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Identification of the Novel Tooth-Specific Transcription Factor AmeloD

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Abstract

Basic-helix-loop-helix (bHLH) transcription factors play an important role in various organs' development; however, a tooth-specific bHLH factor has not been reported. In this study, we identified a novel tooth-specific bHLH transcription factor, which we named AmeloD, by screening a tooth germ complementary DNA (cDNA) library using a yeast 2-hybrid system. *AmeloD* was mapped onto the mouse chromosome 1q32. Phylogenetic analysis showed that *AmeloD* belongs to the achaete-scute complex-like (*ASCL*) gene family and is a homologue of *ASCL5*. AmeloD was uniquely expressed in the inner enamel epithelium (IEE), but its expression was suppressed after IEE cell differentiation into ameloblasts. Furthermore, AmeloD expression showed an inverse expression pattern with the epithelial cell-specific cell–cell adhesion molecule E-cadherin in the dental epithelium. Overexpression of AmeloD in dental epithelial cell line CLDE cells resulted in E-cadherin suppression. We found that AmeloD bound to E-box *cis*-regulatory elements in the proximal promoter region of the *E-cadherin* gene. These results reveal that AmeloD functions as a suppressor of E-cadherin transcription in IEE cells. Our study demonstrated that AmeloD is a novel tooth-specific bHLH transcription factor that may regulate tooth development through the suppression of E-cadherin in IEE cells.

Keywords: yeast two-hybrid assay, ameloblast, E-cadherin, epithelium, cell proliferation, cell migration

Introduction

Mammal tooth development is a classic organogenesis model characterized by reciprocal interactions between the dental epithelium and mesenchyme (Thesleff 1996). In mice, tooth development is initiated at embryonic day 11.5 (E11.5) and followed by a series of morphologically distinctive stages. At E14.5, dental epithelium cells start to differentiate into different dental epithelium cell types, such as the inner enamel epithelium (IEE), outer enamel epithelium, stratum intermedium, and stellate reticulum. IEE cells are progenitors of ameloblasts, and they are able to differentiate into enamel-secreting ameloblasts, a critical process for enamel formation (Miletich and Sharpe 2003; Kuang-Hsien Hu et al. 2014).

Ameloblast development is a stepwise cellular differentiation process. Dental epithelial stem cells, which are marked by the pluripotent factor Sox2, have the potential to differentiate into all the other dental epithelial cell lineages (Juuri et al. 2012; Juuri, Isaksson, et al. 2013; Juuri, Jussila, et al. 2013). In mice, the incisors continuously grow throughout adult life because of the existence of dental epithelial stem cells at the incisor cervical loop region (Kuang-Hsien Hu et al. 2014). IEE cells are highly proliferative and migrate from the cervical loop toward the distal end of the mouse incisor (Wang et al. 2007; Li et al. 2012). Differentiated ameloblasts secrete enamel matrix proteins and form enamel (Fukumoto et al. 2004; Bei 2009). Dental enamel is the hardest tissue in the body and plays significant roles in daily chewing and protecting dentin and the inner pulp tissue (He et al. 2010; He et al. 2011).

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A supplemental appendix to this article is available online.

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Studies with mutant mice have identified a number of factors that function at the different stages of ameloblast development. Sox2, a marker of dental epithelial stem cells, maintains competence for successional tooth formation in mammals (Juuri et al. 2012; Juuri, Jussila, et al. 2013). E-cadherin is dynamically expressed at different stages of ameloblast development and regulates IEE cell migration (Li et al. 2012). The transcription factor epiprofin is one of the key protein factors to promote IEE cell proliferation and differentiation (Nakamura et al. 2004; Nakamura et al. 2008). However, there is limited knowledge available on regulatory mechanisms of stage-specific ameloblast development. Therefore, identification of new factors regulating stage-specific ameloblast development is necessary and important.

In mammals, bHLH proteins are important regulators for neurogenesis, myogenesis, heart development, and hematopoiesis (Ross et al. 2003; Jones 2004). bHLH proteins can be divided into class I and class II: class I proteins are ubiquitously expressed, while class II proteins, such as MyoD and NeuroD, are expressed in a tissue-specific manner. Class I E proteins, such as E12, form a heterodimer with class II bHLH proteins through their HLH domains and activate or suppress gene transcription (Massari and Murre 2000).

In this study, we identified a tooth-specific bHLH protein, AmeloD, which is uniquely expressed in IEE and Hertwig epithelial root sheath (HERS) cells. Overexpression of AmeloD in epithelial cell lines resulted in E-cadherin suppression. Functionally, AmeloD directly binds to the *E-cadherin* proximal promoter and recruits chromatin repressive complex. Our results suggest that AmeloD is an important regulator for tooth development.

Materials and Methods

Cell Culture and Transfection

CLDE cells were cultured in keratinocyte-SFM medium (Thermo Fisher Scientific) (Yoshizaki et al. 2014). MDCK cells, COS-7 cells, and 293T cells were purchased from ATCC and cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Thermo Fisher Scientific). DNA was transfected into cells with Lipofectamine LTX. Small interfering RNAs (siRNAs) were purchased from Dharmacon and transfected into cells with an RNA interference (RNAi) transfection kit (ThermoFisher). For adenovirus-associated expression vectors transfection, CLDE cells were infected at a multiplicity of infection of 100, and MDCK cells were transfected at a multiplicity of infection of 200.

Cell Proliferation Assay and ChIP Analysis

Cell proliferation assay was performed with a CCK-8 kit (Dojindo). For chromatin immunoprecipitation (ChIP), CLDE cells were crosslinked by 1% formaldehyde solution for 10 min at room temperature. Chromatin was sonicated to 200 to 300 bp with a Bioruptor for 22 cycles. Chromatin prepared from 1×10^7 cells was used for each ChIP. The ChIP reactions were done with a ChIP-IT High Sensitivity Kit (Active Motif). DNA purified from ChIP assays was applied for quantitative polymerase chain reaction (qPCR) reactions, and data were normalized by percentage of input.

Detailed methods of yeast two-hybrid (Y2H) screening, complementary DNA (cDNA) library construction and screening, AmeloD full-length sequence and analysis, in situ hybridization, and AmeloD antibody specificity tests are included in the Appendix Materials and Methods.

Results

Isolation of the Novel bHLH Factor AmeloD from a Tooth Germ cDNA Library

We sought to identify novel tooth-specific bHLH proteins regulating tooth development. For this purpose, we screened an E13.5 rat tooth germ cDNA library using the Y2H system, with the E12 bHLH domain as bait. After 2 rounds of screening, 19 positive clones were identified, and most were previously identified genes, such as *MyoD, dHand*, and *Twist1*. One of the positive clones was found to encode a novel bHLH protein, and we named this gene *AmeloD* (NCBI gene accession MG575629), as the *AmeloD* transcript was specifically expressed during ameloblast development. To obtain a full-length mouse *AmeloD* cDNA clone, we screened a mouse E19 molar cDNA library with a rat AmeloD probe. We obtained a mouse *AmeloD* cDNA clone containing 1,539 bp (Appendix Fig. 1A), including 5' untranslated sequences, and a coding sequence encoding 188 amino acids, including 3' untranslated sequences. Phylogenetic analysis showed that *AmeloD* belongs to the *ASCL* gene family and is homologous to *ASCL5* (Appendix Fig. 1B). *ASCL5* has been identified at only the genomic level: its expression patterns and functions are unknown. The *ASCL5* (*AmeloD*) gene structure consists of 2 exons (Fig. 1A): untranslated exon1 (178 bp) and coding sequence containing exon2 (1,748 bp) containing 188 amino acid coding sequences. The *AmeloD* gene is mapped to mouse chromosome 1 q32 (Appendix Fig. 1C, D). The *AmeloD* cDNA starts at the residues 210 in exon 2. The 5' RACE analysis from the 5' part of the *AmeloD* cDNA showed the 5' mRNA extension to the residue 22 in exon1 (data not shown). The amino acid sequences of the AmeloD bHLH domain are highly conserved among those of other ASCL proteins, while 5' and 3' sequences are more specific (Appendix Fig. 1B).

To characterize the function of the AmeloD protein, we developed an AmeloD polyclonal antibody. Western blot analysis showed that the molecular weight of AmeloD protein is about 25 kDa. AmeloD antibody can recognize the exogenous AmeloD protein (Cos7 cells with *AmeloD* overexpression) and the endogenous AmeloD protein in mouse tooth lysate (Appendix Fig. 2A, B). To test the antibody specificity, we used scrambled and AmeloD-specific peptides to neutralize AmeloD antibody and test the antibody function in Western

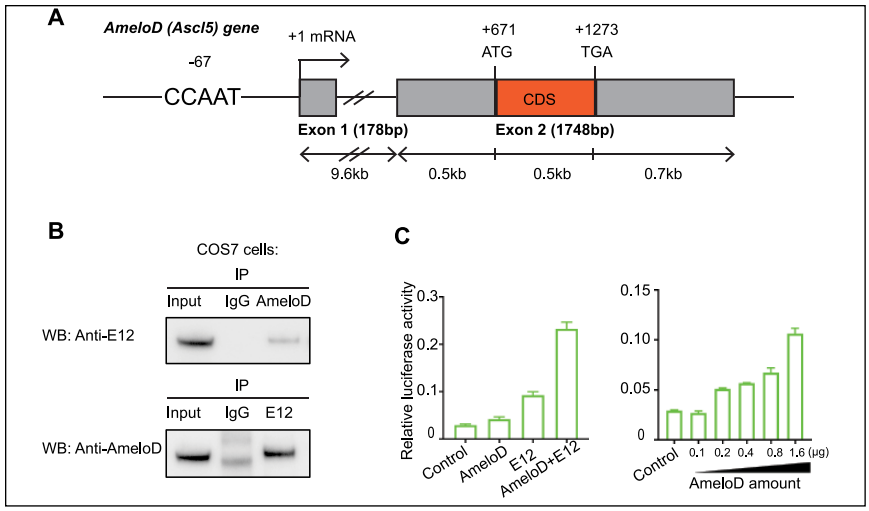

Figure 1. Identification of a novel basic-helix-loop-helix protein AmeloD. (**A**) Gene structure of *AmeloD*. The solid boxes represent exons. The location of the CCAAT box is 67 bp upstream of the transcriptional start site. Exon 1 encodes the 5′-untranslated sequence, and exon 2 encodes the coding sequence of *AmeloD*. (**B**) Co-immunoprecipitation of AmeloD and E12. Co-transfection of *AmeloD* and *E12* expression plasmid DNA into COS7 cells, followed by co-immunoprecipitation and Western blot. AmeloD and E12 proteins physically interact with each other. (**C**) MCK-luc reporter assay with AmeloD. AmeloD and E12 synergistically activate the E-box luciferase reporter (left panel), and gradually increasing the *AmeloD* DNA amount increased the luciferase activity (right panel). Values are presented as mean ± SD ($n = 3$).

blot and immunofluorescence staining of postnatal day 1 (P1) mouse incisor. Our results showed that AmeloD-specific peptides totally blocked the AmeloD antibody function, and we did not observe any blocking effect when AmeloD antibody was neutralized by the scrambled peptide (Appendix Fig. 2C, D). Co-immunoprecipitation assay showed that AmeloD and E12 form a heterodimer (Fig. 1B). Co-transfection of HEK293 cells with AmeloD and E12 expression vectors and with a muscle creatine kinase gene reporter (*MCK*-luc) vector—which is an E-box-containing myogenic enhancer luciferase reporter and can be activated by MyoD-E12 heterodimer (Markus et al. 2002)—showed that AmeloD and E12 synergistically activated the MCK-luc reporter (Fig. 1C, left panel). Gradually increasing the AmeloD expression levels also increased luciferase activity (Fig. 1C, right panel). Together, these results suggest that AmeloD is a novel bHLH protein that physically interacts with E12 protein and binds to the E-box element.

AmeloD Is a Tooth-Specific bHLH Factor and Expressed Uniquely in IEE Cells during Ameloblast Development

Northern blot analysis with RNA from different tissues of P1 mice showed that *AmeloD* was specifically expressed in molars and incisors (Fig. 2A). In situ hybridization with anti-sense (*AmeloD-AS*) probes showed that *AmeloD* expression started in the E11 incisor region (Fig. 2B). Later, *AmeloD* was strongly expressed in IEE cells of E14 and E17 mouse molar sections. Interestingly, at the P7 stage, *AmeloD* was detected in only the third molar, not in the second or first molar (Fig. 2B, lower panel). At the P7 stage, IEE cells are already differentiated into ameloblasts in the first and second molars but not yet in the third molars. *AmeloD* sense (*AmeloD-S*) probe was used as a control and showed no *AmeloD* signals in the mouse molar sections at different developmental stages (Fig. 2B, right panel). Immunofluorescence staining of mouse molar sections and the P1 incisor sagittal section also showed that AmeloD was specifically expressed in IEE cells but not in differentiated ameloblasts (Fig. 2C, D). AmeloD is also expressed in the HERS cells during mouse molar development (Figs. 2C, 3A). HERS cells are rapidly proliferating cells located in the cervical loop region of an enamel organ during mouse molar development (Li et al. 2017; Wang and Feng 2017). Immunofluorescence staining with Sox2, a dental epithelium stem cell marker, Ki67, a cell proliferation marker, and amelogenin, an ameloblast differentiation marker, in P2 mouse incisor serial sections showed that a large population of the AmeloD-positive cells were also Ki67 positive; however, AmeloD staining did not co-localize with Sox2 or amelogenin staining (Appendix Fig. 3A). Next, qPCR analysis showed that *AmeloD* was highly expressed from E14.5 to P1 molars and in the IEE cells of mouse incisor. The *AmeloD* expression level was dramatically downregulated once cells went into the differentiation stage (Fig. 2E). In contrast, *ameloblastin* was highly expressed in the P3 and P7 molars. The gene expression analysis also showed that *AmeloD* has an inverse expression pattern to *ameloblastin* in incisors and is highly expressed at IEE cells (Fig. 2E). These results suggest that AmeloD may regulate ameloblast development at the progenitor stage.

AmeloD Inhibits E-cadherin Expression in IEE Cells

Based on the expression pattern of AmeloD during mouse incisor development, we hypothesize that AmeloD might function in progenitor ameloblast. We used CLDE cells as a model to study the function of AmeloD; CLDE cells are dental epithelium cells derived from the cervical loop region of E17.5 mouse incisor (Yoshizaki et al. 2014). IEE cells are rapidly proliferating cells with high cell motility (Seidel et al. 2010). However, neither overexpression nor siRNA knockdown of AmeloD had an effect on cell proliferation rate, indicating that AmeloD does not regulate cell proliferation (Appendix Fig. 3B). Although CLDE cells have a very low level of AmeloD expression and we could not detect AmeloD protein expression in CLDE cells (data not shown), we did detect *AmeloD* expression knockdown by siRNAs with qPCR (Appendix Fig. 3C).

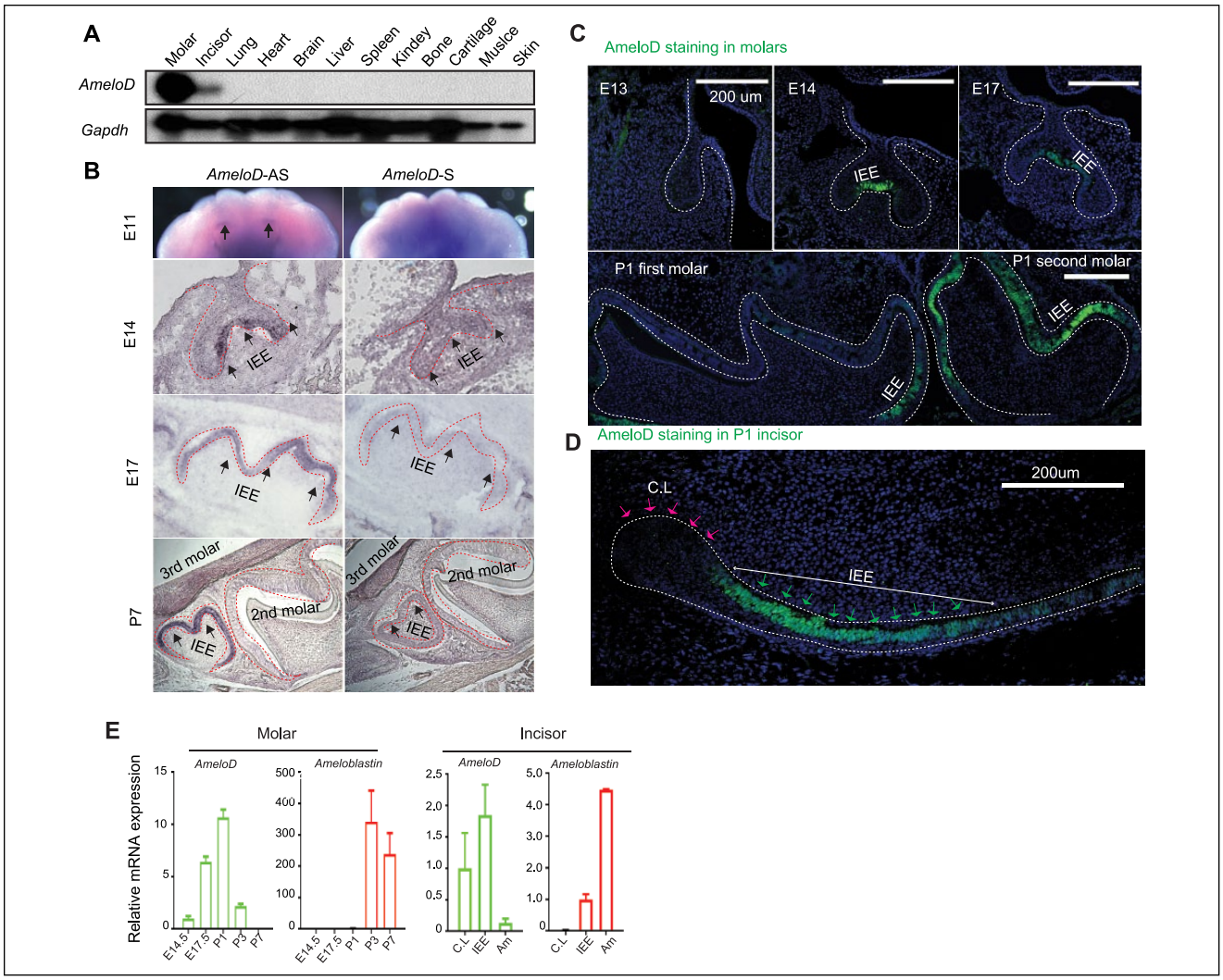

Figure 2. Expression pattern of AmeloD. (**A**) Northern blot analysis of *AmeloD* expression in different tissues from the postnatal day 1 (P1) mouse, and the results show that *AmeloD* is uniquely expressed in the mouse molars and incisors. (**B**) In situ hybridization of *AmeloD* expression in the embryonic day 11 (E11) maxilla and E14, E17, and P7 mouse molars (secondary and third molars). AS, anti-sense probe; S, sense probe. The arrow indicates the expression of *AmeloD* transcript. (**C**) Immunofluorescence staining of AmeloD expression with AmeloD antibody (green) in E13.5, E14.5, E17.5, and P1 molar sections (first and second molars). (**D**) Immunofluorescence staining of AmeloD with anti-AmeloD antibody (green) in the sagittal section of the P1 mouse incisor. The red arrows indicate the cervical loop (CL) region of mouse incisor, and the green arrows indicate IEE cells. Scale bar: 200 μm. (**E**) Quantitative polymerase chain reaction gene expression analysis of *AmeloD* and *ameloblastin* expression during mouse molar and incisor development. Values are presented as mean ± SD ($n = 3$). IEE, inner enamel epithelium. Am, ameloblast.

E-cadherin expression levels are highly dynamic during ameloblast development and related to the motility function of IEE cells (Li et al. 2012). Immunofluorescence staining of AmeloD and E-cadherin in P1 mouse molar and incisor sections showed that AmeloD and E-cadherin had an inverse expression pattern in IEE cells. In IEE cells, AmeloD was highly expressed, but there was no E-cadherin expression (Fig. 3A, B, white arrowheads). However, when IEE cells were differentiated into ameloblasts, AmeloD expression was downregulated, but the E-cadherin expression level was upregulated again (Fig. 3A, B, yellow arrowheads). The inverse expression pattern of AmeloD and E-cadherin in IEE cells suggests that AmeloD may function as a suppressor of E-cadherin expression during ameloblast development. We tested this hypothesis by infecting CLDE cells with adeno-GFP and adeno-AmeloD expression vectors. Indeed, after AmeloD infection, the morphology of CLDE cells changed from a cuboidal cell shape to spindle-shaped fibroblast-like cells (Fig. 3C, upper panel). Immunofluorescence staining results showed that the overexpression of AmeloD did suppress the expression of the cell–cell adhesion proteins E-cadherin and β-catenin (Fig. 3C, middle and lower panel); this result was further confirmed by Western blot analysis (Fig. 3D) and gene expression analysis (Fig. 3E). Overexpression of adeno-AmeloD in another epithelial cell line, MDCK cells, also resulted in the suppression of E-cadherin and β-catenin expression (Appendix Fig. 4A, B). Thus, AmeloD suppressed E-cadherin expression in CLDE and MDCK cells.

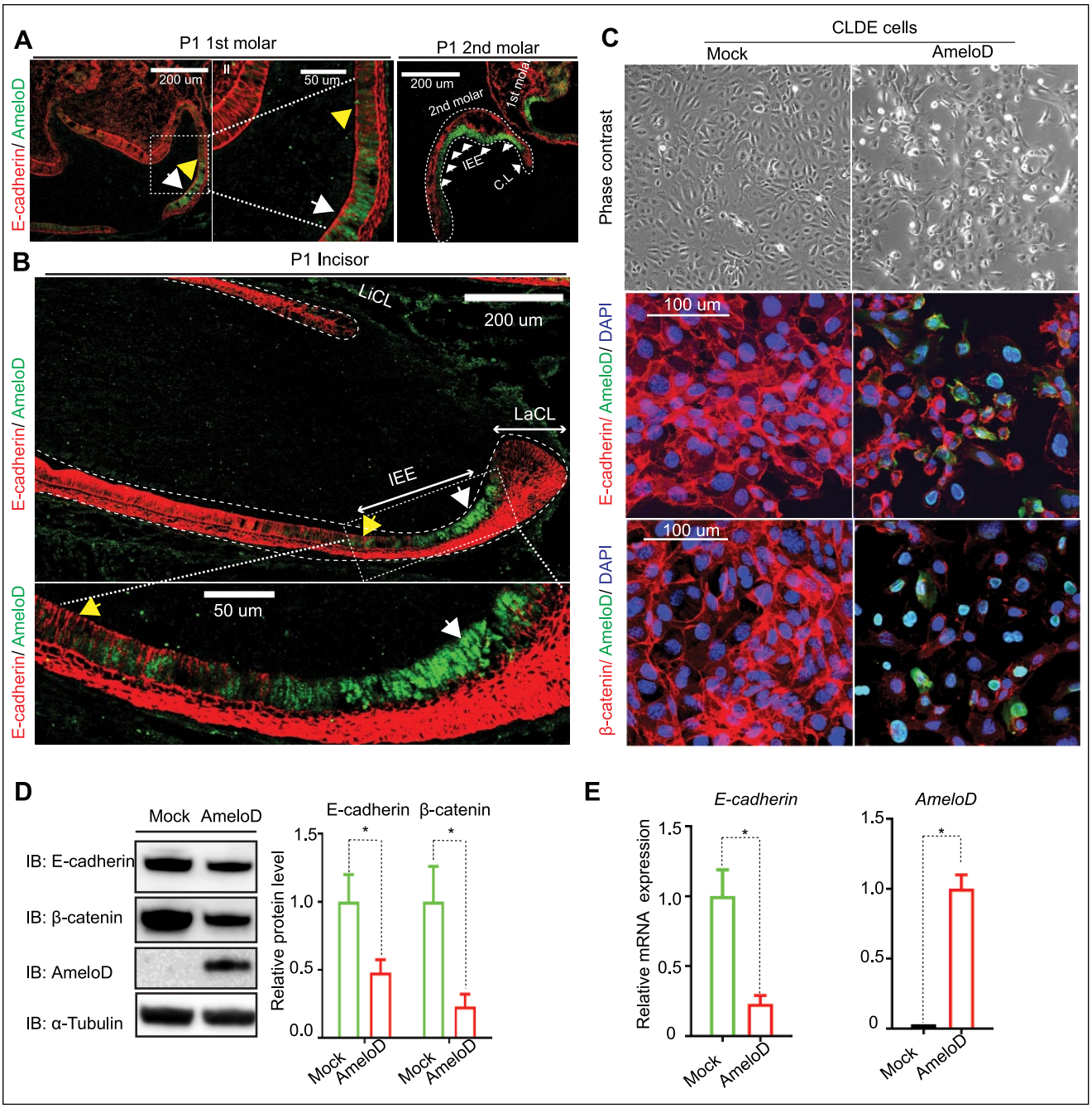

Figure 3. AmeloD inhibits E-cadherin expression in progenitor ameloblasts. Immunofluorescence staining of AmeloD (green) and E-cadherin (red) in postnatal day 1 (P1) mouse (**A**) molars and (**B**) incisors. The white arrows indicate cells that have a high level of AmeloD expression (green color) and a low level of E-cadherin expression (red color), and the yellow arrows indicate cells that have a low level of AmeloD expression and a high level of E-cadherin expression. (**C**) Overexpression of AmeloD in CLDE cells suppressed E-cadherin expression. Phase contrast images showed that *AmeloD* overexpression resulted in morphologic changes in CLDE cells (top panel); immunofluorescence staining of AmeloD (green) with E-cadherin (red) or AmeloD (green) with beta-catenin (red) showed that overexpression of *AmeloD* in CLDE cells resulted in E-cadherin and beta-catenin degradation. (**D**) CLDE cells were transfected by control and *AmeloD* constructs, with Western blot analysis of E-cadherin and beta-catenin protein levels in CLDE cells after 72 h of transfection. Quantitative analysis was done by Image J. (**E**) Quantitative polymerase chain reaction gene expression analysis of *E-cadherin* and *AmeloD* expression in CLDE cells 72 h after transfection of control and *AmeloD* constructs. Data are shown as mean ± SD ($n = 3$). Statistical significance is shown by a *t* test. $^*P < 0.05$.

AmeloD Binds to the E-cadherin Proximal Promoter through E-boxes

To investigate the mechanism by which AmeloD suppresses E-cadherin transcription, we performed ChIP analysis using CLDE cells infected with the adeno-AmeloD vector. Two sets of primers were designed for the ChIP analysis: primers P1-P2 were designed to amplify the *E-cadherin* proximal promoter (−100 bp), which has 2 adjacent E-box elements that are important for binding of bHLH proteins (Yang et al. 2004;

Sideridou et al. 2011); primers P3–P4 were designed to amplify part of *E-cadherin* exon2 and served as a no-binding control. Antibodies against AmeloD and IgG were used for ChIP reactions. We found that AmeloD binds to the *E-cadherin* proximal promoter at a significantly higher level than that of the no-binding and IgG controls (Fig. 4A). Next, we compared H3K27me3 or PRC2 complex Ezh2 enrichment levels at the *E-cadherin* proximal promoter in CLDE cells transfected with adeno-GFP and adeno-AmeloD expression vectors. Our results revealed that AmeloD overexpression significantly increased the H3K27me3 and Ezh2 enrichment level at the *E-cadherin* proximal promoter (Fig. 4B). Since H3K27me3 generally serves as a repressive marker for gene transcription, our results indicate that AmeloD inhibits *E-cadherin* transcription by recruiting the PRC2 repressive complex to *E-cadherin* proximal promoter. Co-immunoprecipitation analysis of AmeloD with Ezh2 or Suz12, which are the 2 core components of the PRC2 complex, did not show direct physical interactions between AmeloD and these 2 proteins (data not shown), suggesting that recruiting of the PCR2 complex to *E-cadherin* promoter was mediated by other factors. In fact, there are conserved E-box elements in the proximal promoter of *E-cadherin* among different species (Fig. 4C). Furthermore, AmeloD suppressed the activity of the *E-cadherin* promoter luciferase reporter containing 3 E-boxes but did not suppress the activity of the *E-cadherin* promoter reporter containing E-box mutations (Fig. 4D). Similar results were obtained when co-transfecting a *Snail2* expression vector, a well-known E-cadherin suppressor, with WT and E-box mutant *E-cadherin* luciferase reporters. These results further demonstrated that the proximal promoter E-box elements are necessary for *E-cadherin* suppression by AmeloD. In summary, AmeloD directly binds to the *E-cadherin* proximal promoter through E-box elements and inhibits E-cadherin expression.

Discussion

In this study, we identified a novel bHLH protein, AmeloD, which is uniquely expressed in teeth but not in other tissues (Figs. 1, 2A). By in situ hybridization, immunofluorescence staining, and gene expression analysis, we demonstrated that AmeloD is uniquely expressed in proliferating IEE cells and has an inverse expression pattern with E-cadherin (Figs. 2, 3A, B; Appendix Fig. 3A). By overexpression of AmeloD or knockdown AmeloD in CLDE cells, we did not observe that AmeloD regulates cell proliferation (Appendix Fig. 3B, C), suggesting that AmeloD might regulate a different function of IEE cells.

During mouse incisor development, the dental epithelial stem cell-derived progenitor cells migrate from the cervical loop toward the distal region (Dassule et al. 2000; Harada et al. 2002; Klein et al. 2008; Juuri et al. 2012; Juuri, Jussila, et al. 2013). To facilitate this migration process, the epithelium cell-specific cell–cell adhesion protein E-cadherin is partially downregulated in the IEE cells, and its expression level is upregulated again in the differentiated ameloblasts (Li et al. 2012). The inverse expression pattern of AmeloD and E-cadherin prompted us to explore a potential regulatory relationship between them. Functional analysis of AmeloD revealed that AmeloD suppressed E-cadherin expression in CLDE cells; overexpression of AmeloD in CLDE cells resulted in *E-cadherin* downregulation at the mRNA and protein levels (Fig. 3C–E). ChIP-qPCR analysis showed that AmeloD preferentially binds to the proximal promoter of *E-cadherin*, which has conserved E-box elements among different species (Fig. 4A, C). In fact, several other *E-cadherin* repressive factors, such as bHLH proteins Twist1 and E47, also bind to E-box elements at the *E-cadherin* promoter and repress E-cadherin expression during epithelium-mesenchyme transition (Yang et al. 2004; Yang et al. 2010). Furthermore, by ChIP-qPCR analysis, we found that AmeloD overexpression resulted in a higher enrichment level of histone repressive marker H3K27me3 and PRC2 core complex Ezh2 at the *E-cadherin* proximal promoter (Fig. 4A, B), suggesting that AmeloD represses E-cadherin expression by recruiting the histone repressive complex to the *E-cadherin* promoter. By in vitro co-immunoprecipitation analysis of AmeloD with Ezh2 or Suz12 (data not shown), we did not find direct binding of AmeloD to Ezh2 or Suz12, suggesting that recruiting of Ezh2 to the *E-cadherin* promoter was not directly through AmeloD; instead, some other protein factors mediated an indirect association between AmeloD and the PRC2 complex, and future studies should focus on identifying the protein complex.

In the developing mouse molar, AmeloD is also expressed in HERS cells (Figs. 2C, 3A), which are derived from the elongation of the enamel organ and play an important role in cementum formation and proper root development (Li et al. 2017). Contrary to AmeloD's inverse expression pattern with E-cadherin in IEE cells, AmeloD and E-cadherin are coexpressed in the progenitor HERS cells, suggesting that AmeloD might have distinct functions in IEE and HERS cells. Crown development and root formation are tightly regulated by multiple signaling pathways, such as Wnt and Tgfβ (Wang and Feng 2017). Transcription factors such as Bcl11b, epiprofin, and Pitx2 have been found primarily to regulate ameloblast differentiation and enamel formation (Nakamura et al. 2008; Golonzhka et al. 2009; Li et al. 2014), while Dkk1, Nfic, and c-Fos were found to regulate root developmental process (Huang and Chai 2012; Jussila and Thesleff 2012; He et al. 2016; Li et al. 2017; Wang and Feng 2017). In our current study, we found that AmeloD suppresses E-cadherin expression and promotes IEE cell motility. Based on its expression pattern and in vitro functional studies, we propose that AmeloD regulates enamel and root development in mice. In a separate study by our group, we showed that AmeloD knockout mice developed mild to moderate tooth developmental defects, such as enamel hypoplasia and short tooth roots (Chiba et al. 2018, unpublished data). Knockout AmeloD protein in mice did not totally block tooth development, indicating that there might be functional compensation between AmeloD and other bHLH proteins. In fact, some other bHLH proteins, such as Mitf and Tfe3, were functionally redundant in osteoclast development (Steingrimsson et al. 2002). One limitation of our current study is that most of the functional studies of AmeloD are through overexpression systems. To fully understand the molecular function of AmeloD, it would be ideal to combine knockdown/knockout experiments in cell lines with in vivo knockout mice studies and

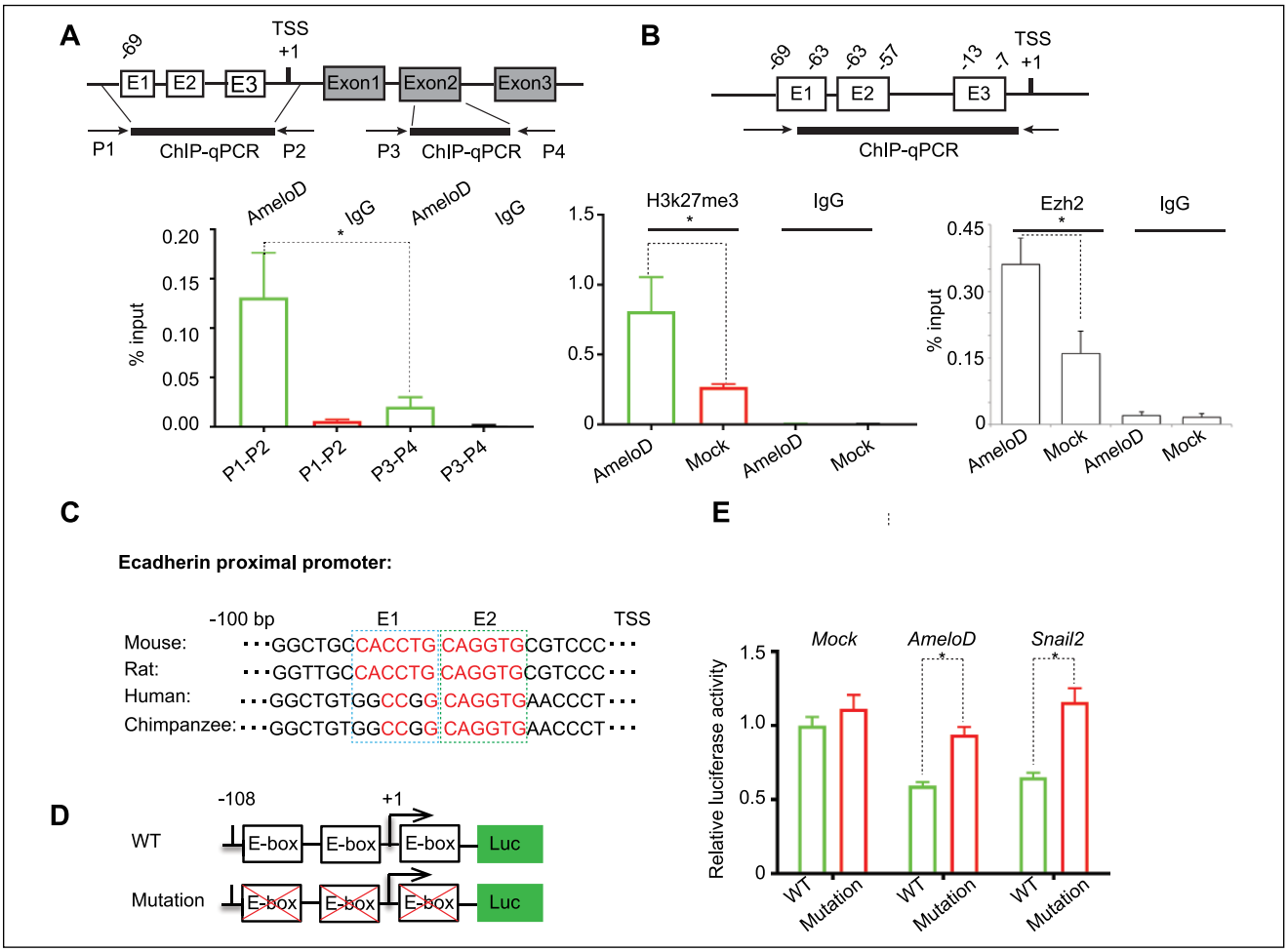

Figure 4. AmeloD binds to *E-cadherin* proximal promoter and inhibits *E-cadherin* expression. (**A**) ChIP-qPCR results showed that AmeloD binds to the *E-cadherin* proximal promoter. Nuclear extracts were prepared from CLDE cells transfected by adeno-AmeloD for 72 h. Antibodies for AmeloD and IgG were used for ChIP assay. Primer set P1-P2 was designed to amplify the proximal promoter region (−69 bp to TSS) of the mouse *E-cadherin* gene, and the primer set P3-P4 was designed to amplify 100 bp of the *E-cadherin* gene exon 2. (**B**) *Left panel*: ChIP-qPCR results showed that binding of AmeloD to the *E-cadherin* promoter increased the repressive marker H3K27me3 enrichment. The H3k27me3 enrichment level was relative to the percentage of input. Nuclear extracts prepared from CLDE cells were transfected by adeno-GFP and adeno-AmeloD expression vectors for 72 h. Antibodies for H3K27me3 and IgG were used for immunoprecipitation. *Right panel*: ChIP-qPCR results showed that binding of AmeloD to the *E-cadherin* promoter increased the PCR2 complex component Ezh2 enrichment. The Ezh2 enrichment level was relative to the percentage of input. Nuclear extracts prepared from CLDE cells were transfected by adeno-GFP and adeno-AmeloD expression vectors for 72 h. Antibodies for Ezh2 and IgG were used for immunoprecipitation. (**C**) The upper panel shows that the consensus E-box element (E1 and E2) appears at the *E-cadherin* proximal promoter among different species; the lower panel shows the diagram of the wild-type (WT) and mutated *E-cadherin* luciferase reporter. (**D**) Luciferase assay showed that AmeloD inhibits the WT *E-cadherin* luciferase reporter activity but not the mutated *E-cadherin* luciferase reporter. Transcription factor snail2 served as a positive control, and an empty construct served as a negative control. Data are shown as mean ± SD ($n = 3$). Statistical significance is shown by a *t* test. $^*P < 0.05$. ChIP, chromatin immunoprecipitation; qPCR, quantitative polymerase chain reaction.

study the cell lineage-specific functions (IEE vs. HERS) of AmeloD in mouse tooth development.

In summary, we have identified a novel tooth-specific bHLH factor, AmeloD. AmeloD is uniquely expressed in IEE cells and suppresses E-cadherin expression. Our studies reveal that AmeloD is an important regulator of tooth development.

Author Contributions

B. He, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript; Y. Chiba, S. de Vega, K. Tanaka, C. Rhodes, contributed to design and acquisition, critically revised the manuscript; H. Li, contributed to conception, data acquisition and analysis, critically revised the manuscript; K. Yoshizaki, M. Ishijima, contributed to data acquisition and analysis, critically revised the manuscript; K. Yuasa, M. Ishikawa, K. Sakai, contributed to data acquisition, critically revised the manuscript; P. Zhang, contributed to data analysis and interpretation, critically revised the manuscript; S. Fukumoto, contributed to conception and design, critically revised the manuscript; X. Zhou, contributed to data interpretation, critically revised the manuscript; Y. Yamada, contributed to conception,

design, data acquisition and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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