RESEARCH REPORTS
Biological

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Synergistic Roles of Amelogenin and Ameloblastin

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APPENDIX

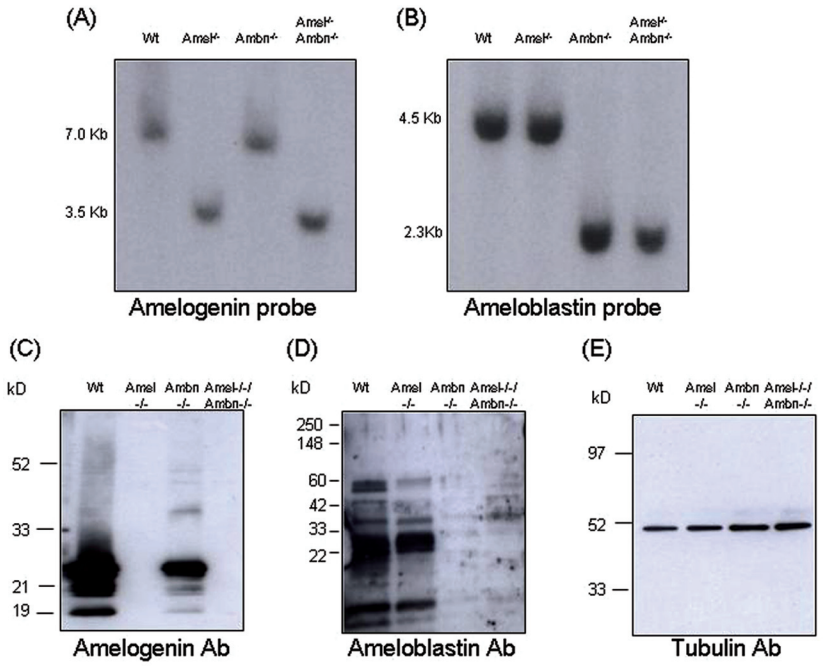
MATERIALS & METHODS

Protein Analysis

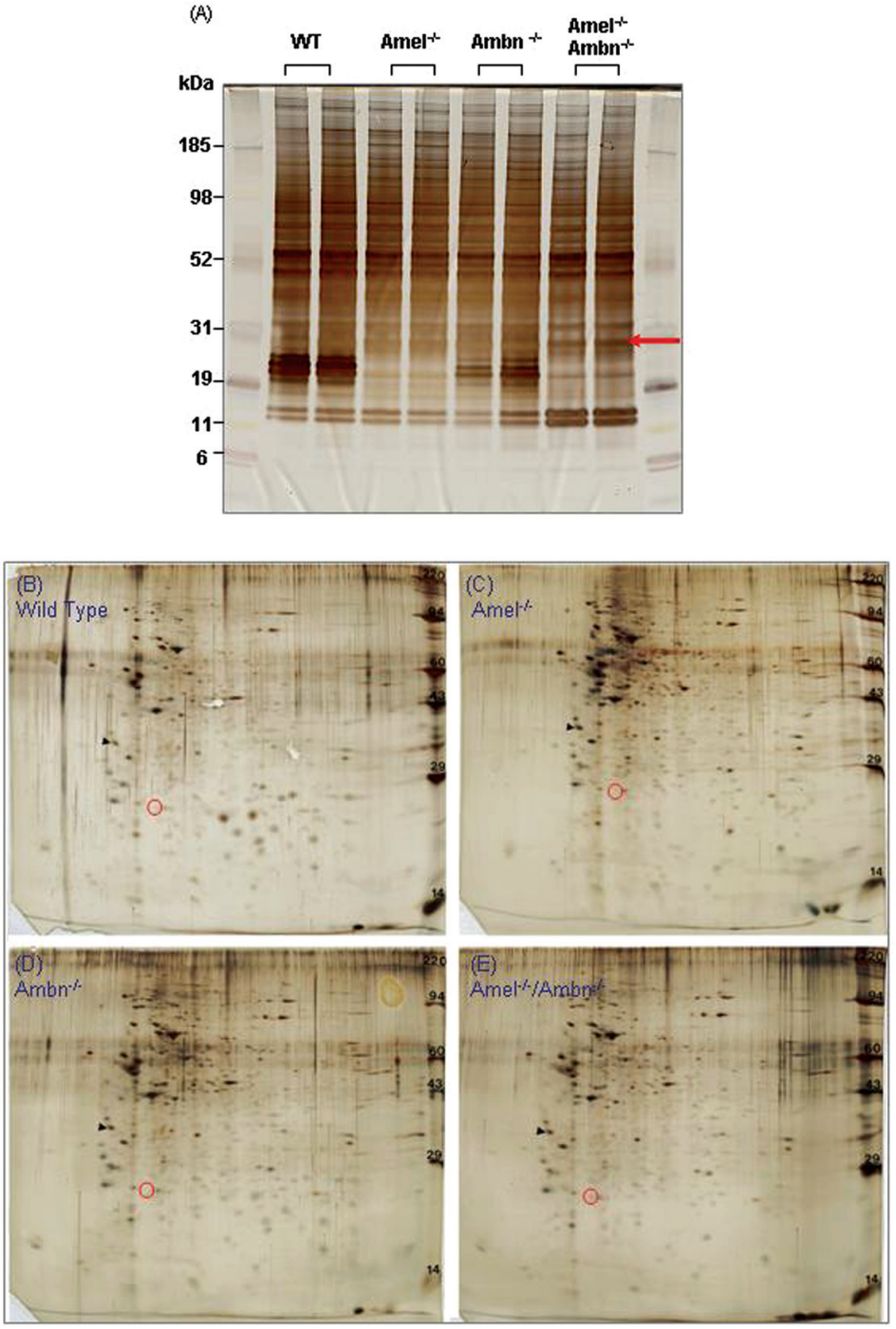
Developing molars were dissected from P3 mice. The samples were suspended in 50 µL of lysis buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$). The suspension was vortexed and centrifuged, and the protein samples were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For silver staining, gels were stained with Silver Quest (Invitrogen, Carlsbad, CA, USA).

Two-dimensional Gel Electrophoresis

The identical samples were used from the previous experiment. Two-dimensional gel electrophoresis was performed according to a method previously described (O'Farrell et al., 1977). Molecular-weight standards (220,000, 94,000, 60,000, 43,000, 29,000, and 14,000) appeared as bands on the right side of the gels. One isoelectric point marker, added to each sample as an internal standard, was marked with a black arrowhead. The gels were dried between sheets of cellophane paper, with the acid edge to the left.



Appendix Figure 1. Generation of *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice. **(A,B)** Southern blot analysis of genomic DNA from wild-type and mutant mice. **(A)** For Southern blotting of amelogenin mice, genomic DNA was digested with *Pst*I, and hybridized with the flanking probe as described in MATERIALS & METHODS. Wild-type and mutant alleles were detected as 7.0- and 3.5-kb fragments, respectively. **(B)** For Southern blotting of ameloblastin mice, genomic DNA was digested with *Xba*I and hybridized with the flanking probe. The wild-type and mutant alleles were detected as 4.5- and 2.3-kb fragments, respectively. **(C,D,E)** Western blot analysis of amelogenin **(C),** ameloblastin **(D),** and tubulin **(E)** of the tooth lysate from wild-type, *Amel*$^{-/-}$, *Ambn*$^{-/-}$, and *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice. P3 mandibular first molars were dissected and subjected to lysis with 100 µL of lysis buffer, and a 20-µL quantity was separated by SDS-PAGE and immunoblotted with polyclonal anti-amelogenin (C), anti-ameloblastin (D), and tubulin (E) antibodies.



Appendix Figure 2. Proteomic analysis of tooth lysates from the wild-type and mutant mice. **(A)** Silver staining of total lysates from P3 mandibular molars from wild-type, *Amel X*$^{-/-}$, *Ambn*$^{-/-}$, and *Amel X*$^{-/-}$/*Ambn*$^{-/-}$ mice separated by SDS-PAGE. Arrow indicates the 24-kDa protein band that is up-regulated in *Amel X*$^{-/-}$/*Ambn*$^{-/-}$ mice. **(B-E)** Two-dimensional gel electrophoresis of total lysates from P3 mandibular molars from wild-type, *Amel X*$^{-/-}$, *Ambn*$^{-/-}$, and *Amel X*$^{-/-}$/*Ambn*$^{-/-}$ mice. An isoelectric point marker, added to each sample as an internal standard, was marked with a black arrowhead. Red circle indicates a 24-kDa protein spot that stains densely in the lysate from *Amel X*$^{-/-}$/*Ambn*$^{-/-}$ mice.

In-gel Digestion and Matrix-assisted Laser Desorption Ionization (MALDI) Analysis of Coomassie-stained Proteins

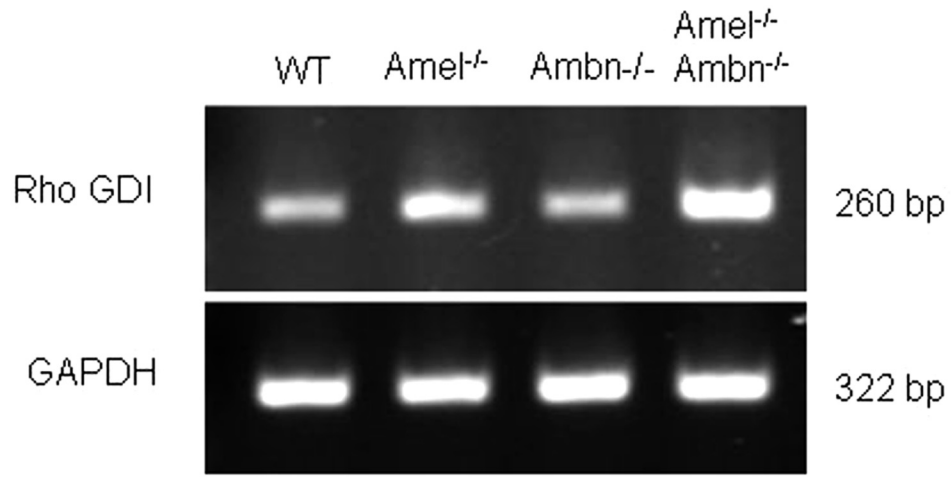
MALDI-mass spectroscopic analysis and N-terminal sequencing were carried out by Kendrick Laboratories, Inc. (Madison, WI, USA) and the Center for Biologics Evaluation and Research, Food and Drug Administration Biotechnology Core Facility Services (Bethesda, MD, USA). MALDI mass spectroscopic analysis was performed on the digest with a PerSeptive Voyager DE-RP mass spectrometer (Applied Biosystems, Inc., Foster City, CA, USA) in a linear mode. We used the amino acid sequence obtained by N-terminal protein sequencing to search for sequence homology against the PIR International Database and SWISS-PROT, using the BLAST and FASTA programs.

Western Blotting Analysis

Western blot analysis was performed for amelogenin and ameloblastin in wild-type, *Amel X*$^{-/-}$, *Ambn*$^{-/-}$, and *Amel X*$^{-/-}$/*Ambn*$^{-/-}$ mice. P3 and P7 mandibular first molars were dissected and subjected to lysis with 100 μL of lysis buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$), and a 20-μL quantity was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with polyclonal anti-amelogenin or ameloblastin antibodies. The blots were incubated with anti-amelogenin or anti-ameloblastin antibodies, and the signals were detected with an ECL kit (Amersham Biosciences, Buckinghamshire, UK). The anti-ameloblastin and anti-amelogenin antibodies were generated as previously reported (Gibson *et al.,* 2001; Fukumoto *et al.,* 2004). Anti-tubulin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RNA Isolation and RT-PCR

Developing molars were dissected from P7 mice. Total RNA was isolated by means of the RNeasy kit and treated with DNase I (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from 1 μg of RNA at 42°C



Appendix Figure 3. Altered RhoGDI mRNA expression in wild-type and mutant mice. RT- PCR analysis showed that RhoGDI mRNA was slightly up-regulated in P7 developing molars of *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice. *GAPDH* was used as control.

for 90 min with Oligo(dT)$_{12\text{-}18}$ primer and Superscript II reverse-transcriptase (Invitrogen). PCR amplification was performed by the following primers:

(Rho GDI) 5′-TGTGACTCGACTGACCTTGG -3′ and 5′-AAC-TCATACTCCTCGG CCCT-3′ (NM_133796.2); and (GAPDH) 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-GCATGGACTGTG-GTCATGA-3′ (XM193532).

PCR was performed with 26 cycles, 90°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. All reactions were carried out in a Perkin Elmer gene PCR system 3600 (Perkin Elmer, Wellesley, MA, USA).

RESULTS

Generation of the Amelogenin and Ameloblastin Double-null (*Amel X*$^{-/-}$*/Ambn*$^{-/-}$) Mice

We generated the *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice by crossing *Amel X*$^{-/-}$ mice with *Ambn*$^{-/-}$ mice. The genotypes of wild-type, *Amel X*$^{-/-}$, *Ambn*$^{-/-}$, and *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice were confirmed by Southern blotting of genomic DNA. Briefly, for amelogenin null mice, the genomic DNA was digested with *Pst*I, and the blots were hybridized with a flanking probe as described previously (Gibson *et al.,* 2001). The wild-type and amelogenin mutant alleles were detected as 7.0- and 3.5-kb fragments (Appendix Fig 1A). For ameloblastin mutant mice, Southern blot analysis of genomic DNA, samples that were digested with *Xba*I, was performed by the utilization of a flanking probe containing exons 7-9 (Fukumoto *et al.,* 2004). The wild-type and ameloblastin mutant alleles were identified as 4.5- and 2.3-kb fragments (Appendix Fig 1B). Southern blot analysis confirmed disruption of both amelogenin and ameloblastin genes in *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice (Appendix Figs. 1A, 1B). We performed Western blot analysis to examine the protein expressions of amelogenin and ameloblastin in the mutant mice (Appendix Figs. 1C, 1D, 1E). No amelogenins were detected by Western blot analysis with the anti-amelogenin antibody (Appendix Fig. 1C) in *Amel X*$^{-/-}$ and *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice.Ameloblastin protein was not detected by Western blot analysis with the ameloblastin antibody (Appendix Fig. 1D) in *Ambn*$^{-/-}$ and *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice.

Rho GDI Expression is Increased in *Amel X-/-/Ambn-/-* Ameloblasts

The protein extracts of lower first molars from P3 mice were resolved on an SDS-PAGE gel (Appendix Fig. 2A). Silver staining of the protein gel showed an intense band with a 24-kDa molecular weight in the *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice. For further characterization of the 24-kDa band in *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice, the protein extract was subjected to two-dimensional gel electrophoresis. Gels were stained with Coomassie brilliant blue and analyzed for differentially expressed proteins in the wild-type, Amel$^{-/-}$, Ambn$^{-/-}$, and *Amel X*$^{-/-}$*/Ambn*$^{-/-}$ mice (Appendix Figs. 2B-2E). The relative spot intensities were WT = 1, Amel X$^{-/-}$ = 1.05, Ambn$^{-/-}$ = 1.51, and Amel X$^{-/-}$/Ambn$^{-/-}$ =2.03. These spots were eluted for the MALDI mass spectrum analysis for protein identification, and these proteins were identified as Rho GDI.

APPENDIX REFERENCE

O'Farrell PZ, Goodman HM, O'Farrell PH (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* 12:1133-1141.