agctgccaggaacacacagctgcccggga--acacacacagctgccgggaacacacac	546
Human	287A.A.G.....C.....AC.....	346
Chimp	547	acagctgccgggaacacacacacacacagctgcccgggaacacacacacacacacac	606
Human	347G.....	406
Chimp	607	CATTTCATTCAGAGTTTCTG	626
Human	407	426
Chimp	1	TTGATCAACTGCGGTCTTTATTCTTTTGTGCTCGAATTTTACCATTCTAGATTAAAA	60
Human	1	60
Chimp	61	AGCTTCGCTCCCTTAAGACACAACCTTAGCCTAGTTTAAACAACAGCTCATTCAATTCG	120
Human	61	120
Chimp	121	TTCTTTCAATTTTCACAAAAGTCTTATTGAACATGTGCTATGCAAGAGCGCTTTGCA	180
Human	121	180
Chimp	181	GGTACAAAGCAAGAAACACACAGAGCTGCCTCAAGGCACTGTGTATGGCTAAAT	240
Human	181	240
Chimp	241	GTCAAAATGAACATAACAATCAATGTTTACAAGCTCAGAAAAAATCCCTTCCCTACT	300
Human	241T.....	300
Chimp	301	CTGACGACAGACAGAAATGATTTCAACCTTAAACGAGCCTTATGTGCAAGGAGGACAC	360
Human	301T.....	360
Chimp	361	TGCCCCCGAAAGACATTCACAGCCTAATCCCAAGAAAGCCAGGAAACACAGTTAGTTTCATG	420
Human	361T.....	420
Chimp	421	GCAAGGGAAATTAAGGCTACTCATCAGCTGACCTTAAGATTGAGATCAAGACAAAGCC	480
Human	421	480
Chimp	481	TTTTAAAAAGGAAACAGGCAAGAGGGGTGAGAGTTGGAGAACGCCCTGTGGCGAAG	540
Human	481	540
Chimp	541	GAAGCCGACATAGGAGTGTGACATCAGCTTTGGAGATAGAAAGGGTCCCAAGTCA	600
Human	541C.....	600
Chimp	601	TGAATGTAG--GCAGCCTCAAGAAGCCGAAAGGGCCAGGAAAGGAAACCTCCCTAGAG	657
Human	601GCA.....	660
Chimp	658	CTGACGTCTtttttttttAATTGACAAGTAAAGATGATATACATTTCTGGGCCATGTGA	717
Human	661C.....	720
Chimp	718	TAATTTAATACATTTCATTTAATGTGCAAGATTAAATCAGGTTAATTGGGATAGCCATCA	777
Human	721C.....	780
Chimp	778	CCTTAAATATTGTCTTTCTTTATGCTAGAAACATTAGAATTCCTCCCTCTAGCTATT	837
Human	781C.....	840
Chimp	838	CTGAAATGTGCAATAGATTATTGTAATGATAGCCACCCCACTGATC	884
Human	841	887
Chimp	17	GTGTTGAGGAAGCACCAGCTCTCCCTGCCCTTTGGACACTGTCATTCAGGTCTAA	76
Human	1T.....T.....T.....C.....	60
Chimp	77	CACTGGACCTTGTGGTACAGTCAACAGAAATAAGCCCTCCCAAAATGTCATGG	136
Human	61C.....T.....	113
Chimp	137	TCCATCTCTGGGACCTGGGAAGATGTCACTTACAAGGCAAGGTTACTTTGCAAGTGG	196
Human	114	173
Chimp	197	AGTCAAGTCAAGGCTGGAGATAGGTTGGAAAGTGGTTATCTCAGATTATCCAGGTGG	256
Human	174A.....T.....	233
Chimp	257	TTCCAATATAATTACCGTGTGGCTCTTAAAGATAGAGACCTTTCTGCTGTGAGGTC	316
Human	234A.....	293
Chimp	317	AGAGAGAGTGTGACGAGATGGAAGAGGGCCAGAGAGATGCAATTTTCTGCTTTGA	376
Human	294G.....	353
Chimp	377	AGATGAAGAAAGGAGCCATGAGCCAAAGGAAATAGTGCCTGGAAGCCAGGAATAC	436
Human	354	400
Chimp	437	CCCTGGCTGGCAGCCAGCAAGAAACAGGACCTCAGTCTCAACCAACAATGGGCTGAA	496
Human	401	449
Chimp	497	CCCTGCCAATTACTTAAGTGAAGCAGGAGCAGATTCTCCCTAAAGCTTCCGAAAGCCA	556
Human	450A.....	509
Chimp	557	GGCAATCTGCCAACCTCGATTTTGGCTGGAGAAACCTATGTTGGATTCTGACCTA	616
Human	510C.....G.....	569
Chimp	617	CAGAACTTAAGTAAGTTTGTAGCTGTGATAAGCTGTGTTTGTGTCATTTGTGACAG	676
Human	570	629
Chimp	677	CAGTAGTAGGAACGAATTCATTAGGAGCTGCTGTGCTGGATGCAGCCAGCTGG	736
Human	630	689
Chimp	737	ACGCTTAAAGGCCCTAACCCCTCCTCTCTGCTGACTCCCTTCTTAGAGGCTGGGGGC	796
Human	690	749
Chimp	797	AAAGTACTCGCACCTCCCTGAGTGCAGGAGCCACGGCTCAGCTCAGGTTGCGA	856
Human	750A.....	809
Chimp	857	GGAAACCTGCTGAGGACTTTGGGGCATTGCTTCCCTGATGAGAAAGACAGCATG	916
Human	810A.....	869
Chimp	917	GTGGCAGTCTTTGACCTCTCCCTCTGCTGTAAGTATTGCAGTGCAGCACTGTA	976
Human	870	929
Chimp	977	GAAGGAAGTGGGAAAGATCCGACAGATGACCTGATGATGATGATGATGATGATGATG	1036
Human	930T.....A.....	989
Chimp	1037	TCTCCGGAGTCAATGGCTGGCATTACCACTGTCTGAGTGTATTTCTGCTACTTAC	1096
Human	990T.....G.....C.....	1049
Chimp	1097	ACACCCAGAAGCAGGAGGAGCCATCGCAGATGCGTAAACCCGATCGGTAGCAGA	1156
Human	1050	.G.....	1109
Chimp	1157	CAGTCAAGTACATGACTGCATAGGCTCCCTGACAGCTCAGGGCTCTGATCAGGAAG	1216
Human	1110	1169
Chimp	1217	GGAGGTGAGTCTGCAACTCTGCGCAAGGATGAGAAAGTTGGCTGATCTCCACTCAGAG	1276
Human	1170	1229

148

149 **Supplementary Figure.** BLAST alignment between the chimpanzee and human amplified sequences for
150 intron 6 (a), 9 (b) and 19 (c). Dots represent identities, lowercase letters show low complexity sequences.

151 We also used the CNCC ATAC-seq data to map peaks in putative enhancers associated with Hh

152 genes. To do so, we downloaded the GeneHancer database⁹⁷ (v4_12) and extracted the 530

153 putative enhancers associated with Hh genes. For each putative enhancer, we investigated

154 chimpanzee and human ATAC-seq peaks 10 kb around it (Supplementary Table 28).

155

156 EVC2 effect on hedgehog signaling output

157 **Stable cell line generation**

158 *Evc2*^{-/-} NIH/3T3 Flp-In cells were generated using CRISPR/Cas9 genome editing using the guide
159 sequence 5'-gatatttcaaaaatgctcac-3', and were described previously in Pusapati et al.⁹¹.

160 Doxycycline-inducible Evc2 expression was generated by cloning mouse Evc2 (N-terminally
161 tagged with 3xHA (downstream of signal sequence) and C-terminally tagged with 1D4) into the
162 pCDH-Lenti-TRE-rtta3G-BLAST plasmid (gifted from Atul Kumar). Lenti-virus was generated
163 by transfection of this construct in combination with psPAX2 (Addgene plasmid number 12260)
164 and pVSVG (Addgene plasmid number 14888) into 293T cells. Lenti-viral supernatant was then
165 added to *Evc2*^{-/-} NIH/3T3 Flp-In cells for 48 hours before selection with puromycin
166 (Calbiochem).

167 Generation of pooled cell lines expressing low, medium and high amounts of mouse Evc2 was
168 carried out by cloning mouse Evc2 C-terminally tagged with TEV-YFP-FLAG into the pMSCV
169 plasmid (Addgene plasmid number 24828). The pMSCV construct was then transfected into
170 Bosc23 helper cells in order to generate retroviral supernatants⁹⁸. Virus was added to *Evc2*^{-/-}
171 NIH/3T3 Flp-In cells after 24 and 48 hours by centrifugation for 1 hour at 800g. Stably infected
172 cells were then selected by puromycin. High multiplicity of infection was evident by the death of
173 only a small fraction (~5%) of cells. These cells were sorted into pools of 100,000 cells each by
174 fluorescence activated cell sorting in order to isolate low, medium and high expressing pooled
175 lines.

176

177 **Cell culture and Hedgehog signaling assays**

178 Both *Evc2*^{-/-} NIH/3T3 Flp-In cells and Dental Pulp Stem Cells (DPSCs) were grown in high
179 glucose Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) containing
180 10% Fetal Bovine Serum (FBS) (Sigma), 2 mM L-glutamine (Gemini Biosciences), 1× MEM
181 nonessential amino acids solution (Gibco), penicillin (40 U/ml) and streptomycin (40 µg/ml)
182 (Gemini Biosciences), and 1 mM sodium pyruvate (Gibco).

183 In order to test for Sonic Hedgehog ligand responsiveness, cells were ciliated by growing to
184 confluency and then exchanging the media to low serum (0.5% FBS) DMEM containing the
185 aforementioned supplements for either 24 hours (NIH/3T3 Flp-In cells) or 48 hours (DPSCs).
186 Sonic Hedgehog ligand (recombinant protein generated as in Bishop et al.⁹⁹) and doxycycline
187 (Sigma, D9891) were added 24 hours prior to cell lysis and Western Blotting.

188 Primary antibodies used for Western Blotting include: mouse monoclonal anti-GLI1 (Cell
189 Signaling, Cat# 2643; RRID: AB_2294746), mouse monoclonal anti-alpha tubulin (clone DM1A,
190 Sigma-Aldrich, Cat#T6199; RRID: AB_477583), rabbit polyclonal anti-P38 (Abcam, Cat# ab7952;
191 RRID: AB_306166), rabbit anti-GFP (Novus Biologicals, NB600-308), rabbit polyclonal anti-SUFU
192 (produced in rabbits (Josman Laboratories) and affinity purified before use), rabbit polyclonal anti-
193 EVC2 (produced in rabbits (Cocalico Biologicals) and affinity purified before use). Secondary
194 antibodies used for Western Blotting include: Peroxidase AffiniPure Donkey Anti-Mouse IgG (H+L)
195 (Jackson ImmunoResearch Laboratories, Cat#715-035-150; RRID:AB_2340770), Peroxidase
196 AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Cat#111-035-
197 144; RRID: AB_2307391) and Peroxidase AffiniPure Donkey Anti-Goat IgG (H+L) (Jackson
198 ImmunoResearch Laboratories, Cat#705-035-003; RRID: AB_2340390).
199

200 EVC2 CNCC-specific KO mice

201 All mice were maintained and used in compliance with the Institutional Animal Care and Use
202 Committee (IACUC) of the University of Michigan in accordance with the National Institutes of
203 Health guidelines for Care and Use of animals in research, and all experimental procedures were
204 approved by the IACUC of the University of Michigan. All mice were housed in ventilated cages
205 with free access to food and water. Ventilating cages were in rooms in 18-23 C with 40-60%
206 humidity. Housing room has a 12 hours dark/light cycle with light starting from 6 am.

207 Two loxP sites were inserted to intron 12 and 14 of the *Evc2* locus, respectively. Cre-dependent
208 recombination caused truncation at exon 15 to generate no functional protein⁶³. Neural crest-
209 specific *Evc2* mutant mice were generated by crossing *Evc2* floxed mice with *Wnt1-Cre* mice¹⁰⁰.

210 Animals were harvested at postnatal day 28 (P28) for structural analyses.

211 Micro-CT scanning of fixed skulls was taken at the University of Michigan using a micro-CT
212 system (mCT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were as following:
213 voxel size 12 μ m, 55 kVp, 109 mA, 0.5 mm AL filter, and integration time 500 ms.

214 Heterozygous mice were used as controls because they are phenotypically indistinguishable from
215 wildtype mice⁶³.

216 To quantify skull morphological differences between controls and *Evc2* KO P28 mice, surface
217 models were first generated based on micro-CT data. Next, each Ellis-van Creveld craniofacial
218 phenotype was examined between control and KO mice and compared to phenotypes that
219 separate humans and chimpanzees, as described above and in Gokhman et al⁴⁸. For the *increased*
220 *forehead height* phenotype, we examined frontal bone height. *Tapering of the lower face* was
221 equated to ventral rotation of the lower face and micrognathia.

222 DICOM files/images obtained from micro-CT were used to generate 3D model using ITK-SNAP
223 (www.itksnap.org). 3D slicer (www.slicer.org) was then used for placements of anatomical
224 landmarks. The mandible associated measurements were determined using landmarks from
225 Extended Data Fig. 6c, i.e., mandible length (1-4), condyle head width (3-4), gonial angle (the
226 angle of 5-7 and 4-8) and mandible height (2-6). The thickness of the palate bone was
227 determined at the palate bone at first molar levels. The root length and enamel thickness were
228 determined in molar 1 of controls and KOs. Width of the nasal bone was determined at the most
229 anterior part of nasal bones. These data were combined with previous measurements from *Evc2*
230 KO mice, including of soft tissues^{63,64,68}.
231

Panel	Species	Accession	Sequence	Position	
a	Chimp	1	GGGGTTCCTAAGGGGTAGAGTCAATATCACAGACGAGCAACCAAAAGTAACAGCGGG	60	
	Human	1T.....	60	
	Chimp	61	ACTATTCAGGGTGGGGCAGGGAAGCCACGAGGAAAGTCTCCCGAGCATGCGAGGAG	120	
	Human	61A.....	120	
	Chimp	121	CCAGGCTCTGGGAACCAACAACCTCCCTCTCaaagccagccagccaggaacacac	180	
	Human	121T.....G.....	180	
	Chimp	181	acagctgcccaggaacacacacagctgcccaggaacacacacagctgcccagga---ac	236	
	Human	181G.....ACAC..	240	
	Chimp	237	acacacagctgcccggaaacacacacagctgcccggaaacacacacagctgcccggga	296	
	Human	241A.....G.....A.....A.....	298	
	Chimp	297	acacacacagctgcccggaaacacacacacagctgcccggaaacacacacacacac	356	
	Human	299T.....	358	
	Chimp	357	G 357		
	Human	359	. 359		
	Chimp	371	gcccggaaacacacacagctgcccaggaacacacacagctgcccaggaacacacacagct	430	
	Human	167A..G.....G.....G.....G.....C.....	226	
	Chimp	431	GCCC-GGA-acatacacacacagctgcccggaaacacacacagctgcccaggaacacac	488	
	Human	227A..A..C..C.....A.....G.....G.....G.....	286	
	Chimp	489	agctgcccaggaacacacacagctgcccggga--acacacacagctgcccggaaacacac	546	
	Human	287A..A..G.....C.....A.....AC.....	346	
	Chimp	547	acagctgcccggggCTTCAGTCTCTGAGGCTCTGCTGAGGACATGGAAGCTTCCATT	606	
	Human	347T.....	406	
	Chimp	607	CATTTCATTAGACTTTCTG 626		
	Human	407T..... 426		
	b	Chimp	1	TTGATCAACTGCGGTTCTTATTCTTTTGTGATCGAATTTACCATTCTAGATTAATAA	60
		Human	1T.....	60
		Chimp	61	AGTCTCCGTCGCCCTTAAGACACAACTTAGCCTAGTTTTTAACAACAGCTCATTCTTTC	120
		Human	61T.....	120
		Chimp	121	TTCCTTTCATTCTCAAAAAGTGTATTGAACTGTGCTATGCAACGAGCGCTTTGCA	180
		Human	121T.....	180
Chimp		181	GGTTACAAAGCAAGAAAGAAACACAAGAGCTGCCTCAAGGCAGTGTGTATGGCTAAAT	240	
Human		181T.....	240	
Chimp		241	GTCAAATGACTAAACAATCAATGTTGTACAAGCTCAGAAAACAAAATCCCTTCCCTACT	300	
Human		241T.....	300	
Chimp		301	CTGAGACAGACAGAATGGATTTTCAACCTTAAAGCAAGCCCTTATGTGGCAGGGAGACAG	360	
Human		301T.....	360	
Chimp		361	TGCCCCCGCAAGACATTACACAGCTAATCCCCAGACGGCAACACAGTGTAGTTTCAAG	420	
Human		361T.....	420	
Chimp		421	GCAAGGGGAATTAAGGTACTCATCAGCTGACCTTAAGATTGAGATCAAGACAAGGCC	480	
Human		421T.....	480	
Chimp		481	TTTTAAATAGGAAACAGGCAAGAGGGGTACAGATTTGGAGAAGCCCTGTGGCGAAG	540	
Human		481T.....	540	
Chimp		541	GAAGCCGACATAGGAGTATGACATCAGCTTTGGAGATAGAAAGGGGTCCCAAGTCAG	600	
Human		541T.....	600	
Chimp		601	TGAATGTAG--GCAAGCTCAAGAAGCGGAAAGGCCAGGAAGGGAACCTCCCTAGAG	657	
Human		601GCA.....	660	
Chimp		658	CTGACGTCTTTTTTTAATTGACAAGTAATAGATGTATACATTTTCTGGGCCATGTGA	717	
Human		661C.....	720	
Chimp		718	TAAATTAATACATTTCATTAAATGTGCAAAAGATTAATCAGGTTAATGGGATAGCCATCA	777	
Human		721C.....	780	
Chimp		778	CCTTAAATATTGCTCTTTCTTTATGCTAGAAACATTAGAATTCCTCCCTCTAGCTATT	837	
Human		781C.....	840	
Chimp		838	CTGAAATGTGCAATAGATTATTGTAATGATAGCCACCCACTGATC 884		
Human		841T..... 887		
c	Chimp	17	GTGTTGAGGAAGCACCAGCTCCTCCCTGCCCCGTTTGGACACTGTCTTCCAGTGTAA	76	
	Human	1T.....T.....C.....	60	
	Chimp	77	CACTGGCACCTTGTGGTACAGTCAACAGATAATGACCCCTCCCAAAATGTCCATGG	136	
	Human	61C.....T.....	113	
	Chimp	137	TCCCTCCTGGACCTGCGAAGATGTCACTTACAAAGGCAAGGTTACTTTGCAAGTGGG	196	
	Human	114T.....	173	
	Chimp	197	AGTCAGGTCAGGGCCCTGGAGATAGGGTGGAAAGTGTATCTCAGATTATCCAGGTGG	256	
	Human	174A.....T.....	233	
	Chimp	257	TTCCAAATATAATTACCCTGTGGTCTTAAGAGTAGAGACCTTCTCCCTGTGTGAGGTC	316	
	Human	234A.....A.....T.....	293	
	Chimp	317	AGAGAGAGATGTGACAGGATGGAAGAAGGGCCAGAGATGCAATTTCCTGGCTTTGA	376	
	Human	294G.....	353	
	Chimp	377	AGATGAAGAAAGGAGCCATGAGCCAAGGAATGTAGGTGCCACTGGAAGCCAGGAATTC	436	
	Human	354T.....	400	
	Chimp	437	CCCTGGCTGGCAGCCAGCAAGAAACAGGACCTCAGTCTCAACCAATGGGCTGAA	496	
	Human	401T.....	449	
	Chimp	497	CCCTGCCAATTACTTAAGTAGCAGGAAGCAGATTCTCCCTTAAGCTTCCGAAAGGCA	556	
	Human	450A.....	509	
	Chimp	557	GGCAATCTGCCAACACCTCGATTTTGGCTGGGAAACCCATGTTGGATTTCTGACCTA	616	
	Human	510C.....T.....G.....	569	
	Chimp	617	CAGAAGTTAAGTAAGTTTGTAGCTGTGTAAGTGTGCTTTGTGGTCAATTTGTGACAG	676	
	Human	570T.....	629	
	Chimp	677	CAGTAGAGAAACAGATTCATTGAGGAGTCGGTGTGCTGTGGTGCAGCCAGCTGG	736	
	Human	630T.....	689	
	Chimp	737	ACGCTTAAAGCCCTTAACCCCTCCTTCCTGCTGACTCCCTTCTTAGAGGCTGGGGGC	796	
	Human	690T.....	749	
	Chimp	797	AAAGTACTCGCACCTCCCTGACAGTAGAGGGCCACGGGTGAGTCTCAGGTTGCGA	856	
	Human	750A.....	809	
	Chimp	857	GGAAACCTGCTGAGGACTTGGGGCATTGCTTCTTGTAGAGAAGACAGGACATG	916	
	Human	810T.....G.....	869	
Chimp	917	GTGGCAGTCTTTGACCTCTCCCTCCTGCTGAAAGTATTGACAGTGAAGTGTGA	976		
Human	870T.....	929		
Chimp	977	GAAGAACTGGGAAAGAAATGCCAGAACATGACCTGATGCTCAGTGCAGCAGCACC	1036		
Human	930T.....A.....G.....	989		
Chimp	1037	TCTCCGAGTCAATGGGCTGGCATCACCAGTGTCAAGTTGATTTTCTGCTACTTAC	1096		
Human	990T.....G.....C.....	1049		
Chimp	1097	ACACACCAGAAGCAGGAGCCCATCGCAGATGCTATATAAACCAGTCCGGTAGCAGA	1156		
Human	1050G.....	1109		
Chimp	1157	CAGTCAGTACATGACTGCATAAGGCTCCGTCACAGCCCTCAGGGCTCTGTATCAGGAAG	1216		
Human	1110T.....	1169		
Chimp	1217	GGAGTGTGCTGCAACTCTGGCCAAAGATGAGAAGTTGGCTGATCTCCACTCAGAGA	1276		
Human	1170T.....	1229		

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233 **Supplementary Figure. BLAST alignment between the chimpanzee and human amplified**
234 **sequences for intron 6 (a), 9 (b) and 19 (c).** Dots represent identities, lowercase letters show
235 low complexity sequences.

236 Supplementary Information References

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