An evolutionarily conserved helix mediates ameloblastin-cell interaction

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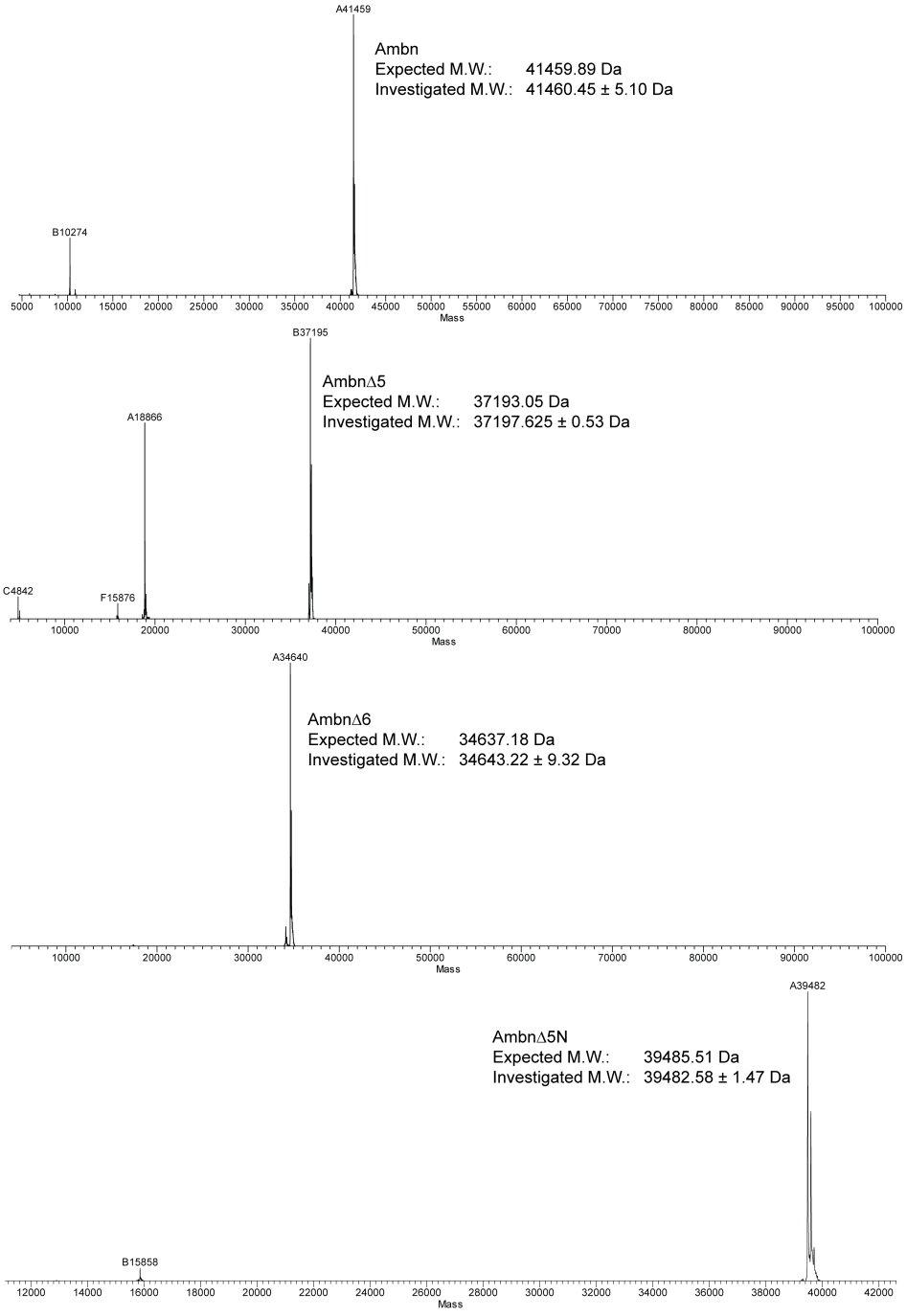


Figure S1. Mass spectra of Ambn (M.W. = 41459.89 Da), AmbnΔ5 (M.W. = 37193.05 Da), AmbnΔ6 (M.W. = 34673.18 Da) and AmbnΔ5N (M.W. = 39485.51 Da). Mass spectra confirmed that the purified recombinant proteins are the target proteins. Mass spectra were collected by Thermo LTQ Orbitrap XL LC-MS in Pasarow Mass Spectrometry Laboratory, University of California Los Angeles, CA, USA. M.W.: molecular weight.

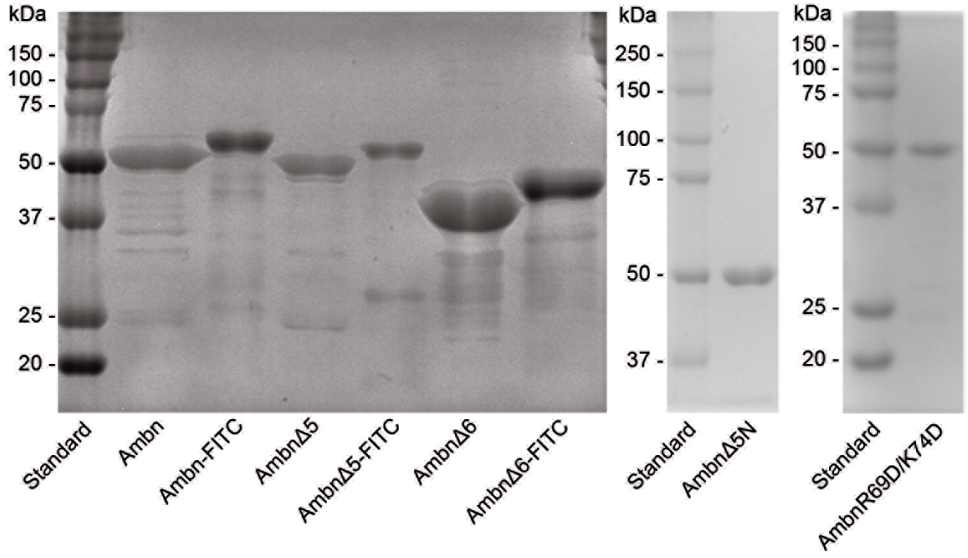


Figure S2. SDS-PAGE of recombinant Ambn, Ambn-FITC, AmbnΔ5, AmbnΔ5-FITC, AmbnΔ6, AmbnΔ6-FITC, AmbnΔ5N and AmbnR69D/K74D. The bands confirmed that Ambn and its variants were qualified for biochemical and biophysical experiments, and that they did not degrade during the FITC labelling procedure at room temperature.

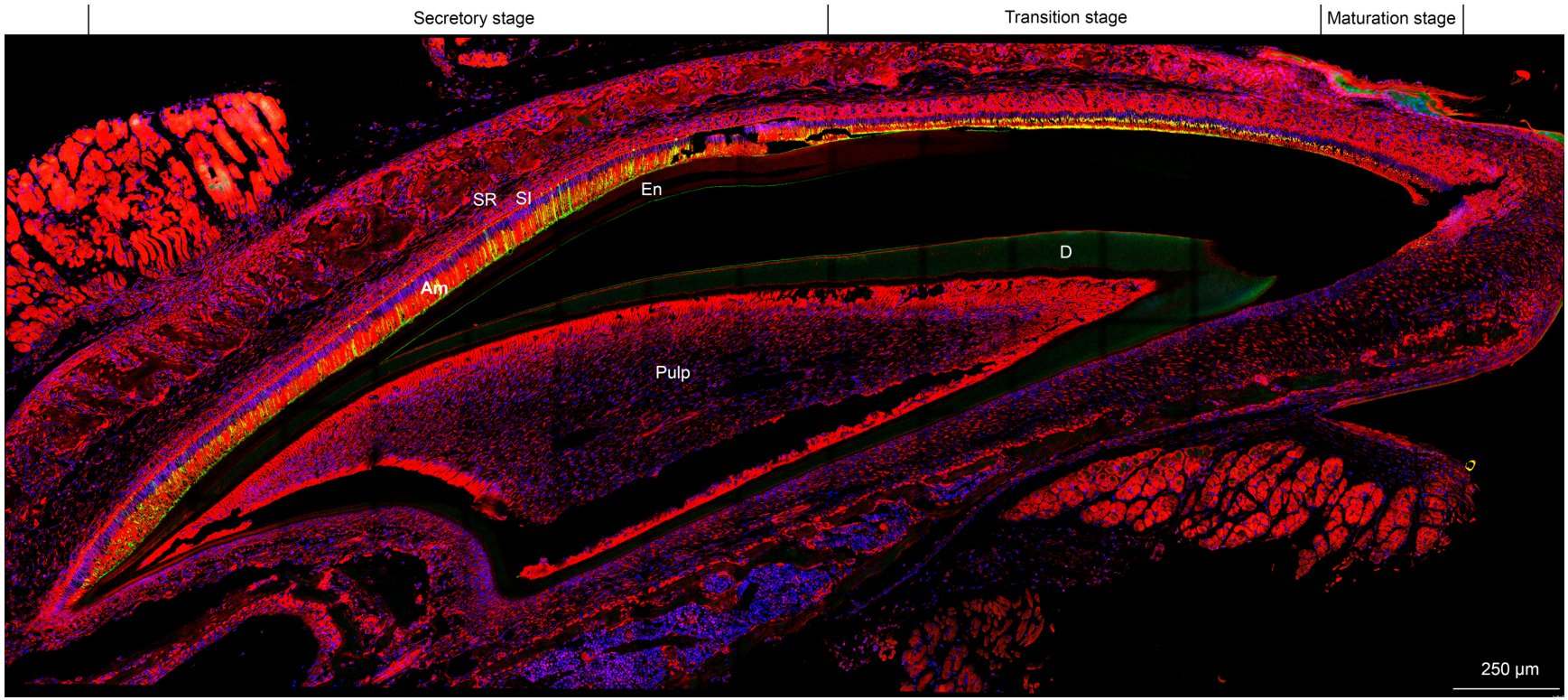


Figure S3. Localization of Ambn *in vivo*. Immunofluorescence of a mouse mandibular incisor (P8) showed the localization of Ambn (Green) *in vivo*. Ambn is secreted from secretory stage to transition stage, and accumulated at the interface between ameloblast cells and enamel matrix. Ambn colocalizes with cell membrane (red, stained with DiD) at the interface from secretory stage to transition stage. Green, Ambn; Red, membrane; Blue, nucleus. Am, ameloblast; D, Dentin; En, enamel; SI, stratum intermedium; SR, stellate reticulum. See methods for details.

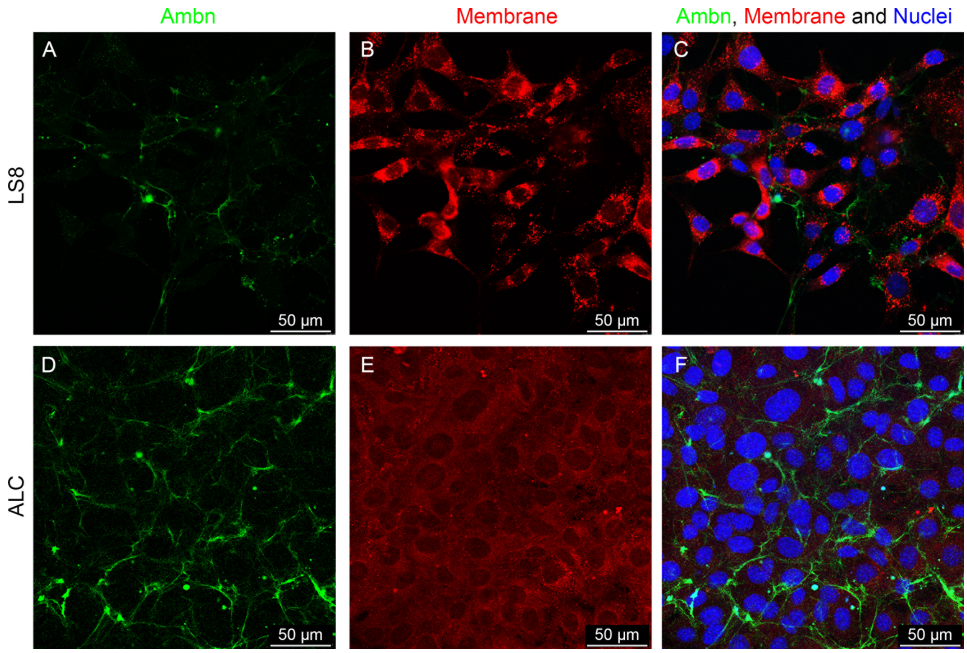


Figure S4. Confocal images of LS8 and ALC cells incubated with 5 μM Ambn-FITC. When the cell density is higher, the Ambn-cell adhesion is clearer even with the same concentration of Ambn-FITC as that used in the low-density cell culture experiment.

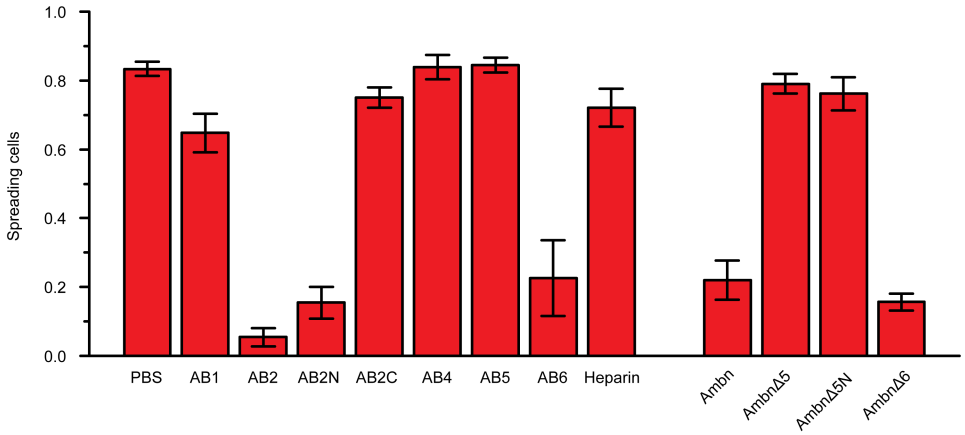


Figure S5: Ambn-derived peptides and heparin inhibit the adhesion of Ambn to TCMK-1, an epithelial cell from mouse kidney. Peptides AB2 and AB2N inhibit TCMK-1 cell spreading on the Ambn-coated plates most significantly. Ambn and AmbnΔ6 inhibit the adhesion of TCMK-1 to the Ambn-coated plates. In contrast, AmbnΔ5 and AmbnΔ5N, without the AH-motif, lose their ability to inhibit cell adhesion.

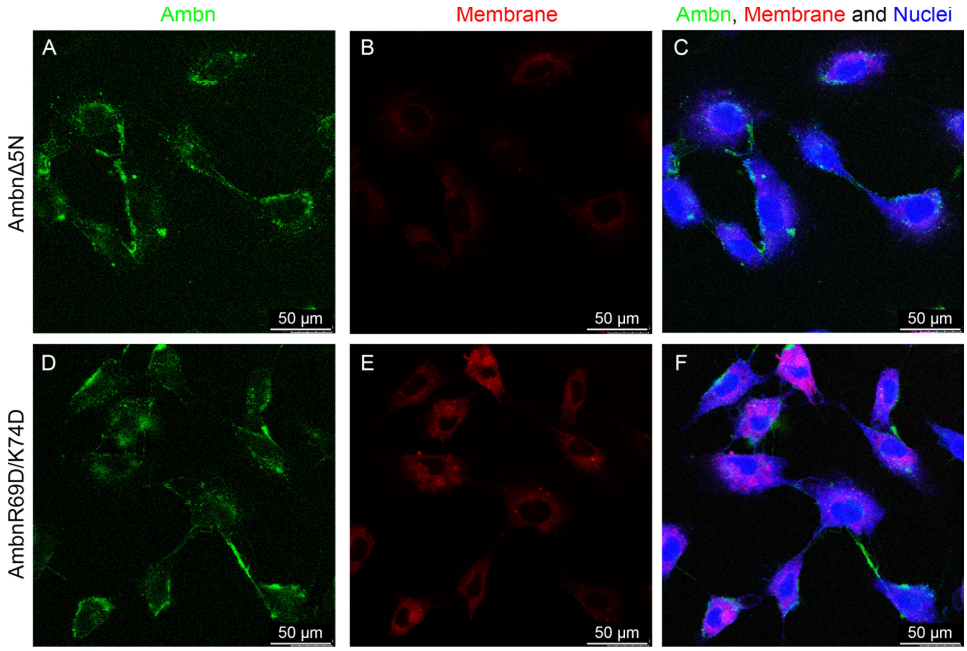


Figure S6: Disrupting the AH motif affect Ambn-cell adhesion. Confocal images of ALC cells incubated with 5 µM AmbnΔ5N-FITC or AmbnR69D/K74D-FITC. Red: DiD-stained membrane; Green: FITC-labelled Ambn variants. The brightness of green was increased for better visibility of AmbnΔ5N-FITC or AmbnR69D/K74D-FITC.

Table S1. The sequences of mouse Ambn-derived peptides and their corresponding exons.

Peptides	Exons	Sequence
AB1	Exon 3&4	^{27}VPAFPQQPGAQGMAPPGMASLSLETMRQLGSLQGLNALSQ^{66}
AB2	Exon 5	^{67}YSRLGFGKALNSLWLHGLLPPHNSFPWIGPREHETQQ^{103}
AB4	N-terminus of exon 6	^{104}PSLQPHQPGLKPFLQPTAATGVQVTPQKPGPQPPMHPGQLPLQ^{146}
AB5	Exon 7 and C-terminus of exon 6	^{147}EGELIAPDEPQVAPSENPPTPEVPIMDFADPQFPTVFQIAR^{187}
AB6	Exon 13	^{240}YGTLFPRFGGFRQTLRRLNQNSPKGGDFTVEVDSPVSVTKGPEK^{283}
AB2N	N-terminus of exon 5	^{67}YSRLGFGKALNSLWLHGLLP^{86}
AB2C	C-terminus of exon 5	^{87}PHNSFPWIGPREHETQQ^{103}

Table S2. The distribution of 18 amino acid amphipathic helix (AH) motifs among enamel matrix proteins. Protein sequences of human, mouse and pig full-length Ambn, Amel, Enam and Amtn were from the GenBank database. The potential AH-forming sequences were screened by Heliquest as described previously (Su et al. 2019b).

Protein	*Mus musculus*		*Homo sapiens*		*Sus scrofa*	
	Sequence	No of AH	Sequence	No of AH	Sequence	No of AH
Amel	Isoform 1 NP_001075447.1	0	Isoform 1 NP_001133.1	0	NP_999071.1	0
	Isoform 2 NP_033796.1	0	Isoform 2 NP_872622.1	0		
	Isoform 3 NP_001277300.1	0	Isoform 3 NP_872621.1	0		
Ambn	Isoform 1 NP_001290360.1	2	Isoform CRA_a EAX05623.1	1	AAA85588.1	1
	Isoform 2 NP_033794.1	2	Isoform CRA_b EAX05624.1	1		
Enam	Isoform CRA_a EDL05358.1	0	Isoform X1 XP_006714119.1	1	NP_999406.1	0
	Isoform CRA_b EDL05359.1	0	Isoform 2 NP_001355062.1	1		
	Isoform CRA_c EDL05360.1	0				
Amtn	NP_082069.1	0	Isoform 1 NP_997722.1	0	XP_020956200.1	0
			Isoform 2 NP_001273660.1	0		

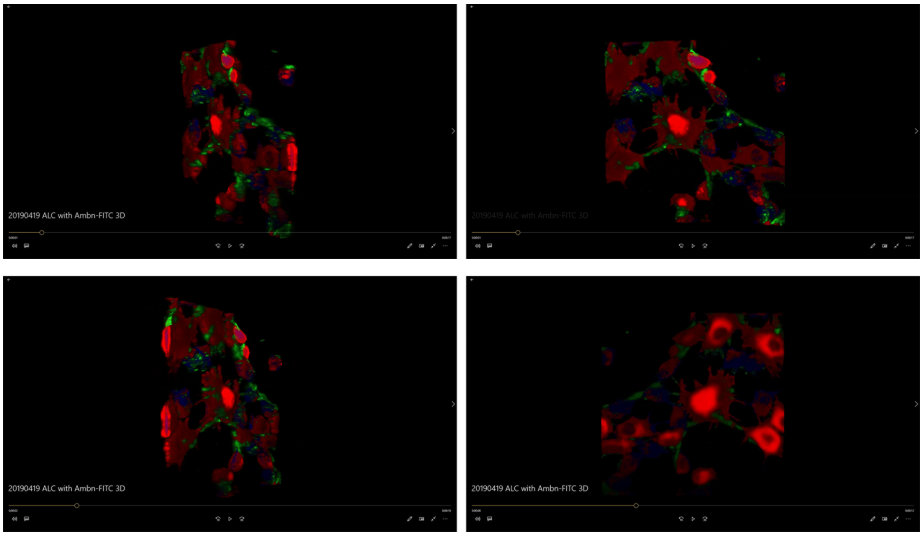
Notes:
1. The AH motifs on the signal peptides were not included.
2. The two potential AH motifs of mouse Ambn overlap.
3. Human enamelin has a potential AH motif with a proline at the c-terminal (Details in Table S3).

Table S3. Amino acid sequences, hydrophobicity (H), hydrophobic moment (μH), net charge and number of proline (Pro) of the potential amphipathic helix (AH) motifs of enamel matrix proteins.

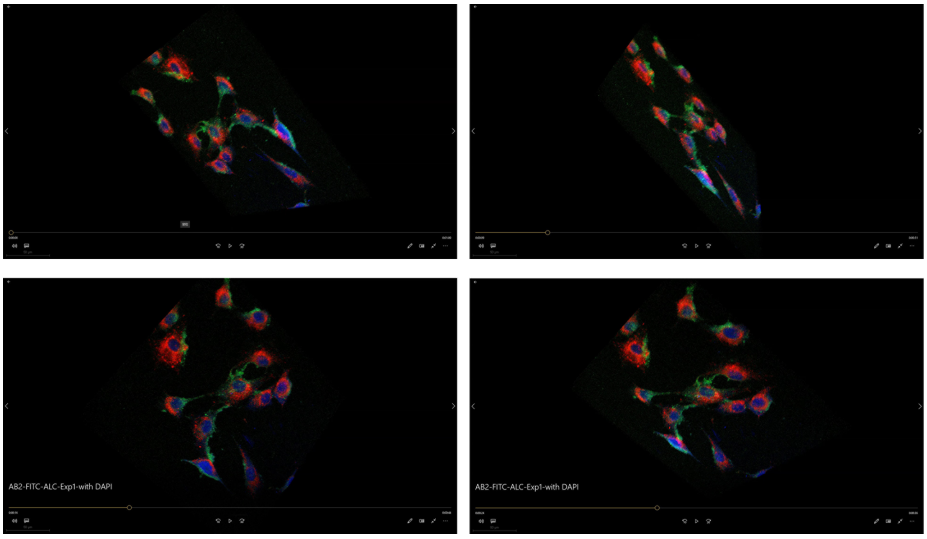
All the potential AH sequences contain a hydrophobic face according to Heliquest analysis (Gautier et al. 2008).

Protein	Sequence id	Species	Potential AH motif	H	μH	Charge	Pro
Ambn	NP_001290360.1 Isoform 1	Mouse	^{58}LQGLNALSQYSRLGFGKA^{75}	0.392	0.233	2	0
			^{69}RLGFGKALNSLWLHGLLP^{86}	0.709	0.129	2	1
	NP_033794.1 Isoform 2		^{58}LQGLNALSQYSRLGFGKA^{75}	0.392	0.233	2	0
			^{69}RLGFGKALNSLWLHGLLP^{86}	0.709	0.129	2	1
Ambn	EAX05623.1 Isoform CRA_a	Human	^{8}RYGFGKSFNSLWMHGLLP^{25}	0.627	0.172	2	1
	EAX05624.1 Isoform CRA_b		^{26}RYGFGKSFNSLWMHGLLP^{43}	0.627	0.172	2	1
Ambn	AAA85588.1	Pig	^{64}RFGFGKSFNSLWMHGLLP^{81}	0.673	0.155	2	1
Enam	XP_006714119.1 Isoform X1	Human	^{782}FNRNIWDQATHLQKAPAR^{799}	0.231	0.454	2	1
	NP_001355062.1 Isoform 2		^{564}FNRNIWDQATHLQKAPAR^{581}	0.231	0.454	2	1

Video S1. 3D confocal images of ALC cells incubated with 5 μM Ambn-FITC. Green: Ambn; Red: membrane; Blue: nucleus. The video showed that Ambn-cell adhesion more clearly and it also confirmed that the Ambn-cell adhesion is specific (please see Video S1 online).



Video S2. 3D confocal images of ALC cells incubated with 20 μM AB2-FITC. Green: Ambn; Red: membrane; Blue: nucleus. The video showed that AB2 adhered to ALC cells, while the pattern is different (please see Video S2 online).



Supplementary methods and materials

Protein expression and purification: Recombinant mouse Ambn was expressed and purified following our published protocol (Su et al. 2019a). Briefly, the proteins were expressed in BL21(DE3) and purified using nickel affinity chromatography. The His and thioredoxin tags were cleaved by enterokinase and were removed using high-performance liquid chromatography. Ambn p.Y67_Q103del (exon 5 deleted; AmbnΔ5), Ambn p.R69_P86del (amphipathic helix-forming motif at N-terminus of exon 5 deleted; AmbnΔ5N), Ambn p.P104_V168del (exon 6 deleted; AmbnΔ6), and Ambn p.R69D/K74D (R69D/K74D) were created using Q5 Site-Directed Mutagenesis Kit, and expressed and purified as described above. The proteins were characterized using SDS-PAGE and mass spectroscopy (Figures S1 and S2).

Mouse tissue immunofluorescence-DiD labeling and imaging: All animal studies were conducted following protocols approved by the USC Institutional Animal Care & Use Committee (IACUC). Post-natal day 8 (P8, with day of birth assigned day zero) mice were euthanized and mandibles were dissected. The mandibles were fixed in 4.0% paraformaldehyde for 24 hours and decalcified in 10% EDTA containing 0.1% glutaraldehyde for 8-10 days. Mandibles were dehydrated and embedded in paraffin. 7.0 μm thick sections were cut from the paraffin blocks, taking care to maintain the structure of the developing incisor.

Deparaffinization and antigen-retrieval was done by submerging the slides in 10.0 mM sodium citrate (pH 6.0) with 0.05% Tween-20 in a 60°C water bath overnight. Standard protocols were followed for endogenous peroxidase inhibition (0.3% hydrogen peroxide in TBS), and blocking (0.1% bovine serum albumin in TBS). Sections were then incubated for 45 minutes in 0.3% Triton X-100 in TBS. DiD staining was performed with 5 mM DiD (ThermoFisher Scientific) diluted 1:100 in DMSO overnight at 40°C in a hot air oven. Sections

were labeled with anti-Ambn primary antibody M-300 (host: rabbit, Santa Cruz Biotechnology, now discontinued) diluted (1:250) in 0.1% BSA in TBS at room temperature overnight. Anti-rabbit secondary antibody conjugated with Alexa 488 (ThermoFisher Scientific) was used to detect the presence of ameloblastin. DAPI (1:1000) was added to the secondary antibody dilution solution. To prevent the DiD from leaching out, no mounting media were used and coverslips were placed using TBS and sealed with clear nail polish.

The immunofluorescence-labelled tissue sections were imaged using a Leica TCS SP8 confocal microscope with an oil immersion objective HCX PL APO CS × 63 (NA 1.4). Detection of secondary antibody for Ambn was performed at 498-560 nm (excitation 488 nm); detection of DiD was performed at 643-750 nm (excitation 633 nm).

Leica Application Suite X Advanced Fluorescence software (version 1.8.1.13759) was used to analyze the colocalization of Ambn and the cell membrane of ameloblasts.

Cell attachment and spreading assays: The assays were conducted following the published protocols (Humphries 2001; Sonoda et al. 2009). Cell attachment and spreading assays were performed in 96-well cell culture plates (Cellstar, Cat. No: 655180). Each well was coated with 100 µl 5 µg/ml recombinant mouse Ambn diluted in pH7.4 PBS, and blocked with 50 µl 10 mg/ml BSA in in divalent cation-free PBS for 1 h at 37 °C.

For cell attachment assay, after washing, about 2.5×10^4 cells were treated with or without 10 µg/ml AB1, AB2, AB4, AB5, AB6, heparin (Sigma, Cat. No: H7640), Ambn, AmbnΔ5, AmbnΔ6, Ambn Δ5N, or AmbnR69D/K74D. The treated cells were added to plates, diluted with equal volume of pH7.4 PBS buffer, and incubated for 30-40 min at 37°C. Then, the unattached cells were removed by washing the wells gently with 100 µl room temperature PBS for three

times. The attached cells were fixed with 5% (w/v) glutaraldehyde for 20 min, stained with 0.1% crystal violet, and counted under a microscope. In the control PBS group, about 1000-5000 cells were found in each well.

For cell spreading assay, the procedure was similar to the cell attachment assay, except that the cell number used in each well was about 1.0×10^4 and the treated cells were incubated for 90 min for ALC cell, and 2 hours for LS8 and NIH3T3 cells. The percentage of cells that adopted spread morphology was determined using a microscope. For each technical replicate, about 100-400 cells in an image were counted.

Unilamellar lipid vesicle preparation: Large unilamellar vesicles (LUVs) were prepared and characterized using published methods (Su et al. 2019b). Briefly, 100 nm LUVs were prepared with a lipid molar ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) : 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) : 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) : 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol (POPI) : sphingomyelin (SM) = 40:25:15:10:10, to mimic the membrane domain involved in epithelial cell-extracellular matrix (ECM) adhesion (Márquez et al. 2008). The dried lipid mixtures were rehydrated in pH 7.4 buffer containing 10.0 mM HEPES, 50.0 mM KCl, 1.0 mM EDTA, 3.0 mM NaN3.

For membrane leakage assays, LUVs were prepared with 9.0 mM ANTS (8-Aminonaphthalene-1,3,6-trisulfonic acid) and 25.0 mM DPX (p-xylene-bis-pyridinium bromide).

Membrane leakage assay: The 300 μM 100 nm LUVs encapsulating 9.0 mM ANTS and 25.0 mM DPX were mixed separately with 1 μM Ambn, AmbnΔ5N or R69D/K74D and BSA as

negative control. Release of fluorophore ANTS and its quencher DPX was monitored by measuring the increase of ANTS fluorescence intensity using a Quanta Master 4 fluorescence spectro-fluorometer. Excitation and emission wavelengths were 380 nm and 520 nm. All the split widths were 5 nm. All data were normalized to the fluorescence intensity when all the vesicles were disrupted by Triton X-100.

Circular dichroism spectroscopy: Two μM Ambn, AmbnΔ5N or R69D/K74D were mixed with 300 μM, 100 nm LUVs in pH 7.4, 10.0 mM Tris-HCl buffer and kept at room temperature for 5.0 min before measurements. Far-UV circular dichroism spectra were recorded on a J-815 circular dichroism spectrometer (JASCO) over a wavelength range of 190-260 nm using a 1 mm path length quartz cell.

Intrinsic tryptophan fluorescence spectroscopy: Samples were prepared as described for CD. Tryptophan fluorescence spectra were recorded on a Quanta Master 4 fluorescence spectro-fluorometer. The excitation wavelength was 295 nm, the emission wavelength range was 300-400 nm, and all the split widths were 5 nm. The changes of emission maximum were analyzed to reveal the tertiary structural changes of Ambn.

Amphipathic helix prediction: Protein sequences of human, mouse and pig full-length Ambn, Amel, Enam and Amtn were extracted from the GenBank database. The potential amphipathic helix-forming sequences were screened by Heliquest as described previously (Gautier et al. 2008).

3D Visualization of ALC cells with Ambn-FITC or AB2-FITC: 3D visualization of ALC cells was created by Leica Application Suite X Advanced Fluorescence software (version 1.8.1.13759). 163 z-stack images with 0.1 μm step size were used for creating 3D image of ALC

cells with Ambn-FITC. 17 z-stack images with 0.3 μm step size were used for creating 3D image of ALC cells with AB2-FITC.

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