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Kyoto University
Anti–USAG-1 therapy for tooth regeneration through enhanced BMP signaling

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Uterine sensitization–associated gene-1 (USAG-1) deficiency leads to enhanced bone morphogenetic protein (BMP) signaling, leading to supernumerary teeth formation. Furthermore, antibodies interfering with binding of USAG-1 to BMP, but not lipoprotein receptor–related protein 5/6 (LRP5/6), accelerate tooth development. Since USAG-1 inhibits Wnt and BMP signals, the essential factors for tooth development, via direct binding to BMP and Wnt coreceptor LRP5/6, we hypothesized that USAG-1 plays key regulatory roles in suppressing tooth development. However, the involvement of USAG-1 in various types of congenital tooth agenesis remains unknown. Here, we show that blocking USAG-1 function through USAG-1 knockout or anti–USAG-1 antibody administration relieves congenital tooth agenesis caused by various genetic abnormalities in mice. Our results demonstrate that USAG-1 controls the number of teeth by inhibiting development of potential tooth germs in wild-type or mutant mice missing teeth. Anti–USAG-1 antibody administration is, therefore, a promising approach for tooth regeneration therapy.

INTRODUCTION
Like beaks, nails, horns, and several eccrine glands, teeth are ectodermal organs. Tooth morphogenesis is regulated by a signal transduction network involving interactions between the epithelium and mesenchyme (1–3). Interactions involving positive and negative loops among bone morphogenetic protein (BMP), fibroblast growth factors, Sonic hedgehog, and Wnt pathways regulate the morphogenesis of individual teeth (1, 4). While the number of teeth is usually strictly controlled in individual species (5), it can increase or decrease congenitally in about 1% of individuals (6–8). Conditions of decreases and increases in the usual number of teeth are called tooth agenesis and supernumerary teeth, respectively. Analyses of mouse models have begun to clarify the genetic factors and molecular and pathological mechanisms underlying these conditions (4, 9).

Investigations of single-gene knockout (KO) mice have demonstrated that loss of function of Usag-1, also referred to as Sclerostin domain containing 1 (SOSTDC1), ectodin, or Wnt modulator in surface ectoderm (WISE), CCAAT/enhancer-binding protein beta (CEBPB), Sparyt homolog 2 (SPRY2), sparyt homolog 3 (SPRY3), or Epiprofin (EPFPN), result in the production of supernumerary teeth (10–14). Results from these studies suggest that de novo tooth formation may be regulated by a single candidate gene. Supernumerary teeth may result from the rescue of arrested teeth germ (10, 15); we have previously reported the transformation of the residual deciduous incisor into supernumerary teeth in USAG-1-deficient mice (10). USAG-1 is a bifunctional protein that antagonizes BMP and Wnt, the two signaling molecules essential for tooth development (4, 9).

The importance of BMP in supernumerary tooth formation was demonstrated by transplantation of incisor explants supplemented with BMP7 in USAG-1−/− mice, which induced the development of supernumerary teeth (16). Hence, the administration of candidate molecules can result in whole tooth formation in suitable conditions. Furthermore, it has been suggested that BMP signaling is essential for morphogenesis of extra teeth (16, 17), while Wnt signaling is important for supernumerary tooth formation (15, 18). However, it is unknown whether BMP or Wnt signaling is required for the determination of tooth number.

Tooth agenesis is the result of arrested tooth development. Several genes responsible for congenital tooth agenesis, such as Msx1, Runx2, Ectodysplasin A (EDA), or Pax9 (4, 6, 7), have been identified primarily using KO mouse models (19–24). We previously reported that tooth development arrested in Runx2−/− mice, a mouse model for congenital tooth agenesis (24), was rescued in Runx2+/−/USAG-1−/− mice, a supernumerary mouse model (25). While a clear link between USAG-1 and rescue of congenital agenesis has been established, it remains unknown whether local inhibition of USAG-1 function is sufficient to rescue tooth development. Clinical applications of targeted molecular drugs based on antibody preparations for a variety of diseases, such as rheumatoid arthritis and cancer, are increasingly common (26, 27). The genetic mechanisms of supernumerary tooth formation suggest that a targeted molecular therapy for tooth regeneration can be a viable therapeutic approach.

This investigation aimed to generate and use a monoclonal anti–USAG-1 antibody, rather than genetic inhibition, for the local arrest and recovery of tooth development. To this end, we also performed experiments to determine whether BMP or Wnt signaling is dominant during tooth development.

RESULTS
Tooth formation recovery using murine models
Phenotypic changes in an Msx1−/−/USAG-1−/− mouse generated by mating mouse models of congenital tooth agenesis and supernumerary teeth were investigated. The development of both the maxilla and the mandible was arrested in the early stages. However, a cleft palate was additionally observed in USAG-1+/+Msx1−/− mice

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(Fig. 1, F and G). Although mouse offspring with a USAG-1^{−/−}/Msx1^{−/−} background should have theoretically been obtained with one-sixteenth incidence, only 3 of 151 littermate mice had the USAG-1^{−/−}/Msx1^{−/−} genotype (Fig. 1A). Histological evaluation revealed that all USAG-1^{−/−}/Msx1^{−/−} mice had normal third maxillary molar teeth (Fig. 1, H and I).

Next, we analyzed EDA1^{−/−}/USAG-1^{−/−} mice. As EDA1 is located on the X chromosome, female EDA1^{−/−}/USAG-1^{−/−} and male EDA1^{+/−}/USAG-1^{−/−} mice are null for USAG-1 and EDA1. These double KO mice had normal teeth, hyperdontia, or fused mandibular molars, whereas 75% of the female USAG-1^{+/−}/EDA1^{−/−} and male USAG-1^{+/−}/EDA1^{−/−} mice had molar hypodontia in the mandible (Fig. 1, J to R′, and fig. S2). Hair loss behind the ear and tail kink, which are the typical phenotypes associated with tabby mice, were present in all USAG-1 and EDA1 double KO mice (Fig. 1V). These results suggest that Usag-1^{−/−} can rescue congenital tooth agenesis during early tooth development and promote morphogenesis of the whole tooth structure arrested in the late stage.

**Usag-1–neutralizing antibody recovers missing teeth and generates a whole tooth**

To investigate whether inhibition of USAG-1 function rescues congenital tooth agenesis, we purified five mouse USAG-1 monoclonal antibodies (#12, #16, #37, #48, and #57) using a bioactive human USAG-1 recombinant protein derived from *Escherichia coli* as an antigen and USAG-1^{+/−} mice. USAG-1 is suggested to inhibit Wnt and BMP signals via direct binding to BMP and the Wnt coreceptor LRP5/6 (28, 29). Therefore, these five antibodies were categorized into three different classes, based on their interference abilities of the binding to both BMP and Wnt (#57), BMP (#12 and #37), or Wnt (#16 and #48) (Fig. 2, A and B). We confirmed that all antibodies could bind the mouse and human USAG-1 recombinant proteins (Fig. 2C), although #16 and #48 showed low affinity (Fig. 2, D and E).

These results enabled the investigation of the function of USAG-1 with respect to BMP and Wnt signaling pathways for the determination of the number of teeth.

Each USAG-1–neutralizing antibody was systemically administered to EDA1 pregnant mice. Low birth and survival rates were observed in mice administered USAG-1–neutralizing antibodies #12, #16, or #48 (Fig. 3A). USAG-1–neutralizing antibodies #16, #37, #48, and #57 rescued molar hypodontia in the mandible of EDA1^{−/−} mice compared with control mice (Fig. 3, B and C, and fig. S3). USAG-1–neutralizing antibody #37 reversed hypodontia at a high rate and in a dose-dependent manner (Fig. 3B). In addition, USAG-1–neutralizing antibodies #12, #16, #37, and #57 led to the production of supernumerary teeth in the maxillary incisor, mandibular incisor, or molar of EDA1 KO/hetero mice (Fig. 3, B and C, and fig. S3).

**Fig. 1. Recovery of tooth formation in double KO mice with congenital tooth agenesis and supernumerary teeth.** (A) Number of mice with indicated genotypes. (B to F) Frontal hematoxylin and eosin–stained sections of the left maxillary incisor and third molar (M3) in USAG-1^{−/−}/Msx1^{−/−} mice immediately after birth. (J) Summary of tooth phenotypes in 8-month-old F2 generation EDA1/USAG-1 double-mutant mice. (K to R) Representative tooth phenotypes in dry skulls of 8-month-old F2 generation EDA1/USAG-1 double-mutant mice. (S to V) Ear hair, tail hair, and tail tip phenotypes. ST, supernumerary teeth; FT, fused teeth; Def., defect of teeth. Photo credit: H. Kiso, Kyoto University.
Unexpectedly, USAG-1–neutralizing antibody #57 induced the formation of supernumerary teeth in the maxillary incisor, mandibular incisor, or molar of wild-type mice at a high rate and a dose-dependent manner (Fig. 3, B and C, and fig. S3). However, fused molars were observed instead of supernumerary teeth in the maxillary molar region (Fig. 3C and fig. S3). Both antibodies neutralized BMP signaling antagonistic function, at least in vitro (Fig. 3, B and C, and fig. S3). These results indicate that BMP signaling is essential for determining the number of teeth in mice. Furthermore, a single systemic administration of a neutralizing antibody can generate a whole tooth.

**USAG-1–neutralizing activity generates a whole tooth by affecting BMP signaling**

To determine the epitope of USAG-1–neutralizing antibodies #37 and #57, we performed epitope mapping using 169 linear peptides, including 20 sequential amino acids (Fig. 4, A and D). USAG-1–neutralizing antibody #37 specifically reacted with six overlapping peptides (D16–D21) spanning the region Q129 EWRCVNDKTRTQR 148, suggesting that the epitope is localized within the central 10-residue segment containing the sequence VNDKTRTQR (Fig. 4B). Although the three-dimensional (3D) structure of USAG-1 is unknown, its high sequence homology with sclerostin (SOST) that belongs to the same BMP antagonist DAN family enabled us to build a homology model of mouse USAG-1 using the nuclear magnetic resonance structure of SOST (Fig. 4E) (28). It was revealed that the epitope recognized by antibody #37 lies on the surface-exposed edge strand of the central β sheet of USAG-1, consistent with the ability of #37 to recognize native USAG-1. This region is located far from the NXI motif, which is the binding site for LRP5/6 (Fig. 4E) (29), suggesting that this antibody does not block USAG-1 interaction with the Wnt coreceptor LRP5/6. Antibody #37 did not affect the Wnt1-antagonizing activity of USAG-1 (Fig. 2B). In contrast to #37, antibody #57 did not show reactivity toward any of the USAG-1–derived overlapping peptides (Fig. 4C), indicating that it recognizes a 3D epitope present on the USAG-1 surface.

It has been established that the endogenous Wnt pathway inhibitor SOST exerts its inhibitory effect by binding to the “E1” domain of Wnt coreceptor LRP6 (30). As described in the previous section, the conservation of the LRP6-binding motif NXI in USAG-1 strongly suggests that it binds to the same domain of LRP6 as well. We evaluated the USAG-1 binding to the human LRP6 ectodomain fragments of varying lengths. As shown in Fig. 5A, stoichiometric binding of USAG-1 was observed with the E1–E2 domain fragment of LRP6, confirming the prediction that the binding site was located in the E1 domain. In contrast, no binding was observed with E1–E4 or E3–E4 fragments. The lack of binding with E1-containing E1–E4 fragment can be explained by the fact that the NXI-binding surface of E1 is occluded in the context of the whole ectodomain of LRP6, which shows a highly curved “C-shape” in the electron microscopic images (31). We then investigated whether the USAG-1–neutralizing antibodies...
can interfere with the LRP6–USAG-1 interaction. As shown in Fig. 5B, near-complete inhibition was observed with antibody #16, while #48 exhibited partial inhibition. This finding was consistent with their ability to inhibit the Wnt-modulating activity of USAG-1 (Fig. 2B). Three other antibodies (#12, #37, and #57) did not affect the binding of USAG-1 to LRP6 E1-E2, corroborating their inability to counteract the Wnt-modulating capability of USAG-1 (Fig. 2B). On the basis of these results, we conclude that neutralizing the antagonizing effect of USAG-1 on BMP rather than Wnt signals is more effective in achieving substantial phenotypic changes in mice, i.e., recovering missing teeth or making a whole tooth.

To investigate the functional differences between antibodies #37 and #57 with respect to BMP signaling, we analyzed the cross-reactivity of these antibodies with members of the DAN subfamily (Fig. 5C). We detected a faint signal for SOST in transfected human embryonic kidney (HEK) 293 cells using immunohistochemistry with antibody #57 but not with #37 (Fig. 5D and fig. S4). This weak cross-reactivity with SOST is likely due to the similarities in the 3D structures of SOST and USAG-1 (28). Furthermore, systemic administration of an antibody mixture containing antibodies #12, #16, #37, #48, and #57 increased the number of supernumerary teeth and the size of fused teeth in the mandible of USAG-1−/− mice (Fig. 5, E and F). These results suggest that antibody #57 may inhibit the genetic redundant responsibility for supernumerary tooth formation by affecting SOST, a BMP antagonist.

Last, to confirm that USAG-1–neutralizing activity affects BMP signaling to generate a whole tooth in a nonrodent model, we systemically administered antibody #37 to postnatal ferrets that had both deciduous and permanent teeth. We observed supernumerary tooth formation in maxillary incisor like the third dentition, although a five times higher concentration, three administrations of antibody #37, and immunosuppression were required (Fig. 6, A to D). The supernumerary tooth was likely to have a similar shape to the usual permanent incisor, located to the lingual side of permanent teeth, whereas a shorter root seemed to be growing (Fig. 6, E to G). Therefore, this supernumerary incisor might be categorized as the third dentition (32). Furthermore, phosphorylated Smad-positive cells were observed within pulp of supernumerary tooth (Fig. 6, H and I).

**DISCUSSION**

Single systemic administration of USAG-1–neutralizing antibodies that interfere mainly with BMP signaling (#37 and #57) rescued tooth agenesis in EDA1-deficient mice and led to the efficient formation of a whole tooth in a dose-dependent manner in wild-type mice. To the best of our knowledge, the identification of targeted antibodies that can promote tooth regeneration has not been reported earlier. The antibodies generated in the present study neutralized the antagonistic action of USAG-1 on BMP signaling, and reduced LRP5/6 dosage rescued the USAG-1–null phenotype, including supernumerary tooth formation (15). However, Wnt signaling involvement
cannot be excluded based on these findings because several mice were not born or did not survive. Thus, it is necessary to perform further experiments such as epitope binning involving higher numbers of USAG-1–neutralizing antibodies and detailed analyses of recombinant USAG-1 protein epitopes.

We observed links between several causative genes, including Msx1 and USAG-1, with the recovery of congenital tooth agenesis but not cleft palate in Msx1-deficient mice (Fig. 1). A single systemic administration of USAG-1–neutralizing antibodies targeting only the BMP signaling pathway rescued tooth agenesis in EDA1 deficient mice but did not affect other phenotypes associated with this lineage. Conversely, USAG-1 abrogation only rescued cleft palate development in Pax9-deficient mice, which modulated Wnt but not BMP signaling (33). Small-molecule Wnt agonists also corrected the cleft palate in Pax9-deficient mice (34). This indicates that the USAG-1–neutralizing antibody did not cure all tooth agenesis cases but that the mutations in causative genes for congenital tooth agenesis may constitute biomarkers for patient selection. Nevertheless,
extensive studies are warranted for future clinical applications. EDA controls BMP activity (35), whereas EDAR acts on Wnt target genes (36, 37). Congenital tooth agenesis may be rescued by administering a USAG-1–neutralizing antibody for BMP and not Wnt signaling. Furthermore, a single systemic administration of an EDA agonistic antibody in an EDA-deficient dog after birth rescued congenital tooth agenesis (38). Application of USAG-1–targeted neutralizing antibodies for tooth regeneration must be focused on congenital tooth agenesis with mutations of specific causative genes.

Further, we succeeded in obtaining USAG-1–neutralizing antibodies with the potential to generate a whole new tooth, even in wild-type mice. The phenotypic changes in these mice were similar to those in USAG-1-KO mice, suggesting that this antibody may rescue the rudimental tooth primordia in USAG-1–deficient mice. Human teeth, except for the permanent molars, are diphyodont (32). The first (deciduous) and second (permanent) generation of teeth are sometimes accompanied by a “third dentition” of rudimental teeth that can occur in addition to the permanent teeth (32). On the basis of an analysis of 78 patients with supernumerary teeth, we previously reported that the third dentition is a cause of supernumerary teeth in humans (32). Stimulation of the third dentition by targeted molecular therapy may be a viable approach for whole tooth regeneration. In the current study, we showed that systemic application of a USAG-1–neutralizing antibody could regenerate a whole tooth like the third dentition in ferrets, which are diphyodont animals with the similar dental pattern to human. However, the clinical application of this modality will require further investigation in nonrodent models, such as suncuses, dogs, or pigs, in addition to ferrets.

The development of a treatment method using cell-based tissue engineering is common in mainstream regenerative medicine. Although extensive research has been done in the field of tooth regeneration using tissue engineering techniques (39, 40), none of the available therapies are clinically applicable due to cost and safety issues. Although it is considered necessary to generate a new original tooth germ, in our investigation, we observed the presence of rudimental tooth primordia. Therefore, we did not have to create new tooth primordia even in the wild-type animals. The growth of tooth primordia is inhibited by USAG-1. Besides, congenital tooth agenesis associated with various genetic abnormalities is caused by arrested tooth development. For this reason, the conventional tissue engineering approach is not suitable for tooth regeneration. Our study outcomes show that cell-free molecular therapy targeting USAG-1 is effective in the treatment of a wide range of congenital tooth agenesis and the induction of third dentition.
MATERIALS AND METHODS

Study design
This study’s main objectives included the generation and use of a monoclonal anti–USAG-1 antibody to locally arrest and recover tooth development in mice. We also performed experiments to determine whether BMP or Wnt signaling modulated tooth development. This study was approved by the Animal Research Committee of Kyoto University (reference number: Med Kyo 11518), KAC Co. Ltd. (reference number: 19-1103), and the Recombinant DNA Experiment Safety Committee of Kyoto University (reference number: 180211). Experiments were performed in accordance with approved guidelines. All experiments were repeated at least three times. Sample sizes were chosen empirically to ensure adequate statistical power. All valid measurements were included in our analysis. No outliers were excluded. Primary data are provided in the figures or the Supplementary Materials.

Animals
USAG-1−/− mice with a 106-bp deletion in exon 1 were produced using the CRISPR-Cas system with a C57BL/6J genetic background (fig. S1) (Macrogen Co. Ltd., Seoul, South Korea). Dental anomalies similar to those described in previous reports (10), including incisal supernumerary teeth, fused maxillary molars, and supernumerary mandibular molars, were observed in USAG-1−/− mice. EDA1-deficient mice (Tabby6: C57BL/6J A^w1-Eda^t/a/J) were obtained from the Jackson Laboratory (JAX stock #000338). Msx1-deficient mice with a 129S4/SvJae genetic background were provided by the Mutant Mouse Resource and Research Centers (MMRRC stock #000068-UCD). We interbred heterozygous USAG-1 and Msx1 mice and analyzed the F2 generation. To eliminate the influence of the mouse background, only F2 progeny USAG-1−/−/Msx1−/− mice were analyzed. Polymerase chain reaction was performed using KOD FX NEO polymerase (KFX-201; TOYOBO, Osaka, Japan) and specific primers. Embryos were obtained by timed mating; day E0 started from midnight, before finding a vaginal plug. Outbred pregnant ferrets were purchased from Marshall BioResources Japan Co. Ltd. A subgroup of the offspring was maintained in immunosuppressive condition, as previously reported (41).

Plasmid and recombinant proteins
Preparation of PA-tagged mouse USAG-1 recombinant protein from mammalian cells was performed as previously reported (42). Other tagged USAG-1 recombinant proteins, derived from E. coli or baculoviral expression systems (R&D systems Inc., MN, USA;
For determination of alkaline phosphatase (ALP) activity, C2C12 Alkaline phosphatase assay tion was determined. Antibodies were stored at −80°C until use. tion was determined. Antibodies were stored at −80°C until use. fer exchange with phosphate-buffered saline (PBS), and concentra- filter (Amicon Ultra-15; Millipore, Burlington, MA, USA) for buf- tant antibody was eluted using the elution buffer from the MAbTrap Kit was loaded onto a Protein G column (GE Healthcare, Chicago, IL, clonal antibodies were identified. The culture supernatant (10 ml) were screened by ELISA, and those secreting anti–USAG-1 mono- Japan) containing HAT as a supplement. The resultant hybridomas 1 week on GIT medium (Wako Pure Chemical Corporation, Osaka, (1.0 × 104  cells per well in a 48-well plate) were transiently transfected porter assay system (Promega, Madison, WI, USA). Briefly, HEK293 cells we used the TOP reporter system based on the dual-luciferase re- Luciferase reporter assay To assess the neutralizing effects of the anti–USAG-1 antibodies on Wnt/β-catenin signaling modulated by recombinant mouse USAG-1, we used the TOP reporter system based on the dual-luciferase re- reporter assay system (Promega, Madison, WI, USA). Briefly, HEK293 cells (1.0 × 10^4 cells per well in a 48-well plate) were transiently transfected with constitutively active herpes simplex virus thymidine kinase promoter-driven Renilla luciferase (20 ng per well) as an internal control, a β-catenin–responsive firefly luciferase reporter plasmid TopFlash (50 ng per well) (Millipore), and Wnt1 expression plasmid (1 ng per well) using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). After 4-hour incubation, the plasmids and the transfection reagent in DMEM supplemented with 10% FBS were replaced with a fresh medium containing recombinant mouse USAG-1 protein (1 µg/ml). Cells were harvested after 20 to 24 hours, and both firefly and Renilla luciferase activity were measured in duplicate or triplicate according to the manufacturer’s instructions. The firefly luciferase activity was normalized against the Renilla luciferase activity.

### Epitope mapping

Epitope mapping was performed by Kinexus Co Ltd. (Vancouver, Canada). Briefly, SPOT synthesis of two copies of a peptide array (15-mer peptide scan of a protein with 183 amino acids; human Sostdc1 without signal peptide) was performed on a cellulose membrane. Two of the synthesized copies of the peptide array were incubated with primary mouse USAG-1 antibodies (0.3 g/ml), and the bound antibody was detected by incubating the arrays with the detection reagent (1:25,000 dilution; HRPalpaca anti-mouse antibody) and subsequent treatment with electrochemiluminescence reagent.

### Immunoprecipitation

Reactivity of each monoclonal antibody (mAb) with native USAG-1 in solution was evaluated by immunoprecipitation. Briefly, 5 µg of purified anti–USAG-1 mAbs was incubated with 15 µl of Protein A-Sepharose (GE Healthcare) for 2.5 hours at 15° to 25°C, followed by a brief wash with PBS. The beads were incubated with the culture supernatants of the Expi293F cells transiently transfected with either mouse or human USAG-1 containing N-terminal PA tag (42). After extensive washing with PBS, the bound proteins were eluted from the beads by adding SDS sample buffer and then analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) using 5 to 20% gradient gel under nonreducing conditions.

### Bio-layer interferometry

Binding kinetics of anti–USAG-1 antibodies were analyzed using bio-layer interferometry with Octet RED system (ForteBio, Fremont, CA, USA). Binding assays were performed in 96-well microtiter plates at 25°C with orbital sensor agitation at 1000 rpm. Amine re-active (AR2G) sensors were immobilized with each antibody dissol- ed at 10 to 20 µg/ml in 10 mM sodium acetate buffer (pH 6.0) followed by quenching with 1 M ethanamine (pH 8.5). Purified mouse USAG-1 was serially diluted in a running buffer [20 mM Hepes and 150 mM NaCl (pH 7.2) containing 0.005% Tween 20] and added to different wells (final volume: 200 µl). The binding was monitored by dipping the sensors into the wells for 120 s, followed by dissociation in the running buffer for 120 s. After each binding experiment cycle, antibody-immobilized biosensors were regeneration by dipping in a regeneration buffer [10 mM glycine–HCl (pH 3.0)]. The $K_d$ values were determined using Octet Data Analysis Software 7.1 (ForteBio) using a 1:1 global fitting model.

### LRP6-binding assay

Binding between USAG-1 and LRP6 ectodomain was evaluated as follows. The soluble human LRP6 ectodomain fragments containing different regions (E1-E4, residues 1 to 1244; E1-E2, residues 1 to 629; E3-E4, residues 630 to 1244) were C-terminally His-tagged and transiently expressed in Expi293F cells as described previously (44). After immobilizing onto Ni–NTA beads, they were further incubated with the culture supernatants of the Expi293F cells stably expressing mouse USAG-1 established previously (42). The bound USAG-1 was eluted together with the LRP6 fragments by SDS and analyzed by nonreducing SDS-PAGE. For the assessment of the ability of anti–USAG-1 antibodies to compete with LRP6 binding, Protein A beads were incubated with each antibody (step 1), followed by incubation with USAG-1 (step 2), and lastly with LRP6 E1-E2 fragment (step 3) to allow the formation of a ternary complex. The bound proteins were analyzed by nonreducing SDS-PAGE. The diminished intensity of the signal corresponding to the LRP6 E1-E2
fragment indicated the overlap of the binding sites for the antibody and LRP6.

Analysis of teeth phenotypes
Pregnant EDA1 mice at E13 of gestation (4 to 6 weeks) were intra-peritoneally injected with anti-USAG-1 antibodies (16 μg/g). Offspring were analyzed at 5 weeks of age. After removing the skin, dissected maxillae and mandibles from the heads of the offspring were soaked in 0.02% proteinase K prepared in PBS at 37°C for 4 days and cleaned with 5% H2O2 at 15° to 25°C for 5 min. Last, they were rinsed in H2O and air-dried. Neutones were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (7 mm) were cut and stained with hematoxylin and eosin. Offspring of ferrets at 1 and 3 weeks after birth or 1, 3, and 5 weeks were intraperitoneally injected with anti-USAG-1 antibodies (16 or 80 μg/g). They were analyzed by taking photographs and micro-computed tomography (micro-CT).

Micro-CT analysis
We performed 3D micro-CT scans (inspeXio SMX-100CT; Shimadzu, Kyoto, Japan) on the maxillary incisors of ferrets, 13 weeks after birth. We converted CB files [512 × 512 pixels, 8 bits; voxel size, x:y:z = 1:1:1 (~0.06 mm per side)] to TIFF files, and 3D images were reconstructed and analyzed using computer imaging software (VGSTUDIO MAX; Volume Graphics GmbH, Heidelberg, Germany).

Immunocytochemistry
Immunocytochemistry was performed using standard techniques. Briefly, HEK293 cells were seeded on poly-l-lysine–coated coverslips (Matsunami Glass Ind. Ltd., Osaka, Japan). FLAG-tagged DAN family protein expression plasmids were transfected (1 μg per well) into the cells using Lipofectamine 3000. After transfection (24 hours), the cells were fixed with 4% paraformaldehyde/PBS (Sigma-Aldrich) for 30 min. Next, the cells were washed with PBS three times and incubated in blocking buffer (10% bovine serum albumin/PBS) for 1 hour, followed by incubation in the mouse monoclonal anti-USAG-1 antibody or anti-FLAG antibody (4 ng/ml) (Sigma-Aldrich) in the blocking buffer overnight at 4°C. To visualize the immunoreactivity, the cells were incubated with Cy3-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA)/PBS (1:400) after being washed three times with PBS. Nuclear staining was performed using 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific).

Immunohistochemistry
Paraffin-embedded sections of ferret were immunostained with primary rabbit polyclonal antibodies against phosphorylated Smad 1/5/8 (1:50; Merck KGaA, Darmstadt, Germany) and secondary biotinylated anti-rabbit/mouse antibodies (Nichirei Bioscience, Tokyo, Japan), as previously described (11, 32). Sections were then counter-stained with hematoxylin, dehydrated in a graded series of ethanol and xylene, and covered with coverslips.

Statistical analysis
Data are shown as means ± SEs. For comparing multiple conditions, a one-way analysis of variance (ANOVA) was performed, followed by two-tailed Dunnett’s multiple comparisons test. Statistical significance of differences was assessed as follows: *P < 0.05, **P < 0.01. Statistical analyses were performed using the SAS statistical software, version 9.4 (SAS Institute, Cary, NC).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/7/eabf1798/DC1

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