ABSTRACT:
A highly specialized cytoskeletal protein keratin 75 (K75), expressed primarily in hair follicles, nail beds and lingual papillae, was recently discovered in dental enamel - the most highly mineralized hard tissue in the human body. Among many questions this discovery poses, the fundamental question regarding the trafficking and secretion of this protein, lacking a signal peptide, is of an utmost importance. Here we present evidence that K75 is expressed during the secretory stage of enamel formation and is present in the forming enamel matrix. We further show that K75 is secreted together with major enamel matrix proteins amelogenin and ameloblastin and it was detected in Golgi and the ER-Golgi intermediate compartment (ERGIC) but not in rER. Inhibition of ER-Golgi transport by brefeldin A did not affect the association of K75 with Golgi, while ameloblastin accumulated in rER and its transport from rER into Golgi was disrupted. Together these results indicate that K75, a cytosolic protein lacking signal sequence, is secreted into the forming enamel matrix utilizing portions of conventional ER-Golgi secretory pathway. To the best of our knowledge, this is the first study providing insights into mechanisms of keratin secretion.

Keratins comprise a large and diverse family of epithelial cytoskeletal proteins which evolved from two ancestral genes during the expansion of tetrapods into the land environments (1). There are 54 keratins in humans, divided into two major groups, type I (acidic) and type II (basic), which assemble into heterodimers (2). Based on their location and function, keratins are further divided into epithelial, hair follicle and hair keratins (2). Keratins organize into heavily cross-linked networks of intermediate filaments, which provide mechanical rigidity to the cells and play important roles in the cell-cell contacts, as components of the desmosomal complexes (3,4). In addition to the major role in the cytoskeleton recent studies suggest that keratins can have other important functions. Specifically, keratin 17 was detected in cell nuclei, where it was shown to regulate gene expression (5). Hair follicle type II keratin 75 (K75, AKA K6hf) is expressed in ectodermal appendages of amniotes, such as hair and feather follicles, nail beds and lingual papillae (6-8). A single amino acid substitution
A161T in this protein causes a common skin disorder, *pseudofofliculitis barbae*, colloquially called barber’s itch (9). We recently demonstrated that K75 is present in dental enamel forming cells, ameloblasts, and enamel (10). Moreover, it was found that dental enamel in the individuals carrying the *pseudofofliculitis barbae*-causing polymorphism have altered structure and mechanical properties and are more susceptible to caries (10). Recently a number of other epithelial keratins were discovered in enamel and association between mutations these keratins and a higher susceptibility to caries has been established (11). Furthermore, in our recent study K75 was identified in forming porcine enamel by western blot and mass spectrometry (12). Importantly, unlike other transient enamel matrix proteins (EMPs), K75 was not degraded by resident enamel proteases KLK4 and MMP20, strongly suggesting that it incorporates into mature extracellular enamel matrix (EEM).

Although hairs and teeth evolved independently, they share a number of regulatory pathways involved in their morphogenesis (13,14). For example, expression of K75 and several other keratins in ameloblasts, enamel forming cells, is regulated by DLX3 transcription factor (10), which plays the major role in organogenesis of hairs and teeth (15-17). Mutations in DLX3 cause Tricho-Dento-Osseus syndrome (TDO), manifested by severe hair and tooth defects. Specifically, enamel in TDO patients lacks the rod pattern, which is the hallmark of mammalian enamel (18).

Mature enamel is the hardest tissue in the body; it comprises 95% carbonated hydroxyapatite mineral and approximately 1% organic matrix. The basic building blocks of enamel, enamel rods, are arrays of elongated crystals aligned with their crystallographic c-axes (19-21). The rods are woven into a very complex 3D structure, which is responsible for the unique mechanical resilience of this material (20,22-25). While mature enamel is a highly mineralized material, it starts as a hydrogel containing arrays of tiny crystallites (26-29). Enamel formation (amelogenesis) is carried out by specialized epithelial cells, ameloblasts, in a highly coordinated multistep process (30). Enamel matrix deposition begins at the surface of forming dentin by so-called presecretory ameloblasts. They differentiate into secretory ameloblasts, polarized cells with the nuclei located at the basal (proximal) pole and the Tomes' processes, specialized cellular secretory apparatus responsible for the enamel rod formation, at the apical (distal) pole (31-33). The secretory EEM mainly consists of three proteins- amelogenin (AMELX) (34), comprising ~90% of the matrix, ameloblastin (AMBN) (35) and enamelin (ENAM) (36), and metalloproteinase MMP20 (37), which play important roles in the regulation of mineralization (38-45). All enamel matrix proteins (EMPs) possess a signal sequence and are secreted via the conventional ER-Golgi secretory pathway (46,47).

Interestingly, several articles reported interactions between EMPs and cytosolic keratins, suggesting the presence of novel trafficking mechanisms in ameloblasts (48,49). Once the full thickness of enamel is deposited, the maturation stage begins, at which the majority of EMPs is removed and replaced by thickening enamel crystals (43). Only a small fraction of a heavily cross-linked fibrous material remains in the mature enamel (28). This material comprises rod sheaths, enveloping enamel rods, and enamel tufts, protein rich structures in the inner enamel (50-52) which contain K75 and other keratins (10,11).

Our discovery of K75 in enamel (10,12) raises a number of questions regarding its roles in enamel formation and its functional properties. One question, with the potential impact beyond the enamel research, is of particular interest, namely when and how this cytosolic protein lacking lead sequence enters the extracellular space. There are only a few reports in the
literature describing keratins outside cells (53,54), but these studies do not address the secretory pathway question. It is possible that K75 ends up in the enamel matrix as a part of cellular debris embedded in the matrix during enamel secretion (55). Alternatively cytosolic proteins can be secreted via unconventional protein secretion mechanisms (56-58) which include Golgi bypass (56,59), secretory autophagy (60,61), secretory lysosomes (62) and endosomes (63). On the other hand, studies show that a number of proteins lacking the signal sequence can enter classical secretory pathway via posttranslational translocation into rER (64,65).

To gain insights into the mechanisms of K75 trafficking and secretion we carried out a number of experiments to characterize the expression pattern and the trafficking pathway of this protein during enamel formation. Importantly, unlike the majority of trafficking studies, we preformed our experiments in vivo, since ameloblasts, highly specialized polarized secretory cells, cannot maintain their differentiated phenotype in vitro.

**Results.**

*K75 is expressed by secretory ameloblasts and is present in forming enamel.* Taking advantage of the constantly growing murine incisors, containing all stages of enamel development (Figure 1A), we were able to observe K75 expression pattern during amelogenesis and to compare it to AMELX and AMBN, two major EMPs with well-established expression profiles (46,66,67). Figure 1B shows immunofluorescence (IF) images taken from three boxed areas in Figure 1A. The IF results indicate that only AMELX is expressed at the presecretory stage of amelogenesis and all three proteins are expressed during the secretory stage (Figure 1B). At the transition between secretory and maturation stages AMELX and K75 expression cease, while AMBN expression persists though the maturation stage (Figure 1B). The expression profiles of AMELX and AMBN observed in this study are consistent with earlier findings (46,66,67). Importantly, our results clearly demonstrate that K75 protein is exclusively expressed during the secretory stage of amelogenesis. Higher magnification imaging of secretory stage ameloblasts revealed that the K75 distribution in secretory ameloblasts was remarkably different from other typical cytokeratins, such as K14 (Figure 1C,D). While IF of K14 revealed a network of filaments throughout the ameloblast, K75 was found in large granular bodies located in the distal parts of ameloblasts and a distinct signal, organized into a band in the central portion of the cytoplasm. Strong K75 signal was also present in Tomes’ processes. In situ hybridization (ISH) studies revealed that *Krt75* has a unique expression pattern in the craniofacial region. Its expression is confined to cells of hair follicles, lingual papillae and enamel organ (Figure 1E, Suppl. Fig. 1A-B). The presence of K75 in forming molar and incisal enamel from 14 days-old rats was also detected by western blot (WB) (Figure 1F). A band around 70 KDa was observed in both molar and incisal enamel and in the case of molar enamel a higher molecular weight band between 110 and 160 KDa, likely corresponding to a K75 dimer with an acidic keratin was detected (Figure 1F). The fact that K75 is observed at higher than nominal K75 MW of 59 KDa suggests that it undergoes posttranslational modifications, potentially glycosylated in forming enamel (68). The 70 KDa band was further analyzed, using mass spectrometry (MS) which identified with 100% confidence five K75 fragments covering 8% of its sequence (Suppl. Fig. 1C). Importantly no enamel matrix proteins were identified in this band. Overall, these results corroborate our earlier observation of K75 in enamel (10,12) and revealed that K75 expression is associated with secretory ameloblasts.

*K75 is present in membrane-delineated vesicles in secretory ameloblasts.* To better assess the intracellular localization of K75 in the secretory ameloblasts, immunogold transmission
electron microscopy (IG-TEM) studies were carried out. Ameloblasts are polarized secretory cells with extensive rER and Golgi networks throughout the cell body (Figure 2A; Suppl. Fig. 2-4). In addition to typical perinuclear rER and Golgi structures, a large cylindrical Golgi complex aligned with the cell axis exists in the mid-portion of an ameloblast (31,33) (Suppl. Fig. 2 and 3). The cis face of this central Golgi complex is mainly oriented towards the plasma membrane and the trans face towards the core of the cell, rich in vesicles. The space between the cis Golgi and plasma membrane is lined with the rER network. The very distal end of the ameloblast cell body, bordering the Tomes’ process, is free of Golgi and rich in rER and secretory vesicles (33) (Suppl. Fig. 4). Tomes’ processes are free of rER and Golgi cisternae but are filled with small secretory granules and contain a tubulo-vesicular network (69,70) (Fig. 2D; Suppl. Fig. 4). The IG-TEM analysis revealed K75 signal in the membrane-delineated vesicles containing electron dense material in the cell bodies and Tomes’ processes (Figure 2B-D; Suppl. Fig. 3 and 4). Two distinct groups of vesicles were identified based on their sizes. Large vesicles of various shapes (700±556 nm) were observed primarily in the apical region of the cell body (Figure 2A,B; Suppl. Fig. 3). Smaller circular vesicles were observed throughout the ameloblast body (89±20 nm) (Figure 2A,C, Suppl. Fig. 3) and in Tomes’ process (102±35 nm) (Figure 2A,D). The differences between the two groups were not statistically significant, suggesting that they belong to the same population. Our observations indicate that the small vesicles are covered with one single membrane bilayer, and no membrane delineated compartments were found within the vesicles, suggesting that these are not multivesicular bodies or autophagosomes. K75 signal was also detected in the EEM surrounding the Tomes’ processes (Figure 2D, Figure 3G,J). Our IG-TEM studies failed to identify association of K75 with tonofilaments, while other keratins, i.e. K14, were clearly associated with them (Suppl. Fig. 5A). Furthermore, we were not able to identify this protein in multivesicular bodies. Collectively, these results suggest that K75 is translocated into vesicles in the cell bodies of ameloblasts and is exocytosed via Tomes’ processes. To the best of our knowledge, this is the first observation of a keratin, a cytosolic protein lacking signal sequence, in secretory vesicles.

K75 co-localized with two major EMPs. AMBN and AMELX, in secretory ameloblasts.

The discovery of K75 in membrane delineated secretory vesicles prompted us to carry out co-localization studies of K75 with AMBN and AMELX. IF revealed significant levels of co-localization of K75 with the EMPs in cell bodies and Tomes’ processes (Figure 3A,B) as well as discrete structures, resembling strings of beads co-localizing with AMBN in the enamel rod sheaths (Figure 3C). Importantly, all three proteins are present in structures, resembling enamel tufts, at the DEJ in forming molars (Figure 3B white arrowheads).

A quantitative IF co-localization analysis in the bodies of secretory ameloblasts revealed that close to ~90% of K75 co-localizes with AMBN or AMELX, and ~50% of K75 overlaps with both EMPs (Figure 3D). In contrast, only about 30% of either AMBN or AMELX signal overlapped with K75 (Suppl. Fig. 5B,C,F).

In addition to IF co-localization studies a high-resolution IG-TEM analysis was also carried out. The results revealed that K75 co-localizes with AMBN and AMELX in small and large vesicles in the bodies of ameloblasts, and in the Tomes’ processes (Figure 3E-J, Suppl. Fig. 3 and 4). The quantitative analysis of small secretory vesicles in the Tomes’ processes revealed that the co-localization of K75 with AMBN, as well as with AMELX, is more than 90%. Similarly, more than 90% of the vesicles containing either AMBN or AMELX contained
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K75 (Suppl. Fig. 5D,E,G). Overall, the results of the IF and IG-TEM co-localization studies strongly indicate that K75, despite the lack of signal sequence, enters the main secretory pathway and is co-transported with the EMPs.

**K75 co-localizes with ERGIC and Golgi markers.** To investigate the trafficking route of K75 in secretory ameloblasts, IF co-localization of K75 and markers for rER (CP1), ERGIC (ERGIC53, AKA LMAN1), Golgi (Golga 5/G5) and lysosomes (LAMP1) was studied. In parallel co-localization experiments with the same markers were conducted with AMBN, which is secreted via the classical secretory mechanism (47). K75 and AMBN had low levels of co-localization with rER (Figure 4A,B). However, a significant degree of overlap for both AMBN and K75 was observed with ERGIC and Golgi (Figure 4E,F; Suppl. Fig. 6). In the ameloblasts cell bodies ERGIC signal was present in large granules which were often attached to cis-Golgi face (Suppl. Fig. 3, Suppl. Fig. 6). Interestingly, a strong ERGIC signal overlapping with K75 and AMBN was present in the apical portions of ameloblasts and in the Tomes’ processes (Figure 4E,F).

**ER-Golgi trafficking inhibition using Brefeldin A (BFA) does not affect co-localization of K75 with Golgi, while significantly reducing its co-localization with ERGIC.** To further assess the details of K75 secretory route we conducted trafficking inhibition experiments using BFA. BFA, a potent inhibitor of rER-Golgi transport, is widely used in the studies of protein trafficking (46,71). Mice were injected with BFA dissolved in ethanol. No obvious changes were found between one-hour ethanol vehicle group, used as controls, when compared to the non-treated specimens (Fig. 5 A-D vs 4A-D). A quantitative co-localization analysis of secretory ameloblasts in the vehicle group showed that 14±2.5% of AMBN and 6±1% of K75 co-localized with rER marker CP1 (Figure 5A,B,I). While no significant overlap between K75 and CP1 is expected, since K75 is lacking the signal peptide, low co-localization levels of AMBN and CP1 can be due to a very short residence time of AMBN molecules in rER as it was previously reported (46). In contrast substantial levels of co-localization of K75 (54±16%) and AMBN (45.3±8%) with Golgi marker G5 were found (Figure 5,C,D,J). In the BFA treated group, one hour after injection, the co-localization pattern of AMBN changed dramatically. Specifically, AMBN signal associated with the central Golgi complex significantly decreased (8.5±2%; p<0.01) while its overlap with the rER significantly increased (30±5%; p<0.01) (Figure 5E,G,I,J). These results demonstrate that the disruption of rER-Golgi transport by BFA leads to the accumulation of AMBN in rER and its depletion form Golgi, which is anticipated for an extracellular protein secreted via the classical rER-Golgi dependent pathway (57,72). The treatment with BFA, however, had a more limited effect on the localization of K75 in ER and Golgi. Co-localization of K75 and CP1 in the treatment group remained at low levels comparable to the control (10±1%) (Figure 5F, I); while the quantitative analysis revealed no significant changes in co-localization of K75 and G5 between the control and treatment groups (41±14% vs. 54±16%; p=0.34) (Figure 5H,J). Co-localization studies using antibody against another Golgi marker GM130 yielded similar results, namely the BFA treatment lead to a dramatic decrease in overlap of AMBN and GM130, while the colocalization of K75 and GM130 remained essentially unchanged (Suppl. Fig. 7).

The distribution of ERGIC53 and its co-localization with K75 and AMBN in the vehicle controls was similar to untreated samples (Figure 4E,F and 6A,B). After one hour treatment with BFA large granules positive for ERGIC53 disappeared and instead a diffuse ERGIC53 signal was detected throughout the ameloblast body (Figure 4E,F and 6A,B vs. 6C,D and Suppl.
The treatment also led to a significant decrease of ERGIC53 signal in the Tomes’ processes. These observations prompted to conduct quantitative analysis of ERGIC53 co-localization with K75 and AMBN after BFA treatment. The quantitative co-localization analysis revealed significant reduction in K75 co-localization with ERGIC53 (29.5±5.8% vs. 9.9±1.6%; p=0.003) (Figure 6E). At the same time the co-localization of AMBN and ERGIC53 remained unchanged; it was 21.8±1.1% in vehicle control vs. 26.5±6.3% BFA treatment, p=0.5 (Figure 6E).

To rule out potential effects of the carrier (ethanol) we conducted similar experiments using PBS as the carrier which lead to similar results, however in a shorter timeframe, possibly due to a slower degradation of BFA when using ethanol as a solvent (Suppl. Fig. 9).

TEM analysis of the animals treated with BFA in ethanol revealed dilation of the rER, further confirming disruption of rER-Golgi trafficking (Suppl. Fig. 10). IG-TEM of the control group detected AMBN in small vesicles, larger compartments of irregular shapes, potentially ERGIC, and Golgi apparatus, while no AMBN signal was detected in the rER (Suppl. Fig. 10). After the treatment with BFA we observed the accumulation of AMBN signal in rER, however no signal was observed in the Golgi apparatus Suppl. Fig. 10), consistent with the results of IF studies. Overall, the results of BFA inhibition experiments indicate that while AMBN trafficking from rER to Golgi was blocked, as anticipated for a typical secreted protein, however the BFA treatment did not affect association of K75 with Golgi.

**Discussion**

Presence of heavily cross-linked keratin matrix in enamel was first proposed in the late 19th century and a number of more recent papers suggested the presence of keratins in enamel (52). Yet, the unequivocal evidence demonstrating the presence of keratin in enamel was lacking. Our recent studies for the first time demonstrate presence of several keratins, including K75, in enamel matrix, and demonstrate that mutations in these genes cause structural and functional defects of enamel (10-12,73). The present results further corroborate our earlier observations of K75 in enamel (10,12) and revealed that K75 expression is associated with secretory stage ameloblasts.

One of the arguments against the presence of keratins in enamel matrix was the fact that these are cytosolic proteins which lack signal sequence, and therefore cannot be secreted. There are several potential scenarios of translocation of cytosolic proteins into the extracellular space – they can be dislodged into the matrix as a part of cellular debris (55), secreted via canonical ER-Golgi pathway (64,65) or a number unconventional protein secretion mechanisms (56). Our co-localization studies demonstrate that secretion of K75, at least in part, utilizes the canonical secretory apparatus. Specifically, K75 is present in secretory vesicles together with two major EMPs AMELX and AMBN, which possess the signal sequence and are secreted via classical secretory pathway (46,47). K75 was also found in ERGIC and Golgi apparatus, cellular compartments associated with the classical secretory pathway. To the best of our knowledge, there are only a few other examples of cytosolic proteins lacking signal peptide, which utilize conventional secretory pathway (64,65,74,75). Although there were a couple of reports showing keratins extracellularly (53,54), the mechanisms by which these keratins proteins enter the extracellular space were not previously studied. Our study is the first to demonstrate that K75 is co-secreted with AMBN and AMELX and that it utilizes, at least partially, the canonical ER-Golgi secretory pathway.

The fact that K75 co-localizes with AMBN and AMELX in secretory vesicles and the enamel matrix opens a possibility of interactions between...
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K75 and EMPs. Ravindranath et al. reported interactions between AMELX and K5 and K14 (48,49). Further studies are needed to clarify this question. In another study using yeast-two hybrid system it was found that tuftelin, another protein expressed by ameloblasts, interacts with K6 and K5 (76).

ER-Golgi trafficking inhibition experiments using BFA provide further insights into the secretory pathway of K75 in ameloblasts. While BFA treatment which disrupts rER-Golgi transport lead to the accumulation of AMBN in dilated rER and its disappearance from Golgi as previously described (46), BFA treatment did not significantly affect the co-localization of K75 with Golgi. At the same time, even though ERGIC53 signal distribution in the cells changed, the degree of AMBN co-localization with ERGIC53 remained unchanged, while co-localization of K75 and ERGIC53 decreased significantly. Hence, although K75 utilizes portions of the canonical ER-Golgi secretory pathway it behaves differently than typical secretory proteins containing signal sequence. According to our model, K75 enters the classical secretory pathway not via rER, as most secreted proteins, but it is translocated via ERGIC. Once in the ERGIC it is co-transported with other secretory cargo through the Golgi apparatus and secreted from the Tomes’ processes. At the same time, the fact that the BFA treatment, which leads to breakdown of ERGIC, does not significantly affect K75 co-localization with Golgi apparatus suggests that either BFA treatment prevents K75 from clearing Golgi or there is another ERGIC independent mechanism of K75 translocation into the Golgi. To the best of our knowledge, this is the first demonstration of a trafficking route for a cytosolic protein which utilizes portions of conventional rER-Golgi secretory pathway and it is very different from other unconventional pathways utilized by cytosolic proteins (57,58).

Our observation of ERGIC53 in association with EMPs, in distal rER rich region of ameloblast and in the Tomes’ processes - the ameloblasts secretory apparatus, raises the possibility that some elements of the ERGIC system can be involved in a secretory pathway that bypasses Golgi. Earlier studies of ameloblasts revealed an extensive tubulovesicular system in the distal portions of ameloblasts and the Tomes’ processes (69,70) which can potentially be a part of ERGIC. One possible scenario is that some of these ERGIC structures are produced in the distal rER rich zone (Suppl. Fig. 4 Suppl. Fig. 6) and transported into the Tomes’ process directly. There are several examples of Golgi bypass (56,57,59), however it is possible that, in ameloblasts, secreted proteins can simultaneously be transported via the conventional rER-Golgi pathway, as well as through the Golgi bypass mechanism (59) involving ERGIC.

It is important to emphasize that ameloblasts are highly specialized super-secretory cells and their secretory pathways are best studied \textit{in vivo}. The fact that in tissue cultures ameloblast-derived cells never attain their secretory phenotype presents a major challenge for studies of ameloblast cell biology. This constrain potentially applies to other cell types. The vast majority of cell trafficking and secretion studies are conducted in tissue culture systems, and often the data obtained \textit{in vitro} are not in a good agreement with \textit{in vivo} observations (57), potentially because cells \textit{in vitro} are not able to differentiate properly. We anticipate, that the mechanisms of secretion revealed in this study are not unique to ameloblasts but are much more universal and other cell types with high secretory activity might utilize them as well.

Conclusions:

Overall, these results further support our previous observations of K75 in ameloblasts and enamel and demonstrate that it is expressed
during the secretory stage of amelogenesis. Furthermore, our data demonstrate that the majority of K75 is co-transported together with EMPs and is secreted from the Tomes’ processes. Using cell compartment markers, we detected K75 in ERGIC and Golgi apparatus. We further conducted experiments with ER-Golgi trafficking inhibitor BFA, which revealed that while the treatment disrupted co-localization of K75 with ERGIC, its association with Golgi remained unchanged. Collectively, these results demonstrate that K75, a cytosolic protein lacking signal sequence, utilizes portions of the classical secretory pathway. Our results also suggest that some of the secreted proteins might utilize the Golgi bypass mechanism via ERGIC associated vesicles. Together these results provide novel insights into the K75 secretion processes and cellular trafficking mechanisms in ameloblasts and potentially other systems.

**Experimental Procedures**

All methods were performed in accordance with the relevant guidelines and regulations. Four-week old Sprague Dawley rats (Charles River, MA) and 4-week old mice (C57BL/6J, Jackson Laboratory, ME) were used in the study. All animal procedures described were approved by the University of Pittsburgh IACUC.

**Tissue preparation for ISH; IF and IG TEM studies:**

Four-week old Sprague Dawley rats (Charles River, MA) and 4-week old mice (C57BL/6J, Jackson Laboratory, ME) were euthanized with CO2. Mandibles were dissected and immediately submersed in 50 ml volumes of 4% paraformaldehyde in 10mM PBS, for IF or TEM, or Karnovsky fixative (2% glutaraldehyde, 2% formaldehyde in 10mM PBS), for TEM at 4°C in less than 3 min after euthanasia. After 24-48 hours of fixation at 4°C, samples were placed into the demineralization solution, containing 0.1 M EDTA (pH 7.2-7.4) for one to two weeks. The demineralization solution was changed every other day.

For IG-TEM experiments animals were anesthetized with isoflurane and perfused through the left ventricle first with cold PBS for 30 seconds then with cold 4% paraformaldehyde in 0.1M phosphate buffer or cold 1% Glutaraldehyde in 0.1M phosphate buffer for 15-20 minutes. The mandibles were dissected and further fixed in the same fixative solution for another 8-12 hours at 4°C, followed by demineralization, as described above.

For IF, whole dehydrated using Leica ASP 300S automatic processor (Leica Biosystems, Buffalo Grove, IL) and paraffin embedded using a standard protocol (77).

For TEM studies, after demineralization mandibular bone around the incisors was trimmed and the molars were removed. The distal fragments of the jaws containing apical portions of the incisors were further cross-sectioned into 1-1.5 mm thick pieces, processed and embedded in LR White or Embed 812 (cat# 14381 and 14120, EMS, Hatfield, PA) according to published protocols (47). In brief, for Embed 812 processing, incisor pieces were post-fixed in 1% ferrocyanide reduced osmium tetroxide for one hour, washed in PBS, dehydrated in graded ethanol and infiltrated with propylene oxide. The samples were embedded in Embed-812 and cured at 65°C for 2 days. For LR White processing, some of the samples were post-fixed in osmium tetroxide while others were not, as osmication reduces the immunoreactivity of the samples. Incisor pieces were washed in PBS, dehydrated in graded ethanol, embedded in LRWhite and cured at 60°C for 1-2 days.

For ISH, mice 1 day postnatal were euthanized with CO2 and fixed with 4% paraformaldehyde in 10mM PBS for 24 hours and embedded in paraffin as described above. For IF and ISH, the paraffin blocks were sectioned using a Leica RM 2225 microtome (Leica Biosystems, Buffalo, IL) into 10 µm thick
sections using a stainless steel microtome knife (Leica 818, Leica, Germany). The sections were mounted on 3-Triethoxysilylpropylamine (440140, Millipore-Sigma, MO) coated glass slides. For TEM the resin blocks were sectioned into 70 nm thick sections using Leica EM UC7 ultramicrotome (Leica Biosystems, Buffalo, IL) equipped with diamond knife (EMS, Hatfield, PA). The sections were mounted on carbon coated Ni grids (EMS, Hatfield, PA).

**IF studies:**
An optimized IF procedure (77,78) developed in our laboratory, aimed at reducing false positives, was used. Briefly, after de-paraffinization, sections underwent trypsin treatment for 5-30 min at 37°C or heating treatment in 10 mM sodium citrate buffer (pH 6.0) for 10-20 min for antigen retrieval. The sections were blocked in 10% serum from the secondary AB host animal in TRIS buffer solution (TBS) for 1 hour at room temperature and incubated with primary ABs at 4°C overnight, followed by incubation with secondary ABs for 45 min at room temperature. The sections were washed with TBS and incubated with 1.5% Sudan Black B (199664, Sigma, MO) in 70% Ethanol to minimize autofluorescence and counter-stained with DAPI. Primary ABs used include rabbit anti-AMELX, 1:100 (ABT260, Abcam, MA), goat anti-AMBN, 1:100 (sc-33102, Santa Cruz, CA), rabbit anti-AMBN, 1:100 (sc-50534, Santa Cruz, CA), rabbit anti-Golga 5 (G5), 1:100 (NBP1-83352, Novusbio, CO), rabbit anti-GM130, 1:100 (Ab52649, Abcam, MA), rabbit anti-ERGIC53, 1:100 (sc-66880, Santa Cruz, CA), monoclonal rabbit anti calreticulin peptide 1 (CP1), 4μg/ml (CPTC-CALR-1-s, Developmental Studies Hybridoma Bank, IA), rat anti-LAMP1, 4μg/ml (1D4B-s, Developmental Studies Hybridoma Bank, IA), rabbit anti-K5, 1:2000 (905501, Biolegend, MA), rabbit anti-K14, 1:1000 (905301, Biolegend, MA), guinea pig anti-K75, an antibody widely used in IF studies (6,10,79), 1:100 (20R-2647, Fitzgerald, MA). Guinea pig serum (88R-1015, Fitzgerald, MA), other normal sera or IgGs purchased from Santa Cruz Biotech Inc. or Jackson Immunoresearch Inc. were used as isotype controls. Secondary ABs used included AlexaFlour-555/Cy3/Cy5 conjugated donkey anti-primary AB host IgG (H+L), 1:500 purchased from Jackson Immunoresearch, PA. Mounted slides were analyzed using Nikon A1 confocal microscope system (Nikon Instruments Inc., NY) in Center for Biologic Imaging (CBI) at the University of Pittsburgh. Co-localization analysis was performed using NIS-Elements software (Nikon Instruments Inc., NY) provided with the microscope K75, AMBN and AMELX co-localization analysis was performed on the samples collected from 3 rats per group. The statistical analysis of the co-localization data was performed using Microsoft Excel and Origin software packages (OriginLab Corporation, Northhampton, MA).

**In situ hybridization.**
The ISH was performed following a published protocol (80,81). Briefly, the sections of the P1 mice were de-paraffinized, rehydrated, post-fixed, and underwent the pre-hybridization in the first day. Digoxigenin (DIG)-labeled RNA probes to the sense and antisense strands of mouse Krt75 partial cDNA (421 bp) corresponding to exon 9 including the c-terminal tail domain and part of the 3' noncoding region (1494 to 1996 bp of Ref Seq NM 133357.3) were hybridized to the sections. The sections were blocked in 10% sheep serum for 2 hours, incubated with 1:2000 anti-DIG ALP AB in 1% sheep serum mixture overnight at 4°C on the second day, washed and incubated in developing solution (1681451, Boehringer, CT) from the third day. The reaction was stopped by washing in PBS. The slides mounted in toluene (4112, ThermoFisher, MA) and were observed under a light microscope in the bright field mode.

**Western blot (WB):**
The enamel matrix was collected from apical portions of continually growing incisors and
unerupted 1st molars from 14 day-old Wistar rats.
For incisal enamel matrix preparation, mandibles of 14 day-old Wistar rats were dissected and the basal bone was removed to expose unerupted portions of incisors under dissecting microscope. The cellular components of enamel organ including ameloblast layer were carefully removed and the forming enamel tissue was scraped from the dentin surface. The matrix was transferred to Tris buffered saline (TBS) (Thermo-Scientific) with the proteinase inhibitor cocktail (Thermo Scientific) at 1:100 ratio. Molar enamel preparation was carried out according to published procedure(82). Maxillary and mandibular unerupted 1st molars were extracted and pulp tissue was removed under the dissecting microscope. The crowns were cleaned of surrounding tissues and enamel matrix proteins were extracted using a published method (82) with slight modifications. Briefly, the dissected molars were incubated with 0.17 M HCL / 0.98% formic acid for 2 hrs at 4°C, and the supernatant was concentrated using 3K centrifugal filtering unit (Millipore). Whiskers were processed according to a published procedure(83). Briefly, whiskers were plucked from the rat snout and the hair follicles were collected in 200 µL of the protein extraction buffer, with the proteinase inhibitor cocktail. The hair follicles were vortexed, and incubated at 37°C overnight. For WB the samples were centrifuged and the supernatant was collected for analysis. The concentration of the proteins in the samples was detected using BCA assay. Ten µg of proteins were loaded in Bis-Tris 4-12% Gel (Life technologies) and subjected to SDS-PAGE. The protein bands were transferred from the gel to a PVDF membrane (Life Technologies), and bands containing proteins of interest were detected using ECL substrate (#32106, Thermo Scientific). Primary ABs used were anti-K75 guinea pig IgG conjugated with HRP, 1:2000 (706-035-148, Jackson Immunoresearch) and goat anti-rabbit IgG conjugated with HRP, 1:2000 (#32460 Thermo Scientific)

Mass spectrometry.
For mass spectrometry the bands of interest were dissected in the gels and submitted to the University of Pittsburgh Biomedical Mass Spectrometry Center for analysis.

Immuno-Gold TEM (IG-TEM) studies:
The IG-TEM studies were performed on 70 nm thick sections mounted on carbon coated Ni grids. The immunolabeling was performed according to published procedures (46,47). Briefly, grids were blocked in the same blocking solution as in IF protocol described above for one hour, incubated with primary antibody for one hour, washed with TBS, then incubated with secondary antibody for one hour, washed and counter-stained with 2% Uranyl acetate for 10m. Primary ABs’ concentrations 10 times higher than in IF assays described above were used. Six nm colloidal gold conjugated donkey anti-guinea pig IgG, 1:30 (706-195-148, Jackson Immunoresearch, PA), 12 nm colloidal gold conjugated donkey anti-rabbit IgG, 1:30 (711-205152, Jackson Immunoresearch, PA) were used as secondary ABs. Guinea pig serum (88R-1015, Fitzgerald, MA) and rabbit serum (011-000-001, Jackson Immunoresearch, PA) were used as isotype controls. All grids were observed with JEM 1400Plus TEM (JEOL USA Inc., MA) at 80 kV, located in CBI at University of Pittsburgh. To assess the distribution of K75 in vesicles of different sizes in the ameloblast cell bodies and the Tomes’ processes, sections from 3 rats were labeled with anti-K75 ABs and analyzed. The vesicles containing at least 1 gold particle were counted and measured and at least 50 vesicles per animal were analyzed. For IG-TEM colocalization of K75 with EMP in Tomes’ processes, LR White sections from 3 rats were double labeled for K75 and either AMBN or
AMELX. Vesicles with at least 1 gold particle were counted and more than 400 vesicles were analyzed.

**Brefeldin A experiments:**
BFA powder (B-8500, LC Laboratories, MA) dissolved in DMSO at 25mg/ml concentration was used as a stock solution. BFA stock solution was further dissolved in 30% ethanol or PBS to a final concentration of 0.5 mg/ml prior to the experiments. Four-week old mice were used in this study; all animals were anesthetized by isoflurane. Three mice received intraperitoneal injection of 0.5mg BFA in ethanol and three mice injected with ethanol only were used as vehicle control. The mice were euthanized one hour after the injection. In another set of experiments, three mice were injected with 0.5 mg BFA in PBS. These animals were euthanized 15min, 30min, 60 min after the injection. One mouse was injected with the vehicle (control) and was euthanized 60 min after the injection. The animals were euthanized in CO₂ one hour after the injection and fixed in 4% paraformaldehyde according to IF or TEM procedures as described above. Independent two sample t-test was used to analyze the colocalization differences between the vehicle control and BFA/Ethanol treated mice (n=3).

**Quantitative IF co-localization analysis:**
Quantitative co-localization analysis was performed using a well-established methodology (84,85). The co-localization analysis was conducted using a proprietary Nikon software NIS Elements (Nikon, Melville, NY). The quantitative co-localization analysis was conducted on sections from 3 animals. Co-localization was calculated based on the number of pixels in confocal slices from a z-stack which contain two channels. The co-localization was assessed according to the established procedures (84-86), using Pearson correlation analysis and Manders coefficients, which represent a ratio of overlapping pixels to the total number of pixels in each channel. For background adjustment adjacent sections were treated with naïve sera (isotype control) and signal intensity from these sections was used to determine the background fluorescence. The co-localization analysis was conducted using a proprietary Nikon software NIS Elements (Nikon, Melville, NY). The quantitative co-localization analysis was conducted on sections from 3 animals. The data was analyzed using t-test assuming unequal variance in OriginPro 2015 software package (Origin Labs, Northampton, MA).
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Authors contributions:
EB conceived the study; EB and XY designed the experiments; EB, XY, MM and OD contributed to the development of the project through regular discussions of the results. XY- conducted the bulk of the IF and IG-TEM experiments, MM designed the ISH probes, YY and HY conducted WB experiments, EB, XY and HY analyzed the data, EB and XY drafted the manuscript and EB, XY, MM OD and HY revised it.
References:

1. Vandebergh, W., and Bossuyt, F. (2012) Radiation and Functional Diversification of Alpha Keratins during Early Vertebrate Evolution. *Mol. Biol. Evol.* **29**, 995-1004

2. Moll, R., Divo, M., and Langbein, L. (2008) The human keratins: biology and pathology. *Histochem Cell Biol* **129**, 705-733

3. Fuchs, E., and Cleveland, D. W. (1998) A structural scaffolding of intermediate filaments in health and disease. *Science* **279**, 514-519

4. Herrmann, H., and Aebi, U. (2004) Intermediate filaments: Molecular structure, assembly mechanism, and integration into functionally distinct intracellular scaffolds. *Annu. Rev. Biochem.* **73**, 749-789

5. Hobbs, R. P., Jacob, J. T., and Coulombe, P. A. (2016) Keratins Are Going Nuclear. *Dev. Cell* **38**, 227-233

6. Winter, H., Jacobs, M., Rogers, M. A., Schweizer, J., Langbein, L., Praetzel, S., Leigh, I. M., and Tidman, N. (1998) A Novel Human Type II Cytokeratin, K6hf, Specifically Expressed in the Companion Layer of the Hair Follicle. *J. Invest. Dermatol.* **111**, 955-962

7. Wang, Z., Wong, P., Langbein, L., Schweizer, J., and Coulombe, P. A. (2003) Type II Epithelial Keratin 6hf (K6hf) Is Expressed in the Companion Layer, Matrix, and Medulla in Anagen-Stage Hair Follicles. *J. Invest. Dermatol.* **121**, 1276-1282

8. Lin, S. J., Wideliz, R. B., Yue, Z. C., Li, A., Wu, X. S., Jiang, T. X., Wu, P., and Chuong, C. M. (2013) Feather regeneration as a model for organogenesis. *Dev. Growth Differ.* **55**, 139-148

9. Winter, H., Schissel, D., Parry, D. A. D., Smith, T. A., Liovic, M., Lane, E. B., Edler, L., Langbein, L., Jave-Suarez, L. F., Rogers, M. A., Wilde, J., Peters, G., and Schweizer, J. (2004) An unusual Ala12Thr polymorphism in the 1A alpha-helical segment of the companion layer-specific keratin K6hf: Evidence for a risk factor in the etiology of the common hair disorder pseudofolliculitis barbae. *J. Invest. Dermatol.* **122**, 652-657

10. Duverger, O., Ohara, T., Shaffer, J. R., Donahue, D., Zerfas, P., Dullnig, A., Crecelius, C., Beniash, E., Marazita, M. L., and Morasso, M. I. (2014) Hair keratin mutations in tooth enamel increase dental decay risk. *J. Clin. Invest.* **124**, 5219-5224

11. Duverger, O., Carlson, J. C., Karacz, C. M., Schwartz, M. E., Cross, M. A., Marazita, M. L., Shaffer, J. R., and Morasso, M. I. (2018) Genetic variants in pachyonychia congenita-associated keratins increase susceptibility to tooth decay. *PLoS Genet.* **14**, e1007168

12. Chiba, R., Okubo, M., Yamamoto, R., Saito, M. M., Kobayashi, S., Beniash, E., and Yamakoshi, Y. (2019) Porcine keratin 75 in developing enamel. *J. Oral Biosci.*

13. Biggs, L. C., and Mikkola, M. L. (2014) Early inductive events in ectodermal appendage morphogenesis. *Semin. Cell Dev. Biol.* **25**, 11-21

14. Sharpe, P. T. (2001) Fish scale development: Hair today, teeth and scales yesterday? *Curr. Biol.* **11**, R751-R752

15. Price, J. A., Bowden, D. W., Wright, J. T., Pettenati, M. J., and Hart, T. C. (1998) Identification of a mutation in DLX3 associated with tricho-dento-osseous (TDO) syndrome. *Hum. Mol. Genet.* **7**, 563-569

16. Duverger, O., Lee, D., Hassan, M. Q., Chen, S. X., Jaisser, F., Lian, J. B., and Morasso, M. I. (2008) Molecular consequences of a frameshifted DLX3 mutant leading to Tricho-Dento-Osseous syndrome. *J. Biol. Chem.* **283**, 20198-20208

17. Duverger, O., Zah, A., Isaac, J., Sun, H. W., Bartels, A. K., Lian, J. B., Berdal, A., Hwang, J,
14

and Morasso, M. I. (2012) Neural Crest Deletion of Dlx3 Leads to Major Dentin Defects through Down-regulation of Dspp. J. Biol. Chem. 287, 12230-12240

18. Li, Y., Han, D., Zhang, H., Liu, H. C., Wong, S. W., Zhao, N., Qiu, L. X., and Feng, H. L. (2015) Morphological analyses and a novel de novo DLX3 mutation associated with tricho-dento-osseous syndrome in a Chinese family. Eur. J. Oral Sci. 123, 228-234

19. Nanci, A. (2007) Ten Cate's Oral Histology: Development, Structure, and Function, Elsevier Health Sciences

20. Stifler, C. A., Wittig, N. K., Sassi, M., Sun, C.-Y., Marcus, M. A., Birkedal, H., Beniash, E., Rosso, K. M., and Gilbert, P. U. P. A. (2018) X-ray Linear Dichroism in Apatite. J. Am. Chem. Soc. 140, 11698-11704

21. Glimcher, M. J., Daniel, E. J., Travis, D. F., and Kamhi, S. (1965) Electron optical and X-ray diffraction studies of the organization of the inorganic crystals in embryonic bovine enamel. J. Ultrastruct. Res. 12, 15-77

22. Bajaj, D., and Arola, D. D. (2009) On the R-curve behavior of human tooth enamel. Biomaterials 30, 4037-4046

23. Baldassarri, M., Margolis, H. C., and Beniash, E. (2008) Compositional determinants of mechanical properties of enamel. J. Dent. Res. 87, 645-649

24. Chai, H., Lee, J. J. W., Constantino, P. J., Lucas, P. W., and Lawn, B. R. (2009) Remarkable resilience of teeth. Proc Natl Acad Sci USA 106, 7289-7293

25. Macho, G. A., Jiang, Y., and Spears, I. R. (2003) Enamel micro structure - a truly three-dimensional structure. J. Hum. Evol. 45, 81-90

26. Hu, Y., Smith, C. E., Cai, Z., Donnelly, L. A.-J., Yang, J., Hu, J. C.-C., and Simmer, J. P. (2016) Enamel ribbons, surface nodules, and octacalcium phosphate in C57BL/6 Amelx−/− mice and Amelx+/− ionization. Mol. Genet. Genomic Med. 4, 641-661

27. Daculsi, G., and Kerebel, B. (1978) High-resolution electron-microscope study of human enamel crystallites - size, shape, and growth. J. Ultrastruct. Res. 65, 163-172

28. Travis, D. F., and Glimcher, M. J. (1964) The structure and organization of, and the relationship between the organic matrix and the inorganic crystals of embryonic bovine enamel. J. Cell Biol. 23, 447-497

29. Fincham, A. G., Moradian-Oldak, J., Diekwisch, T. G. H., Lyaruu, D. M., Wright, J. T., Bringas, P., and Slavkin, H. C. (1995) Evidence for Amelogenin "Nanospheres" as Functional Components of Secretory-Stage Enamel Matrix. J Struct Biol 115, 50-59

30. Lacruz, R. S., Habelitz, S., Wright, J. T., and Paine, M. L. (2017) Dental Enamel Formation and Implications for Oral Health and Disease. Physiol. Rev. 97, 939-993

31. Garant, P. R., and Nalbandian, J. (1968) Observations on the ultrastructure of ameloblasts with special reference to the Golgi complex and related components. J. Ultrastruct. Res. 23, 427-443

32. Katchburian, E., and Holt, S. J. (1972) Studies on the Development of Ameloblasts. J. Cell Sci. 11, 415-447

33. Warshawsky, H. (1968) The fine structure of secretory ameloblasts in rat incisors. Anat Rec 161, 211-229

34. Snead, M. L., Lau, E. C., Zeichner-David, M., Fincham, A. G., Woo, S. L. C., and Slavkin, H. C. (1985) DNA sequence for cloned cDNA for murine amelogenin reveal the amino acid sequence for enamel-specific protein. Biochem. Biophys. Res. Commun. 129, 812-818
Krebsbach, P. H., Lee, S. K., Matsuki, Y., Kozak, C. A., Yamada, R. M., and Yamada, Y. (1996) Full-length sequence, localization, and chromosomal mapping of ameloblastin - A novel tooth-specific gene. J. Biol. Chem. 271, 4431-4435

Hu, C.-C., Fukae, M., Uchida, T., Qian, Q., Zhang, C. H., Ryu, O. H., Tanabe, T., Yamakoshi, Y., Murakami, C., Dohi, N., Shimizu, M., and Simmer, J. P. (1997) Cloning and Characterization of Porcine Enamelin mRNAs. J. Dent. Res. 76, 1720-1729

Bartlett, J. D., Simmer, J. P., Xue, J., Margolis, H. C., and Moreno, E. C. (1996) Molecular cloning and mRNA tissue distribution of a novel matrix metalloproteinase isolated from porcine enamel organ. Gene 183, 123-128

Beniash, E., Simmer, J. P., and Margolis, H. C. (2005) The effect of recombinant mouse amelogenins on the formation and organization of hydroxyapatite crystals in vitro. J Struct Biol 149, 182-190

Fang, P. A., Conway, J. F., Margolis, H. C., Simmer, J. P., and Beniash, E. (2011) Hierarchical self-assembly of amelogenin and the regulation of biomineralization at the nanoscale. Proc Natl Acad Sci U S A 108, 14097-14102

Margolis, H. C., Beniash, E., and Fowler, C. E. (2006) Role of macromolecular assembly of enamel matrix proteins in enamel formation. J. Dent. Res. 85, 775-793

Yamazaki, H., Tran, B., Beniash, E., Kwak, S. Y., and Margolis, H. C. (2019) Proteolysis by MMP20 Prevents Aberrant Mineralization in Secretory Enamel. J. Dent. Res. 98, 468-475

Fincham, A. G., Moradian-Oldak, J., and Simmer, J. P. (1999) The Structural Biology of the Developing Dental Enamel Matrix. J Struct Biol 126, 270-299

Simmer, J. P., and Hu, J. C. C. (2002) Expression, structure, and function of enamel proteinases. Connect. Tissue Res. 43, 441-449

Paine, M. L., Zhu, D.-H., Luo, W., Bringas, P., Goldberg, M., White, S. N., Lei, Y.-P., Sarikaya, M., Fong, H. K., and Snead, M. L. (2000) Enamel Biomineralization Defects Result from Alterations to Amelogenin Self-Assembly. J Struct Biol 132, 191-200

Tao, J., Fijneman, A., Wan, J., Prajapati, S., Mukherjee, K., Fernandez-Martinez, A., Moradian-Oldak, J., and De Yoreo, J. J. (2018) Control of Calcium Phosphate Nucleation and Transformation through Interactions of Enamelin and Amelogenin Exhibits the “Goldilocks Effect”. Crystal Growth & Design 18, 7391-7400

Nanci, A., Zalzl, S., Lavoie, P., Kunikata, M., Chen, W. Y., Krebsbach, P. H., Yamada, Y., Hammarstrom, L., Simmer, J. P., Fincham, A. G., Snead, M. L., and Smith, C. E. (1998) Comparative immunochemical analyses of the developmental expression and distribution of ameloblastin and amelogenin in rat incisors. J. Histochem. Cytochem. 46, 911-934

Zalzl, S. F., Smith, C. E., and Nanci, A. (2008) Ameloblastin and amelogenin share a common secretory pathway and are co-secreted during enamel formation. Matrix Biol. 27, 352-359

Ravindranath, R. M., Basilrose, R. M., Sr., Ravindranath, N. H., and Vaitheesvaran, B. (2003) Amelogenin interacts with cytokeratin-5 in ameloblasts during enamel growth. J Biol Chem 278, 20293-20302

Ravindranath, R. M., Tam, W. Y., Bringas, P., Jr., Santos, V., and Fincham, A. G. (2001) Amelogenin-cytokeratin 14 interaction in ameloblasts during enamel formation. J Biol Chem 276, 36586-36597

Robinson, C., and Hudson, J. (2011) Tuft protein: protein cross-linking in enamel development. Eur. J. Oral Sci. 119 Suppl 1, 50-54
Secretion of Keratin 75 by Ameloblasts

51. Robinson, C., Fuchs, P., and Weatherell, J. A. (1977) Fate of matrix proteins during development of dental enamel. *Calcif. Tissue Res.* **22**, 185-190

52. Duverger, O., Beniash, E., and Morasso, M. I. (2016) Keratins as components of the enamel organic matrix. *Matrix Biol.* **52-54**, 260-265

53. Alix-Panabieres, C., Vendrell, J.-P., Slijper, M., Pelle, O., Barbotte, E., Mercier, G., Jacot, W., Fabbro, M., and Pantel, K. (2009) Full-length cytokeratin-19 is released by human tumor cells: a potential role in metastatic progression of breast cancer. *Breast Cancer Res.* **11**

54. Chan, R., Rossitto, P. V., Edwards, B. F., and Cardiff, R. D. (1986) Presence of proteolytically processed keratins in the culture-medium of mcf-7 cells. *Cancer Res.* **46**, 6353-6359

55. Goldberg, M., Vermelin, L., Mostermans, P., Lécolle, S., Septier, D., Godeau, G., and Legeros, R. Z. (1998) Fragmentation of the Distal Portion of Tomes' Processes of Secretory Ameloblasts in the Forming Enamel of Rat Incisors. *Connect. Tissue Res.* **38**, 159-169

56. Rabouille, C. (2017) Pathways of Unconventional Protein Secretion. *Trends Cell Biol.* **27**, 230-240

57. Nickel, W., and Rabouille, C. (2009) Mechanisms of regulated unconventional protein secretion. *Nat. Rev. Mol. Cell Biol.* **10**, 148-155

58. Rabouille, C., Malhotra, V., and Nickel, W. (2013) Diversity in unconventional protein secretion. *J. Cell Sci.* **125**, 5251

59. Grieve, A. G., and Rabouille, C. (2011) Golgi Bypass: Skirting Around the Heart of Classical Secretion. *Cold Spring Harb. Perspect. Biol.* **3**

60. Ponpuak, M., Mandell, M. A., Kimura, T., Chauhan, S., Cleyrat, C., and Deretic, V. (2015) Secretory autophagy. *Curr. Opin. Cell Biol.* **35**, 106-116

61. Davis, S., Wang, J., and Ferro-Novick, S. Crosstalk between the Secretory and Autophagy Pathways Regulates Autophagosome Formation. *Dev. Cell* **41**, 23-32

62. Blott, E. J., and Griffiths, G. M. (2002) Secretory lysosomes. *Nat. Rev. Mol. Cell Biol.* **3**, 122-131

63. Simons, M., and Raposo, G. (2009) Exosomes – vesicular carriers for intercellular communication. *Curr. Opin. Cell Biol.* **21**, 575-581

64. Stefanovic, S., and Hegde, R. S. (2007) Identification of a targeting factor for posttranslational membrane protein insertion into the ER. *Cell* **128**, 1147-1159

65. Shao, S. C., and Hegde, R. S. (2011) Membrane Protein Insertion at the Endoplasmic Reticulum. in *Annu. Rev. Cell. Dev. Biol.* (Schekman, R., Goldstein, L., and Lehmann, R. eds.). pp 25-56

66. Begue-Kirn, C., Krebsbach, P. H., Bartlett, J. D., and Butler, W. T. (1998) Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. *Eur J. Oral Sci.* **106**, 963-970

67. Bleicher, F., Couble, M. L., Farges, J. C., Couble, P., and Magloire, H. (1999) Sequential expression of matrix protein genes in developing rat teeth. *Matrix Biol.* **18**, 133-143

68. Snider, N. T., and Omary, M. B. (2014) Post-translational modifications of intermediate filament proteins: mechanisms and functions. *Nature Reviews Molecular Cell Biology* **15**, 163-177

69. Uchida, T., and Warshawsky, H. (1992) Zinc iodide-osmium tetroxide impregnation of the "tubulo-vesicular system" in Tomes' process of the rat incisor ameloblast. *Anat Rec* **232**, 325-339

70. Yoshida, H., Inoue, M., Inoue, S., and Akisaka, T. (1996) Ultrastructure of kitten molar
secretory ameloblasts after quick-freezing and freeze-substitution. J. Electron Microsc. 45, 483-490
71. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure. J. Cell Biol. 116, 1071-1080
72. Nickel, W. (2003) The mystery of nonclassical protein secretion. Eur. J. Biochem. 270, 2109-2119
73. Duverger, O., Cross, M. A., Smith, F. J. D., and Morasso, M. I. (2019) Enamel Anomalies in a Pachyonychia Congenita Patient with a Mutation in KRT16. J. Invest. Dermatol. 139, 238-241
74. Hayoun, D., Kapp, T., Edri-Brami, M., Ventura, T., Cohen, M., Avidan, A., and Lichtenstein, R. G. (2012) HSP60 is transported through the secretory pathway of 3-MCA-induced fibrosarcoma tumour cells and undergoes N-glycosylation. FEBS J. 279, 2083-2095
75. Merendino, A. M., Bucchieri, F., Campanella, C., Marciano, V., Ribbene, A., David, S., Zummo, G., Burgio, G., Corona, D. F. V., de Macario, E. C., Macario, A. J. L., and Cappello, F. (2010) Hsp60 Is Actively Secreted by Human Tumor Cells. PLoS ONE 5
76. Paine, C. T., Paine, M. L., and Snead, M. L. (1998) Identification of tuftelin- and amelogenin-interacting proteins using the yeast two-hybrid system. Connect Tissue Res 38, 257-267; discussion 295-303
77. Yang, X., and Beniash, E. (2019) Immunofluorescence Procedures for Developing Enamel Tissues. in Odontogenesis: Methods and Protocols, Methods in Molecular Biology (Papagerakis, P. ed.), Springer. pp In Press
78. Yang, X., Vidunas, A., and Beniash, E. (2017) Optimizing Immunostaining of Enamel Matrix: Application of Sudan Black B and Minimization of False Positives from Normal Sera and IgGs. Front. Physiol. 8
79. Langbein, L., Yoshida, H., Praetzel-Wunder, S., Parry, D. A., and Schweizer, J. (2010) The keratins of the human beard hair medulla: the riddle in the middle. J. Invest. Dermatol. 130, 55-73
80. Verdelis, K., Szabo-Rogers, H. L., Xu, Y., Chong, R., Kang, R., Cusack, B. J., Jani, P., Boskey, A. L., Qin, C. L., and Beniash, E. (2016) Accelerated enamel mineralization in Dspp mutant mice. Matrix Biol. 52-54, 246-259
81. Szabo-Rogers, H. L., Geetha-Loganathan, P., Whiting, C. J., Nimmagadda, S., Fu, K., and Richman, J. M. (2009) Novel skeletogenic patterning roles for the olfactory pit. Development 136, 219-229
82. Yamakoshi, Y., Richardson Amelia, S., Nunez Stephanie, M., Yamakoshi, F., Milkovich Rachel, N., Hu Jan C.-C., Bartlett John, D., and Simmer James, P. (2012) Enamel proteins and proteases in Mmp20 and Klk4 null and double-null mice. Eur. J. Oral Sci. 119, 206-216
83. Winter, H., Hofmann, I., Langbein, L., Rogers, M. A., and Schweizer, J. (1997) A Splice Site Mutation in the Gene of the Human Type I Hair Keratin hHa1 Results in the Expression of a Tailless Keratin Isoform. J. Biol. Chem. 272, 32345-32352
84. Dunn, K. W., Kamocka, M. M., and McDonald, J. H. (2011) A practical guide to evaluating colocalization in biological microscopy. American Journal of Physiology - Cell Physiology 300, C723-C742
85. Bolte, S., and Cordelières, F. P. (2006) A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224, 213-232
86. Manders, E. M. M., Verbeek, F. J., and Aten, J. A. (1993) Measurement of co-localization of
objects in dual-colour confocal images. *J. Microsc.* **169**, 375-382
Figure 1. Characterization of K75 expression in developing enamel tissue. (A) Sagittal section of an apical portion of rat incisor in bright field. Presecretory, secretory and maturation stages are identified based on the morphological changes of ameloblasts. Three white boxes indicate areas shown in panel B. (B) AMELX, K75 and AMBN expression at the presecretory, secretory and late secretory-early maturation stages of amelogenesis. AMELX was found early in the presecretory and secretory ameloblasts; it ceased at the secretory-maturation transition. K75 and AMBN were expressed throughout the secretory stage. K75 expression ceased at secretory-maturation transition, while AMBN expression continued into the maturation stage. High magnification IF images of K75 (C) and K14 (D) in the mouse secretory ameloblasts. K14 showed a typical fibrillar network of tonofilaments, while K75 was present in form of granules mainly located in the distal half of the cell body and in the form of long bands located in the central cytoplasm. (E) In situ hybridization of Krt75 in mouse 1st molar at P1. Krt75 mRNA signal was present in ameloblasts, stratum intermedium and also in odontoblasts. (F) Western blot of hair follicle extract (HF), molar (M) and incisal (I) secretory enamel from 14 day-old rat using guinea pig anti-K75 antibodies, which were used for IF studies; Ag and Ab – molar secretory enamel lane stained with AMELX and AMBN antibodies, respectively, Mc– molar secretory enamel lane stained with Coomassie Brilliant Blue. The scale on the left represents estimate molecular weight in KDa. AB-amebolast; D-dentin, EEM- extracellular enamel matrix; N- nucleus; OD- odontoblast; P-pulp; SI- stratum intermedium; TP- Tomes’ process.
Figure 2. Distribution of K75 in secretory ameloblasts by IG-TEM (A) Schematic depiction of secretory ameloblasts. The double arrows represent the regions in which different populations of vesicles were detected. The numbers represent mean diameters of the vesicles for each population and the regions of the cell in which these vesicles exist are indicated by double arrows. Note that the sizes of the small vesicles in the cell body and in the Tomes' processes are not statistically different. For size measurements five ameloblasts were selected and 10-20 vesicles per group per ameloblast were measured. (B) Large membrane delineated vesicle positive for K75. (C) Small size K75 positive vesicles in the cell body. (D) Small K75 positive vesicles in Tomes' process. Arrowheads point toward the small vesicles containing K75. Note that one of the vesicles is undergoing exocytosis. rER – rough ER. The size of the gold particles is 6 nm. EEM – Extracellular enamel matrix; GA – Golgi apparatus; rER – rough endoplasmic reticulum.
Figure 3. Triple IF labeling of K75, AMELX and AMBN visualized by laser scanning confocal microscopy of secretory ameloblasts and enamel in incisors (A) and molars (B) from 4-week old rat. Note the bright overlap structures at the DEJ resembling the enamel tufts (white arrowheads). Large arrow identifies the sequence of enamel deposition. (C) High magnification image showing co-localization of K75 and AMBN in secretory enamel matrix. Note that K75 signal consists of arrays of compact clusters and overlaps with AMBN signal. (D) Pie chart showing the extent of the overlap of K75 with AMELX and AMBN based on the quantitative analysis of the incisal secretory ameloblast bodies from three 4-week old rats. (E-G) Co-localization of K75 (6 nm) with AMELX (12 nm) and AMBN (12 nm) in secretory ameloblasts of 4-week old rats by IG-TEM: (E,H) Small vesicles in the cell bodies; (F,I) large vesicles in the cell bodies, and (G,J) small vesicles in Tomes' processes. AB - ameloblast; CL- cervical loop; D- dentin; EEM- extracellular enamel matrix; P- pulp; OD- odontoblast; N- nucleus; OS- occlusal surface; SI- stratum intermedium; TP- Tomes' process. Data from 3 animals was used for IF and IG-TEM co-localization analyses.
Figure 4. IF co-localization of AMBN (A,C,E,G) and K75 (B,D,F,H) with different cellular organelle markers in the secretory ameloblasts from 4 weeks old mice. (A, B) Co-localization with rER marker CP1. Note that the co-localization for both proteins is very low. (C,D) Overlap with Golgi marker G5. G5 signal appears as two well organized long strips roughly parallel to each other, flanking the core of ameloblast body. Both AMBN and K75 signals organized into bands adjacent to the two strips of Golgi complexes in the ameloblast core, partially overlapping with G5. Furthermore, large AMBN and K75 positive large granules were closely associated the Golgi cisternae, potentially translocating their contents into Golgi for further processing. (E,F) Overlap with ERGIC marker ERGIC53. ERGIC53 signal was associated with large granules in the ameloblast cell bodies where it co-localized with AMBN and K75. ERGIC53 signal was also found in the distal portions of ameloblasts and Tomes' processes. Prominent overlap of K75 and AMBN with ERGIC53 was evident. (G,H) Overlap with lysosome marker LAMP1. LAMP1 signal was present throughout ameloblast cell bodies in the form granules of different shapes and sizes. There was a very limited overlap of K75 with LAMP1, while AMBN was more prominently associated with LAMP1. Scale bar: 10 μm. AB - ameloblast; SI- stratum intermedium; TP- Tomes' process.
Secretion of Keratin 75 by Ameloblasts

Figure 5. IF analysis of secretory ameloblasts from 4-week old mouse incisors after BFA treatment. Vehicle controls (A-D) - 1 hour after the vehicle injection. (E-H) Corresponding co-labelling images after 1 hour BFA treatment. (E) Co-localization of AMBN with CP1. Considerable overlap between AMBN and CP1 was shown, indicating that the classic secretory protein AMBN accumulated in ER after BFA treatment. (F) Co-localization of K75 with CP1 was not substantially affected by BFA, although the K75 labeled large granular vesicles could not detected anymore. (G) Co-localization of AMBN with G5. After BFA treatment, overlap of AMBN with Golgi complex dramatically decreased, indicating that trafficking from ER to Golgi was blocked by BFA. (H) Co-localization of K75 with G5. K75 signal was still associated with the central Golgi complex, indicating that K75 potentially utilized an unconventional pathway to translocate into Golgi apparatus. Box graphs show the overlap of AMBN and K75 with CP1 (I) and G5 (J) in the BFA treatment groups and vehicle controls. Filled diamonds represent the values of the overlap (%). Open squares represent mean and the central crossbars represent median values, the end crossbars represent SD. Significant differences (p ≤ 0.05) existed between groups marked by different letters. Scale bar: 10 μm.
**Figure 6.** IF analysis of secretory ameloblasts from 4-week old mouse incisors after BFA treatment. Vehicle controls (A, B) - 1 hour after the vehicle injection and BFA treatment (C,D) – 1 hour after BFA injection. Box graphs show the overlap of AMBN and K75 with ERGIC53 (E) in the BFA treatment groups and vehicle controls. Filled diamonds represent the values of the overlap (%). Open squares represent mean and the central crossbars represent median values, the end crossbars represent SD. Significant differences (p≤0.05) existed between groups marked by different letters. Scale bar: 10 μm.
 Trafficking and secretion of Keratin 75 by ameloblasts in vivo.
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