Ctip2/Bcl11b controls ameloblast formation during mammalian odontogenesis

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The transcription factor Ctip2/Bcl11b plays essential roles in developmental processes of the immune and central nervous systems and skin. Here we show that Ctip2 also plays a key role in tooth development. Ctip2 is highly expressed in the ectodermal components of the developing tooth, including inner and outer enamel epithelia, stellate reticulum, stratum intermedium, and the ameloblast cell lineage. In *Ctip2*$^{-/-}$ mice, tooth morphogenesis appeared to proceed normally through the cap stage but developed multiple defects at the bell stage. Mutant incisors and molars were reduced in size and exhibited hypoplasticity of the stellate reticulum. An ameloblast-like cell population developed ectopically on the lingual aspect of mutant lower incisors, and the morphology, polarization, and adhesion properties of ameloblasts on the labial side of these teeth were severely disrupted. Perturbations of gene expression were also observed in the mandible of *Ctip2*$^{-/-}$ mice: expression of the ameloblast markers *amelogenin*, *ameloblastin*, and *enamelin* was down-regulated, as was expression of *Msx2* and *epiprofin*, transcription factors implicated in the tooth development and ameloblast differentiation. These results suggest that Ctip2 functions as a critical regulator of epithelial cell fate and differentiation during tooth morphogenesis.

amelogenesis | cellular differentiation | enamel | tooth development | transcription factor

Tooth development is a model system for study of coordinated molecular interactions between ectoderm and the underlying, neural crest-derived mesenchyme. Tooth development is initiated with a thickening of the oral ectoderm at embryonic day 10.5 (E10.5) that gives rise to the dental lamina, which in turn expands into underlying mesenchyme, forming the tooth bud (bud stage, E12.5). The cap stage (E14.5) follows and includes folding of the dental ectoderm in a process that is regulated by a transient signaling center, the primary enamel knot. The enamel knot expresses signaling molecules that stimulate proliferation of surrounding epithelium and mesenchyme. The early bell stage of tooth development (E15.5–E16.5) is characterized by continued epithelial expansion and differentiation into the inner (IEE) and outer enamel epithelium (OEE), stratum intermedium (SI), and stellate reticulum (1–6).

During the mid- to late-bell stage of odontogenesis (E16.5–E19.5), 2 tooth-specific cell types are formed: ameloblasts, which differentiate from the IEE and secrete enamel, and odontoblasts, which derive from dental mesenchyme and produce dentin (5, 7, 8). Ameloblasts also synthesize and secrete the enamel matrix proteins, amelogenins, and nonamelogenins, which are assembled into a structural framework (9, 10). Ameloblasts transport calcium and phosphate ions into the extracellular matrix, which results in nucleation and growth of hydroxyapatite crystals.

Enamel formation on mouse incisors is an asymmetric process resulting from differential distribution of ameloblasts around these teeth during development. The lingual side of mouse incisors lacks ameloblasts and is enamel-free, while ameloblasts localize on the labial side of the developing incisor and promote enamel formation on this aspect of the tooth. In contrast, odontoblasts are found on both sides of the developing incisor (11).

Several transcription factors have been implicated in tooth development, including Pax9, Pitx2, Runx2, Msx1, Msx2, and others (reviewed in ref. 12). Mutations in *Pax9* and *Msx1* cause oligodontia in humans (13, 14), and *Msx1*-mutant mice exhibit arrest of tooth development at early stages (15). Msx2 regulates proliferation of the enamel organ and cusp morphogenesis, and terminal differentiation of ameloblasts (16).

Ctip2/Bcl11b is a transcriptional repressor (17–19) that plays critical roles in the development and function of several organ systems, including the central nervous (20, 21), immune (22, 23), and cutaneous (24) systems. Germline deletion of *Ctip2* is associated with perinatal lethality (22), demonstrating the essentiality of Ctip2 for life.

Although a tooth developmental defect has not been reported in *Ctip2*$^{-/-}$ mice, the epidermal defects associated with loss of Ctip2 (24), together with a high level of Ctip2 expression in dental tissues of ectodermal origin (Fig. 1) prompted us to investigate this possibility. We report herein that lack of Ctip2 compromises tooth development. *Ctip2*$^{-/-}$ molars were characterized by a hypoplastic stellate reticulum and poorly developed cusps at later stages. Ameloblast-like cells developed inappropriately on the lingual side of lower incisors in *Ctip2*$^{-/-}$ mice, while ameloblasts on the labial side were smaller, disorganized, nonpolarized, and exhibited low levels of expression of ameloblast-specific genes, such as *amelogenin*, *ameloblastin*, and *enamelin*. Expression of *Msx2* was also downregulated in *Ctip2*$^{-/-}$ mice, and ChIP studies revealed that *Msx2*, and other genes involved in ameloblast development and function, are likely direct targets of Ctip2. Collectively, these data suggest that Ctip2 is essential for the terminal differentiation of ameloblasts and proper tooth formation.

Results

Ctip2 Expression in Developing Jaw and Tooth. Antibody staining revealed high levels of Ctip2 expression in the oral ectoderm of the first branchial arch at E9.5 (Fig. 1*A*). Ctip2 expression continued to be detected in oral ectoderm, with lower levels in the first branchial arch mesenchyme at E10.5 (Fig. 1*B*). At E12.5, Ctip2 expression was detected in the thickening of the oral ectoderm, the dental epithelium of future molars (Fig. 1*C*),

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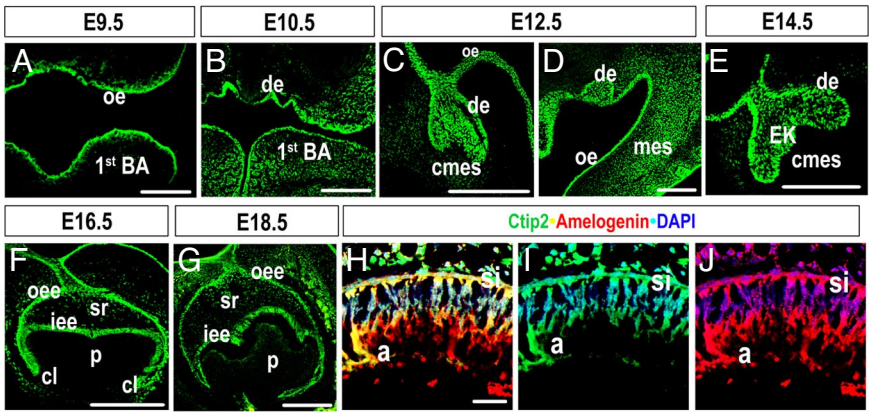

Fig. 1. Ctip2 expression profile during tooth development. (*A–J*) IHC in coronal sections of mouse embryonic heads at indicated developmental ages. Ctip2 staining appears green in all panels. (*A*) Expression of Ctip2 in the ectoderm of the 1st BA and future oe at E9.5. (*B*) Expression of Ctip2 in de at E10.5 and across the 1st BA. (*C* and *D*) Ctip2 expression in the oe, de, and cmes of a developing lower molar (*C*) and upper incisor (*D*) at E12.5. (*E–G*) Ctip2 expression in the developing lower molar at E14.5 (*E*), E16.5 (*F*), and E18.5 (*G*). (*H–J*) Double-label IHC of coronal sections of a lower incisor at E18.5 using antibodies against Ctip2 and amelogenin and counterstained with DAPI. The IHC data presented in this figure have been reproduced 4–6 times over the course of ≈2 years. [Scale bars: (*A*, *B*, *D*, *G*) 200 μm; (*C*, *E*, *F*) 100 μm; (*H*) 20 μm.] a, ameloblast; BA, branchial arch; cl, cervical loop; cmes, condensing mesenchyme; dc, dental cord; de, dental epilethium; iee, inner enamel epithelium; oe, oral epilethium; oee, outer enamel epithelium; p, papilla, si, stratum intermedium; sr, stellate reticulum.

incisors (Fig. 1*D*), and surrounding mesenchyme (Fig. 1 *C* and *D*), and to a lesser extent in condensing dental mesenchyme (Fig. 1*C*). Substantial Ctip2 expression was noted in the dental epithelium and enamel knot at E14.5 (Fig. 1*E*), and at E16.5, when the dental epithelium had differentiated into IEE and OEE, Ctip2 expression was strongly detected in both structures and in the cervical loop and stellate reticulum (Fig. 1*F*). Expression of Ctip2 in the ectoderm persisted at E18.5 (Fig. 1 *G–J*), but was not appreciably detected in mesenchyme-derived tissues, including dental papilla.

Ctip2 Expression in Ameloblasts. Ctip2 was highly expressed in the IEE (Fig. 1 *F* and *G*), which gives rise to ameloblasts. Double-labeling immunohistochemistry (IHC) revealed colocalization of Ctip2 and the ameloblast marker amelogenin in the late bell stage (Fig. 1 *H–J*). Ameloblasts appeared as columnar cells with elongated nuclei and Ctip2 localized intensely in these nuclei (Fig. 1 *H–J*).

Disruption of Tooth Development in *Ctip2*$^{-/-}$ Mice. *Ctip2*$^{-/-}$ mice die perinatally (22, 24) but do not exhibit an obvious developmental delay or size difference from WT mice during development (24). Three-dimensional, microCT analyses of 5 *Ctip2*$^{-/-}$ mice at P0 indicated normal morphology of the craniofacial skeleton and dentitional initiation and organization that were indistinguishable from WT mice (Figs. S1 and S2). However, closer examination of *Ctip2*$^{-/-}$ mice revealed several defects at the individual stages of tooth development. Both incisors (Fig. 2 *A–J*) and molars (Fig. 2 *K–P*) of the *Ctip2*$^{-/-}$ mice were slightly smaller than those of WT mice at all stages. The bud of mutant incisors was smaller with an elongated dental cord at E14.5 (Fig. 2 *A* and *B*), a defect persisted at E16.5 (Fig. 2 *C* and *D*). Ameloblasts began to differentiate and elongate, and became polarized at E16.5 in WT mice (Fig. 2 *C*, *G*, and *I*), whereas the same cells appeared disorganized and unpolarized in mutants (Fig. 2 *D*, *H*, and *J*). Ameloblasts developed only on the labial side of WT incisors (Fig. 2 *C* and *I*), while epithelial cells populated the lingual aspect (Fig. 2 *C* and *G*). However, cells resembling ameloblasts were found on both labial (Fig. 2 *D* and *J*) and lingual (Fig. 2 *D* and *H*) sides of the incisors in *Ctip2*$^{-/-}$ mice. The absence of lingual/labial asymmetry of ameloblasts observed in Fig. 2 was further established with expression of the ameloblast-specific proteins on the lingual side of *Ctip2*$^{-/-}$ incisors (white arrows in Fig. 3 *D*, *J*, and *P*).

An expansion of the epithelium was also present on the lingual side of the developing, mutant incisor (Fig. 2*H*, green asterisk), adjacent to the ectopic, ameloblast-like cells in *Ctip2*$^{-/-}$ mice (Fig. 2*H*, red asterisk). The stellate reticulum on the labial side of the mutant incisors was indiscernible (Fig. 2*J*, black asterisk), which resulted in a close association between the OEE and IEE, and stratum intermedium, hindering identification of the individual structures of the developing tooth. The OEE of *Ctip2*$^{-/-}$ mice was hypoplastic and the odontoblasts appeared structurally disorganized, but this effect was less severe than the disruption of cells of the ameloblast lineage (Fig. 2 *D*, *F*, and *J*). Thus, loss of Ctip2 appeared to affect the ameloblast lineage preferentially, as the formation of ameloblasts, the boundary of the ameloblast development, and ameloblast specification were all disrupted in *Ctip2*$^{-/-}$ mice, with corresponding effects on the labial/lingual asymmetry, and enamel secretion. TUNEL staining of *Ctip2*$^{-/-}$ incisors (Fig. S3*B*) and molars (Fig. S3*D*) at E16.5 was indistinguishable from that of WT mice (Fig. S3 *A* and *C*, respectively) at this developmental stage, as was cellular proliferation within the developing molar, as assessed by staining with anti-phosphohistone H3 (PH3) (compare Fig. S3 *E* and *F*). These results suggest that the tooth developmental defects in *Ctip2-null* mice are not a result of alterations in cell death or proliferation.

Ctip2$^{-/-}$ molars were slightly smaller than those of WT mice at the bud stage, with a slightly elongated dental cord (Fig. 2 *K* and *L*). At E16.5, *Ctip2*$^{-/-}$ molars appeared smaller and underdeveloped, the enamel knot was not well defined, and the stellate reticulum was hypoplastic (Fig. 2 *M* and *N*), all of which persisted up to E18.5 (Fig. 2 *O* and *P*). These findings suggest that Ctip2 may play a role in the function of the enamel knot, and in the development and maintenance of stellate reticulum during tooth morphogenesis.

Ctip2 Is Implicated in Ameloblast Differentiation. The role of Ctip2 in ameloblast differentiation was studied by examining expression of 3 ameloblast markers in WT and *Ctip2*$^{-/-}$ mice: amelogenin, ameloblastin, and enamelin. *Ctip2*$^{-/-}$ mice exhibited strongly down-regulated expression of amelogenin expression at E16.5 (Fig. 3 *U* and *V*), and all markers at E18.5 (Fig. 3 *A–R*), the latter of which was confirmed by RT-qPCR (Fig. 3*S*).

DEVELOPMENTAL BIOLOGY

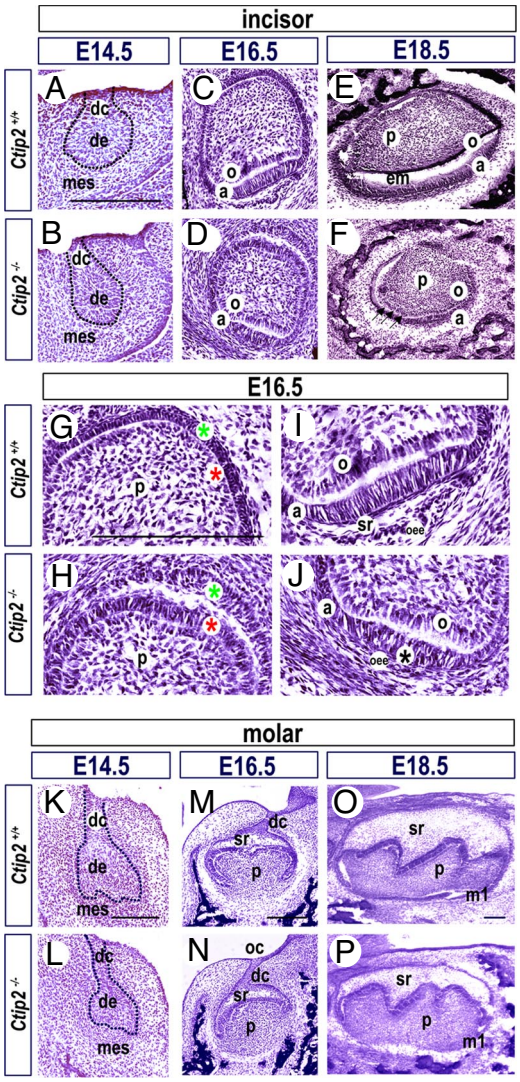

Fig. 2. Defects in tooth development in *Ctip2*$^{-/-}$ mice. H & E staining in coronal sections of WT (*A*, *C*, *E*, *G*, *I*, *K*, *M*, *O*) and *Ctip2*$^{-/-}$ (*B*, *D*, *F*, *H*, *J*, *L*, *N*, *P*) mice at E14.5 (*A*, *B*, *K*, *L*), E16.5 (*C*, *D*, *G–J*, *M*, *N*), and E18.5 (*E*, *F*, *O*, *P*). (*G–J*) Higher magnification of (*C*) and (*D*), respectively, highlighting the lingual (*G* and *H*) and labial (*I* and *J*) sides of a developing incisor. Note the elongated dental cord (*B*, *L*, *N*), reduced and disorganized ameloblast layer (*D*, *F*, *J*), and loss of lingual/labial asymmetry (*G–J*) in *Ctip2*$^{-/-}$ mice. The black asterisk (*J*) indicates a reduced stellate reticulum on the labial side of a developing incisor; the red asterisks represent ectopic ameloblast-like cells on the lingual-side mutant incisors (*H*) and lack of these cells in WT incisors (*G*); and the green asterisks indicate the epithelial expansion on the lingual side of mutant incisors (*H*) and the corresponding cells in WT mice (*G*). All histology studies presented in this figure are representative of at least 4 independent mice of each genotype. [Scale bars: (*A–F*, *K–P*) 100 μm; (*G–J*) 200 μm.] de, dental cord; em, enamel matrix; m1, first molar; mes, mesenchyme; o, odontoblast.

At E16.5, *Ctip2*$^{-/-}$ ameloblasts appeared somewhat elongated, but with short cell bodies containing small and randomly distributed nuclei (Fig. 3 *U–Z*). Strong ß-tubulin expression was detected in the well-defined processes at the apical surface of WT ameloblasts (Fig. 3*W*), consistent with accumulation of microtubules that accompanies polarization. ß-tubulin expression was also detected in *Ctip2*$^{-/-}$ ameloblasts; however, these cells did not appear to elongate to form apical processes properly (Fig. 3*X*). Nuclei of WT ameloblasts were elongated along the apical-basal axis, and positioned primarily on the basal side, adjacent to the stratum intermedium (Fig. 3 *W* and *Y*; note nuclei predominantly localized at positions "1" and "2" in Fig. 3*Y*). Nuclei of ameloblasts in WT mice were rarely observed on the apical processes of these cells in sections from multiple, independent WT mice (Fig. 3 *W* and *Y*; see relative lack of nuclei at position labeled "3" in Fig. 3*Y*). In contrast, nuclei of *Ctip2*$^{-/-}$ ameloblasts were ≈30% shorter than WT ameloblasts at this developmental stage, and the mutant nuclei appeared rounded and located randomly throughout the ameloblast layer, including on the apical aspect of the mutant ameloblasts (Fig. 3*X* and position "3" in Fig. 3*Z*), indicative of a lack of ameloblast elongation or polarization in the mutant mice.

At E18.5, WT ameloblasts express enamel-related proteins and form strong, cell adhesion-based contacts involving numerous adhesion molecules, which contribute to the structural integrity of ameloblast cell layer. However, *Ctip2*$^{-/-}$ ameloblasts exhibited low levels of all 3 enamel proteins of ameloblast (Fig. 3 *B*, *D*, *F*, *H*, *J*, *L*, *N*, *P*, and *R*), with clearly altered cell-cell adhesion in the labial apical regions (*white stars* in Fig. 3 *F*, *L*, and *R*), and decreased expression of *laminin 5-3a* (Fig. 3*S*), which controls adhesion, differentiation, and integrity of the ameloblast cell layer (16, 25).

Considered together, these results suggest that loss of Ctip2 resulted in dysregulation of genes encoding proteins that are crucial for ameloblast development, function, and structural integrity.

The results of ChIP assays performed in tissues derived from E18.5 mandibles suggested that regulation of *amelogenin*, *enamelin*, and *laminin 5-3a* expression by Ctip2 was likely because of direct or indirect interaction of Ctip2 with the corresponding promoters. Ctip2 was found to be present on proximal and distal regions of *laminin 5-3a* and *enamelin* promoters (as defined in Table S1), and the distal region of the *amelogenin* promoter (Fig. 3*T*). Ctip2 was not detected on either the proximal or distal regions of the *ameloblastin* promoter, suggesting that Ctip2 may regulate expression of this gene indirectly or from a regulatory elements that is located outside the regions that were examined in the present study.

Ctip2 Is a Member of the Ameloblast Gene Network. A limited number of transcription factors have been implicated in later stages of ameloblast formation. Msx2 regulates the terminal differentiation of ameloblasts through control of *laminin 5-3a* expression, and *Msx2*$^{-/-}$ ameloblasts (16) phenotypically resemble those of *Ctip2* mutants (Fig. 2). *Msx2*$^{-/-}$ mice exhibit defects in cusp morphogenesis resulting from reduced proliferation of the enamel organ (16), which also resembles the tooth phenotype of *Ctip2*$^{-/-}$ mice. We found that Ctip2 and Msx2 exhibited overlapping domains of expression in the dental epithelium of incisors (not shown) and molars at E14.5 (Fig. 4*A*) and E16.5 (Fig. 4*D*), and loss of Ctip2 resulted in a 2-fold down-regulation of *Msx2* mRNA (Fig. 4*G*), which was consistent with results of IHC studies, particularly at E16.5 (compare Figs. 4 *E* and *F*). Ctip2 mutants appeared to express lower levels of Msx2 within the dental epithelium of the developing molar, as well as in the oral epithelium (compare Figs. 4 *E* and *F*). Ctip2 was found to be present on proximal and distal regions of the *Msx2* promoter (Fig. 4*H*), suggesting that *Msx2* may be a direct target of Ctip2 in ameloblasts.

Epiprofin/Klf14/Sp6 is expressed in both developing ameloblasts and differentiated odontoblasts, and controls proliferation and differentiation of the dental epithelium (26). Epiprofin expression was down-regulated in *Ctip2*$^{-/-}$ mice (Fig. 4*H*), and Ctip2 was present on the proximal region of the *epiprofin* promoter (Fig. 4*H*), suggesting that epiprofin may also be a direct, transcriptional target of Ctip2 in the developing mandible. Considered together, these results appear to place *Msx2* and *epiprofin* downstream of Ctip2 during tooth morphogenesis. Sp3 controls enamel production through regulation of expression of

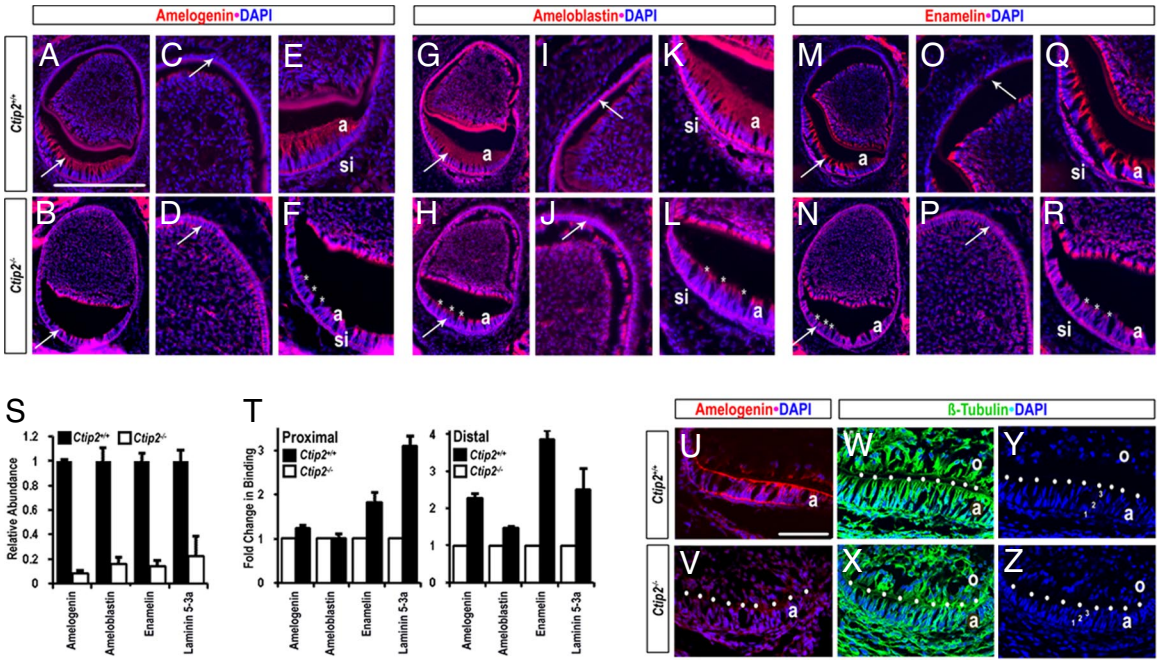

Fig. 3. Ameloblast defects in *Ctip2*$^{-/-}$ mice. IHC on coronal sections of a mandibular incisor using indicated antibodies in WT (*A*, *C*, *E*, *G*, *I*, *K*, *M*, *O*, *Q*, *U*, *W*, *Y*) and *Ctip2*$^{-/-}$ (*B*, *D*, *F*, *H*, *J*, *L*, *N*, *P*, *R*, *V*, *X*, *Z*) mice at E16.5 (*U–Z*) and E18.5 (*A–R*). All sections were counterstained with DAPI. Amelogenin- (*A–F*), Ameloblastin- (*G–L*), and Enamelin- (*M–R*) expressing ameloblasts were severely reduced in *Ctip2*$^{-/-}$ mice. A secondary ameloblast-like layer was present on the lingual side of the *Ctip2*$^{-/-}$ mice (*white arrows* in *D*, *J*, and *P*). Ameloblasts were reduced in size (*white arrows* in *B*, *H*, and *N*) with deformed or absent adhesion points on the labial side of incisors in the *Ctip2*$^{-/-}$ mice (*white stars* in *F*, *H*, *L*, *N*, and *R*). (*S*) RT–quantitative PCR (RT-qPCR) analyses comparing levels of *Amelogenin*, *Ameloblastin*, *Enamelin*, and *Laminin 5-3a* expression in mandibular tissue of WT and *Ctip2*$^{-/-}$ mice at E18.5. Expression levels of all 4 genes were significantly decreased in the mutants ($P < 0.05$ in all cases). (*T*) ChIP assays on proximal and distal promoter regions (as defined in Table S1) of the indicated genes conducted using qPCR. The ratio of amplification products present in immunoprecipitates from WT and *Ctip2*$^{-/-}$ mice was determined to indicate the specificity of the ChIP signal in WT mice. Data shown in *A–R* and *U–Z* are representative of 3 and 5 similar experiments, respectively. RT-qPCR and ChIP data presented in (*S*) and (*T*) represent averages from studies using tissue from 3 (ChIP) to 6 (RT-qPCR) independent mice of each genotype. (*U* and *V*) Decreased expression levels of amelogenin in mutant ameloblasts at E16.5. The white dotted line represents the boundary between ameloblasts and odontoblasts in (*V*). (*W* and *X*) Immunostaining with an anti-ß-tubulin antibody and DAPI. *Y* and *Z* indicate the DAPI stained nuclei of the ameloblats indicated in the versions of (*W*) and (*X*), respectively. White dots in (*W–Z*) denote the ameloblast apical boundary. Nuclei of WT ameloblasts were predominantly found on the basal surface (position labeled ''1'' in *Y*) or in the middle of the cell (position ''2''), but rarely on the apical aspect (position ''3''). In contrast, nuclei were randomly distributed throughout the mutant ameloblasts (positions 1–3 in *Z*). [Scale bars: (*A–R*) 200 μm; (*U–X*) 100 μm; (*Y* and *Z*) 50 μm.]

ameloblast-specific genes (27). Expression of Sp3, however, was unaffected in *Ctip2* mutants (Fig. 4*G*), suggesting that Ctip2 is not upstream of Sp3, or the 2 proteins function in parallel pathways to regulate ameloblast function.

Discussion

We have described a unique function of the transcriptional regulatory protein Ctip2 during tooth development and amelogenesis. Ctip2 appears to be necessary for ameloblast formation, location, differentiation, and maintenance.

Ctip2$^{-/-}$ teeth were characterized by blunted cusps and reduced stellate reticuli, while ameloblasts exhibited several structural abnormalities, such as reduced size, poor polarization, compromised adhesion, and loss of the normal labial/lingual asymmetrical distribution of ameloblasts around the mandibular incisors (Figs. 2 and 3), which was not correlated with cranial dysmorphogenesis. *Ctip2*$^{-/-}$ ameloblasts failed to synthesize appreciable amounts of ameloblast-specific proteins, which are required for enamel formation, and Ctip2 appeared to regulate the expression of genes encoding some of these proteins, as well as transcription factors that are implicated in the regulation of ameloblast differentiation and enamel formation (16, 28).

Ctip2-null ameloblasts exhibited a ≈10-fold reduction in the expression of 3 main proteins that define the secretory stage of ameloblasts: amelogenin, ameloblastin, and enamelin. All 3 proteins belong to the secretory calcium-binding phosphoprotein gene family (29). Amelogenin is the principal component of the enamel matrix that is secreted by ameloblasts, while ameloblastin, enamelin, and tuftelin, are essential but much less abundant (30, 31). Amelogenin participates in signal transduction, contributes to ion transport for enamel biomineralization, and to the general architecture of the tooth, as well as possibly playing a role in periodontal regeneration (32). Amelogenin is essential for enamel crystal organization (33), and *Amelogenin*-null mice develop abnormal teeth characterized by a chalky white color and a disorganized, hypoplastic enamel (34). In this context, it is of interest that we have found that ≈25% of adult, heterozygous Ctip2 mice (*Ctip2*$^{+/-}$) exhibit extended and discolored incisors that are also very soft and chalky (data not shown). As amelogenin appears to be a direct target of Ctip2 in the mandible, these findings suggest a possible *Ctip2* gene-dosage effect on amelogenin expression in the developing or adult tooth.

Enamelin comprises only 1 to 5% of enamel, yet plays an essential role in enamel formation by promoting and catalyzing growth of enamel crystals at the mineralization front of the ameloblast surface (28). Enamel crystals are organized into rods, and each rod is the product of a single ameloblast. As enamel crystals grow, ameloblasts are displaced from the growing tooth front, resulting in a thickening of the enamel layer and compromised secretion of enamel proteins. Meanwhile, degradation of extracellular proteins facilitates growth of enamel crystal

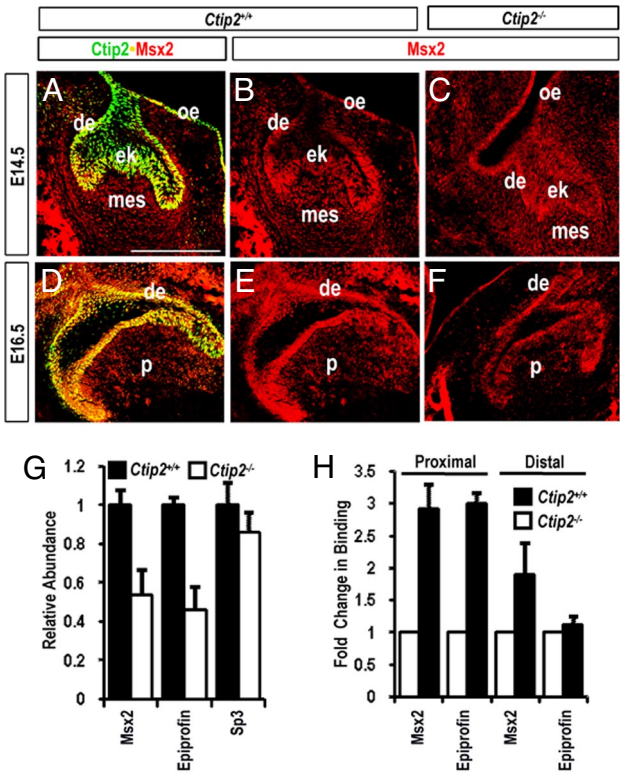

Fig. 4. Ctip2 acts upstream of Msx2 and epiprofin during ameloblast differentiation. (*A–F*) Double-label IHC on coronal sections of lower molars using indicated antibodies in WT mice at E14.5 (*A*) and E16.5 (*D*). Ctip2 and Msx2 are colocalized in the enamel knot, dental and oral epithelia. (Scale bars: 200 μm.) Images shown in panels (*B*) and (*E*) depict Msx2 staining in WT mice at E14.5 and E16.5, respectively. Msx2 expression in *Ctip2*$^{-/-}$ mice at E14.5 (*C*), and E16.5 (*F*). (*G*) Comparative levels of expression of *Msx2*, *epiprofin*, and *Sp3* in WT and *Ctip2*$^{-/-}$ mandibles at E16.5, as determined by RT-qPCR. Expression of *Msx2* and *epiprofin*, but not that of *Sp3*, was reduced in the mutants ($P < 0.05$). (*H*) ChIP assays on the proximal and distal promoter regions (as defined in Table S3) of the indicated genes from WT and *Ctip2*$^{-/-}$ mandibles at E16.5. Data shown in (*A–F*) are representative of 4 similar experiments, whereas the studies presented in (*G*) and (*H*) represent averages of 3 to 5 mice of each genotype.

rods, which continues until the rods come into contact with each other (35).

Mutations at both the *AMELOGENIN* (Xp22.3-p22.1) and *ENAMELIN* (4q21) loci contribute to a heterogenous group of human enamel disorders known as amelogenesis imperfecta (AI). Mutations at the *AMELOGENIN* locus are associated with X-linked AI, whereas those at the *ENAMELIN* locus underlie the genetic basis of autosomal dominant AI (36).

Ameloblastin-null mice exhibit severe enamel hypoplasia that is accompanied by detachment of ameloblasts from the matrix, loss of ameloblast polarity, and re-entrance of ameloblasts into the cell cycle (37). Although loss of ameloblastin in mice recapitulates many phenotypic properties of AI in humans, mutations at the human *AMELOBLASTIN* locus have not been described in AI. Nonetheless, ameloblastin, a cell adhesion molecule, is required for maintenance of the differentiated state of ameloblasts (37), and plays a key role in the function of this cell type.

Ameloblast differentiation is regulated by antagonistic actions of BMP4 and activin A from 2 mesenchymal cell layers flanking the dental epithelium (38, 39). Given the regulation of Amelogenin, Ameloblastin, and Enamelin expression by Ctip2, and the loss of asymmetric distribution of ameloblasts around *Ctip2*$^{-/-}$ incisors, it seems reasonable to speculate that Ctip2 exerts multiple temporally controlled functions during the formation of the tooth by: (*i*) suppressing ameloblast formation on the lingual side of incisors, possibly by acting in the BMP/activin-A signaling pathway; (*ii*) controlling enamel formation and mineralization through regulation of the terminal differentiation of ameloblasts; and (*iii*) maintaining the differentiated state of ameloblasts, perhaps by sustaining expression of ameloblastin.

Ctip2 expression was not observed in the dental papilla and odontoblasts, and the odontoblasts were only mildly deformed in *Ctip2-null* mice (data not shown), which may be a consequence of altered signaling originating from the epithelium. Low levels of Ctip2 expression in the condensing mesenchyme at E12.5 to E14.5 may represent a transient pulse of Ctip2 expression that is necessary to initiate the odontoblast-differentiation program, resulting in expression of BMPs, which in turn induce differentiation of ameloblast precursors in the epithelium.

Our results resonate well with the previous reports of the in vivo function of Ctip2 and a new concept is emerging for the role of this protein in regulating cellular differentiation processes and tissue architecture. In the nervous system, Ctip2 marks postmitotic corticospinal motor neurons (CSMN) and medium spiny neurons (MSN). CSMN neurons fail to form connections with the their targets because of the axonal pathfinding defects in *Ctip2*$^{-/-}$ mice (20). MSNs in *Ctip2* mutants are characterized by dysregulated expression of numerous MSN-specific markers (21). Ctip2 appears to play an important role in the differentiation and function of both of these postmitotic, neuronal populations.

Disruption of the *Ctip2* locus leads to complete blockade of the $\alpha\beta$ T-cell developmental program, and this is a function of the timing of excision. Germline deletion of *Ctip2* results in T-cell development arrest at the double-negative 3 stage, as these immature T cells fail to express T-cell receptors and consequently undergo apoptosis (22). Thus, T cells form in *Ctip2-null* mice but fail to differentiate into mature, $\alpha\beta$ T lymphocytes. Similarly, deletion of *Ctip2* later in T-cell development also produces a differentiation block (23).

Keratinocyte differentiation is defective in *Ctip2*$^{-/-}$ mice, leading to epidermal hypoplasticity and disruption of the epidermal protective barrier (24). Ctip2 does not appear necessary for keratinocyte formation in skin but, rather, the protein seems likely to play an important role in cellular differentiation, and this may be of particular relevance for other, ectodermally derived tissues, such as the dental epithelium and developing tooth. Indeed, ameloblast-like cells formed on the labial side of developing incisors of *Ctip2-null* mice. However, these cells were small, and failed to express appreciable amounts of ameloblast-specific proteins, did not become properly polarized, and exhibited compromised adhesive properties on the labial aspect of developing incisors. Moreover, *Ctip2*$^{-/-}$ mice developed an ectopic population of ameloblast-like cells on the lingual aspect of developing incisors, which was not seen in WT mice. Thus, the ameloblast developmental program clearly initiated in *Ctip2*$^{-/-}$ mice but appeared to arrest during the early stages of cellular differentiation. While the molecular basis for this arrest in ameloblast development in *Ctip2*-null mice remains to be defined, the ameloblast phenotype of *Ctip2* mutant mice is highly reminiscent of the CSMN, MSN, T cell, and keratinocyte phenotypes of *Ctip2*$^{-/-}$ mice, as described above. In all cases, Ctip2 appears to function as a critical regulator of cellular differentiation and maintenance of the differentiated phenotype.

In summary, our results provide clear evidence that Ctip2 plays an important role in controlling tooth development, and these studies specifically implicate Ctip2 in formation, differentiation, labial-lingual patterning of enamel formation, and maintenance of the ameloblast cell lineage. Thus, craniofacial development joins development of the immune (22) and nervous (20,

21) systems, and skin (24) on the expanding list of known biological functions of Ctip2.

Materials and Methods

Mice. Generation of *Ctip2*$^{-/-}$ mice on an ICR genetic background has been described (24). All animal experiments were conducted with the approval of the Oregon State University Institutional Animal Care and Use Committee.

Immunohistochemistry and Histology. Theiler staging criteria were used to verify that embryos of equivalent stages were compared in all IHC and histology studies as previously described (40, 41). Antibodies used, sources, and dilutions are detailed in Table S2.

Real-Time PCR. Mandibles were dissected from E18.5 mice and stored in RNAlater reagent (Qiagen) at 4 °C. Total RNA was extracted using RNAeasy kit (Qiagen), and first stand synthesis was carried out using oligo(dT) primers and SuperScript III (Invitrogen) reverse transcriptase. cDNA was amplified with gene-specific primers on an ABI 7500 Real-Time PCR system and SYBR green methodology. Amplification of all targets by qPCR was normalized to that of GAPDH, which was used as an internal control. Primer sequences are shown in Table S3.

ChIP. ChIP was performed as described (42), and primer sequences can be found in Table S1.

MicroCT analyses. Computerized tomography (microCT) scanning was conducted on a Scanco mCT 40 (SCANCO Medical AG) to study the ex-vivo morphology of the craniofacial skeleton and dentitional initiation and organization of *Ctip2*$^{-/-}$ animals at P0. Heads were placed in a consistent rostral-caudal orientation within a 12-mm diameter scan tube to enable the imaging of the incisors in an informative orientation. The entire skull was scanned at 12 μ per voxel resolution generating $\approx$1,000 cross-sectional images. Renderings were generated and individual microCT slices extracted using software supplied by the manufacturer.

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