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Original Article

Depletion of CTCF induces craniofacial malformations in mouse embryos

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Abstract: Increasing evidence implicates chromatin structure and epigenetic regulation in various human developmental disorders, including facial abnormalities and intellectual disability. Mutations in CCCTC-binding factor (CTCF) demonstrate its role in craniofacial development, but early lethality precludes the use of *Ctcf* mutant mice for phenotypic investigations. In this study, we deleted *Ctcf* specifically in neural crest cells, the multipotent cells that give rise to many structures of the skeleton and connective tissues in the developing head. Although the pharyngeal arches were initially morphologically normal, many of the neural crest cell-derived skeletal and non-skeletal components were truncated in the *Wnt1-Cre; Ctcf*$^{fl/fl}$ mutant mice. The expression level of chondrogenic and osteogenic-related genes were significantly decreased. Our results implicate CTCF in two distinct events in craniofacial development; first, in the regulation of outgrowth and morphogenesis by cell survival and proliferation, and second, in the differentiation of the facial skeleton. Our findings highlight the important contribution of CTCF to craniofacial pathologies.

Keywords: CCCTC-binding factor (CTCF), neural crest cells, craniofacial malformation, embryogenesis

Introduction

The craniofacial architecture comprises interconnected bones, cartilage, and connective tissues, the correct formation of which is necessary for the proper encasement of the brain and sensory organs [1]. Craniofacial development in vertebrates involves a complex series of molecular and morphogenetic processes, beginning with the ventrolateral migration of multipotent cranial neural crest cells from the dorsal brain as the mesenchymal source for the facial primordia [2]. Specifically, distinct populations of cranial neural crest cells from the diencephalon, midbrain, and hindbrain form the skull vault and facial and pharyngeal skeletons [3]. Whereas rostral cranial neural crest cells give rise to the frontonasal prominence that becomes the mid- and upper face, the more posterior cells fill the pharyngeal arches that develop into the maxilla, mandible, and lateral skull. The second pharyngeal arch contributes to the ear and neck skeleton [1, 4]. A clearer understanding of neural crest development is important, as neural crest cells are involved in a variety of craniofacial birth defects, including cleft lip and palate.

Craniofacial formation requires precise regulation of the genes important for developmental specification, migration, and differentiation [2]. Increasing numbers of genes involved in chromatin structure and epigenetic regulation are implicated in a variety of developmental process and developmental disorders [5]. However, recent studies have mainly focused on factors related to DNA methylation and histone modification [5]. CCCTC-binding factor (CTCF), an epigenetic component essential for regulating the three-dimensional organization of the genome, is a zinc finger DNA binding protein that has been associated with various cellular processes, including transcriptional regulation and insulation, X chromosome inactivation, and RNA splicing [6]. CTCF plays roles in tissue development and the differentiation of various cell types, ranging from embryonic stem cells to neural, cardiac, and muscle cells [7]. Moreover, *Ctcf* is expressed in the developing vertebrate cranial neural crest [8]. Mutations in CTCF are

responsible for specific phenotypes in humans, such as syndromic craniofacial deformities and growth retardation related to microcephaly, cleft palate, and dental anomalies [9-11]. However, the role of CTCF in regulating external and internal structures during craniofacial development is not known. Therefore, we utilized transgenic mice with conditional knockout (cKO) of *Ctcf* in neural crest cells to document the role of CTCF in craniofacial development. Our results demonstrate that the essential association between CTCF and neural crest cells-derived as a crucial regulator of the head document.

Materials and methods

Mice generation and breeding

The mice utilized in this study carried a conditional *Ctcf* allele (*Ctcf*$^{fl/fl}$) described in our previous study. *Ctcf*$^{fl/fl}$ and *Ctcf*$^{fl/+}$ mice were crossed with Tg (*Wnt1-Cre*) 11Rth/MileJ mice to obtain neural crest-specific conditional knockout mutants and controls, respectively [12, 13]. All animal protocols were approved by the Institutional Animal Care and Use Committee at Yonsei University College of Medicine.

Gross anatomical and histological analyses

For gross anatomical observation, embryos were dissected at embryonic day 9.5 (E9.5), E15.5, and E18.5 and fixed in 4% paraformaldehyde overnight. All of the samples were observed under a stereomicroscope (Leica S8 APO). Fixed E15.5 embryos were paraffin-embedded, sectioned (12 μm), and stained with hematoxylin (Sigma) for 2 min and eosin (Sigma) for 3 min. The tissue sections were observed under a light microscope (Leica DM2500) after mounting with Permount mounting medium (Thermo Fisher Scientific).

Alizarin red and alcian blue staining

Embryos were eviscerated and treated with hot water (65°C) before fixation in 95% ethanol overnight and then in acetone overnight to remove fat. The specimens were subsequently stained with 0.03% alcian blue (Sigma) solution for cartilage staining and washed with 70% ethanol for three h. Next, they were incubated in a 1% potassium hydroxide (KOH; Sigma) solution until they became transparent. The specimens were transferred into 0.05% alizarin red (Sigma) overnight for bone staining. The specimens were cleared by placing them in 1% KOH-20% glycerol for two days and incubated with 1% KOH in 50% glycerol until image processing.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Skull, maxilla, and mandible tissues from *Wnt1-Cre; Ctcf*$^{fl/+}$ and *Wnt1-Cre; Ctcf*$^{fl/fl}$ mice were quickly dissected in cold DEPC-treated PBS, followed by RNA isolation with the Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA synthesis was performed using a ImProm-II™ cDNA Synthesis Kit (Promega) with oligo (dT) primers. RT-qPCR was performed using the SensiFAST SYBR Hi-ROX Mix (BIOLINE) and an ABI7500 real-time PCR instrument system (Thermo Fisher Scientific). The primer sequences of the genes are as follows: *Col1a1*, 5'-GCAACAGTCGCTTCACCTACA-3' and 5'-CAATGTCCAAGGGAGCCACAT-3'; *Sox9*, 5'-CCTTCAACCTTCCTCACTACAGC-3' and 5'-GGTGGAGTAGAGCCCTGAGC-3'; *OPN*, 5'-TCTGATGAGACCGTCACTGC-3' and 5'-TCTCCTGGCTCTCTTTGGAA-3'; *Aggrecan*, 5'-AAGTTCCAGGGTCACTGTTAC-3' and 5'-AAGTTCCAGGGTCACTGTTAC-3'; *ActB*, 5'-GGCTGTATTCCCCTCCATCG-3' and 5'-CCAGTTGGTAACAATGCCATGT-3'. Student's t-tests were used to determine statistical significance. $P<0.05$ was considered as significant.

Results

Ctcf deficiency results in defective outgrowth and morphogenesis of craniofacial organs

To investigate the role of CTCF in neural crest-derived craniofacial development, we conditionally deleted *Ctcf* from neural crest cells using *Wnt1-Cre* mice [12, 13]. *Wnt1-Cre; Ctcf*$^{fl/fl}$ embryos were indistinguishable from control *Wnt1-Cre; Ctcf*$^{fl/+}$ embryos at E9.5, when they have pharyngeal arches and small maxilla (**Figure 1**). The fact that there was no defective growth of the facial primordia after the migration of cranial neural crest cells to the maxilla and pharyngeal arches suggests that the generation and migration of neural crest cells were not affected. However, defective growth was observed at E15.5. *Wnt1-Cre; Ctcf*$^{fl/fl}$ embryos exhibited smaller heads with an defective anterior skull, resulting in an exposed brain (**Figure**

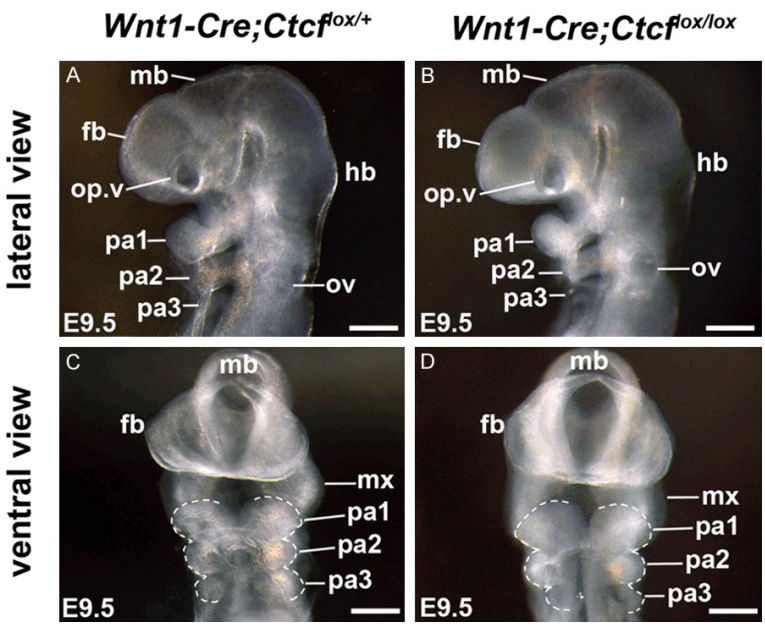

Figure 1. Gross morphology of E9.5 *Wnt1-Cre; Ctcf*$^{fl/+}$ and *Wnt1-Cre; Ctcf*$^{fl/fl}$ embryos. Lateral (A, B) and ventral (C, D) views of *Wnt1-Cre; Ctcf*$^{fl/+}$ (control) and *Wnt1-Cre; Ctcf*$^{fl/fl}$ (*Ctcf* cKO) embryos at E9.5. The overall structure of the head where the prospective skull will form and the formation of pharyngeal arches are not different in control and *Ctcf* cKO. The maxilla primordia, which is important for craniofacial organ formation, is also well formed in the control and *Ctcf* cKO. Abbreviations: fb, forebrain; mb, midbrain; hb, hindbrain; op.v, optic vesicle; pa, pharyngeal arch; ov, otic vesicle; mx, maxilla. Scale bars, 250 μm.

2G, **2I**; red asterisks) as well as shortened mandibles and maxilla; the maxilla did not come in contact with each other (**Figure 2H**, **2I**; yellow line).

Histological analyses revealed that the left and right palatal shelves failed to fuse at the midline in the *Wnt1-Cre; Ctcf*$^{fl/fl}$ mice, unlike in the control mice (**Figure 2**, compare **2J** and **2K** with **2C** and **2D**), resulting in a complete cleft palate. The palatal shelves were also not elevated in the mutant (**Figure 2J**, **2K**). The size of the developing tongue (width and height) was smaller in the *Wnt1-Cre; Ctcf*$^{fl/fl}$ mice than in the *Wnt1-Cre; Ctcf*$^{fl/+}$ controls; the upper part of the tongue was generally wider than the lower region in the *Wnt1-Cre; Ctcf*$^{fl/+}$ tongue but not in the *Wnt1-Cre; Ctcf*$^{fl/fl}$ tongue (**Figure 2C**, **2E**, **2J**, **2K**; red lines). Tooth development is generally at the cap stage at this phase of development. Whereas the molars of maxilla and mandibles in control mice had a normal cap shape, those of the *Wnt1-Cre; Ctcf*$^{fl/fl}$ mutants exhibited thickening of the dental epithelium (**Figure 2F**, **2M**; green dotted line), and Meckel's cartilage was not observed under the tooth germ.

Real-time qPCR was performed for quantitative analysis of genes important for chondrogenesis and osteogenesis in the E15.5 *Wnt1-Cre; Ctcf*$^{fl/+}$ and *Wnt1-Cre; Ctcf*$^{fl/fl}$ mutant head with skull and craniofacial tissues (**Figure 2N**). The expression of *Aggrecan* and *Sox9*, chondrogenic markers, were significantly decreased in *Wnt1-Cre; Ctcf*$^{fl/fl}$, compare to the *Wnt1-Cre; Ctcf*$^{fl/+}$ (**Figure 2N**). In addition, it was confirmed that the expression level of osteogenic marker *Osteopontin* (*OPN*) and *Col1a1* was down-regulated in the *Wnt1-Cre; Ctcf*$^{fl/+}$ than *Wnt1-Cre; Ctcf*$^{fl/+}$ (**Figure 2N**).

Taken together, these results indicate that CTCF is required for proper outgrowth and morphogenesis of the skull, palate, tongue, teeth, and Meckel's cartilage and suggest that CTCF regulates craniofacial development after neural crest cell migration.

Ctcf deficiency causes extensive loss of the craniofacial skeleton

At E18.5, *Wnt1-Cre; Ctcf*$^{fl/fl}$ embryos had dramatically smaller heads than *Wnt1-Cre; Ctcf*$^{fl/+}$ controls, with absent anterior skulls and hypoplasia of maxilla, mandibles, and ear pinnae (**Figure 3A**, **3B**). We stained the skeletons of E18.5 embryos to obtain a detailed understanding of these structural defects. The zygomatic processes of the maxilla and upper part of the maxilla were retained in *Wnt1-Cre; Ctcf*$^{fl/fl}$ mice (**Figure 3C-F**). More caudally, the jugal and squamosal bones were reduced, and the palatine and pterygoid were absent (**Figure 3C**, **3D**). The cranial base in the *Wnt1-Cre; Ctcf*$^{fl/fl}$ embryos was also severely affected; the orbitosphenoid, presphenoid, and rostral half of the basisphenoid were missing, and the alisphenoid was reduced (**Figure 3C**, **3D**). Rostrally, the upper region of the nasal bone in the

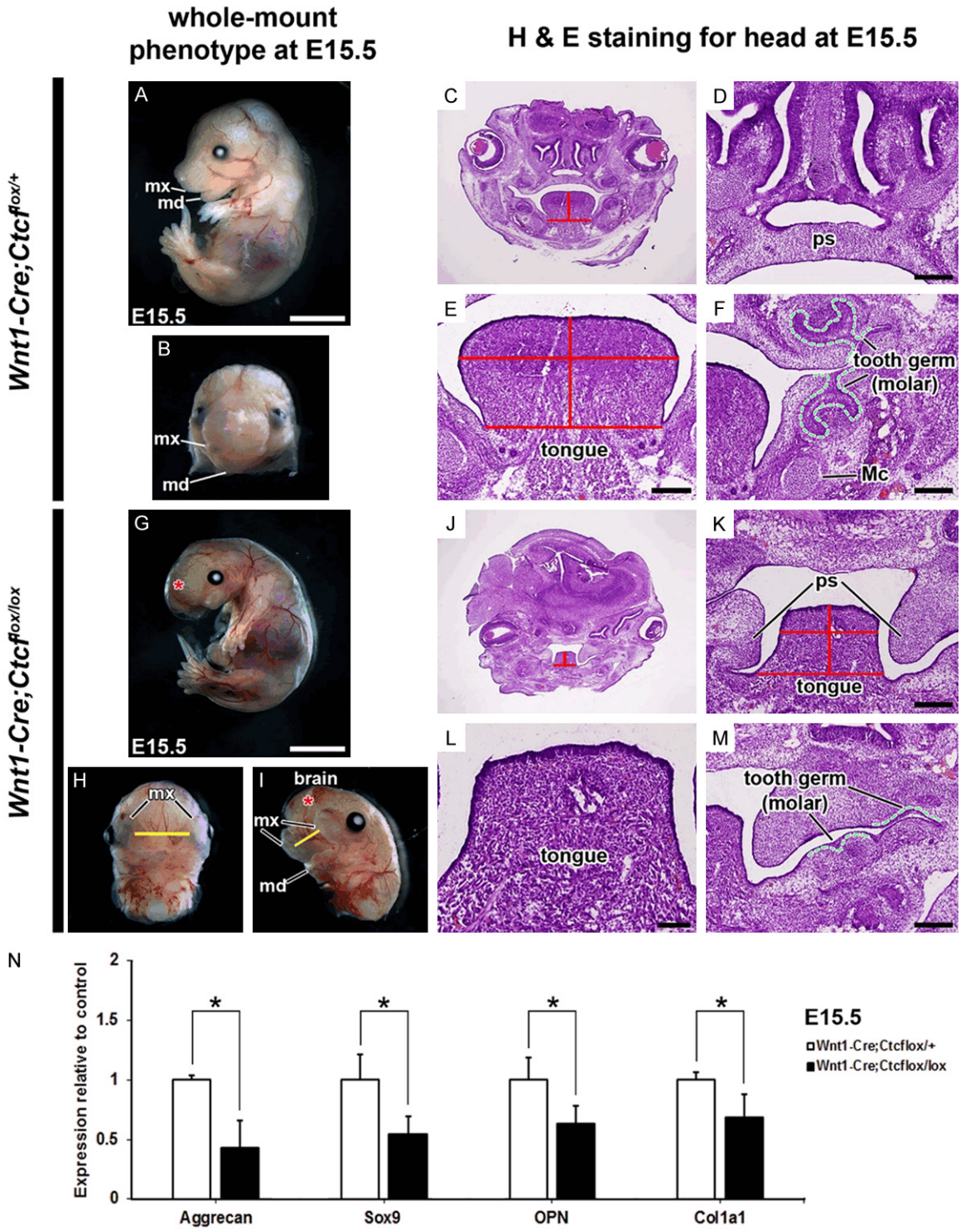

Figure 2. Anatomical and histological analyses of E15.5 *Wnt1-Cre; Ctcf*$^{fl/+}$ and *Wnt1-Cre; Ctcf*$^{fl/fl}$ embryos. Altered expression level of skeleton-related genes in mutant skull and craniofacial tissues. A, B, G, H, I. Gross images of *Wnt1-Cre; Ctcf*$^{fl/+}$ (control) and *Wnt1-Cre; Ctcf*$^{fl/fl}$ (*Ctcf* cKO) embryos at E15.5. The skull vault is defective in the *Ctcf* cKO embryo, and hypoplasia of the maxilla and mandible is apparent. Each maxilla part is separated, compare to the control. C-F, J-M. H&E-stained tissue sections of the head showing the morphology of the palate, tongue, and tooth at E15.5. The palatal shelves of the *Ctcf* cKO embryos are not fused and vertically oriented. The width and height of the tongue in *Ctcf* cKO are much smaller, the overall shape is more rectangular. The tooth development in the control embryo is at cap stages, whereas the tooth germ in *Ctcf* cKO shows dental placode status. Meckel's

cartilage is not observed in the *Ctcf* cKO. N. Expression level of *Aggrecan*, *Sox9*, *Osteopontin* (*OPN*), and *Col1a1* in control and *Ctcf* cKO. The expression level of chondrogenic-related genes such as *Aggrecan* and *Sox9* was significantly decreased in *Ctcf* cKO. The expression of *Opn* and *Col1a1*, osteogenic-related genes, are lower in *Ctcf* cKO than in control. Abbreviations: mx, maxilla; md, mandible; ps, palatal shelves; Mc, Meckel's cartilage. Horizontal red lines, upper and lower parts of the tongue; vertical red lines, tongue height; green dotted lines, margin of the dental epithelium and mesenchyme. Scale bars, 500 μm. *P<0.05.

Wnt1-Cre; Ctcf$^{fl/fl}$ mice was much smaller than in *Wnt1-Cre; Ctcf$^{fl/+}$* controls (**Figure 3E**, **3F**). Near the ears, the otic capsules appeared normal, but the underlying tympanic rings were absent in the *Wnt1-Cre; Ctcf$^{fl/fl}$* mutants (**Figure 3E**, **3F**). By contrast, the three occipital bones, namely, the basioccipital, interparietal, and supraoccipital bones, which derive from paraxial mesoderm, were less affected (**Figure 3C-F**). The dentary was hypoplastic and short and the coronoid, condylar, and angular processes were not distinguishable in the *Wnt1-Cre; Ctcf$^{fl/fl}$* mutants, unlike in the *Wnt1-Cre; Ctcf$^{fl/+}$* controls (**Figure 3G**, **3H**). To examine the expression level of chondrogenic and osteogenic genes in the E18.5 *Wnt1-Cre; Ctcf$^{fl/+}$* and *Wnt1-Cre; Ctcf$^{fl/fl}$* mutant head with skull and craniofacial tissues, RT-qPCR was performed at E18.5 (**Figure 3I**). The expression of *Aggrecan* and *Sox9*, chondrogenic markers, were significantly reduced in *Wnt1-Cre; Ctcf$^{fl/fl}$*, compare to the *Wnt1-Cre; Ctcf$^{fl/+}$* (**Figure 3I**). Also, the expression level of osteogenic marker *Osteopontin* (*OPN*) and *Col1a1* in the *Wnt1-Cre; Ctcf$^{fl/fl+}$* was significantly lower than *Wnt1-Cre; Ctcf$^{fl/+}$* (**Figure 3I**).

The defects of the skull vault were limited to the absence or decrease in the size of the neural crest-derived frontal bone in the craniofacial region, whereas the parietal and intraparietal bones, which are mesoderm-derived, were less affected. The reduction in the size of external ear pinnae, which derive from ectoderm, is likely to be a secondary phenotype resulting from the failure of most of the head skeleton to form despite the presence of cranial neural crest cells in the facial primordia (see **Figure 1**). Thus, the skeletal defects of the craniofacial region in *Wnt1-Cre; Ctcf$^{fl/fl}$* mice reflect the requirement for CTCF in post-migratory cranial neural crest cells within the facial primordia.

Discussion

Craniofacial morphogenesis appears to result from reciprocal interactions between intrinsic and extrinsic environments and the neural crest mesenchyme, where timing is an essential component [1]. Despite advances in the identification of genes influencing cranial neural crest cell patterning [5], little is known about the influence of chromosome organizers. In particular, the effects of regulators of chromatin conformation, such as CTCF in distinct subpopulations of neural crest cells, on the patterning programs that give rise to the specific structure of skeletal elements are poorly understood.

Our study demonstrates a crucial role for CTCF in neural crest-derived craniofacial formation and development. The loss of *Ctcf* in neural crest cells eliminated or reduced all of the neural crest cell-derived craniofacial elements, whereas the mesoderm-derived structures were less affected (**Figures 2**, **3**). Specifically, *Ctcf* deficiency perturbed the development of skeletal components in and around the skull vault, palate, and tympanic ring as well as non-skeletal parts, such as the tongue and teeth. In addition, the dentary, Meckel's cartilage, jugal bone, and the zygomatic process of the maxilla were present but reduced, which might reflect the influence of CTCF on the development of pharyngeal arch-derived organs (**Figure 3**). These results suggest that CTCF may be involved in the differentiation of neural crest-derived structures during embryogenesis.

Neural crest cell specification and migration from the neural tube largely depend on signaling molecules, such as Wnt, Bmp, and Fgf, which promote the expression of neural crest specifier genes, such as *Snail2*, *Foxd3*, *Sox9*, and *Sox10* [14]. However, the normal morphology of the facial primordia and pharyngeal arches in *Wnt1-Cre; Ctcf$^{fl/fl}$* mutants at E9.5 suggests that CTCF does not affect these processes. Rather, CTCF may impact processes after neural crest cell specification during the outgrowth of maxillary and frontonasal skeletal elements and tooth and pharyngeal arch-derived organ development, which are regulated by Fgf, Bmp, and Shh signaling [14]. Interest-

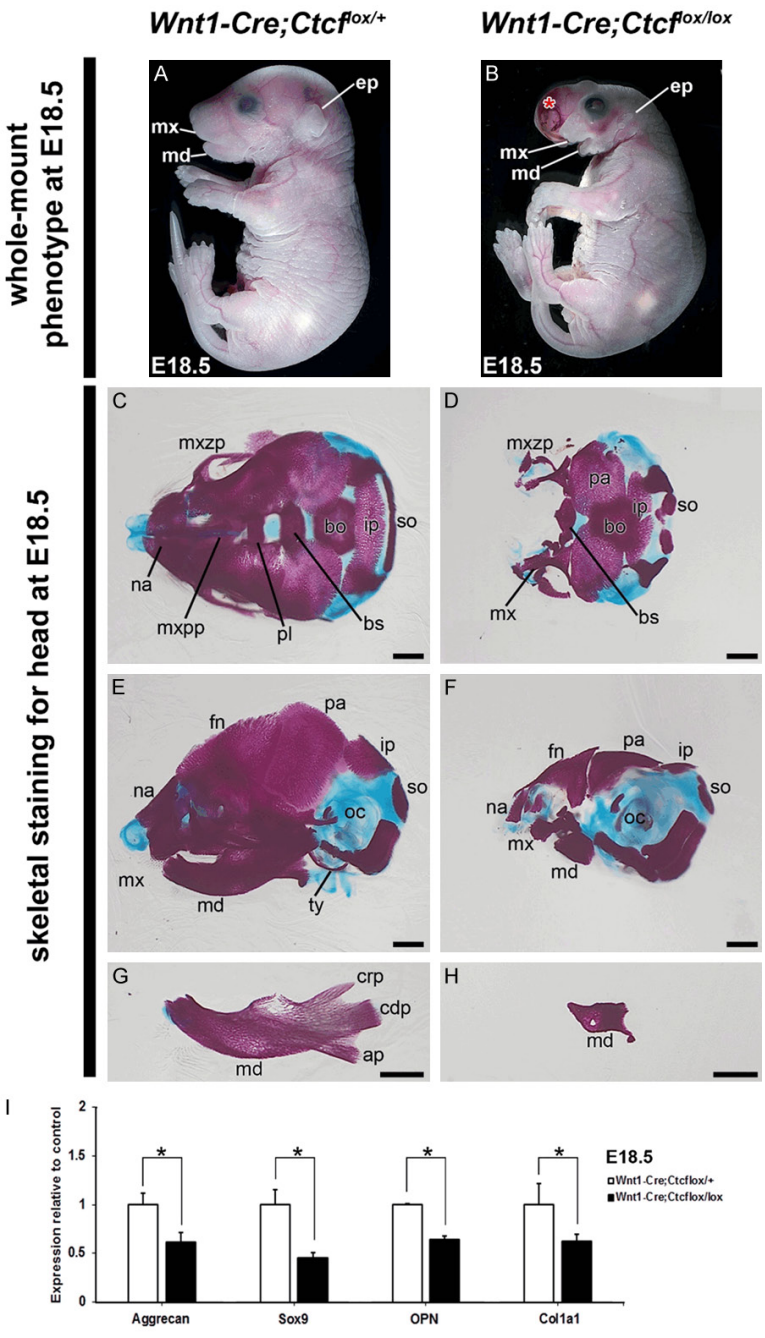

Figure 3. Comparison of gross morphology and skeletal structures in the heads of E18.5 *Wnt1-Cre; Ctcf*$^{fl/+}$ and *Wnt1-Cre; Ctcf*$^{fl/fl}$ embryos. (A-H) Anatomical whole-mount structure of E18.5 *Wnt1-Cre; Ctcf*$^{fl/+}$ (control) and *Wnt1-Cre; Ctcf*$^{fl/fl}$ (*Ctcf* cKO) embryos. (A, B) The brain of the *Ctcf* cKO embryo is exposed without the skull and shorter maxilla and mandible in the smaller head; the malformation of the ear pinna is apparent. Dorsal (C, D) and lateral (E, F) views of skeletal elements in control and *Ctcf* cKO heads at E18.5. (G, H) The dentary bone is dramatically smaller in the *Ctcf* cKO embryo than in the control at E18.5. Coronoid, condylar, and angular processes cannot be distinguished. (I) Altered expression level of *Agreecan*, *Sox9*, *OPN*, and *Col1a1* in head of the control and *Ctcf* cKO at E18.5. The expression of *Agreecan* and *Sox9* in *Ctcf* cKO, chondrogenic genes, are significantly downregulated in the *Ctcf* cKO than in the mutant. The expression of *OPN* and *Col1a1*, osteogenic genes, are decreased in the *Ctcf* cKO, compare to the control. Abbreviations: mx, maxilla; md, mandible; ep, ear pinna; na, nasal bone; mxzp, zygomatic process of maxilla; mxpp, palatal process of maxilla; pl, palatine; bs, basisphenoid; bo, basioccipital; ip, interparietal; so, supraoccipital; fn, frontal; oc, otic capsule; pa, parietal; ty; tympanic ring; crp, coronoid process; cdp, condylar process; ap, angular process. Scale bars, 1 mm. *P<0.05.

ingly, the craniofacial phenotype of *Wnt1-Cre; Ctcf*$^{fl/fl}$ is similar to mutants with a deficiency in smoothened (*Smo*), a downstream effector of Shh signaling [15]. The facial malformations in these mice result from increased death and decreased proliferation of neural crest cells. The expression of *Shh* in *Ctcf* mutants is strongly decreased in the limb mesenchyme [16]. Global depletion of *Ctcf* is embryonic lethal around the preimplantation stage (E3.5) [17, 18] and is associated with widespread apoptosis mediated by the downregulation of *p53* and upregulation of Puma (*Bbc3*), both of which are the targets of CTCF [7, 16]. Thus, CTCF might be required for cell survival and cell proliferation in the developing craniofacial region, and dysregulation of the p53-dependent pathway and restricted Shh signaling may account for the aberrant formation of the head in CTCF mutants.

The anteroposterior identities of neural crest cell subpopulations influence the development and assembly of the craniofacial skeleton. In early embryogenesis, the pattern of *Hox* expression is an essential molecular distinguishing characteristic of these subpopulations along the antero-

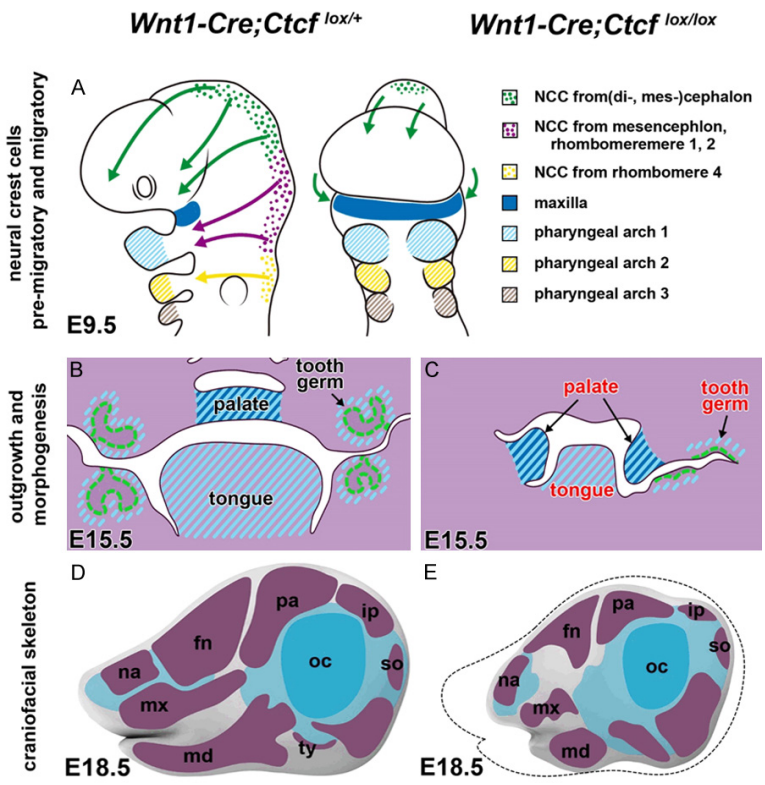

Figure 4. Schematic summary for the effects of *Ctcf* during neural crest-derived craniofacial development. A. Effect of *Ctcf*-deficiency during the neural crest migration. Morphologically, morphology of embryo with maxilla and pharyngeal arches is normal in both Wnt*1-Cre; Ctcf*$^{fl/+}$ (control) and *Wnt1-Cre; Ctcf*$^{fl/fl}$ (*Ctcf* cKO). B, C. The phenotype of *Ctcf* cKO in developing craniofacial region at E15.5. *Ctcf* cKO demonstrates disrupted morphogenesis, including the defective skull, cleft palate, hypoplasia tongue, dental placode, and Meckel's cartilage at E15.5. D, E. The neural crest cells-derived craniofacial skeleton of control and *Ctcf* cKO at E18.5 At E18.5, *Ctcf* cKO shows smaller head with the hypoplasia of anterior skulls and hypoplasia of mandibles and maxilla.

posterior axis and determines the rostrocaudal orientation of the pharyngeal arches [1]. CTCF regulates the transcriptional activation of *Hox* clusters by establishing an inheritable domain [19, 20]. However, *Hox* genes are not expressed in pharyngeal arch 1 or (dien-, mesen-) cephalic neural crest cells that contribute to the formation of the craniofacial region. Therefore, we speculate that CTCF does not regulate *Hox* genes during craniofacial development. However, neural crest cells of the frontonasal region and in the first arch that originate in the midbrain express *Otx2*, a homeodomain transcription factor. Mutation of *Otx2* affects the development of the frontonasal and distal mandibular elements [1]. CTCF is composed of multiple domains that bind to different motifs and various regulatory proteins [6, 20]. Thus, CTCF may be associated with inhibition of *Hox* as an insulator and activation of *Otx2* in neural crest cells during the development of the craniofacial structures.

In summary, our study demonstrates crucial information supporting a role for CTCF in neural crest-derived craniofacial formation and development. The fact that there was no defective growth of the facial primordia after the migration of cranial neural crest cells to the maxilla and pharyngeal arches suggests that the generation and migration of neural crest cells were not affected. The removal of the *Wnt1-Cre; Ctcf*$^{fl/fl}$ head structure reveals that the neural crest-derived skeletal components in and around the palate (pterygoid, palatine, presphenoid, palatal process of maxilla, vomer) and tympanic ring in the ear region, as well as the non-skeletal parts such as the tongue and tooth, are perturbed. In addition, the dentary, Meckel's cartilage, jugal bone, and zygomatic process of the maxilla are present but reduced (**Figure 4**), which might reflect the influence of CTCF on the development of pharyngeal arch-derived organs. Therefore, we conclude that CTCF is dispensable for the generation and migration of neural crest cells but is essential for later steps of craniofacial development. Further studies are needed to explore the underlying genome-wide molecular mechanisms of how CTCF modulates the craniofacial skeleton by epigenetic regulation. Such studies will help to understand the fundamental mechanisms of CTCF-associated craniofacial defects in humans.

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Disclosure of conflict of interest

None.

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