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Truncated amelogenin and LRAP transgenes improve Amelx null mouse enamel

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Abstract

Amelogenin is the most abundant enamel protein involved in enamel mineralization. Our goal was to determine whether all three regions of amelogenin (N-terminus, C-terminus, central core) are required for enamel formation. Amelogenin RNA is alternatively spliced, resulting in at least 16 different amelogenin isoforms in mice, with M180 and LRAP expressed most abundantly. Soon after secretion by ameloblasts, M180 is cleaved by MMP20 resulting in C-terminal truncated (CTRNC) amelogenin. We aimed to determine whether the 2 transgenes (Tg), LRAP and CTRNC together, can improve LRAPTg/*Amelx*−/− and CTRNCTg/*Amelx*−/− enamel thickness and prism organization, which were not rescued in *Amelx*−/− enamel. We generated CTRNCTg/LRAPTg/*Amelx*−/− mice and analyzed developing and mature incisor and molar enamel histologically, by microCT, SEM and microhardness testing. CTRNCTg and LRAPTg overexpression together significantly improved the enamel phenotype of LRAPTg/*Amelx*−/− and CTRNCTg/*Amelx*−/− mouse enamel, however enamel microhardness was recovered only when M180Tg was expressed, alone or with LRAPTg. We determined that both LRAP and CTRNC, which together express all three regions of the amelogenin protein (N-terminus, C-terminus and hydrophobic core) contribute to the final enamel thickness and prism organization in mice.

Keywords

enamel; ameloblasts; amelogenin; Mmp20; secretory stage

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Author contributions

MKP designed project. YX, AR and MKP performed experiments, collected and analyzed data. MKP wrote the paper. All authors read, contributed to discussion and approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

1. Introduction

Amelogenin proteins make up 90% of the enamel matrix secreted by ameloblasts during the secretory stage and include at least 16 alternative splice products [1]. Amelogenin proteins are cleaved during the secretory stage by matrix metalloproteinase-20 (MMP20) [2], resulting in the accumulation of different amelogenin cleavage products in the developing enamel matrix of between ~5–30 kDa [3–5]. Ablation of the murine amelogenin gene leads to non-prismatic, thin enamel, around 10% the thickness of WT, similar to human AI with an amelogenin mutation [6]. The two most abundant amelogenin splice variants are M180, (mouse protein, 180 amino acids) and LRAP (59 amino acids). M180 and LRAP amelogenin may have different functions and their expression is regulated differently [7], however both splice variants have identical, highly conserved N- and C-termini [8] (Fig. 1). M180 is the largest and most abundant amelogenin splice variant, and is also thought to be the most crucial, rescuing approximately 50% the thickness of knockout enamel [9], and improving enamel prism organization [10, 11]. The LRAP isoform, which is highly expressed in secretory stage enamel, albeit at a lower level than M180, is believed to contribute to enamel structure and thickness in cooperation with full-length M180 [9] but not if expressed alone [12] in *Amelx*−/− mice. We aimed to determine whether expression of all three regions of amelogenin, when expressed as CTRNCTg and LRAPTg, are involved in enamel formation.

Although overexpression of an M180 transgene with a C-terminus deletion (CTRNCTg) did not rescue *Amelx*−/− mouse enamel [13], cleaved M180 may interact with LRAP to guide mineralization *in vivo* since M180 is cleaved at the C-terminus early in the secretory stage by Mmp20. We therefore hypothesize that transgenes for cleaved M180 (CTRNCTg) and LRAP overexpressed together in *Amelx*−/− mice can improve rescue over either transgene alone, due to a cumulative effect on enamel phenotype from the presence of all three domains of amelogenin.

2. Results

2.1 Endogenous and transgenic expression of amelogenin isoforms and cleavage products

We compared relative protein expression of M180Tg (~20KD) CTRNCTg (~19KD) and LRAPTg (~7KD) transgenes compared to WT amelogenin using Western blot (Fig. 1). Band intensities were normalized relative to WT in multiple immunoblots (not shown). LRAPTg (~7 kD) was expressed at a higher level in LRAPTg/*Amelx*−/−, M180Tg/LRAPTg/*Amelx*−/− and CTRNCTg/LRAPTg/*Amelx*−/− mice (Fig. 2, lanes 2, 3, 4) than endogenous LRAP in WT mice, as the LRAP band was not visible in this immunoblot (Fig. 2, lane 1). Endogenous M180 and truncated M180 (CTRNC) appeared as a large smear around 17 – 20 kD in WT, which also includes additional amelogenin splice variants and cleavage products (Fig. 2, lane 1). The protein size difference between M180Tg and CTRNCTg, which lacks the last 13 amino acids in M180Tg's C-terminus, is apparent, when comparing CTRNCTg/LRAPTg/*Amelx*−/− to M180Tg/LRAPTg/*Amelx*−/− and M180Tg/*Amelx*−/− of the Western blot (Fig. 2, lanes 3, 4, 5). The CTRNCTg and M180Tg band intensities normalized to WT amelogenin expression of the smear around 17–20 kD indicated similar but slightly lower

expression levels of the transgenes (Fig. 2, lanes 3, 4, 5). This is due to the presence of additional endogenous amelogenin cleavage products in the WT developing enamel.

2.2 Truncated M180 (CTRNC) and LRAP transgenes together significantly improve *Amelx−/−* enamel thickness

We analyzed secretory stage enamel histologically in developing molars from 5 day-old mice. Although the enamel layer was not as thick as WT (Fig. 3A) CTRNCTg/LRAPTg/*Amelx*−/− (Fig. 3B) molars have more enamel than both *Amelx*−/− (Fig. 3D) and LRAPTg/*Amelx*−/− (Fig. 3C) molars, which had little to no enamel.

MicroCT was used to analyze enamel mineral in adult mandibles at a location that was sectioned through both the mature first molar and the developing incisor near the beginning of maturation, when the enamel is not fully mineralized in WT mice (Fig. 4). *Amelx*−/− and LRAPTg/*Amelx*−/− molar and incisor enamel are mostly not visible, too thin to be detectable by the microCT's resolution (Fig. 4B & 4C). Thin layers of enamel were visible in CTRNCTg/*Amelx*−/− molars (Fig. 4D), M180Tg/KO incisors (Fig. 4E, M180Tg/LRAPTg/*Amelx*−/− incisors (Fig. 4F) and CTRNCTg/LRAPTg/*Amelx*−/− incisors (Fig. 4G), while thicker layers of mineralized enamel were visible in M180Tg/*Amelx*−/− molars (Fig 4E), M180Tg/LRAPTg/*Amelx*−/− (Fig. 4F) molars and CTRNCTg/LRAPTg/*Amelx*−/− molars (Fig. 4G). Notably, the enamel in CTRNCTg/LRAPTg/*Amelx*−/− incisors (Fig. 4G) appeared less mineralized than the visible enamel in the other incisors at the same stage.

We also examined enamel mineral in incisors from *Amelx*−/− and transgenic mice by microCT at a later stage of development in the maturation stage, just before eruption, in the last section with alveolar one surrounding the incisor (Fig. 5). In contrast to *Amelx*−/− (Fig. 5B) and LRAPTg/*Amelx*−/− (Fig. 5C) incisors in which enamel was not visible, thin layers of mineralized enamel were visible in incisors from mice with the M180Tg with (Fig. 5F) and without (Fig. 5E) LRAPTg, and both CTRNCTg and LRAPTg (Fig. 5G). WT incisor enamel (Fig. 5A) was noticeably thicker than that of any other genotype, presumably due to the lower transgene expression levels in incisors compared to molars [10], leading to less overall rescue in incisors than occurred in molars (Fig. 4).We measured enamel thickness in adult first molars that were imaged by SEM. Molar enamel thickness of CTRNCTg/LRAPTg/*Amelx*−/− mice improved to 67% the thickness of WT from 19% for *Amelx*−/−), 32% for CTRNCTg/*Amelx*−/− and 31% for LRAPTg/*Amelx*−/− (Fig. 6). M180Tg/LRAPTg/*Amelx*−/− molar was 83% the thickness of WT and significantly thicker than CTRNCTg/LRAPTg/*Amelx*−/− molar enamel (Fig. 6). These data are consistent with histological and microCT analyses which indicate significantly more rescue of *Amelx*−/− enamel thickness with overexpression both CTRNCTg and LRAPTg versus either transgene alone.

2.3 CTRNC and LRAP transgenes together improved enamel structure

Because we wanted to observe the combined effect of truncated M180 and LRAP transgenes on enamel formation, we used SEM to analyze structure of acid-etched incisor enamel (Fig. 7). WT enamel was highly organized with decussating prisms (Fig. 7A). The thin, non-prismatic *Amelx*−/− enamel in adult mouse incisors was not significantly affected by the

addition of truncated M180 transgene (CTRNCTg) (Fig. 7A, D) or LRAPTg (Fig. 7A, C), when expressed alone. However when these transgenes were expressed together (CTRNCTg/LRAPTg/*Amelx*−/−) the enamel had more prism organization (Fig. 7F). This suggests an important role for all three domains (N-terminus, central core, C-terminus) of amelogenin, which were represented by the combination of CTRNCTg and LRAPTg. M180Tg/LRAPTg/*Amelx*−/− enamel was highly organized with decussating enamel prisms and similar structure as WT (Fig. 7E).

2.4 CTRNC and LRAP transgenes did not improve enamel hardness

Microhardness measurements of molar enamel showed that CTRNCTg/LRAPTg/*Amelx*−/− enamel was significantly softer than WT enamel, and enamel of M180/LRAP/*Amelx*−/−mice was not different from WT (Fig. 8). Notably, M180Tg/LRAPTg/*Amelx*−/−incisor enamel structure was more organized (Fig. 7E) and thicker (Fig. 6) than CTRNCTg/LRAPTg/*Amelx*−/−, indicating a crucial role of the C-terminus of M180 in thickness, structure and resulting mechanical properties.

3. Discussion

Although LRAP transgene overexpression upregulated wild-type mouse ameloblast differentiation *in vivo* [14], little is known about the role of the LRAP amelogenin isoform in enamel mineralization during the secretory stage after it is secreted. Like M180, LRAP is cleaved at its C-terminus by MMP20 [15], and while the role of this cleavage is unknown, it is possible that LRAP cleavage products also play a crucial role in enamel formation, albeit at a lower concentration than M180 cleavage products.

Additionally, while many investigators have shown the importance of M180 and its C-terminus both *in vivo* [10, 11, 13, 16] and *in vitro* [17–22], little is understood about the role of cleaved M180 without it's C-terminus (CTRNC) or of the C-terminus itself, although truncated M180 has been suggested to stabilize amorphous calcium phosphate *in vitro* [23, 24]. Interestingly, the C-terminal amino acids of amelogenin were truncated in two reported amelogenesis imperfecta-causing mutations [25, 26].

We showed that C-terminal cleaved M180 transgene (CTRNCTg) and LRAPTg, when overexpressed together in *Amelx*−/− mice, have a pronounced effect on both incisor and molar enamel, improving both thickness and prism organization but not mechanical properties. In previous studies, when express separately, CTRNCTg [13] and LRAPTg [12, 27] had very little effect on the *Amelx*−/− enamel phenotype, although mechanical properties of LRAPTg/*Amelx*−/− enamel were significantly improved over *Amelx*−/− [27]. However, when M180Tg was overexpressed in addition to LRAPTg, both enamel thickness and enamel prism organization were considerably improved [9] over both LRAPTg/*Amelx*−/− [12, 27] and M180Tg/*Amelx*−/− [10] enamel. Although a recent *in vivo* study suggests that M180 is sufficient for thickness and mechanical properties [11], the enamel prismatic architecture was not thoroughly examined at high magnification. Taken together, our data along with that from the literature discussed above provide crucial mechanistic insights regarding the role of the most abundant amelogenins expressed during the secretory stage: M180, cleaved M180 (referred to here as CTRNC) and LRAP.

In vivo, CTRNC may interact with LRAP to promote crystallite elongation during the secretory stage, as suggested by the improved enamel thickness of CTRNCTg/LRAPTg/*Amelx*−/− mice (Fig. 5). Furthermore, as evidenced by the noticeable improvement of enamel structure, CTRNC and LRAP seem to also promote proper crystallite orientation, permitting partial decussating prisms to form (Fig. 7F), however, surprisingly, the enamel hardness was not improved (Fig. 8). When the expression levels of the M180 and LRAP transgenes are similar, the only difference between the LRAPTg/*Amelx*−/− versus the M180Tg/*Amelx*−/− mice is the presence of the hydrophobic core domain in the M180Tg/*Amelx*−/− mice, suggesting an important role for the core domain in crystal elongation and enamel thickness.

Although the improvement in enamel structure was remarkable compared to *Amelx*−/− (Fig. 7B) and either LRAPTg or CTRNCTg overexpressed separately (Fig. 7C&D), the prism organization of enamel in mice with both M180Tg and LRAPTg was even more strikingly organized (Fig. 7E), appearing to have near normal decussating prisms. Furthermore, the presence of M180Tg alone or with LRAPTg resulted in significantly harder enamel, confirming a crucial role of the C-terminus of M180 in enamel structure, resulting mechanical properties and thickness. As mentioned the importance of the C-terminus of M180 has been reported both in vitro [17–22] and in vivo [13, 16].

In conclusion, we demonstrated *in vivo* that CTRNC and LRAP transgenes when overexpressed together can considerably improve the enamel phenotype of *Amelx*−/− mice, although not as drastically as the combination of M180 and LRAP, suggesting important roles for M180, cleaved M180 and LRAP in enamel crystallite formation, elongation and orientation during the secretory stage of enamel formation. Selective ablation of the LRAP splice variant is critically important to developing better understanding of LRAP's role in enamel formation.

4. Methods

4.1. Generation of CTRNC and LRAP double transgenic mice

To generate CTRNCTg/LRAPTg/*Amelx*−/− mice, mice overexpressing both CTRNC [13] and LRAP [12] transgenes from the bovine amelogenin promoter [28] were mated with male *Amelx*−/0 mice [6]. After two generations of mating, both male and female mice were genotyped using PCR primers to detect transgenes [12, 13, 28] as well as amelogenin WT and *Amelx*−/− [6] DNA. To analyze expression of endogenous and transgenic amelogenin in CTRNCTg/LRAPTg/*Amelx*−/− male and female mice and controls (WT, *Amelx*−/−, CTRNCTg/*Amelx*−/−, M180Tg/*Amelx*−/−, LRAPTg/*Amelx*−/−, M180Tg/LRAPTg/*Amelx*−/−), first molars were harvested from 5 day-old mouse pups and protein was extracted. Equal amounts of protein were loaded in each lane and run on a 4–20% SDS-PAGE gel (BioRad). Membranes were immunoblotted with an antibody against full-length *Amelx* (FL-191, Santa Cruz Biotechnology, Santa Cruz, CA), with a goat anti-rabbit secondary antibody (Santa Cruz Biotechnology).

4.2. Histology of developing enamel

5 day-old male and female CTRNCTg/LRAPTg/*Amelx*−/− and control mouse heads were dissected, formalin-fixed, decalcified, paraffin-embedded and sectioned. Sections through developing first molars and incisors with ameloblasts in the secretory stage were carefully chosen, and then deparaffinized in xylene and dehydrated in graded alcohols before H&E staining.

4.3. μCT of adult enamel

Six 6 week-old male and female adult hemi-mandibles were dissected and fixed. μCT was used to compare mineral densities of incisor and molar enamel in specific regions in CTRNCTg/LRAPTg/*Amelx*−/− mice and controls. Adult mouse incisor and molar enamel were assessed for mineralization levels [29]. Hemimandibles with soft tissues removed were scanned in a μCT-40 (Scanco, Brüttisellen, Switzerland) at 70 kV, 114 mA, and 6 μm resolution. Images were processed with μCT-40 evaluation software and ImageJ [30] was used to orient the incisors and molars to clearly observe enamel mineralization in two locations: 1) Within the distal root of the 1st molar and developing incisor in the early maturation stage, and 2) within the maturation stage incisor, the last section before eruption, with the incisor completely enclosed by bone.

4.4. Structure and enamel thickness of adult enamel

Hemi-mandibles were embedded in plastic and polished longitudinally for scanning electron microscopy (SEM) analysis for enamel prism organization and enamel thickness [29]. Mandibles from 6 week-old male and female mice and controls were dissected, and erupted portions of mandibular incisors and molars were washed and dehydrated with graded alcohol and acetone, and embedded in Embed812 resin (Electron Microscopy Sciences, Hatfield, PA), ground in a sagittal plane, and polished to 0.25 μm with diamond suspensions (Electron Microscopy Sciences), etched with 0.1M phosphoric acid for 15 s, gold coated and imaged with a Zeiss Evo LS 10 SEM. Enamel thicknesses were measured from images taken at 1000× in first molars, and enamel structure was visualized in incisors at 2000×. Both thickness and structure were analyzed at the same location of molars and incisors, respectively, in at least 6 samples per genotype.

4.5. Microhardness of adult enamel

Erupted portions of dissected, 6 week-old male and female mandibular incisors and molars from CTRNCTg/LRAPTg/*Amelx*−/− and control mice were washed and dehydrated with graded alcohol and acetone, and embedded sagittally in Embed812 resin (Electron Microscopy Sciences). Samples were then ground and polished to 0.25 μm with diamond suspensions (Electron Microscopy Sciences). The polished samples were tested for enamel microhardness on an M400 HI testing machine (Leco, St. Joseph, MI). Testing was performed with load of 10 g for 5 sec with a Vickers tip, 20 indentations per sample on at least 4 teeth per group and averaged.

4.6. Statistical methods

We used ANOVA with the Tukey *post hoc* test to detect differences ($p < 0.05$) between groups of teeth analyzed for enamel thickness as measured by SEM, and Vickers microhardness (GraphPad Software, San Diego, CA, USA).

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Abbreviations

M180	mouse amelogenin, 180 amino acids
LRAP	leucine rich amelogenin peptide
CTRNC	C-terminal truncated amelogenin
Tg	transgene
Amelx	amelogenin
Mmp20	matrix metalloproteinase-20
KO	knockout mouse
WT	wild-type mouse
μCT	microcomputed tomography
SEM	scanning electron microscopy
AI	amelogenesis imperfecta

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Highlights

- CTRNCTg and LRAPTg overexpression significantly improved the enamel of *Amelx−/−*.
- Enamel microhardness was recovered when M180Tg was expressed alone or with LRAPTg.
- All three regions of amelogenin contribute to final enamel thickness and structure.

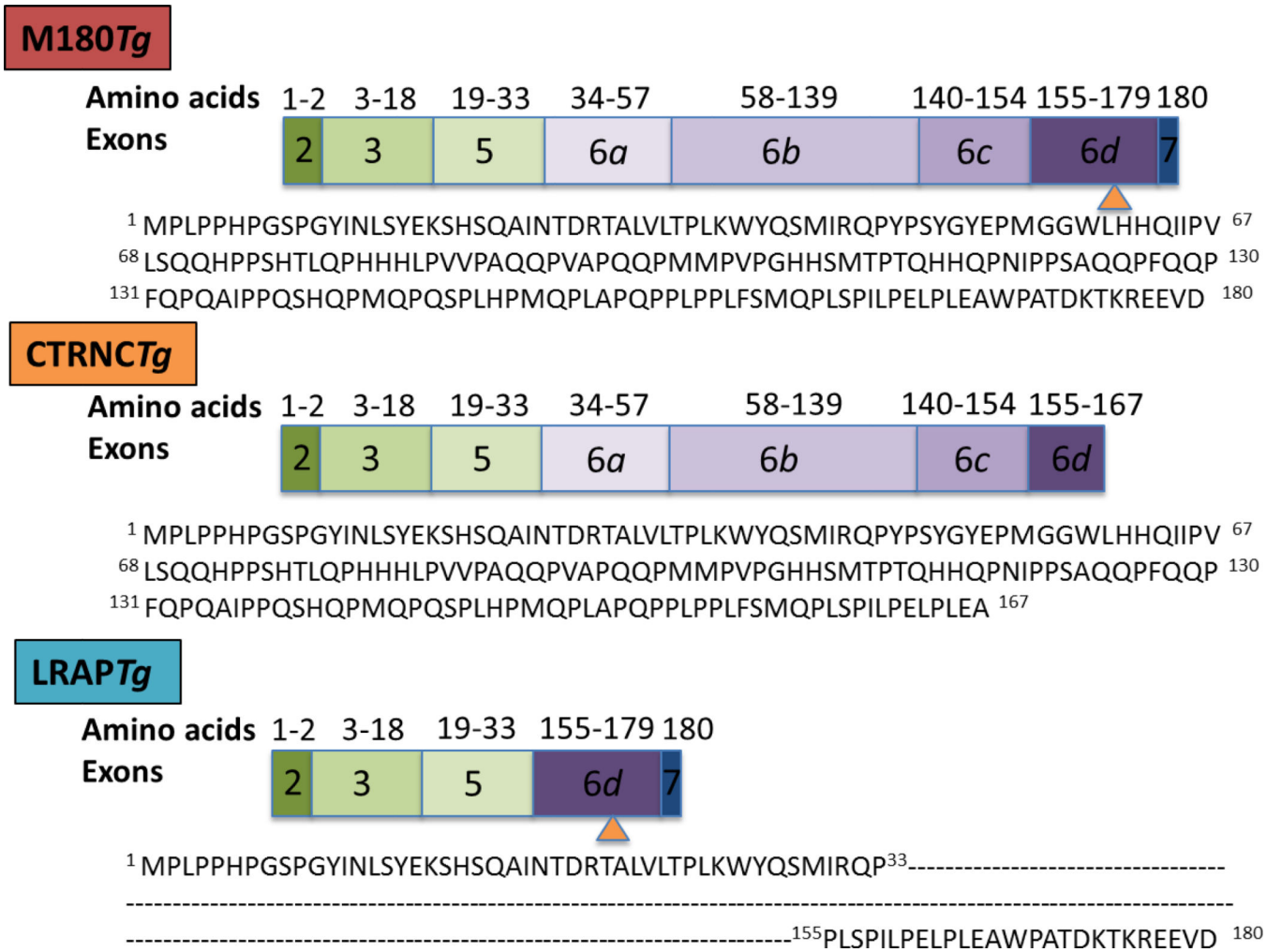

Fig. Schematic of amelogenin transgenes used in this study

M180Tg and LRAPTg represent the major amelogenin isoforms, while CTRNCTg represents the major amelogenin cleavage product. M180Tg contains exons 2, 3, 5, 6a–6d, 7; CTRNCTg contains exons 2, 3, 5, 6a-part of 6d; LRAPTg contains exons 2, 3, 5, 6d, 7. Amino acid numbers and sequences corresponding to transgene exons are shown. Orange arrowhead indicates the site of the first *MMP20* cleavage.

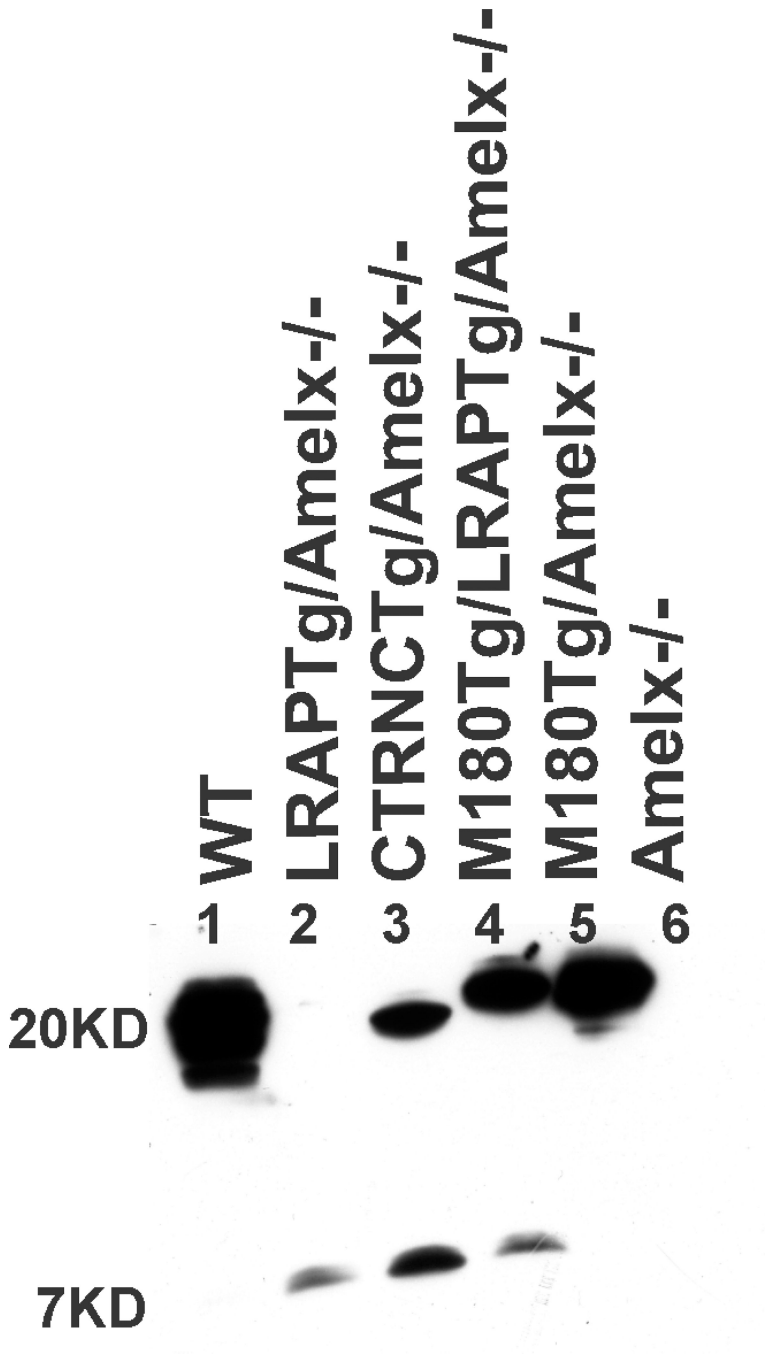

Fig. 2. Amelx protein expression in WT, *Amelx*−/− and transgenic 5 day-old developing molars

WT (lane 1) molars had a smear of amelogenin protein between 17–20 kD representing most of the splice variants and cleavage products expressed during the secretory stage. In this immunoblot, the LRAP band (~7 kD) is not visible (although it was at longer exposure times), but other immunoblots of different WT mice show faint bands around 7 kD representing LRAP. Lanes 2–5 are from Tg mice in *Amelx*−/− backgrounds. Lane 6 is a *Amelx*−/− molar showing no amelogenin protein. LRAPTg expression in double transgenic/*Amelx*−/− mice (lanes 3 & 4) is noticeably higher than that in WT molars. M180Tg (lanes 4

& 5) and CTRNCTg (lane 3) expression was slightly less than to endogenous WT levels (lane 1), presumably due to the presence of less cleavage products.

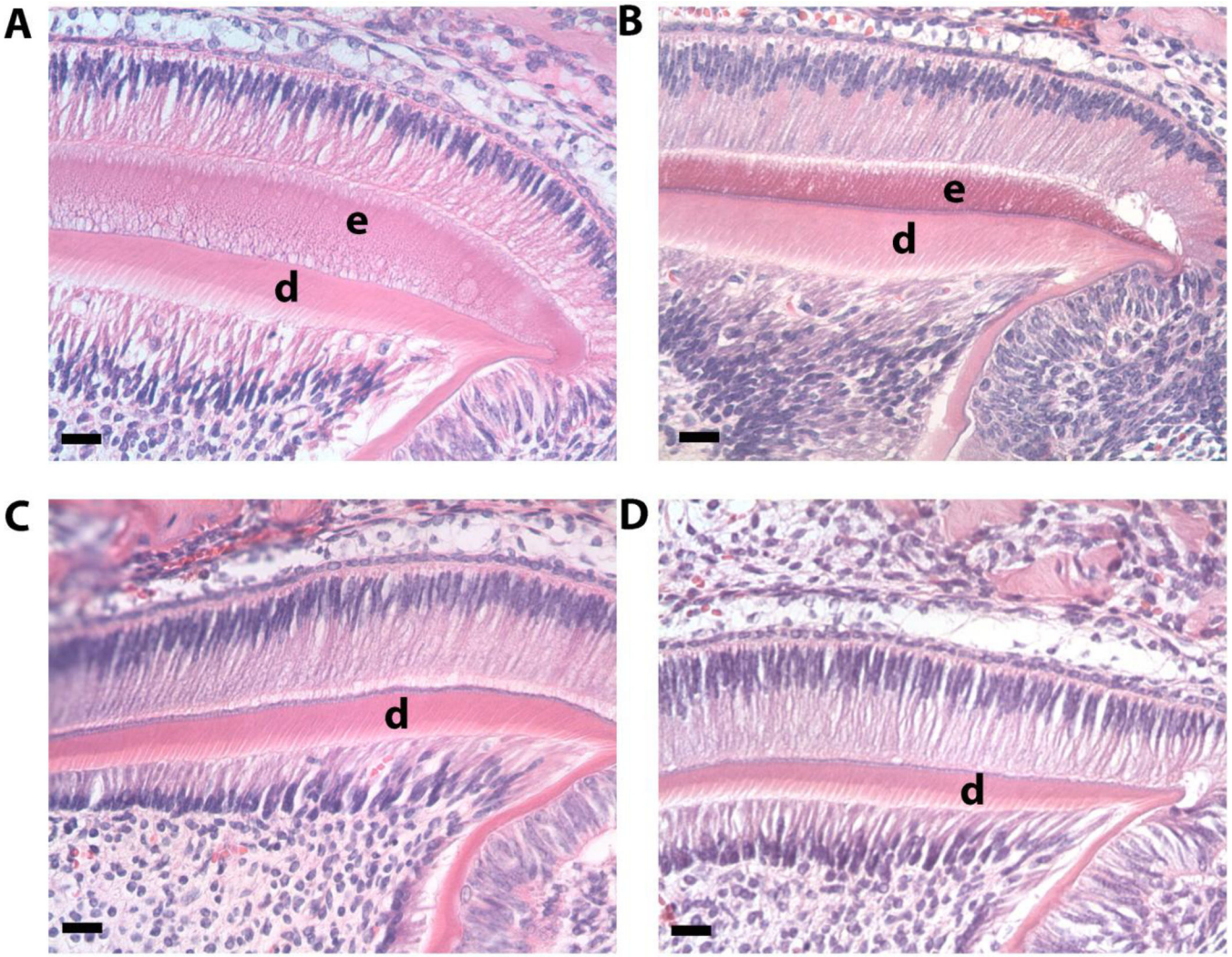

Fig. 3. Histological analysis of developing enamel in 5 day-old 1st molars

Images of H&E stained molars were obtained within the distal cusp of the developing 1st molar at 40× and showed noticeably more enamel in WT (A) and CTRNCTg/LRAPTg/*Amelx*−/− (B) compared to LRAPTg/*Amelx*−/− (C) which showed a thin layer of enamel, and *Amelx*−/− (D), which had no enamel presence. Loss of endogenous amelogenin (B, C, D) and the addition of transgene overexpression (B & C) appeared to have no effect on ameloblasts, odontoblasts or dentin.

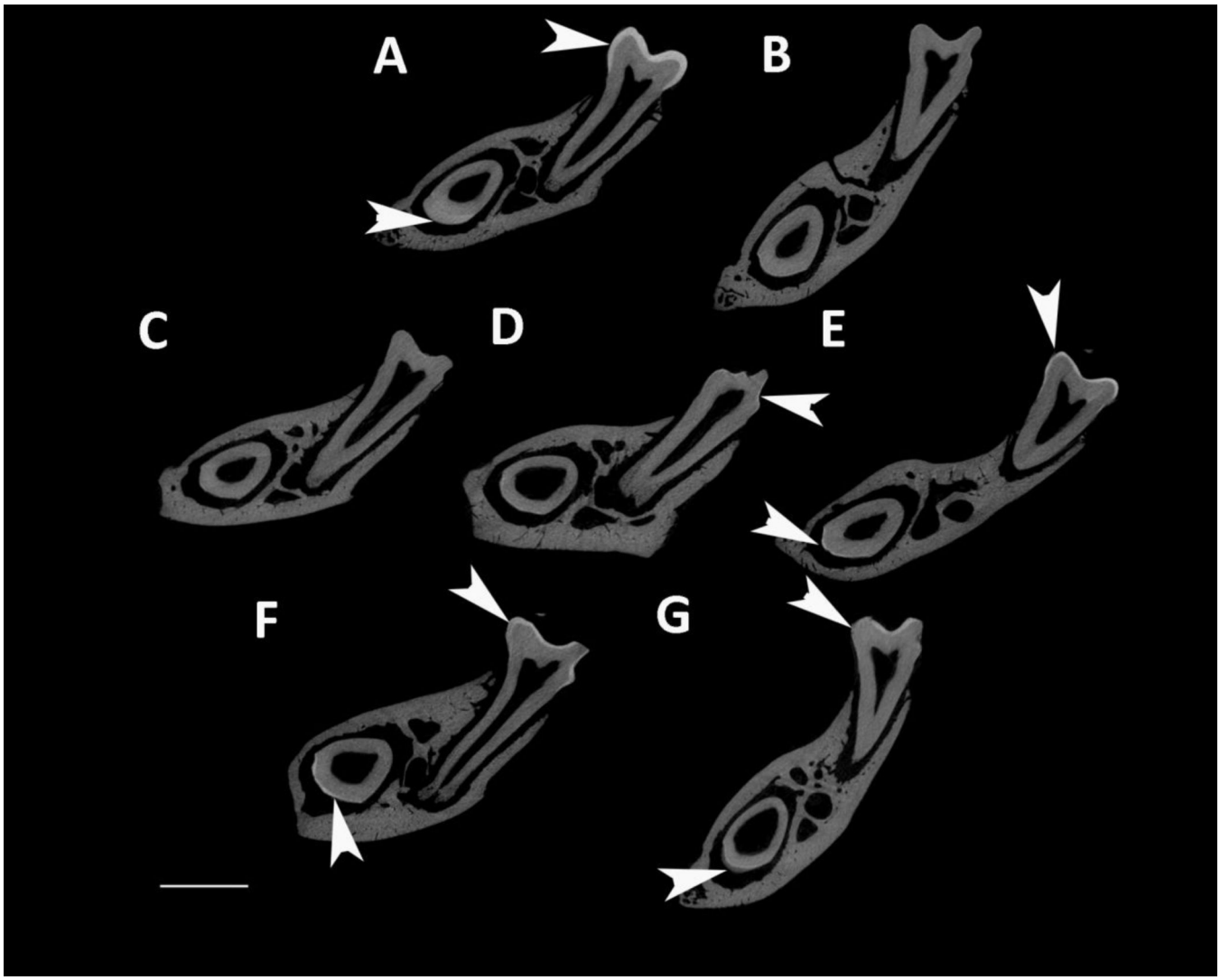

Fig. 4. MicroCT analysis of adult molar and incisor enamel

MicroCT scanned sections were selected in the same location developmentally in each mouse mandible: within the distal root of the first molar, which is adjacent to early maturation-stage of the incisor. Arrowheads point to mineralized enamel layers visible in molars and incisors. Little to no enamel was visible in *Amelx*−/− and LRAPTg/*Amelx*−/− molars and incisors (B & C), and CTRNCTg/*Amelx*−/− incisors (D). Thin enamel layers were visible in CTRNCTg/*Amelx*−/− molars (D), and M180Tg/*Amelx*−/− (E), M180Tg/LRAPTg/*Amelx*−/− (F) and CTRNCTg/LRAPTg/*Amelx*−/− (G) incisors. Thicker enamel layers were visible in WT molars and incisors (A), M180Tg/*Amelx*−/− (E), M180Tg/LRAPTg/*Amelx*−/− (F), and CTRNCTg/LRAPTg/*Amelx*−/− molars (G). Interestingly, incisor enamel in CTRNCTg/LRAPTg/*Amelx*−/− (G) appeared much less mineralized than that in the other genotypes with visible incisor enamel (A, E, F).

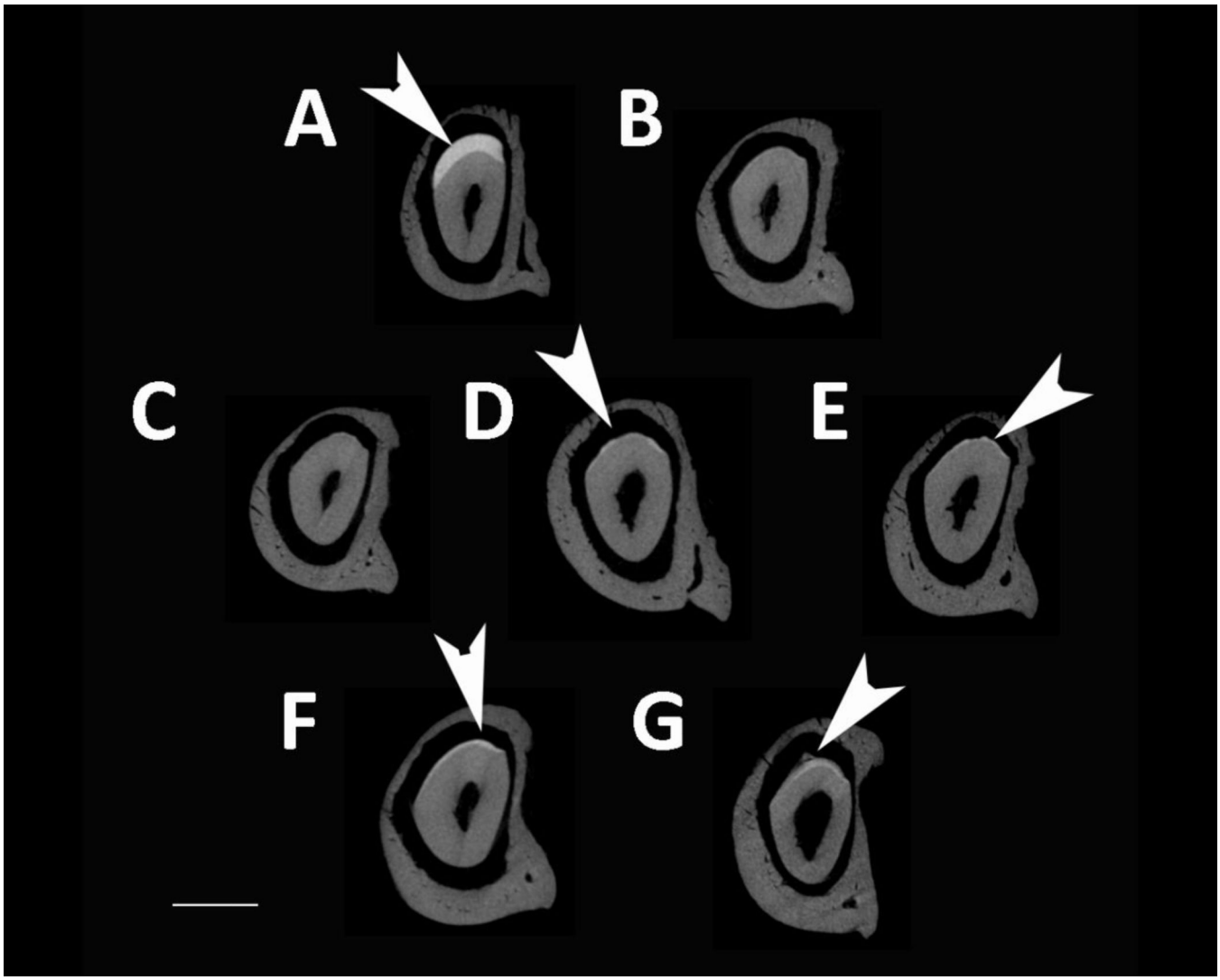

Fig. 5. MicroCT analysis of adult incisor maturation stage enamel

MicroCT scanned sections were selected in the same location developmentally in each mouse mandible: prior to eruption in the maturation-stage of the incisor in the final section that shows the incisors completely surrounded by alveolar bone. Arrows point to mineralized enamel layers visible in incisors. Little to no enamel was visible in *Amelx*−/−, LRAPTg/*Amelx*−/−, CTRNCTg/*Amelx*−/− incisors (B, C, D). Thin mineralized enamel layers were visible in M180Tg/*Amelx*−/− (E), M180Tg/LRAPTg/*Amelx*−/− (F) and CTRNCTg/LRAPTg/*Amelx*−/− (G) incisors. Thicker mineralized enamel layers were only visible in WT incisors (A).

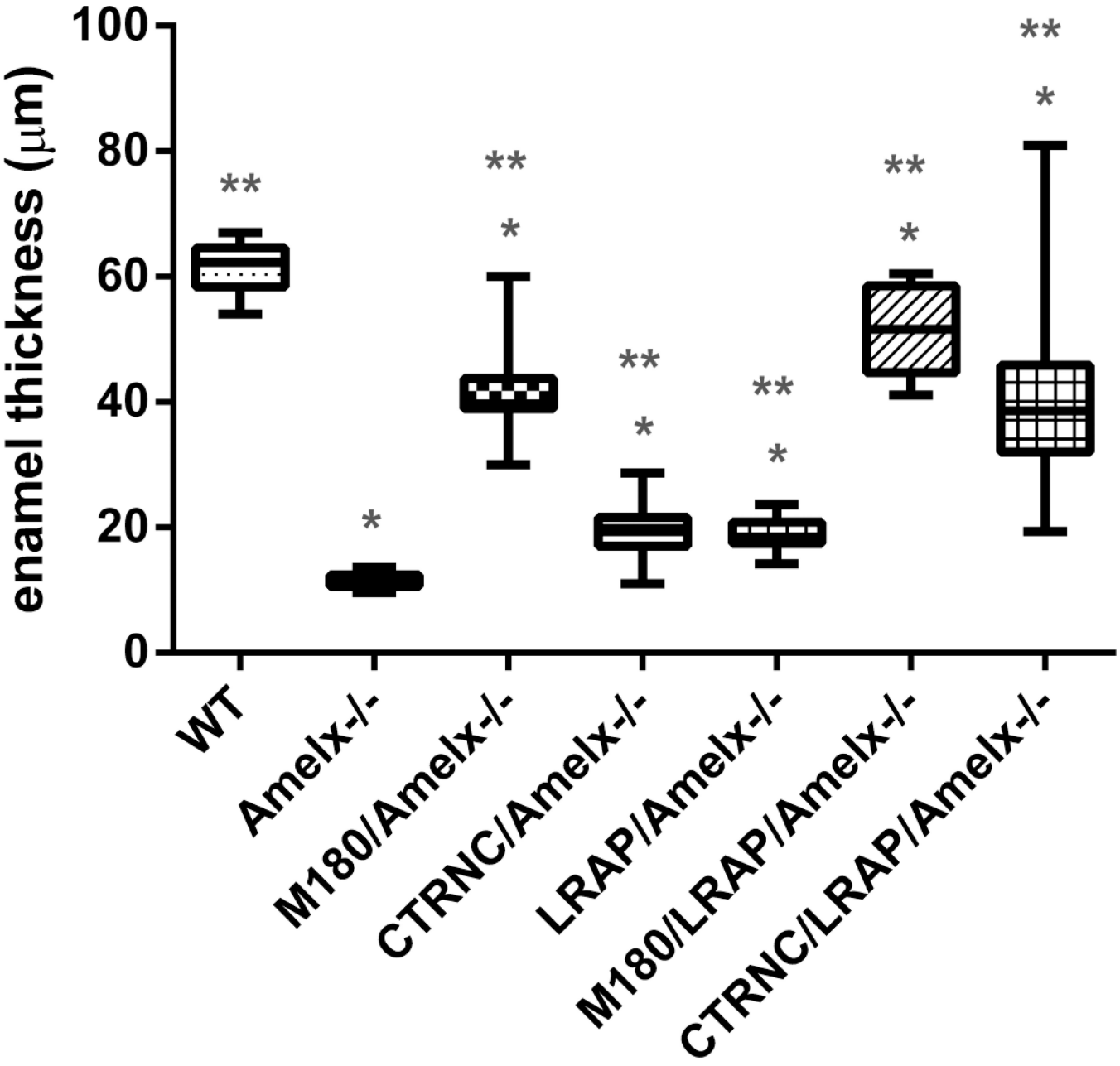

Fig. 6. Molar enamel thickness

Enamel thickness was measured from SEM images of longitudinal sections through distal first molars. All of the transgenic molar enamel was significantly thicker than *Amelx*−/− molar enamel ($p<0.05$), and not as thick as WT enamel ($p<0.05$). However, mice with M180Tg overexpressed alone or with LRAPTg had thicker enamel than those mice without M180Tg (CTRNCTg/*Amelx*−/−, LRAPTg/*Amelx*−/− and CTRNCTg/LRAPTg/*Amelx*−/−). CTRNCTg/LRAPTg/*Amelx*−/− mice had thicker molar enamel than both CTRNCTg/*Amelx* −/− and LRAPTg/*Amelx*−/− mice ($p<0.05$). * = significantly different from WT ($p<0.05$), ** = significantly different from *Amelx*−/− ($p<0.05$). Boxes represent a range of values

between first and third quartile, the line in the center being the median, while the vertical lines represent the maximum and minimum values.

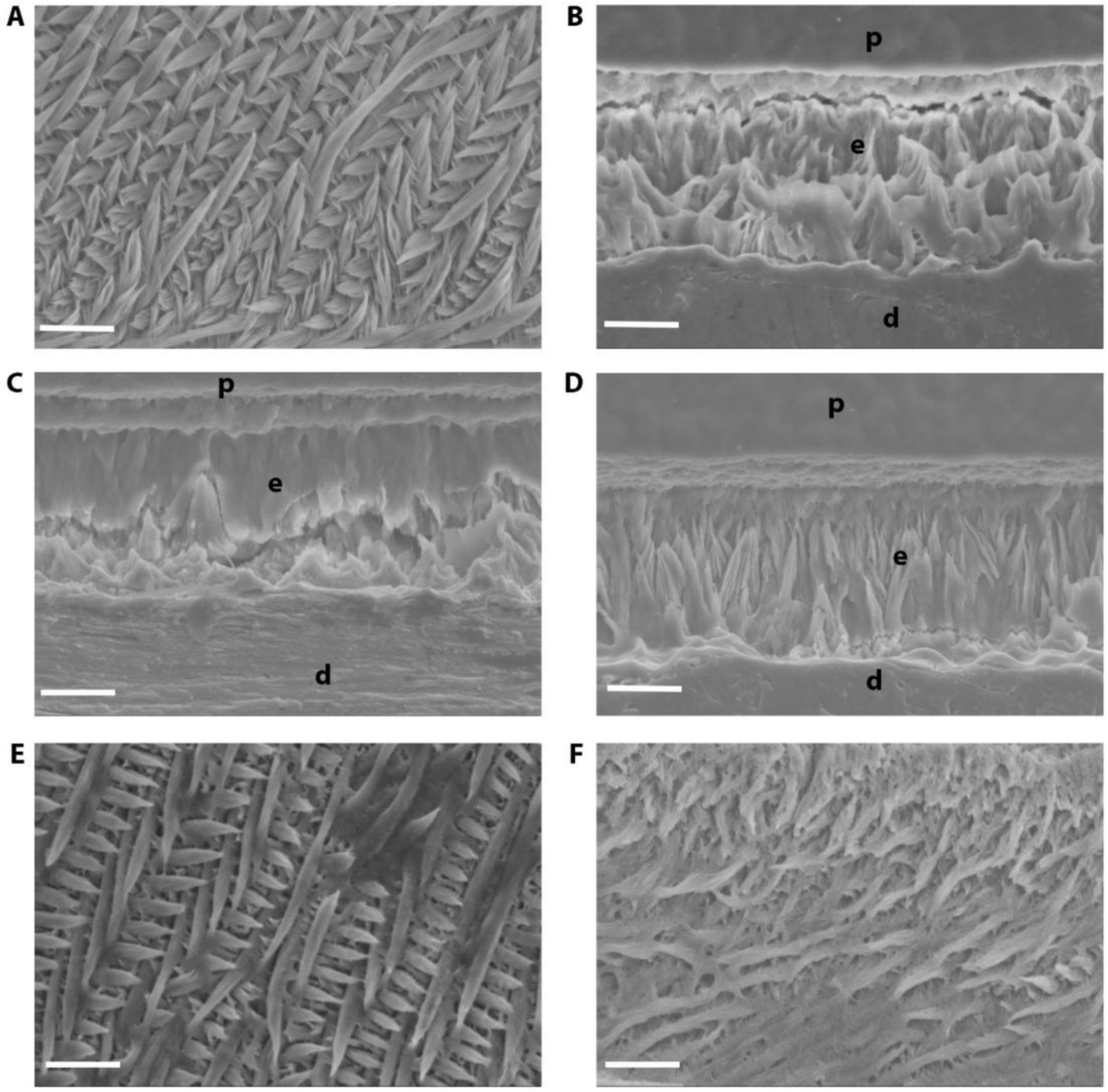

Fig. 7. Incisor enamel structure imaged by SEM

Polished and etched longitudinal sections through mandibular incisor enamel were imaged by scanning electron microscopy in secondary mode at 2000×. While WT (A) exhibits the classic decussating prismatic organization of incisor enamel, *Amelx*−/− (B), LRAPTg/*Amelx*−/− (C), and CTRNCTg/*Amelx*−/− (D) incisor enamel was non-prismatic, with CTRNCTg/*Amelx*−/− enamel exhibiting slightly more organized mineral. CTRNCTg/LRAPTg/*Amelx*−/− (F) incisor enamel had noticeably more mineral organization with some decussating prisms, although not as much decussating prisms as appeared in M180Tg/

LRAPTg/*Amelx*−/− incisor enamel (E). p = plastic embedding material, e = enamel, d = dentin

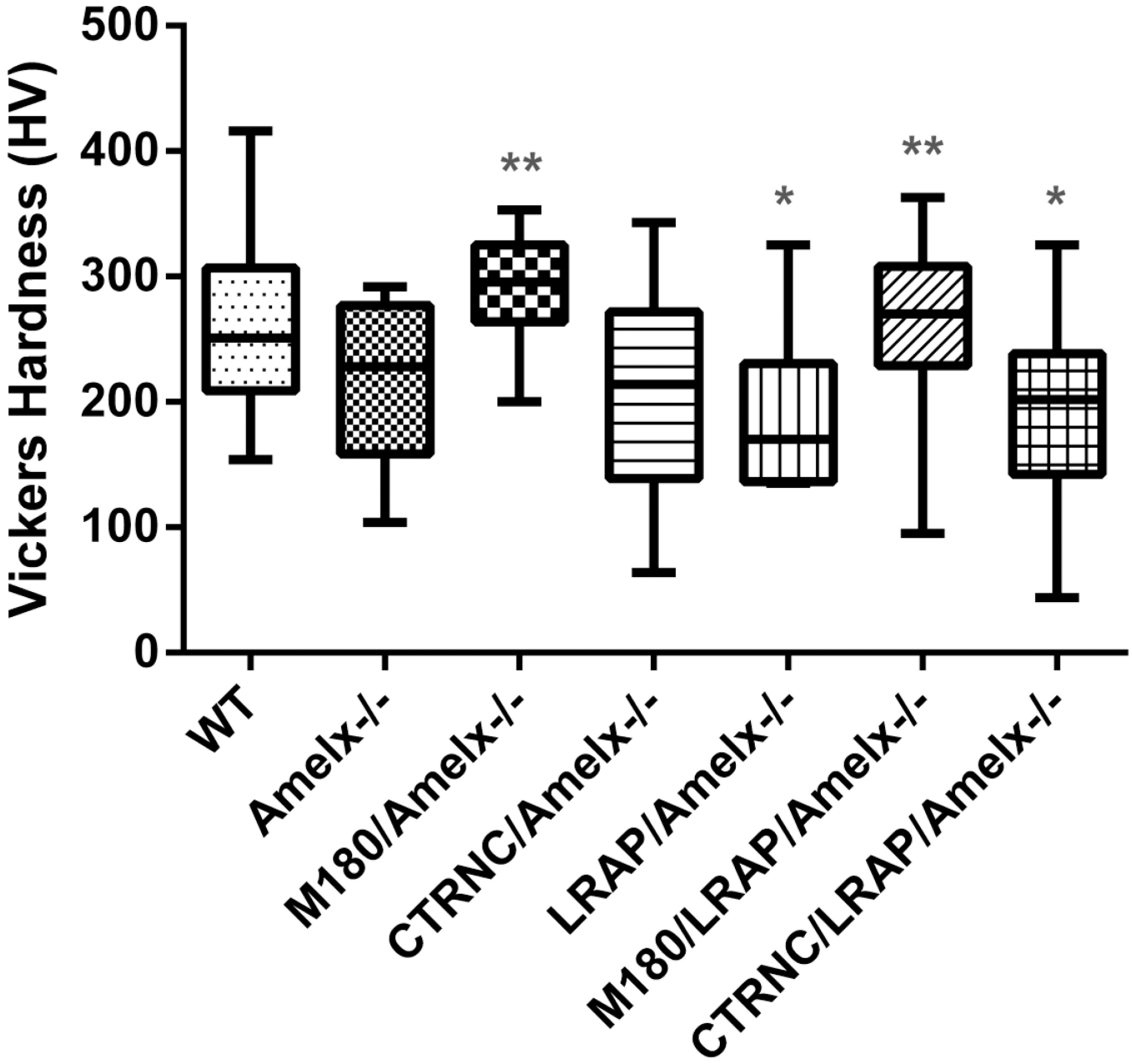

Fig. 8. Vickers Microhardness Testing

Polished longitudinal sections through mandibular molar enamel were used for microhardness testing. If possible, 20 indents per mouse were obtained in adult molar enamel. M180Tg overexpression either alone or with LRAPTg significantly improved enamel hardness over *Amelx*−/− or transgenic mice without M180Tg. * = significantly different from WT ($p<0.05$), ** = significantly different from *Amelx*−/− ($p<0.05$). Boxes represent a range of values between first and third quartile, the line in the center being the median, while the vertical lines represent the maximum and minimum values.