Identification of the Novel Tooth-Specific Transcription Factor AmeloD

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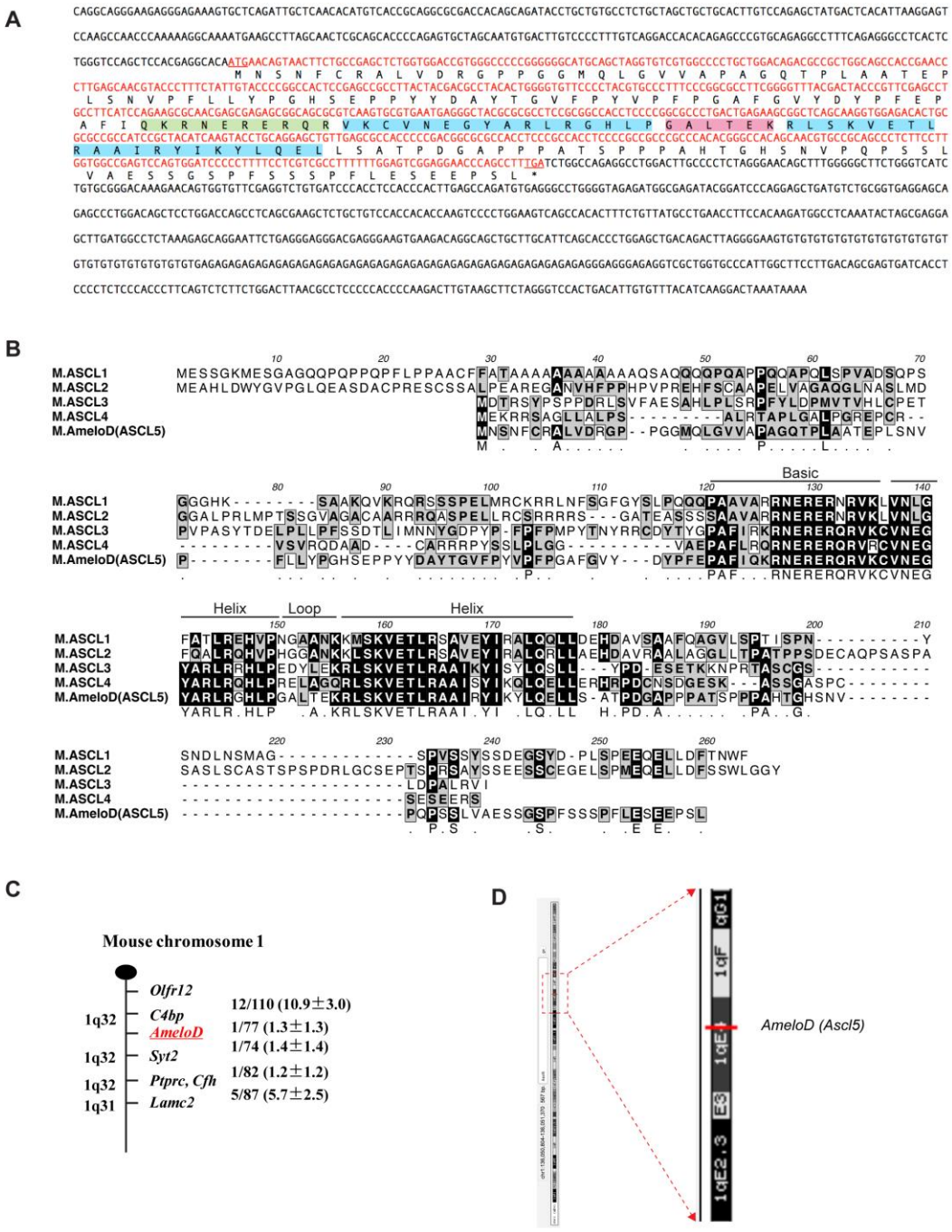
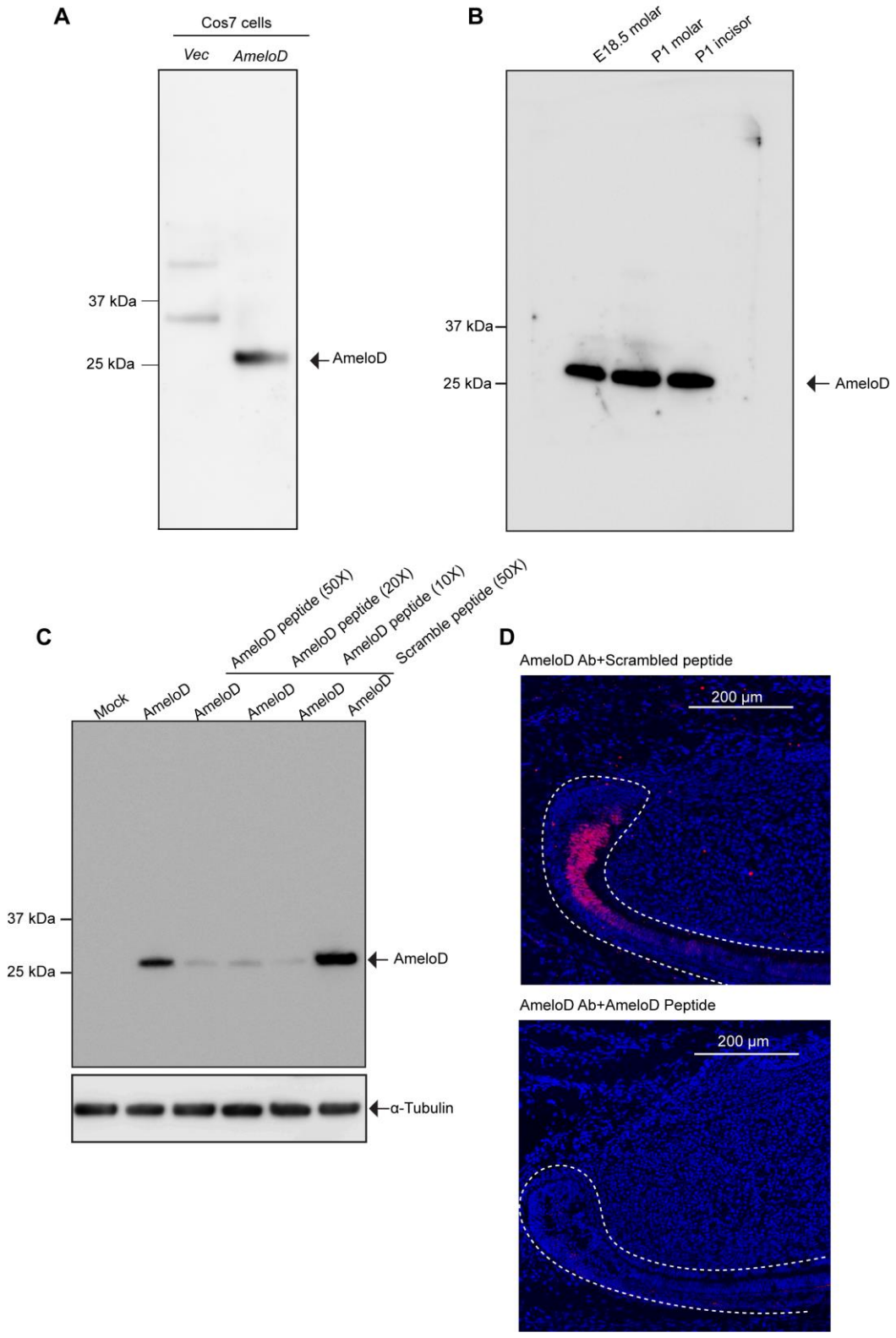
Appendix



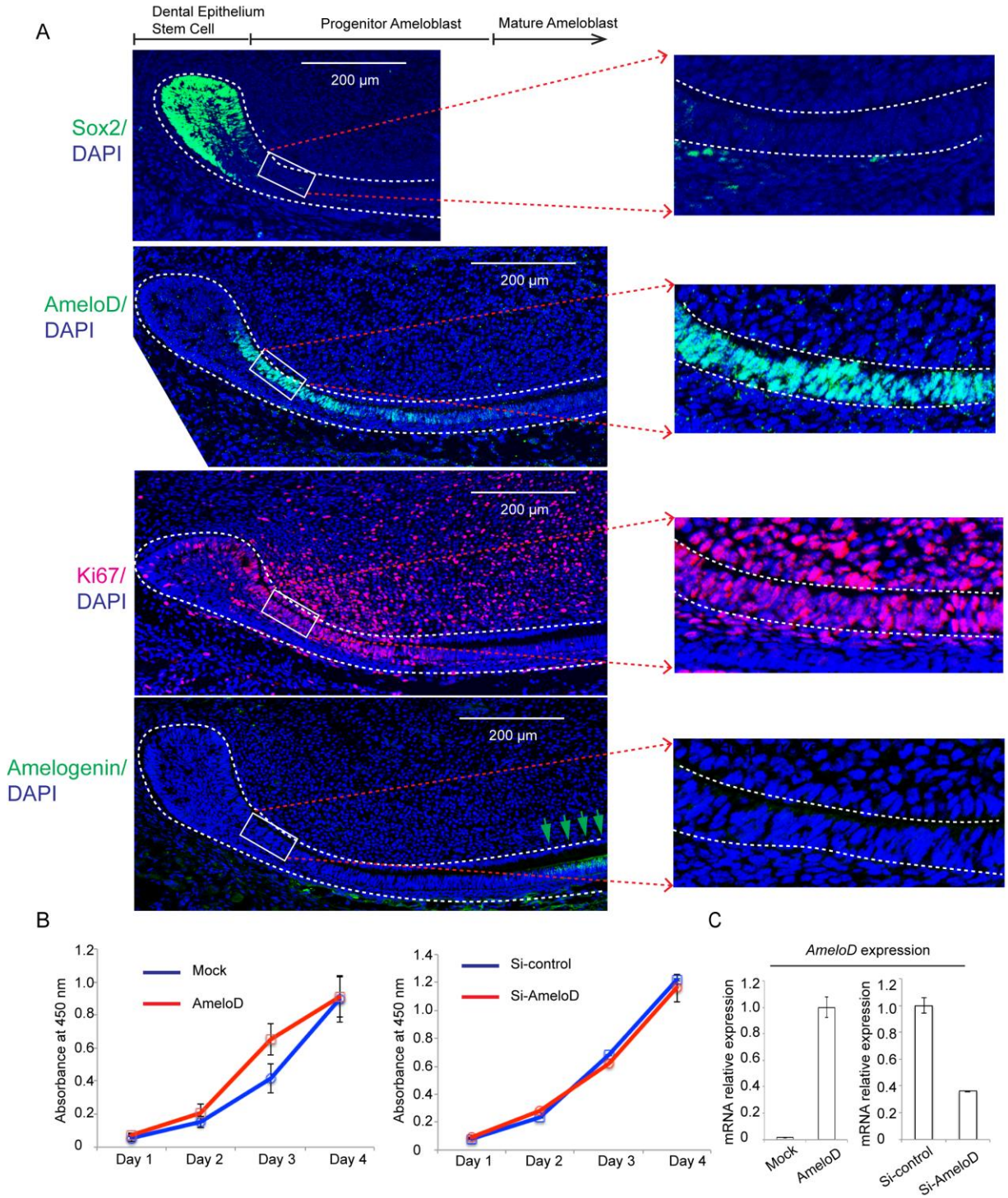
Appendix Figure 1. AmeloD cDNA and amino acid sequences. (A) Full-length cDNA sequence for *AmeloD*. Colored letters indicate the basic-helix-loop-helix domain. Green: basic

domain, Blue: helix domain, Red, loop domain. (B) Protein sequence alignment of AmeloD with ASCL family bHLH proteins. In the bHLH domain region, they have similarities with AmeloD in amino acid sequences. (C) Phylogenic tree of AmeloD. Phylogenetic relationships were analyzed using the distance matrix method based on ClustalW alignments. Right, recombination fractions for adjacent loci. Left, map locations of the human homologs. A value of 0.1 corresponds to a difference of 10% between two sequences. (D) UCSC genome browser snapshot shows that *AmeloD* mapped onto mouse Choromosome 1.

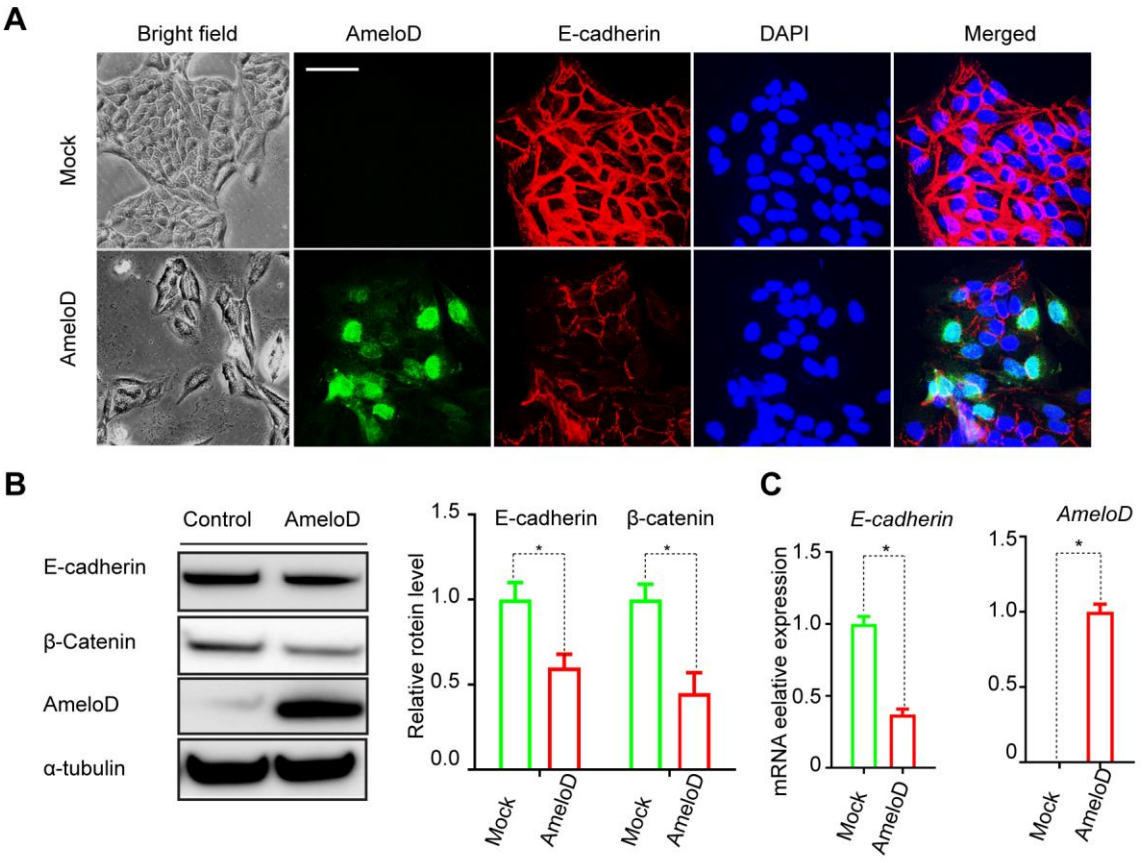


Appendix Figure 2. AmeloD antibody specificity test by Western blot and immunofluorescence. (A) Western blot analysis of AmeloD expression in COS7 cells

transfected by *control* and *AmeloD* expression vectors using Anti-AmeloD antibody. (B) Western blot analysis of AmeloD expression in E18.5 mouse molars, P1 mouse molars, and P1 mouse incisors. (C) AmeloD antibody specificity test in Western blot reactions by using AmeloD-specific peptide and scrambled peptide. The results showed that AmeloD-specific peptide can totally block the AmeloD antibody function in western blot. (D) AmeloD antibody specificity test in immunofluorescence by using AmeloD-specific peptide and scrambled peptide. The results showed that AmeloD-specific peptide can totally block the AmeloD antibody function in immunofluorescence staining.


A
Dental Epithelium Stem Cell
Progenitor Ameloblast
Mature Ameloblast
Sox2/ DAPI
AmeloD/ DAPI
Ki67/ DAPI
Amelogenin/ DAPI
200 μm
B
Mock
AmeloD
Absorbance at 450 nm
Day 1
Day 2
Day 3
Day 4
Si-control
Si-AmeloD
C
AmeloD expression
mRNA relative expression
Mock
AmeloD
Si-control
Si-AmeloD

Appendix Figure 3. Immunostainings of stage-specific markers in P2 mouse incisors. (A) Immunofluorescence staining of Sox2 (Dental epithelium stem cells marker), AmeloD, Ki67 (cell proliferation marker), and Amelogenin (Ameloblast differentiation marker) in P2 mouse incisor serial sections. The staining results showed that AmeloD is uniquely expressed in progenitor Ameloblast, and the AmeloD positive cells are also Ki67 positive. (B) Cell proliferation curve of CLDE cells were transfected with the empty expression vector and *AmeloD* expression vector, and CLDE cells were transfected with scrambled siRNA and *AmeloD*-specific siRNA. (C) qPCR evaluation of *AmeloD* mRNA level in CLDE cells after *AmeloD* overexpression or *AmeloD* knockdown.



Appendix Figure 4. Overexpression of AmeloD in MDCK cells inhibits E-cadherin expression. (A) Overexpression of AmeloD in MDCK cells suppressed E-cadherin expression.

Phase contrast images showed that AmeloD overexpression resulted in morphological changes in MDCK cells (left panel); Immunofluorescence staining of AmeloD (green) with E-cadherin (red) showed that overexpression of AmeloD in MDCK cells resulted in E-cadherin suppression. (B) MDCK cells were transfected by adeno-GFP and adeno-AmeloD and Western blot analysis of E-cadherin and □-catenin protein level in MDCK cells after 72 h of infection. (C) qPCR gene expression analysis of E-cadherin and AmeloD expression in MDCK cells 72 h after infection with adeno-GFP and adeno-AmeloD. Data are shown as mean ± SD (n=3). Statistical significance is shown by t-test (*: $p<0.05$).

Appendix Table. Primers used for q-PCR and ChIP.

Gene name	Forward Primer	Reverse Primer
AmeloD (Ms)	5-CCAGGAGCAGCAGTTCAAACAG-3	5-GAGCCTCCATGACCAGATTC-3
Ameloblastin (Ms)	5-GCGTTTCCAAGAGCCCTGATAAC-3	5-AAGAAGCAGTGTCACATTTCCTGG-3
Gapdh (Ms)	5-TCACCATCTTCCAGGAG-3	5-GCATGGACTGTGGTCATGAG-3
E-cadherin (Ms)	5- ATCGTCCCCGTCCTGCCAATCC -3	5-TTCAGAACCACTGCCCTCGTAATCG-3
E-cadherin (Canis lupus)	5-GACAATGGCCCCATTCCAGA-3	5-GAAGGGAGATGTGTTGGGGG-3
Gapdh (Canis lupus)	5-CAAGGCTGTGGGCAAGGTCATCC-3	5- CTCCAGGCGGCAGGTCAGATCC-3
E-cadherin proximal promoter (P1-P2)	5-CAGGGGTGGAGGAAGTTGAG-3	5-GAGCAGCGCAGAGGCTG-3
E-cadherin proximal promoter (P3-P4)	5-GGGTTACCCTTGGTTTCGGT-3	5-CTGGCAAAGCCATGAGGAGA-3

Appendix Methods

Y2H (Yeast two-hybrid) screening, cDNA library construction and screening

*Y2H screening and cDNA library construction – Saccharomyce*s cerevisiae YRG-2 (*MATa, ura3-53, his3-200, leu-2-3, 112, 112, trp-901, LYS2::UAS-HIS3, URA3 :: UAS-LacZ*), and a GAL4 DNA binding domain expression vector p-BD-GAL4 were obtained from Stratagene (La Jolla CA). The basic helix-loop-helix region of human E12 was amplified by PCR using an antisense primer (5'-TAGCGAATTCTCAGCGGCTGACCACTCGGAG-3') and a sense primer (5'-GCACTGCAGCCCACGGAGGCATACCTTTCA-3'), which corresponds to 883 nt to 1341 nt of the human E12 cDNA sequence. The bait construct, pBD-E12, was generated by inserting the PCR fragment into the EcoRI and PstI sites of pBD-GAL4. For a GAL4 activation domain-tagged cDNA library, poly (A)+ RNA was extracted from the tooth germ tissues of E13.5 rat embryos using a Micro-Fast Track kit (Invitorgen, Carlsbad, CA). An oligo (dT) primed cDNA library was constructed in the HybriZap phage vector (Stratagene). The plasmid (pAD-GAL4) library was obtained by in vivo excision according to the manufacturer's instructions (Stratagene). The library, pAD-TG, had a complexity of 1.5 x 10^6 pfu and an average insert size of about 1.5-kb.

cDNA library screening – To obtain protein capable of binding to the bHLH protein E12, we used the Y2H system monitoring the pAD-GAL4-labeled cDNA library. p-BD-GAL4-E12 and pAD-GAL4 fusion cDNA libraries were transformed into YRG-2 yeast grown in SD /-His /-Leu /-Trp selective medium, followed by transferring to a membrane for yeast β-galactosidase activity test. This step totally produced 2.7 x 10^6 transformants and 19 positive clones. To avoid false results, positive clones were again converted into cDNA in yeast to confirm their transcriptional activation of HIS3 and LacZ expression.

AmeloD full-length sequence and analysis

The pBluescript SK plasmids were excised from the positive phage clones. Both strands of the plasmids were sequenced with synthetic oligonucleotide primers. Sequence analysis was performed using MacVector (Oxford Molecular Limited, UK). The mouse AmeloD cDNA sequence was registered in Gene Bank at NCBI, NIH (GeneBank accession number: MG575629.1). Sequence similarity searches against the non-redundant protein database maintained at NCBI were performed using PSI-BLAST. Phylogenetic relationships were analyzed using the distance matrix method based on ClustalW alignments performed with the MacVector program.

In situ hybridization – *In situ* hybridization of mouse molar sections was performed as described previously (Nakamura et al. 2004). Digoxigenin-labeled probes (antisense and sense) were made by *in vitro* transcription using *AmeloD* cDNAs subcloned into the pBluscript II SK vector as templates in the presence of digoxigenin-labeled dUTP (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The mouse molar sections were prehybridized for 1 h at 50 °C in Hybrisol I (Oncor USA) and then hybridized overnight at 50 °C in Hybrisol I with 100 ng/ml probe in a humidity chamber. After being rinsed in 5X SSC at 60 °C for 20 min, the sections were washed with 50% formamide and 2X SSC at 60 °C for 30 min. Next, they were subjected to RNase digestion for 20 min at 37 °C (1 μg/ml RNase A in buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 0.5 M NaCl, pH 7.5) and then washed with 50% formamide and 2X SSC at 60 °C for 30 min. For the detection of hybridized probes, anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals) was reacted at 1:1000, and the color was developed by incubation with 4-nitro blue tetrazolium chloride and X-phosphate solution. The sense probe was used as a control in these experiments, under the same conditions as the anti-sense probes. No signal was observed with the sense probes.

Antibodies, immunofluorescence, western blot, and Co-IP

Short peptides (RALVDRGPPGG) with high antigenicity in the reserved bHLH region were used to generate AmeloD polyclonal antibodies in rabbits (Research Genetics, USA) and purified by peptide-affinity columns. V5 tagged antibody (R960-25), E-cadherin monoclonal antibody (ECCD-2), and Alexa Fluor 488 and 594 conjugated antibodies were from Life Technologies.

SOX2 antibody (ab97959), Ki67 antibody (ab15580), and AMELX antibody (ab153915) were from Abcam. β-Catenin antibody (D10A8) and HRP-conjugated secondary antibodies were from Cell Signaling Technology. α-Tubulin antibody (T9026) was from Sigma-Aldrich. H3K27me3 antibody was purchased from Abcam. Ezh2 antibody (39933) for ChIP-qPCR was purchased from ACTIVE MOTIF. Immunofluorescence, western blots, luciferase assay, qPCR gene expression analysis, and CO-IP methods were described in our previous publication (Yoshizaki et al. 2014; Yoshizaki et al. 2017).

AmeloD antibody specificity test

Scrambled peptide (VGRLPAGDRPG) and AmeloD-specific peptide (RALVDRGPPGG) were synthesized by PEPTIDE 2.0 and used for AmeloD antibody specificity test. To block the AmleoD antibody function, AmeloD antibodies were diluted in blocking buffer (PBS plus 1% BSA), followed by scrambled peptide and AmeloD-specific peptide incubation with different dilution concentrations at 4 C overnight. Next day, the neutralized antibodies were applied to Western blot or immunofluorescence following standard protocol we described above.

Expression vectors

AmeloD-pCA1F and pEF-V5-HisB-E12 expression vectors were generated in our lab. MCK-luciferase was a gift from Dr. Robert Benezra's lab (Addgene 16062); pGL2Basic-EcadK1 (Addgene 19290) and pGL2Basic-EcadK1/EpaIMUT/EboxMUT/Ebox2MUT (Addgene 19291) constructs were from Dr. Eric Fearon (Hajra et al. 2002).

References

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