**SUPPLEMENTAL MATERIAL**

## MATERIAL AND 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 pH7.4, 150 mM NaCl, 10mM $MgCl_2$). 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, CA).

### *Two-dimensional gel electrophoresis*

The identical samples were used from the previous experiment. Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (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.

### *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) and 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 using a PerSeptive Voyager DE-RP mass spectrometer (Applied Biosystems, Inc., Foster City, CA, USA) in a linear mode. The amino acid sequence obtained by N-terminal protein sequencing was used 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 lysed with 100 µl of lysis buffer buffer (1 % triton X-100, 10 mM Tris-HCL pH7.4, 150 mM NaCl, 10mM $MgCl_2$), and 20 µl were 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 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).

***RNA isolation and RT-PCR***

Developing molars were dissected from P7 mice. Total RNA was isolated using the RNeasy and treated with DNase I (Qiagen, Valencia, CA). First strand cDNA was synthesized from 1 µg of RNA at 42°C for 90 min using Oligo$(dT)_{12-18}$ primer and

Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR amplification was performed by following primers,

Rho GDI, 5'- TGTGACTCGACTGACCTTGG -3' and 5'-AACTCATACTCCTCGG CCCT-3' (NM_133796.2); GAPDH 5'-CCATCACCATCTTCCAGGAG-3' and 5'-GCATGGACTGTGGTCATGA-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 PerkinElmer gene PCR system 3600.

## RESULTS

### *Generation of the Amelogenin and Ameloblastin double null (Amel $X^{-/-}$/$Ambn^{-/-}$) mice*

The *Amel* $X^{-/-}$/*Ambn* $^{-/-}$ mice were generated 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 (Gibson *et al.,* 2001). The wild-type and amelogenin mutant alleles were detected as 7.0- and 3.5-kb fragments (Supplemental Fig 1A). For ameloblastin mutant mice, Southern blot analysis of genomic DNA digested with XbaI and hybridized with 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 (Supplemental Fig 1B). Southern blot analysis confirmed disruption of both amelogenin and

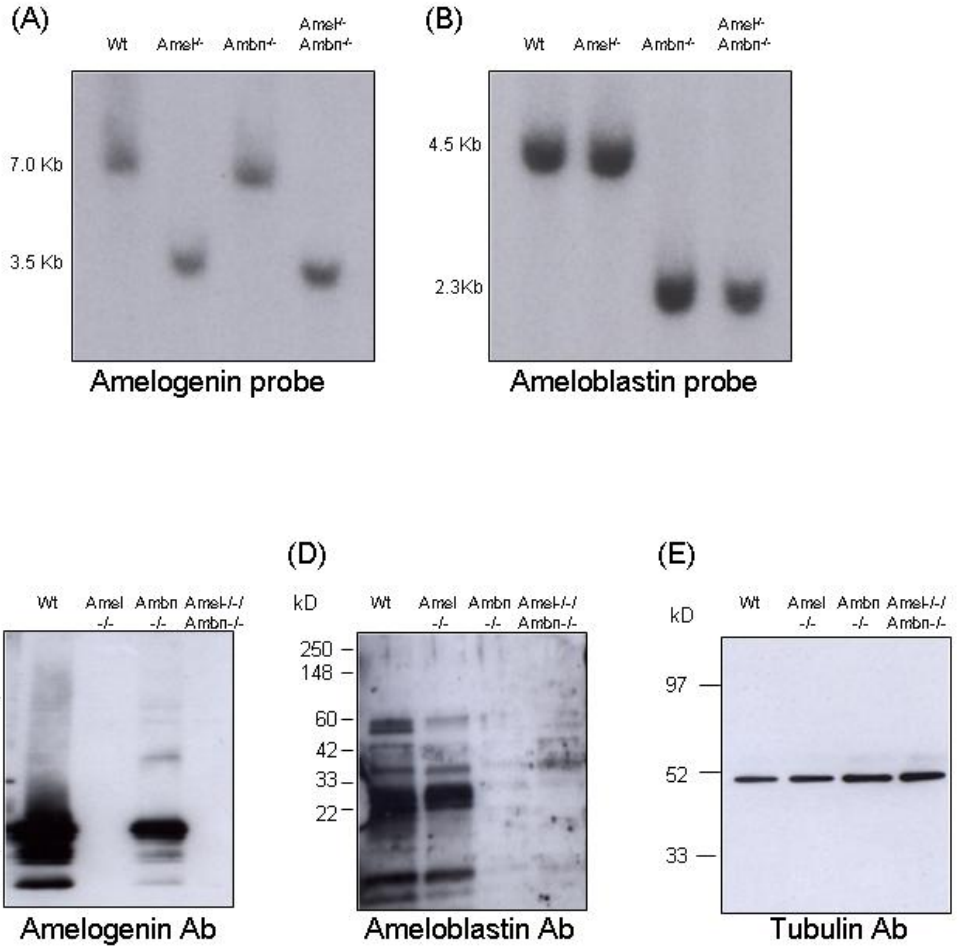
ameloblastin genes in *Amel X$^{-/-}$/Ambn$^{-/-}$* mice (Supplemental Fig 1A and B). Western blot analysis was performed to examine the protein expressions of amelogenin and ameloblastin in the mutant mice (Supplemental Fig. 1C, D and E). No amelogenins were detected by western blot analysis using the anti-amelogenin antibody (Supplemental Fig. 1C) in *Amel X$^{-/-}$* and *Amel X$^{-/-}$/Ambn$^{-/-}$* mice. Ameloblastin protein was not detected by western blot analysis using the ameloblastin antibody (Supplemental 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 molar from P3 were resolved on an SDS-PAGE gel (Supplemental Fig. 2 A). Silver staining of the protein gel showed an intense band with a molecular weight 24 kDa 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 (Supplemental Fig. 2 B-E). 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 to identify the proteins, and these proteins were identified as Rho GDI.

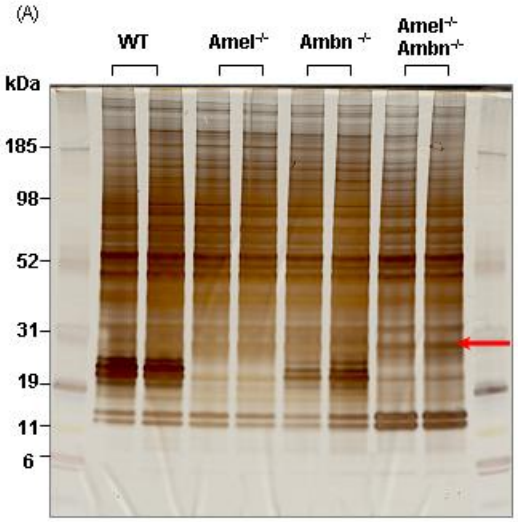
**FIGURES**

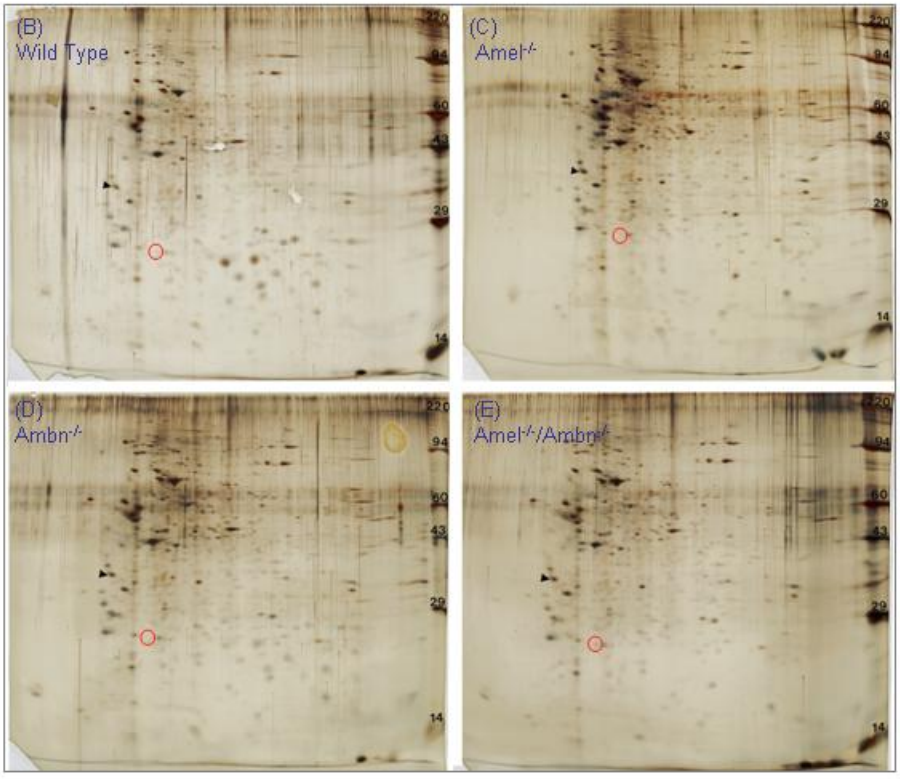
Supplemental Fig. 1



**Supplemental Fig. 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 and 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 lysed with 100 µl of lysis buffer, and 20 µl were separated by SDS-PAGE and immunoblotted with polyclonal anti-amelogenin (C), anti-ameloblastin (D) and tubulin (E) antibodies.

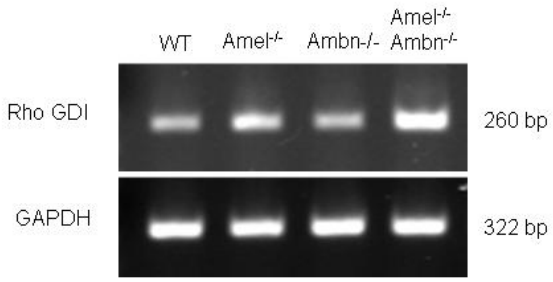
Supplemental Figure 2



**Supplemental Fig. 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*$^{-/-}$, *Amel* $X^{-/-}$/*Ambn*$^{-/-}$ mice separated by SDS-PAGE. Arrow indicates the 24kDa size protein band which 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*$^{-/-}$, *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 24kDa size protein spot that stains dense in the lysate from *Amel* $X^{-/-}$/*Ambn*$^{-/-}$ mice.

Supplemental Fig. 3




**Supplemental Fig. 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 molar of *Amel* $X^{-/-}$*/Ambn*$^{-/-}$ mice. *GAPDH* used as control.